biomedicines

Special Issue Reprint

Latest Research in Post-COVID (Long COVID)

Pathological and Treatment Studies of Sequelae and Complications

Edited by
César Fernández-de-las-Peñas

mdpi.com/journal/biomedicines

MDPI

Latest Research in Post-COVID (Long COVID): Pathological and Treatment Studies of Sequelae and Complications

Latest Research in Post-COVID (Long COVID): Pathological and Treatment Studies of Sequelae and Complications

Editor

César Fernández-de-las-Peñas

MDPI

Basel • Beijing • Wuhan • Barcelona • Belgrade • Novi Sad • Cluj • Manchester

Editor
César Fernández-de-las-Peñas
Universidad Rey Juan Carlos
Alcorcón
Spain

Editorial Office
MDPI AG
Grosspeteranlage 5
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Biomedicines* (ISSN 2227-9059) (available at: https://www.mdpi.com/journal/biomedicines/special_issues/Post-COVID).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-1505-0 (Hbk)
ISBN 978-3-7258-1506-7 (PDF)
doi.org/10.3390/books978-3-7258-1506-7

Contents

About the Editor . . . . . **vii**

César Fernández-de-las-Peñas
Special Issue "Latest Research in Post-COVID (Long COVID): Pathological and Treatment Studies of Sequelae and Complications"
Reprinted from: *Biomedicines* **2024**, *12*, 1188, doi:10.3390/biomedicines12061188 . . . . . **1**

Juan Carlos Sánchez-García, Nuria Pilar Carrascosa Moreno, María Isabel Tovar-Gálvez, Jonathan Cortés-Martín, Antonio Liñán-González, Leticia Alvarado Olmedo and Raquel Rodríguez-Blanque
COVID-19 in Pregnant Women, Maternal—Fetal Involvement, and Vertical Mother-to-Child Transmission: A Systematic Review
Reprinted from: *Biomedicines* **2022**, *10*, 2554, doi:10.3390/biomedicines10102554 . . . . . **6**

César Fernández-de-las-Peñas, Jo Nijs, Randy Neblett, Andrea Polli, Maarten Moens, Lisa Goudman, et al.
Phenotyping Post-COVID Pain as a Nociceptive, Neuropathic, or Nociplastic Pain Condition
Reprinted from: *Biomedicines* **2022**, *10*, 2562, doi:10.3390/biomedicines10102562 . . . . . **20**

Andrea Galassi, Paola Ciceri, Valeria Bono, Lorenza Magagnoli, Matteo Sala, Luisa Artioli, et al.
Growth Differentiation Factor 15 (GDF-15) Levels Associate with Lower Survival in Chronic Kidney Disease Patients with COVID-19
Reprinted from: *Biomedicines* **2022**, *10*, 3251, doi:10.3390/biomedicines10123251 . . . . . **32**

Juan Luis Rodríguez Hermosa, Gianna Vargas Centanaro, María Estela González Castro, Marc Miravitlles, Lourdes Lázaro-Asegurado, Beatriz María Jiménez-Rodríguez, et al.
Severe COVID-19 Illness and α1-Antitrypsin Deficiency: COVID-AATD Study
Reprinted from: *Biomedicines* **2023**, *11*, 516, doi:10.3390/biomedicines11020516 . . . . . **45**

Santiago Larrateguy, Julian Vinagre, Federico Londero, Johana Dabin, Evangelina Ricciardi, Santiago Jeanpaul, et al.
Clinical Variables Related to Functional Capacity and Exertional Desaturation in Patients with COVID-19
Reprinted from: *Biomedicines* **2023**, *11*, 2051, doi:10.3390/biomedicines11072051 . . . . . **58**

Anna Paola Capra, Alessio Ardizzone, Lelio Crupi, Fabrizio Calapai, Michela Campolo, Salvatore Cuzzocrea and Emanuela Esposito
Efficacy of Palmitoylethanolamide and Luteolin Association on Post-Covid Olfactory Dysfunction: A Systematic Review and Meta-Analysis of Clinical Studies
Reprinted from: *Biomedicines* **2023**, *11*, 2189, doi:10.3390/biomedicines11082189 . . . . . **67**

Erika Meléndez-Oliva, Oliver Martínez-Pozas, Juan Nicolás Cuenca-Zaldívar, Jorge Hugo Villafañe, Laura Jiménez-Ortega and Eleuterio A. Sánchez-Romero
Efficacy of Pulmonary Rehabilitation in Post-COVID-19: A Systematic Review and Meta-Analysis
Reprinted from: *Biomedicines* **2023**, *11*, 2213, doi:10.3390/biomedicines11082213 . . . . . **79**

Juan Carlos Sánchez-García, María Rentero Moreno, Beatriz Piqueras-Sola, Jonathan Cortés-Martín, Antonio Liñán-González, Elena Mellado-García and Raquel Rodriguez-Blanque
Physical Therapies in the Treatment of Post-COVID Syndrome: A Systematic Review
Reprinted from: *Biomedicines* **2023**, *11*, 2253, doi:10.3390/biomedicines11082253 . . . . . **103**

Jacopo Maria Fontana, Angelo Alito, Paolo Piterà, Federica Verme, Stefania Cattaldo, Mauro Cornacchia, et al.
Whole-Body Cryostimulation in Post-COVID Rehabilitation for Patients with Obesity: A Multidisciplinary Feasibility Study
Reprinted from: *Biomedicines* **2023**, *11*, 3092, doi:10.3390/biomedicines11113092 . . . . . **117**

Timea Elisabeta Brandibur, Nilima Rajpal Kundnani, Marioara Boia, Daciana Nistor, Daniel Milan Velimirovici, Leonard Mada, et al.
Does COVID-19 Infection during Pregnancy Increase the Appearance of Congenital Gastrointestinal Malformations in Neonates?
Reprinted from: *Biomedicines* **2023**, *11*, 3105, doi:10.3390/biomedicines11123105 . . . . . **129**

Mariann Gyöngyösi, Ena Hasimbegovic, Emilie Han, Katrin Zlabinger, Andreas Spannbauer, Martin Riesenhuber, et al.
Improvement of Symptoms and Cardiac Magnetic Resonance Abnormalities in Patients with Post-Acute Sequelae of SARS-CoV-2 Cardiovascular Syndrome (PASC-CVS) after Guideline-Oriented Therapy
Reprinted from: *Biomedicines* **2023**, *11*, 3312, doi:10.3390/biomedicines11123312 . . . . . **144**

Marzena Romanowska-Kocejko, Agata Jędrzejewska, Alicja Braczko, Klaudia Stawarska, Oliwia Król, Marika Frańczak, et al.
Red Blood Cell Adenylate Energetics Is Related to Endothelial and Microvascular Function in Long COVID
Reprinted from: *Biomedicines* **2024**, *12*, 554, doi:10.3390/biomedicines12030554 . . . . . **158**

Jaume Alijotas-Reig, Ariadna Anunciacion-Llunell, Enrique Esteve-Valverde, Stephanie Morales-Pérez, Sergio Rivero-Santana, Jaume Trapé, et al.
Pituitary–Adrenal Axis and Peripheral Immune Cell Profile in Long COVID
Reprinted from: *Biomedicines* **2024**, *12*, 581, doi:10.3390/biomedicines12030581 . . . . . **173**

Sara Gangi, Laura Bergantini, Paolo Cameli, Irene Paggi, Marco Spalletti, Fabrizio Mezzasalma, et al.
Immunological Similarities and Differences between Post-COVID-19 Lung Sequelae and Idiopathic Pulmonary Fibrosis
Reprinted from: *Biomedicines* **2024**, *12*, 630, doi:10.3390/biomedicines12030630 . . . . . **189**

About the Editor

César Fernández-de-las-Peñas

Dr. César Fernández-de-las-Peñas is a Physical Therapist (PT), PhD in Biomedical Sciences and MD in Sciences (Dr. Med Sci.). He has 25 years of experience as a Professor at Universidad Rey Juan Carlos, Madrid, Spain. He was the Head Division (Chief) of the Department of Physical Therapy, Occupational Therapy, Rehabilitation at Universidad Rey Juan Carlos, for 10 years, creating the first chronic pain clinic in a public university in Spain where he combined clinical practice with teaching and research. He has published up to 760 publications in *JCR* journals. He is a clinical researcher with a particular interest in chronic pain and, more recently, in post-COVID-19. He has published more than 100 papers on long COVID, which is the topic of this Special Issue.

biomedicines

MDPI

Editorial

Special Issue "Latest Research in Post-COVID (Long COVID): Pathological and Treatment Studies of Sequelae and Complications"

César Fernández-de-las-Peñas

Department of Physical Therapy, Occupational Therapy, Physical Medicine and Rehabilitation, Universidad Rey Juan Carlos (URJC), 28922 Alcorcón, Madrid, Spain; cesar.fernandez@urjc.es; Tel.: +34-91-488-88-84

Citation: Fernández-de-las-Peñas, C. Special Issue "Latest Research in Post-COVID (Long COVID): Pathological and Treatment Studies of Sequelae and Complications". *Biomedicines* **2024**, *12*, 1188. https://doi.org/10.3390/biomedicines12061188

Received: 30 April 2024
Accepted: 17 May 2024
Published: 27 May 2024

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pathogen provoked the most unprecedented sanitary outbreak of the current century by causing coronavirus disease 2019 (COVID-19), which has led to approximately 775 million confirmed cases and more than 7 million deaths globally [1]. The COVID-19 outbreak has also prompted one of the most significant explosions of research in the last century, as thousands of papers have been published in a short period of time (four years). In fact, the extensive literature concerning COVID-19 has concentrated on the management of acute cases [2] and the prevention of the spread of the virus, e.g., vaccines [3].

Despite every endeavor to fight against COVID-19, the world is now confronted by another escalating healthcare problem derived from the outbreak: the development of long-lasting symptoms once the acute SARS-CoV-2 infection has passed. The presence of long-lasting symptoms after acute infection has been named long-COVID [4] or post-COVID-19 condition [5]. In fact, more than 100 post-COVID-19 symptoms have been attributed to SARS-CoV-2 infection [6]. Current data suggest that up to 25–30% of COVID-19 survivors report long-lasting post-COVID symptoms at one [7,8] and two [9,10] years after the infection. However, several aspects of this condition remain unknown, such as its underlying mechanisms, its consequences, and treatment strategies for the management of these patients.

This Special Issue of *Biomedicines*, entitled "Latest Research in Post-COVID (Long-COVID): Pathological and Treatment Studies of Sequelae and Complications", focused on these aspects of post-COVID-19 condition, a topic of emerging relevance due to the expected presence of millions of "long-haulers". A total of fourteen papers were published in this Special Issue, with the following topics addressed: (1) the treatment of post-COVID-19 condition; (2) the repercussions of SARS-CoV-2 infection in neonates; (3) risk factors of severe COVID-19; and (4) the phenotyping of post-COVID pain.

Treatment of Post-COVID-19 Condition

The development of treatment strategies for post-COVID-19 condition is an important topic. Among the plethora of post-COVID symptoms that a COVID-19 survivor may suffer from, fatigue and dyspnea are likely the most bothersome. Various studies have reported a prevalence of post-COVID fatigue ranging from 32% [11] to 42% [12] in the first six months after infection, and a prevalence of post-COVID dyspnea ranging from 26% to 41% [13].

The meta-analysis conducted by Meléndez-Oliva et al. reported the moderate to large effects that pulmonary rehabilitation has on post-COVID dyspnea, but not on fatigue, physical function, quality of life, and depressive symptoms; this was in comparison to the typical care interventions (n = 34 trials) [14]. Most studies included in this meta-analysis used exercise and breathing retraining as the main components of pulmonary rehabilitation [14]. Therefore, a potential explanation for this lack of effect on post-COVID fatigue could be that the exercise administered was not personalized to each patient and, accordingly, that the intensity or volume of exercise was not sufficient to reach fatigue

levels; in addition, breathing retraining exercises are mainly focused on the respiratory, but not cardiovascular, system. In fact, the systematic review presented by Sánchez-García et al. found limited evidence that physical therapy interventions related to respiratory musculature and moderate-intensity exercise led to significant improvements in post-COVID fatigue and dyspnea [15]. In fact, it seems that intense exercise, e.g., high-intensity interval training (HIT), moderate-intensity continuous training and strength training, can effectively enhance skeletal muscle deconditioning in patients with post-COVID-19 condition [16]; however, such exercise programs should be individualized due to the presence of post-exertional malaise in this population [17].

In addition to rehabilitation, the treatment of post-COVID-19 condition involves the utilization of other interventions. Among these potential interventions, a study published in this Special Issue found that the administration of micronized Palmitoylethanolamide plus Luteolin (CoUltraPEALut) as an adjuvant treatment with olfactory training effectively aids in the overall recovery of post-COVID olfactory problems (anosmia, hyposmia) [18]. Other medications, such as nonsteroidal anti-inflammatory drugs (NSAID), have been proposed for the management of some symptoms of post-COVID-19 condition. The rationale for applying NSAIDs is based on a reduction in the proinflammatory cytokine response associated with the infection; however, the early application of NSAIDs, at least in the acute COVID-19 phase, has been hypothesized to negatively impact the initial antiviral immune response of the host [19]; however, this hypothesis needs to be further investigated. In this Special Issue, Gyöngyösi et al. describe an improvement in the clinical symptomatology associated with a decrease in the presence of cardiac abnormalities (probably due to ongoing myocardial inflammation) with the prolonged use of NSAIDs in individuals with post-COVID cardiac symptoms [20].

Neonatal Repercussion of SARS-CoV-2 Infection

The risk of the potential perinatal transmission of SARS-CoV-2 has received particular attention from the beginning of the outbreak. With the rapid development of COVID-19 vaccines, questions concerning the safety of vaccination during pregnancy have been raised. Overall, vaccination during pregnancy does not seem to be associated with an increased risk of adverse pregnancy or perinatal outcomes [21].

However, the risk of the transmission of SARS-CoV-2 infection during pregnancy remains unclear. In fact, at the beginning of the pandemic, it was documented that SARS-CoV-2 can result in a high incidence of premature birth, miscarriages or maternal death [22]. This information has changed with further research. Thus, a systematic review published in this Special Issue investigates the possibility of vertical transmission from mother to child [23]. This study found that vertical transmission from mother to child during pregnancy (i.e., transmission via placenta) is not supported by current data, but that vertical transmission at the time of delivery or breastfeeding can be exceptionally possible [23]. Other reviews have also been unable to identify any significant association between acute SARS-CoV-2 infection in early pregnancy (the first 20 weeks of gestation) and adverse fetal, neonatal or maternal outcomes [24,25]. Nevertheless, Rodriguez-Wallberg et al. warned of a 44% increase in the rate of miscarriage rate in recent years [24]. Similar results were also identified by Brandibur et al., who reported that SARS-CoV-2 acute infection during pregnancy was unlikely to cause congenital digestive malformations; however, these authors observed that the number of gastrointestinal malformations was higher during 2022 (n = 47) than during the 3 years (2017–2020) prior to the COVID-19 outbreak (n = 39) [26].

Risk Factors of Severe COVID-19

The identification of individuals at a higher risk of developing severe COVID-19 has received particular attention in the literature. In fact, several studies have investigated whether the presence of deficiencies in gene expression could lead to a higher risk of experiencing the severe form of this condition. For instance, Saengsiwaritt et al. revealed that subjects carrying the C allele of the transmembrane protease serine-2 (TMPRSS2) rs12329760 polymorphism or the T allele of the surface receptor for S1 of the angiotensin-converting

enzyme 2 (ACE2) rs2285666 polymorphism exhibit a higher risk of severe COVID-19 [27]. In this Special Issue, Rodríguez Hermosa et al. found that subjects with alpha-1 antitrypsin deficiency (AATD) are at a higher risk of developing severe COVID-19 [28]. In fact, AATD levels below 116 mg/dL and the presence of a variant of the serine protein inhibitor-A1 (SERPINA1) gene, which could affect alpha-1 antitrypsin (A1AT) protein, were factors associated with the severe form of COVID-19 disease [28].

A study involving patients with chronic kidney disease, a vulnerable population, found that those with a lower estimated glomerular filtration rate and higher levels of Growth Differentiation Factor-15 (GDF-15) presented a higher risk of mortality associated with COVID-19 [29].

Phenotyping of Post-COVID Pain

Pain is an important but underestimated post-COVID symptom experienced by 15–20% of subjects after an acute SARS-CoV-2 infection [30,31]. In this Special Issue, a consensus paper on phenotyping post-COVID pain [32] proposed the application of the 2021 International Association for the Study of Pain (IASP) clinical criteria and grading system for classifying post-COVID pain symptomatology [33]. This consensus paper describes how post-COVID pain symptomatology can fulfill any of the phenotypes proposed by the IASP: nociceptive, neuropathic, nociplastic, and the mixed type [32]. In fact, based on current data, it seems that some patients suffering from post-COVID-19 condition will exhibit a pain phenotype with nociplastic characteristics. Nociplastic pain is defined by the IASP as "pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain" [34]. Based on this definition, the presence of sensitization appears to be a primary mechanism associated with this phenotype. Several musculoskeletal chronic pain conditions are associated with pain sensitization [35]. Thus, nociplastic pain has also been associated with comorbid central-nervous-associated symptoms, e.g., poor sleep quality, fatigue, and cognitive–emotional disturbances, which are all present in post-COVID-19 condition.

Others

In this last section, the remaining papers are summarized. Romanowska-Kocejko et al. observed that the dysregulation of metabolic processes in erythrocytes, in addition to endothelial and microvascular dysfunction, is associated with the decreased delivery of intracellular oxygen in patients with post-COVID-19 condition [36]. In accordance with the hypothesized endothelial problems, another study published in this Special Issue found that pulmonary embolism, as well as the use of a high-flow nasal cannula and prolonged hospitalization, is associated with reduced functional capacity and a higher likelihood of exertional desaturation in patients with post-COVID-19 condition [37]. Thus, the association of endothelial and microvascular dysfunction with reduced intracellular oxygen delivery may partly explain this post-COVID fatigue and limited functional capacity [36]. Similarly, the endothelial disfunction of the brain would explain the presence of post-COVID cognitive symptomatology [38]. In fact, the plethora of cardiovascular post-COVID symptoms that can be observed has been integrated in the term "vascular long-COVID" [39].

The last paper investigated differences in the immune response between patients with post-COVID-19 condition and those with interstitial pulmonary disease [40]. The study revealed a greater depletion of CD4 and natural killer cells in individuals with interstitial pulmonary disease, as well as an increase in CD8 cells. Furthermore, an increase in CD4 and CD8 cells, as a accentuating immune response, was observed in patients with post-COVID-19 condition [40].

As Guest Editor of this Special Issue, I would like to thank the reviewers for their comments, the authors for their valuable contributions, and the *Biomedicines* staff for their collective support and assistance during this process.

Conflicts of Interest: The author declares no conflicts of interest.

References

1. WHO Coronavirus (COVID-19) Dashboard. 2023. Available online: https://covid19.who.int/ (accessed on 28 April 2024).
2. Available online: https://www.covid19treatmentguidelines.nih.gov/management/clinical-management-of-adults/hospitalized-adults--therapeutic-management/ (accessed on 28 April 2024).
3. Wu, X.; Xu, K.; Zhan, P.; Liu, H.; Zhang, F.; Song, Y.; Lv, T. Comparative efficacy and safety of COVID-19 vaccines in phase III trials: A network meta-analysis. *BMC Infect. Dis.* **2024**, *24*, 234. [CrossRef] [PubMed]
4. Fernández-de-las-Peñas, C. Long COVID: Current definition. *Infection* **2022**, *50*, 285–286. [CrossRef] [PubMed]
5. Soriano, J.B.; Murthy, S.; Marshall, J.C.; Relan, P.; Diaz, J.V. WHO Clinical Case Definition Working Group on Post-COVID-19 Condition. A clinical case definition of post-COVID-19 condition by a Delphi consensus. *Lancet Infect. Dis.* **2022**, *22*, e102–e107. [CrossRef] [PubMed]
6. Hayes, L.D.; Ingram, J.; Sculthorpe, N.F. More than 100 persistent symptoms of SARS-CoV-2 (Long COVID): A scoping review. *Front. Med.* **2021**, *8*, 750378. [CrossRef] [PubMed]
7. Chen, C.; Haupert, S.R.; Zimmermann, L.; Shi, X.; Fritsche, L.G.; Mukherjee, B. Global prevalence of post COVID-19 condition or long COVID: A meta-analysis and systematic review. *J. Infect. Dis.* **2022**, *226*, 1593–1607. [CrossRef] [PubMed]
8. Han, Q.; Zheng, B.; Daines, L.; Sheikh, A. Long-term sequelae of COVID-19: A systematic review and meta-analysis of one-year follow-up studies on post-COVID symptoms. *Pathogens* **2022**, *11*, 269. [CrossRef] [PubMed]
9. Fernández-de-las-Peñas, C.; Notarte, K.I.; Macasaet, R.; Velasco, J.V.; Catahay, J.A.; Therese Ver, A.; Chung, W.; Valera-Calero, J.A.; Navarro-Santana, M. Persistence of post-COVID symptoms in the general population two years after SARS-CoV-2 infection: A systematic review and meta-analysis. *J. Infect.* **2024**, *88*, 77–88. [CrossRef]
10. Rahmati, M.; Udeh, R.; Yon, D.K.; Lee, S.W.; Dolja-Gore, X.; McEVoy, M.; Kenna, T.; Jacob, L.; López Sánchez, G.F.; Koyanagi, A.; et al. A systematic review and meta-analysis of long-term sequelae of COVID-19 2-year after SARS-CoV-2 infection: A call to action for neurological, physical, and psychological sciences. *J. Med. Virol.* **2023**, *95*, e28852. [CrossRef] [PubMed]
11. Ceban, F.; Ling, S.; Lui, L.M.W.; Lee, Y.; Gill, H.; Teopiz, K.M.; Rodrigues, N.B.; Subramaniapillai, M.; Di Vincenzo, J.D.; Cao, B.; et al. Fatigue and cognitive impairment in Post-COVID-19 Syndrome: A systematic review and meta-analysis. *Brain Behav. Immun.* **2022**, *101*, 93–135. [CrossRef]
12. Rao, S.; Benzouak, T.; Gunpat, S.; Burns, R.J.; Tahir, T.A.; Jolles, S.; Kisely, S. Fatigue symptoms associated with COVID19 in convalescent or recovered COVID-19 patients: A systematic review and meta-analysis. *Ann. Behav. Med.* **2022**, *56*, 219–234. [CrossRef]
13. Zheng, B.; Daines, L.; Han, Q.; Hurst, J.R.; Pfeffer, P.; Shankar-Hari, M.; Elneima, O.; Walker, S.; Brown, J.S.; Siddiqui, S.; et al. Prevalence, risk factors and treatments for post-COVID-19 breathlessness: A systematic review and meta-analysis. *Eur. Respir. Rev.* **2022**, *31*, 220071. [CrossRef] [PubMed]
14. Meléndez-Oliva, E.; Martínez-Pozas, O.; Cuenca-Zaldívar, J.N.; Villafañe, J.H.; Jiménez-Ortega, L.; Sánchez-Romero, E.A. Efficacy of pulmonary rehabilitation in post-COVID-19: A systematic review and meta-analysis. *Biomedicines* **2023**, *11*, 2213. [CrossRef] [PubMed]
15. Sánchez-García, J.C.; Rentero Moreno, M.; Piqueras-Sola, B.; Cortés-Martín, J.; Liñán-González, A.; Mellado-García, E.; Rodriguez-Blanque, R. Physical therapies in the treatment of post-COVID Syndrome: A systematic review. *Biomedicines* **2023**, *11*, 2253. [CrossRef] [PubMed]
16. Tryfonos, A.; Pourhamidi, K.; Jörnåker, G.; Engvall, M.; Eriksson, L.; Elhallos, S.; Asplund, N.; Mandic, M.; Sundblad, P.; Sepic, A.; et al. Functional limitations and exercise intolerance in patients with post-COVID Condition: A randomized crossover clinical trial. *JAMA Netw. Open.* **2024**, *7*, e24438. [CrossRef] [PubMed]
17. Arienti, C.; Cordani, C.; Lazzarini, S.G.; Del Furia, M.J.; Negrini, S.; Kiekens, C. Fatigue, post-exertional malaise and orthostatic intolerance: A map of Cochrane evidence relevant to rehabilitation for people with post COVID-19 condition. *Eur. J. Phys. Rehabil. Med.* **2022**, *58*, 857–863. [CrossRef] [PubMed]
18. Capra, A.P.; Ardizzone, A.; Crupi, L.; Calapai, F.; Campolo, M.; Cuzzocrea, S.; Esposito, E. Efficacy of Palmitoylethanolamide and Luteolin association on post-COVID olfactory dysfunction: A systematic review and meta-analysis of clinical studies. *Biomedicines* **2023**, *11*, 2189. [CrossRef] [PubMed]
19. Chen, J.S.; Alfajaro, M.M.; Chow, R.D.; Wei, J.; Filler, R.B.; Eisenbarth, S.C.; Wilen, C.B. Nonsteroidal Anti-inflammatory drugs dampen the cytokine and antibody response to SARS-CoV-2 infection. *J. Virol.* **2021**, *95*, e00014-21. [CrossRef] [PubMed]
20. Gyöngyösi, M.; Hasimbegovic, E.; Han, E.; Zlabinger, K.; Spannbauer, A.; Riesenhuber, M.; Hamzaraj, K.; Bergler-Klein, J.; Hengstenberg, C.; Kammerlander, A.; et al. Improvement of symptoms and cardiac magnetic resonance abnormalities in patients with post-acute sequelae of SARS-CoV-2 Cardiovascular Syndrome (PASC-CVS) after guideline-oriented therapy. *Biomedicines* **2023**, *11*, 3312. [CrossRef] [PubMed]
21. Fernández-García, S.; Del Campo-Albendea, L.; Sambamoorthi, D.; Sheikh, J.; Lau, K.; Osei-Lah, N.; Ramkumar, A.; Naidu, H.; Stoney, N.; Sundaram, P.; et al. PregCOV-19 Living Systematic Review Consortium. Effectiveness and safety of COVID-19 vaccines on maternal and perinatal outcomes: A systematic review and meta-analysis. *BMJ Glob. Health* **2024**, *9*, e014247. [CrossRef]
22. Yu, W.; Hu, X.; Cao, B. Viral infections during pregnancy: The big challenge threatening maternal and fetal health. *Matern. Med.* **2021**, *4*, 72–86. [CrossRef]

23. Sánchez-García, J.C.; Carrascosa Moreno, N.P.; Tovar-Gálvez, M.I.; Cortés-Martín, J.; Liñán-González, A.; Alvarado Olmedo, L.; Rodríguez-Blanque, R. COVID-19 in pregnant women, maternal-fetal involvement, and vertical mother-to-child transmission: A systematic review. *Biomedicines* **2022**, *10*, 2554. [CrossRef] [PubMed]
24. Rodriguez-Wallberg, K.A.; Nilsson, H.P.; Røthe, E.B.; Zhao, A.; Shah, P.S.; Acharya, G. Outcomes of SARS-CoV-2 infection in early pregnancy—A systematic review and meta-analysis. *Acta Obstet. Gynecol. Scand.* **2024**, *103*, 786–798. [CrossRef] [PubMed]
25. van Baar, J.A.C.; Kostova, E.B.; Allotey, J.; Thangaratinam, S.; Zamora, J.R.; Bonet, M.; Kim, C.R.; Mofenson, L.M.; Kunst, H.; Khalil, A.; et al. PregCOV-19 Living Systematic Review Consortium. COVID-19 in pregnant women: A systematic review and meta-analysis on the risk and prevalence of pregnancy loss. *Hum. Reprod. Update* **2024**, *30*, 133–152. [CrossRef] [PubMed]
26. Brandibur, T.E.; Kundnani, N.R.; Boia, M.; Nistor, D.; Velimirovici, D.M.; Mada, L.; Manea, A.M.; Boia, E.R.; Neagu, M.N.; Popoiu, C.M. Does COVID-19 infection during pregnancy increase the appearance of congenital gastrointestinal malformations in neonates? *Biomedicines* **2023**, *11*, 3105. [CrossRef]
27. Saengsiwaritt, W.; Jittikoon, J.; Chaikledkaew, U.; Udomsinprasert, W. Genetic polymorphisms of ACE1, ACE2, and TMPRSS2 associated with COVID-19 severity: A systematic review with meta-analysis. *Rev. Med. Virol.* **2022**, *8*, e2323. [CrossRef] [PubMed]
28. Rodríguez Hermosa, J.L.; Vargas Centanaro, G.; González Castro, M.E.; Miravitlles, M.; Lázaro-Asegurado, L.; Jiménez-Rodríguez, B.M.; Rodríguez, R.A.; Moreno Méndez, R.; Torres-Duran, M.; Hernández-Pérez, J.M.; et al. Severe COVID-19 Illness and _1-Antitrypsin Deficiency: COVID-AATD Study. *Biomedicines* **2023**, *11*, 516. [CrossRef] [PubMed]
29. Galassi, A.; Ciceri, P.; Bono, V.; Magagnoli, L.; Sala, M.; Artioli, L.; Rovito, R.; Hadla, M.; Yellenki, V.; D'Arminio Monforte, A.; et al. Growth Differentiation Factor 15 (GDF-15) levels associate with lower survival in chronic kidney disease patients with COVID-19. *Biomedicines* **2022**, *10*, 3251. [CrossRef] [PubMed]
30. Fernández-de-las-Peñas, C.; Navarro-Santana, M.; Plaza-Manzano, G.; Palacios-Ceña Arendt-Nielsen, L. Time course prevalence of Post-COVID pain symptoms of musculoskeletal origin in patients who had survived to SARS-CoV-2 infection: A systematic review and meta-analysis. *Pain* **2022**, *163*, 1220–1231. [CrossRef] [PubMed]
31. Kerzhner, O.; Berla, E.; Har-Even, M.; Ratmansky, M.; Goor-Aryeh, I. Consistency of inconsistency in long-COVID-19 pain symptoms persistency: A systematic review and meta-analysis. *Pain Pract.* **2024**, *24*, 120–159. [CrossRef]
32. Fernández-de-las-Peñas, C.; Nijs, J.; Neblett, R.; Polli, A.; Moens, M.; Goudman, L.; Shekhar Patil, M.; Knaggs, R.D.; Pickering, G.; Arendt-Nielsen, L. Phenotyping Post-COVID pain as a nociceptive, neuropathic, or nociplastic pain condition. *Biomedicines* **2022**, *10*, 2562. [CrossRef]
33. Kosek, E.; Clauw, D.; Nijs, J.; Baron, R.; Gilron, I.; Harris, R.E.; Mico, J.A.; Rice, A.S.C.; Sterling, M. Chronic nociplastic pain affecting the musculoskeletal system: Clinical criteria and grading system. *Pain* **2021**, *162*, 2629–2634. [CrossRef] [PubMed]
34. Kosek, E.; Cohen, M.; Baron, R.; Gebhart, G.F.; Mico, J.A.; Rice, A.S.; Rief, W.; Sluka, A.K. Do we need a third mechanistic descriptor for chronic pain states? *Pain* **2016**, *157*, 1382–1386. [CrossRef] [PubMed]
35. Nijs, J.; George, S.Z.; Clauw, D.J.; Fernández-de-las-Peñas, C.; Kosek, E.; Ickmans, K.; Fernández-Carnero, J.; Polli, A.; Kapreli, E.; Huysmans, E.; et al. Central sensitisation in chronic pain conditions: Latest discoveries and their potential for precision medicine. *Lancet Rheumatol.* **2021**, *3*, e383–e392. [CrossRef] [PubMed]
36. Romanowska-Kocejko, M.; Jędrzejewska, A.; Braczko, A.; Stawarska, K.; Król, O.; Frańczak, M.; Harasim, G.; Smoleński, R.T.; Hellmann, M.; Kutryb-Zając, B. Red blood cell adenylate energetics is related to endothelial and microvascular function in long COVID. *Biomedicines* **2024**, *12*, 554. [CrossRef] [PubMed]
37. Larrateguy, S.; Vinagre, J.; Londero, F.; Dabin, J.; Ricciardi, E.; Jeanpaul, S.; Torres-Castro, R.; Núñez-Cortés, R.; Sánchez-Ramírez, D.; Gimeno-Santos, E.; et al. Clinical variables related to functional capacity and exertional desaturation in patients with COVID-19. *Biomedicines* **2023**, *11*, 2051. [CrossRef] [PubMed]
38. Shabani, Z.; Liu, J.; Su, H. Vascular dysfunctions contribute to the long-term cognitive deficits following COVID-19. *Biology* **2023**, *12*, 1106. [CrossRef] [PubMed]
39. Zanini, G.; Selleri, V.; Roncati, L.; Coppi, F.; Nasi, M.; Farinetti, A.; Manenti, A.; Pinti, M.; Mattioli, A.V. Vascular "Long COVID": A new vessel disease? *Angiology* **2024**, *75*, 8–14. [CrossRef]
40. Gangi, S.; Bergantini, L.; Cameli, P.; Paggi, I.; Spalletti, M.; Mezzasalma, F.; Bargagli, E.; d'Alessandro, M. Immunological similarities and differences between post-COVID-19 lung sequelae and idiopathic pulmonary fibrosis. *Biomedicines* **2024**, *12*, 630. [CrossRef]

biomedicines

MDPI

Systematic Review

COVID-19 in Pregnant Women, Maternal—Fetal Involvement, and Vertical Mother-to-Child Transmission: A Systematic Review

Juan Carlos Sánchez-García [1,2], Nuria Pilar Carrascosa Moreno [3], María Isabel Tovar-Gálvez [1,4,*], Jonathan Cortés-Martín [1,2], Antonio Liñán-González [1,5], Leticia Alvarado Olmedo [1,4] and Raquel Rodríguez-Blanque [1,2]

[1] Research Group CTS1068, Andalusia Research Plan, Junta de Andalucía, 41006 Sevilla, Spain
[2] Nursing Department, Faculty of Health Sciences, University of Granada, 18071 Granada, Spain
[3] Canarias University Hospital, Carretera Ofra S/N, 38320 La Laguna, Spain
[4] Nursing Department, Faculty of Health Sciences in Ceuta, University of Granada, 51001 Ceuta, Spain
[5] Nursing Department, Faculty of Health Sciences in Melilla, University of Granada, Melilla Campus, 52005 Melilla, Spain
* Correspondence: matoga@ugr.es

Abstract: Pregnant women are included in the COVID-19 risk groups even if they do not have any pathology. This requires an analysis of research focused on pregnant women to understand the impact of SARS-CoV-2 on their condition. There is also a need to know whether there is vertical mother-to-child transmission, as well as other consequences in case the pregnant woman is infected and COVID-19 positive. A systematic review was carried out to analyze the existing information on the complications of a pregnant woman infected with the SARS-CoV-2 coronavirus and the possibility of vertical transmission from mother to child, registered in the PROSPERO website and searched in the PubMed, Scopus, CINAHL, and Cochrane Library databases. Finally, 22 articles were included in the review. The review suggests that vertical transmission from mother to child could be exceptionally possible at the time of delivery or breastfeeding, but not through the placenta. It is interesting to point out the good acceptance of vaccination by pregnant women, which may be the reason for the low infectivity. Further research on pregnant women should be carried out to provide evidence on vertical mother-to-child transmission and the role of breast milk in relation to SARS-CoV-2.

Keywords: COVID-19; SARS-CoV-2; pregnancy; vertical mother-to-child transmission; breast milk; vaccination

Citation: Sánchez-García, J.C.; Carrascosa Moreno, N.P.; Tovar-Gálvez, M.I.; Cortés-Martín, J.; Liñán-González, A.; Alvarado Olmedo, L.; Rodríguez-Blanque, R. COVID-19 in Pregnant Women, Maternal—Fetal Involvement, and Vertical Mother-to-Child Transmission: A Systematic Review. *Biomedicines* **2022**, *10*, 2554. https://doi.org/10.3390/biomedicines10102554

Academic Editor: César Fernández De Las Peñas

Received: 4 September 2022
Accepted: 9 October 2022
Published: 13 October 2022

1. Introduction

COVID-19 is a disease caused by a new, already-known virus, called SARS-CoV-2, which caused unexplained pneumonia in late December 2019 and resulted in a global pandemic, which is still present today [1]. Symptoms of the disease can result in a range of symptoms from the need for admission to intensive care units to no symptoms at all. It has been evidenced that it does not act with the same effect on all people and that the most adverse effects appear in those who have some pathology such as respiratory or cardiac diseases [2].

On 11 March, a global pandemic was declared, which meant a great change in the economic, social, and health spheres, negatively affecting vulnerable groups in particular. The people most at risk of developing a serious COVID-19 infection are those over 60 years of age or those with a pathology such as hypertension [3]. Among the groups at risk is the population of pregnant women, whose changing anatomical and physiological status causes alterations that affect their respiratory system, as well as their immune system, cardiovascular function, and even coagulation. Although the risk of contracting the infection is the same as in the general population, their status means that the evolution of the disease may be more severe at the respiratory level [4,5] and may even increase the risk

of presenting with pre-eclampsia and alterations at the placental level [6–8]. We refer to the risks that the mother may suffer but also the fetus, as vertical mother-to-child transmission may occur [9]. Viral infection during pregnancy can seriously harm the fetus, leading to miscarriage, fetal death, intrauterine growth retardation, or the newborn, such as various types of sequelae [10].

Therefore, pregnant women need their own analysis of the mechanism of transmission from mother to fetus, to explore whether or not it really occurs by vertical mother-to-child transmission and the possible complications that may appear at the time of delivery. It is also important to analyze some aspects of the pathology produced by the SARS-CoV-2 virus and whether immunization by vaccination may have had any influence on this possible vertical mother-to-child transmission, thus producing a document that can help in future research [11–14].

The aim of this systematic review was to analyze whether vertical mother-to-child transmission of the SARS-CoV-2 virus occurs in pregnant women in COVID-19 and what the existing maternal–fetal involvement is.

2. Materials and Methods

2.1. Review Protocol

The methodology used for the elaboration of this report followed the Preferred Reporting Items for Systematic reviews and MetaAnalyses (PRISMA) review protocol [15], which consists of a 27-point checklist of the most representative sections of an original article, as well as the process of drawing up these guidelines. This systematic review was carried out following a protocol, available on the web: http://www.crd.york.ac.uk/PROSPERO/ (accessed on 5 August 2022), the registration number of which is ID331580.

2.2. Eligibility Criteria

We considered articles from 1 December 2019 to 1 March 2022 that provided information on COVID-19 in pregnant women and the possibility of fetal transmission, with no restrictions on the language of publication. We accepted any type of article in line with the topic to be addressed. Two researchers independently assessed all the references identified in the search. First, we screened the references according to the title and abstract. Subsequently, articles that met the inclusion criteria in the first phase were read in full text to determine their final inclusion. In cases where there was disagreement between the two researchers on the inclusion of a manuscript, a third researcher was consulted.

Data on quality, patient characteristics, interventions, and relevant outcomes were obtained independently by the authors.

2.3. Sources of Information

The literature search was carried out in the databases Scopus, PubMed, CINAHL, CINAHL, and Cochrane library. A hand search was conducted using reference lists of studies to find other relevant studies. The structured language used was obtained using MeSH terms and Health Sciences (DeCS) descriptors. The descriptors used were “COVID-19”, “SARS-CoV-2”, “pregnancy”, “fetal transmission”, “complications”, and “outcomes”, and the Boolean operators used were AND and OR.

2.4. Search Strategy

Table 1 shows the search strategy that was used to carry out this work, together with the date on which the search was carried out.

Table 1. Search strategies.

Sources of Information	Search String
PUBMED	Search: (COVID-19) OR (SARS- CoV-2) AND (pregnancy) AND (fetal transmission) AND (complications) AND (outcomes). Filters: Full text, publication date 5 years.
SCOPUS	Search within (article title, abstract, keywords): (COVID-19) OR (SARS-CoV-2) AND (pregnancy) AND (fetal transmission) AND (complications) AND (outcomes) AND (Limit-to (DOCTYPE "ar")).
COCHRANE LIBRARY	Search: (COVID-19) OR (SARS- CoV-2) AND (pregnant) AND (outcomes) in title, abstract, keyword.
CINAHL	Search: (COVID-19) OR (SARS- CoV-2) AND (pregnant) AND (rct) AND (outcomes) AND (vertical mother-to-child transmission).

2.5. Risk of Bias in Individual Studies

In order to carry out the methodological evaluation of the articles selected for this study, the design, methodology, and type of study of each paper were analyzed, with the aim of selecting the most specific methodological evaluation scale for each case.

Of the 22 articles, 8 were literature reviews, 8 systematic reviews, 2 cohort studies, 3 case studies, and 1 randomized clinical trial.

Articles with a case study design were assessed using the Rating Scale for Single Participants Designs (SCED). The SCED was constructed including 11 items, of which 10 were used to assess methodological quality and one for the use of statistical analysis. One point was added for each item present, and a maximum score of 11 points could be obtained. Between 9 and 10 indicates very good quality; between 6 and 8 indicates good quality; between 4 and 5 indicates poor quality; and below 4 indicates poor quality. The cut-off point chosen to select the studies was those that obtained a score of 6 points or more.

Table 2 shows the result obtained after applying the methodological evaluation using the SCED scale.

Table 2. Methodological assessment according to the SCED scale.

Author	Article	Numerical Score
Fernandez–Perez et al. [16]	SARS-CoV-2: What it is, how it acts, and how it manifests in imaging studies.	8
Yang et al. [17]	Pregnant women with COVID-19 and risk of adverse birth outcomes and maternal-fetal vertical mother-to-child transmission: A population-based cohort study in Wuhan, China.	6
Ghema et al. [18]	Outcomes of newborns to mother with COVID-19.	6
Resta et al. [19]	SARS-CoV-2 and placenta: New insights and perspectives.	8
Saroyo et al. [20]	Remdesivir treatment for COVID-19 in pregnant patients with moderate to severe symptoms: Serial case report.	7

The AMSTAR-2 (A Measurement Tool to Assess Systematic Reviews) methodological assessment scale was used for the reviews [17]. AMSTAR-2 provides a broad assessment of quality, incorporating imperfections that may have arisen due to improper conduct of the review. AMSTAR-2 was constructed to include 16 domains, which present simple response options: "yes" when the product is positive; "no", if the standard was not met or the existing information was too limited to answer; and "partial yes", in situations where partial adherence to the standard was given. While not providing an overall rating, four levels of confidence emerge: high, moderate, low, and critically low.

Table 3 below shows the results obtained after applying the methodological evaluation using the AMSTAR-2 scale.

Table 3. Methodological assessment according to the AMSTAR-2 scale.

Author	Article	Assessment of Overall Confidence
Wang et al. [21]	Impact of COVID-19 on pregnancy.	High
Diriba et al. [4]	The effect of Coronavirus infection (during pregnancy and the possibility of vertical maternal–fetal transmission: A systematic review.	High
Khedmat et al. [22]	Pregnant women and infants against the infection risk of COVID-19. A review of prenatal and postnatal symptoms.	Moderate
Robaina–Castellanos et al. [23]	Congenital and intrapartum SARS-CoV-2 infection in neonates, hypotheses, evidence, and perspective.	High
Kazemi et al. [24]	COVID-19 and cause of pregnancy loss during the pandemic: A systematic review.	Moderate
Ribeiro et al. [25]	SARS-CoV-2 infection and placental pathology infection.	Moderate
Aghaamoo, Ghods, and Rahmanian [26]	Pregnant women with COVID-19 the placental involvement and consequences.	Moderate
Kant et al. [27]	Clinical features and outcome of SARS-CoV-2 infection in neonates: A systematic review.	Moderate
Ferrer—-Oliveras et al. [28]	Immunological and physiopathological approach of COVID-19 in pregnancy.	Moderate
Barcelos et al. [9]	Vertical mother-to-child transmission of SARS-CoV-2: A systematic review.	Moderate
Auriti et al. [29]	Pregnancy and viral infections: Mechanisms of fetal damage diagnosis and prevention oof neonatal adverse outcomes from cytomegalovirus to SARS-CoV-2.	High
Cavalcante et al. [30]	Maternal immune responses and obstetrical outcomes of pregnant women with COVID-19 and possible health risks of offspring.	Moderate
Jamieson and Rasmussen [31]	An update on COVID-19 and pregnancy.	High
Yoon, Hang, and Ahn [32]	Clinical outcomes of 201 neonates born to mothers with COVID-19: A systematic review and meta-analysis.	High
Morrison et al. [33]	COVID-19: Can we treat the mother without harming her baby?	Moderate
Ryan et al. [34]	Neonates and COVID-19: State of the art neonatal sepsis series.	Moderate

2.6. Selection of Studies

A search was carried out in the different databases using a combination of keywords, obtaining a total of 434 documents. The selected studies were published between 2020 and 2022. Duplicates were eliminated, leaving a total of 276 items. In the selection of articles that could be related to the topic to be addressed, the reviewers carried out a selective reading of the title and abstract of 140 papers. Finally, 22 articles were included in the present review, resulting in the definitive study list shown in Figure 1.

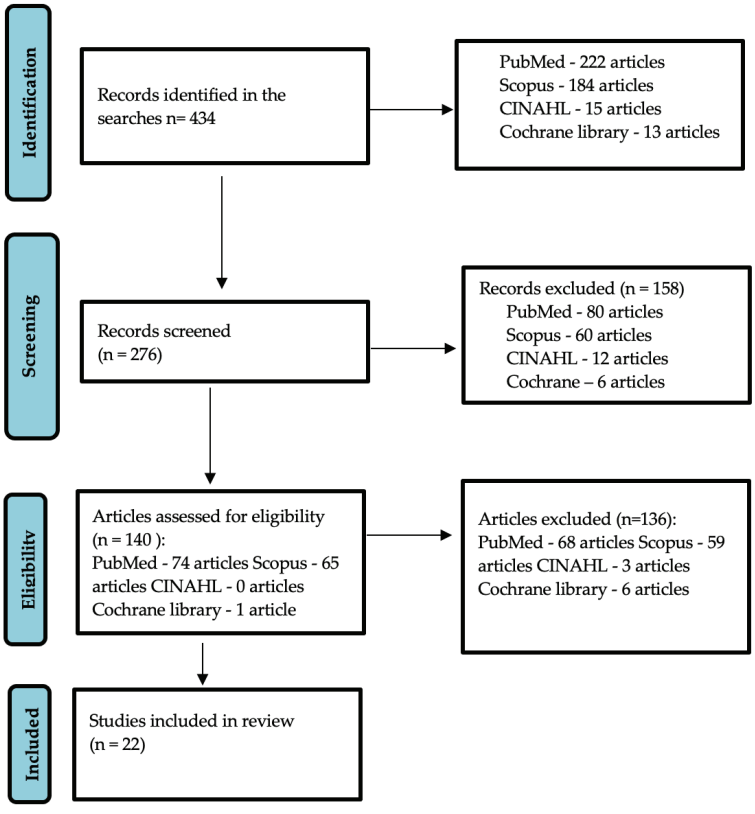


Figure 1. Flow chart (PRISMA guidelines).

3. Results

The main characteristics of all the studies included are shown in Table 4.

3.1. Complications in Pregnant Women

Several studies have been carried out involving pregnant women who tested positive for COVID-19 after a DTAI (Diagnostic Test for Active Infection), in order to study and learn about the most common complications that occurred in them.

Following data collection, the most common symptoms in pregnant women with COVID-19 were found to be fever, cough, fatigue, dyspnoea, diarrhea, and malaise [10]. As the most common complications in these women, we found viral pneumonia [4] and hypertensive disorders such as pre-eclampsia [10].

Yang et al. [9] in their study found a high rate of pregnant women testing positive for COVID-19 who had to undergo a cesarean section due to respiratory distress and fetal intrauterine distress.

In the study by Saroyo et al. [20], they identified that the virus can cause pre-eclampsia, preterm prelabour rupture of membranes, and fetal growth restriction during pregnancy, resulting in circulatory failure for the mother and sometimes for the fetus.

It has also been observed that the infection of pregnant patients with SARS-CoV-2 may increase the risk of maternal mortality since a number of cases were found in Iran, in which they found severe complications in pregnant women, where 1/9 women became ventilator dependent, 1/9 recovered after a long period of hospitalization, and 7/9 died [26].

In the study by Saroyo et al. [20], 4/7 women were observed, who during the first trimester of pregnancy, suffered a miscarriage, and in the second trimester 6/10 pregnant women with COVID-19 had to be intubated and admitted to the ICU (intensive care unit).

There are several ways to diagnose COVID-19 in pregnant women. Generally, it is detected in test samples collected from saliva, upper and lower respiratory tract secretion, urine, and feces, but blood samples can be considered the most important tool for such diagnosis [12].

It is in blood samples where we can find more exhaustive information about the damage caused by this virus in pregnant women, such as those described by Ferrer–Oliveras et al. [28] in their study, in which they refer to the virus-causing deregulation of the proportion of Th17 cells (T-helper lymphocytes) leading to an increase in T-helper cells.

The literature reviewed indicates that most of the cases in which women suffer spontaneous abortions as a result of COVID-19 are caused by placental insufficiency related to the virus [21]. Ferrer–Oliveras et al. [28] shows that this virus induces states of hypercoagulability, leading to thrombotic–hemorrhagic events in pregnant women.

3.2. Fetal/Newborn Complications

To understand the possible complications caused by SARS-CoV-2 at the fetal and neonatal levels, the placenta must first be studied. In their study of the placenta, Resta et al. [19] found the SARS-CoV-2 protein in the placental cells of COVID-19-positive pregnant women. They also found fibrin deposits and inflammatory infiltrates, which produce poor vascular perfusion at the maternal level and growth restriction at the fetal level.

Aghaamoo, Ghods, and Rahmanian [26] report that these fibrin deposits and multiviral infarction in the placenta of pregnant women infected with SARS-CoV-2 may disturb nutrient transport to the fetus. These factors could cause premature contraction and premature rupture of the membrane, causing premature delivery [20].

A study by Ghema et al. [18] showed interesting results in 30 newborns born to COVID-19-positive mothers, with 98% of them being PCR-negative. One of the positives had pneumonia and one died, because of severe sepsis. Although the two subjects had different characteristics and outcomes, a study of their respective placentas showed a similar phenomenon of premature rupture of the membranes. This premature rupture of the membranes can have major neurological repercussions in the life of these subjects such as vasculitis or fetal brain injury [30].

Ghema et al. [18] and Crovetto et al. [35] found through their fetal studies other less frequent complications such as perinatal asphyxia, respiratory failure, multi-organ dysfunction, brain damage, malformation, intrapartum fetal distress, and even death. Another complication in children of COVID-19-infected pregnant women, though rare, is a neonatal inflammatory syndrome whose clinical manifestations are elevated inflammatory biomarkers, organ dysfunction, and in some cases myocardial dysfunction [33].

Based on the studies reviewed, the clinical presentation of the virus in neonates differs from that in adulthood, suggesting that the impact of COVID-19 in neonates may be limited [34]. Short-term outcomes of neonatal infection are positive, but the long-term impact on neurodevelopment is unknown, and the continuous study of these subjects is necessary [30].

3.3. Vertical Mother-to-Child Transmission

There is scientific evidence of transplacental transmission of emerging diseases such as HIV and Ebola [29]. Researchers such as Resta et al. [15] have suggested that this may also be the situation with SARS-CoV-2. The placenta is a protective barrier against disease and infection for the fetus and in the case of SARS-CoV-2, it is considered to prevent transmission of maternal viral infection [34], but it has been observed in the review by Barcelós et al. [9] that it is a potential locus of infection for SARS-CoV-2.

Despite the millions of confirmed cases of COVID-19 worldwide, only one case has been found that met the criteria for vertical mother-to-child transmission. A 23-year-old

pregnant woman, 35 weeks gestational age, was admitted with fever and severe cough with PCR diagnosis of the virus. She underwent a cesarean section for acute fetal distress and tested positive for amniotic fluid. Nasopharyngeal and rectal swabs were collected and found to be positive. In the first days of life, the newborn presented neurological symptoms and a central nervous system disorder diagnosed by magnetic resonance imaging. The placental examination was positive for SARS-CoV-2, suggesting vertical mother-to-child transmission [35].

It has been observed in placental studies that SARS-CoV-2 protein is present, which could produce serious complications in subjects although when tested for COVID-19 have been negative, indicating that no intrauterine transmission has occurred [19]. Jamieson and Rasmussen [31] in their review show that intrauterine transmission is a rare event and is very unusual. However, some newborns after taking the DTAI (Diagnostic Test for Active Infection) for COVID-19 were positive, but it was unknown whether the infection was before, during, or after birth through close contact with an infected person [24].

Khedmat et al. [22] report that in some newborns of positive mothers, high levels of Immunoglobulin M have been found within 2 h of birth, suggesting that in some cases an in utero infection with this virus is possible. There are reassuring results which indicate that vertical mother-to-child transmission rarely affected mortality and had a favorable evolution [32].

Studies were also conducted on the breast milk of those mothers who were infected, as breast milk could be a mechanism of transmission to the neonate; however, these indicated that it was the close contact of the neonate with its mother that caused the infection, as no evidence of SARS-CoV-2 RNA or protein was found in breast milk [27]. However, studies suggest that the mechanisms involved in maternal–fetal transmission are unclear and that transmission is probable, but the incidence is extremely low [33] and most are acquired at delivery or postpartum [25].

Table 4. Table of results.

Author	Objectives	Conclusions
Wang et al. (2021) [21]	To review the clinical manifestations, and maternal and perinatal outcomes of COVID-19 during pregnancy.	There is no evidence of fetal transmission and infants and children experience only mild forms of COVID-19.
Diriba, Awulachew, and Getu (2020) [4]	Assess the effect of coronavirus infection during pregnancy and the possibility of vertical mother-to-child transmission.	None of the studies reported transmission of CoV from mother to fetus in utero, which may be due to a very low expression of angiotensin-2 converting enzyme in early cells at the maternal-fetal interface.
Khedmat et al. (2021) [22]	To study the potential for vertical mother-to-child transmission of COVID-19 in pregnant women. Summary of symptoms and clinical outcomes in mothers and babies, as well as proposed therapies and preventive health solutions.	Babies with respiratory problems may be born to some COVID-19 positive mothers.
Robaina–Castellanos and Riesgo–Rodríguez (2021) [23]	To summarize and analyze the published evidence on the modes of vertical mother-to-child transmission of SARS-CoV-2 (intrauterine or intrapartum).	Congenital and intrapartum SARS-CoV-2 infection in the fetus or newborn is possible, but rare.
Fernandez–Perez et al. (2021) [16]	To inform about how SARS-CoV-2 acts in the body, as well as the imaging studies that help to diagnose it.	It is a virus belonging to the coronavirus family. It affects the respiratory tract and has other adverse side effects that are still under study.
Yang et al. (2020) [12]	Retrospective cohort study conducted in Wuhan with the aim of finding out the most adverse effects that COVID-19 can produce.	This study shows that the two most common adverse effects experienced by pregnant women are premature delivery and cesarean delivery.
Kazemi et al. (2021) [24]	Systematic review aimed at understanding the factors that occur in women who had an abortion after infection.	One factor causing miscarriage may be inflammation of the placenta.
Ribeiro et al. (2021) [25]	To inform about some of the aspects of the pathology.	No specific changes were found in the placentas of pregnant women.
	Placenta in SARS-CoV-2 infection.	The findings show poorer maternal and fetal perfusion in them than in non-pregnant women.
Aghaamoo, Ghods, and Rahmanian (2021) [26]	Investigate possible undesirable maternal and fetal-neonatal consequences of COVID-19.	Detection and follow-up of infected pregnant women reduces the risk of maternal and neonatal death and provides control over complications.
Gratacós et al. (2021) [35]	A population-based study to describe the impact of SARS-CoV-2 infection on pregnancy outcomes.	The rate of pregnancy complications in infected women was similar to that of non-pregnant women.
Ghema et al. (2021) [18]	Study examining 30 newborns of COVID-19 positive women with the aim of providing documented information on mother-to-child transmission.	Mother-to-fetal transmission of the virus was not detected in most of the reported cases, although they were detected positive by PCR.
Kant et al. (2021) [27]	Systematically synthesize the available literature on various modes of transmission (congenital, intrapartum and postpartum), clinical features and outcomes of infection.	Limited evidence suggests that the risk of SARS-CoV-2 infections in newborns is extremely low and postpartum acquisition was the most common mode of infection in newborns.
Ferrer–Oliveras et al. (2021) [28]	This document provides information on research to elucidate potential harmful responses to SARS-CoV-2 and other coronavirus infections.	A severe form of COVID-19 is an immune-mediated hyperinflammatory disorder triggered by a viral infection.

Table 4. *Cont.*

Author	Objectives	Conclusions
Barcelos et al. (2021) [9]	An evaluation of the available evidence on vertical mother-to-child transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).	The risk of vertical mother-to-child transmission of SARS-CoV-2 is very low. Despite the thousands of pregnant women who have been affected, the sample is not sufficient to create the evidence, so further research is needed.
Auriti et al. (2021) [29]	Collects information on possible harm to the fetus in the event of transmission of infection, as well as diagnostic testing.	When transmission of a viral infection occurs, the fetus or newborn may not have any adverse effects at the time, but in the long term it may.
Cavalcante et al. (2021) [30]	Obstetric outcomes of COVID-19 positive women and possible risks.	Possible risk of neurological damage in children of infected women.
Jamieson and Rasmussen (2022) [31]	Update on COVID-19 in pregnancy.	With the development of the vaccine, it has been studied that it is favourable for both mother and fetus and helps to protect both.
Resta et al. (2021) [19]	A case–control study performed in order to highlight any histopathological alterations.	The research demonstrated fetal endothelial distress, as well as the presence of particles attributable to SARS-CoV-2.
Yoon, Hang, and Ahn (2020) [32]	To assess the clinical manifestations and outcomes of newborns of women who had coronavirus 2019 disease during pregnancy.	Evidence suggests that the virus rarely causes fetal and neonatal mortality.
Morrison et al. (2022) [33]	Gathering information on the care that can be provided to the pregnant woman, as well as treatments to treat COVID-19 without harming the fetus.	Pregnant women are very vulnerable to drugs and therefore further research is needed to find out which drugs will not cause any harm to the mother or fetus.
Ryan et al. (2022) [34]	Review of the current available evidence related to the impact of the COVID-19 pandemic on newborns, the effects on their health, the impact on quality of care and indirect influences on their clinical course.	The evidence should be used to continue to promote best practice in neonatal care.
Saroyo et al. (2021) [20]	To know the effect of Remdesivir in pregnant women with COVID-19 and how it influences their recovery.	The Remdesivir protocol for pregnant women with moderate to severe COVID-19 symptoms has resulted in favourable clinical improvement with a shorter recovery period and no adverse effects during the hospitalization period.

4. Discussion

Firstly, it is interesting to highlight that the data on the incidence of cesarean surgeries in pregnant that were positive in the test increased considerably, because they presented respiratory difficulty and developed pre-eclampsia. Thus, these symptoms produced a risk for both the woman and the fetus, confirming that there is a direct relationship between COVID-19 and complications in pregnancy, even if the symptoms presented are not severe [12,17,20,26].

After delivery, the placenta was studied independently and interesting results were obtained, as there were findings of altered coagulation factors in the premature rupture of the placenta, a question related to the alteration in coagulation produced by COVID-19 [12,34,36].

As for vertical mother-to-child transmission, it appears that the placenta continues to act as a barrier as with other viruses, as there is little likelihood of it occurring. In the few studies that provide data from fetuses testing positive, it is not known exactly whether it occurs before, during, or after birth. Breast milk showed no trace of SARS-CoV-2 proteins, so this does not appear to be a mechanism of transmission [22,24,27,31–33]. There are few studies that provide data on the impact of COVID-19 on the fetus, but it appears to be limited as non-specific infectivity test results have been reported. It would be interesting to focus on this topic by relating both maternal vaccination and subsequent breastfeeding as possible protective factors [14,30,34,35,37]. One of the reasons for mother-to-child transmission is the close contact between positive mothers and newborns during breastfeeding, so it can be transmitted through breathing. Therefore, they recommend that all COVID-19-positive mothers practice adequate hand hygiene and pump milk so that the baby can be fed by a healthy caregiver to prevent transmission of the virus. Mothers who prefer skin-to-skin contact should consider the consequences of this, and adopt excellent hand washing and the use of surgical masks to prevent the transfer of Flügge droplets [9,10,34].

Authors such as Wang et al. [21], Robaina–Castellanos et al. [23], and Crovetto et al. [35] focused on finding information about the complications that COVID-19 could cause in pregnant women and at the fetal level, and whether or not there was a possibility of vertical mother-to-child transmission. It has been observed that pregnant women are more likely to be susceptible to SARS-CoV-2 infection than women who are not pregnant, because their immune systems change as a result of pregnancy and respond differently to SARS-CoV-2 [35,38].

The most common COVID-19-related problems in pregnant women are those affecting the respiratory system and blood pressure, leading to hypertensive disorders such as pre-eclampsia [39,40]. Those problems that affect the respiratory system are viral pneumonia in the first place, requiring urgent admission to the intensive care unit, in addition to the fact that in pregnancy there is a 20% increase in the demand for oxygen and at the same time the residual capacity decreases [24]. This respiratory insufficiency can lead to an interruption of the placental flow and cause a miscarriage [34,36]. This problem not only affects the mother, but can also have an impact on the fetus, causing serious complications in its development and nervous system, as seems to be the pattern in adults [29,39,41].

With respect to treatment for infected pregnant women being a challenge compared to the general population, a study was conducted in which five cases of pregnant women treated with Remdesivir (RNA polymerase inhibitor) during hospitalization were studied. All cases showed a shorter duration in hospital with rapid improvement of clinical symptoms and no adverse effects. Although the study showed good results, it has not yet been established as a standardized therapy for treating pregnant women with COVID-19, as its effectiveness needs to be further studied [12,17,42]. Three COVID-19 vaccines are currently available, two mRNA vaccines (Pfizer and Moderna) and one adenoviral vector vaccine (Johnson & Johnson). Although any of the currently licensed vaccines can be administered to pregnant women, the SEGO (Spanish Society of Gynaecology and Obstetrics) states its preference for the mRNA vaccine because there are more safety data than for the adenoviral

vaccine [5,12,43]. Furthermore, the administration of vaccines to a breastfeeding woman poses no risk to either her or her child as they do not contain live microorganisms and therefore have no infective capacity [14,30,31].

During the peak of the pandemic, there was an increase in hospitals performing cesarean sections. This was because most pregnant women had respiratory compromise caused by the infection which would complicate delivery while causing fetal distress, intrauterine growth restriction, or even death [24,31]. The causative factors of fetal loss in the first weeks of gestation are mostly due to inflammatory events affecting the placenta [19,26]. This may cause premature contraction and rupture of the membrane leading to premature delivery and stillbirth [18,23].

The fact that there are neonates showing damage caused by the virus raises suspicions of possible vertical transmission from mother to child. Since the beginning of the pandemic, the vertical mother-to-child transmission of this virus has been debated, with some studies denying this fact and others not ruling out the idea due to the detection of antibodies in the umbilical cord blood of newborns [24,27,29,30]. It would be interesting to be able to perform studies focusing on the analysis of antibodies in cord blood of placental tissue, amniotic fluid, umbilical cord blood, and neonatal blood in the first 12 h of life, in addition to a PCR test using a nasopharyngeal swab [34].

There is no antiviral treatment for COVID-19 in newborns and therefore guidelines for the management of this type of patient should be established, as well as for pregnant women [44]. Although pregnant women are included in the general adult population by age, as was the case in previous pandemics, they used to be excluded from these vaccination programs. However, they are a population group that tends to accept vaccination well, for themselves and their children, so future studies focusing on this population group would promote better adherence to vaccination because of possible reluctance to immunize them against SARS-CoV-2 virus [13,14,45–47], since recent studies show the safety of the administration of the vaccine against COVID-19, with no adverse effects appearing in pregnancy after administration, showing the clear risk of non-vaccination during this period [48–53].

5. Conclusions

Pregnancy increases the risk of severe COVID-19 disease, but the patterns of COVID-19 that affect some people more and others less are not yet known, although adverse fetal outcomes are more associated with women with severe complications.

The analyzed research concludes that the risk of vertical mother-to-child transmission of SARS-CoV-2 from mother to fetus is very low, and is considered a rare but possible event, although more studies focused on this population will be needed to consider it as evidence.

Author Contributions: Conceptualization, R.R.-B. and J.C.S.-G.; data curation, N.P.C.M. and J.C.S.-G.; formal analysis, A.L.-G. and J.C.S.-G.; investigation, R.R.-B. and N.P.C.M.; methodology, R.R.-B., L.A.O. and J.C.-M.; project administration, R.R.-B. and M.I.T.-G.; resources, R.R.-B. and A.L.-G.; supervision, J.C.S.-G.; validation, R.R.-B., N.P.C.M., M.I.T.-G. and L.A.O.; visualization, R.R.-B. and J.C.-M.; writing—original draft, M.I.T.-G., R.R.-B. and J.C.S.-G.; writing—review and editing, R.R.-B., M.I.T.-G. and J.C.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study did not require ethical approval as it did not involve humans or animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. World Health Organization (WHO). Coronavirus Disease (COVID-19) Pandemic. New Coronavirus 2019. 2020. Available online: https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (accessed on 16 May 2022).
2. Rodríguez-Alfonso, B.; Solís, S.R.; Silva-Hernández, L.; Pascual, I.P.; Ibáñez, S.A.; Antón, C.S. 18F-FDG-PET/CT in SARS-CoV-2 infection and its sequelae. *Rev. Esp. Med. Nucl. Imagen Mol.* **2021**, *40*, 299. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8316133/ (accessed on 29 August 2022). [CrossRef] [PubMed]
3. Ministerio de Sanidad—Ciudadanos—Estudio Nacional de Sero-Epidemiología de la Infección por SARS-CoV-2 en España (ENE-COVID). Available online: https://www.sanidad.gob.es/ciudadanos/ene-covid/home.htm (accessed on 29 August 2022).
4. Diriba, K.; Awulachew, E.; Getu, E. The effect of coronavirus infection (SARS-CoV-2, MERS-CoV, and SARS-CoV) during pregnancy and the possibility of vertical maternal-fetal transmission: A systematic review and meta-analysis. *Eur. J. Med. Res.* **2020**, *25*, 39. Available online: https://eurjmedres.biomedcentral.com/articles/10.1186/s40001-020-00439-w (accessed on 29 August 2022). [CrossRef]
5. Della Gatta, A.N.; Rizzo, R.; Pilu, G.; Simonazzi, G. Coronavirus disease 2019 during pregnancy: A systematic review of reported cases. *Am. J. Obstet. Gynecol.* **2020**, *223*, 36–41. Available online: http://www.ajog.org/article/S0002937820304385/fulltext (accessed on 29 August 2022). [CrossRef]
6. Tossetta, G.; Fantone, S.; Delli Muti, N.; Balercia, G.; Ciavattini, A.; Giannubilo, S.R.; Marzioni, D. Preeclampsia and severe acute respiratory syndrome coronavirus 2 infection: A systematic review. *J. Hypertens.* **2022**, *40*, 1629–1638. Available online: https://pubmed.ncbi.nlm.nih.gov/35943095/ (accessed on 22 September 2022). [CrossRef] [PubMed]
7. Bloise, E.; Zhang, J.; Nakpu, J.; Hamada, H.; Dunk, C.E.; Li, S.; Imperio, G.E.; Nadeem, L.; Kibschull, M.; Lye, P.; et al. Expression of severe acute respiratory syndrome coronavirus 2 cell entry genes, angiotensin-converting enzyme 2 and transmembrane protease serine 2, in the placenta across gestation and at the maternal-fetal interface in pregnancies complicated by preterm birth or preeclampsia. *Am. J. Obstet. Gynecol.* **2021**, *224*, 298.e1–298.e8.
8. Eich, M.L.; Menter, T.; Mokwa, N.F.; Grüttner, B.; Müller, A.M. Intrauterine fetal demise in extensive SARS-CoV-2-associated placental maternal vascular malperfusion in the setting of SARS-CoV-2 placentitis (severe acute respiratory syndrome coronavirus 2). *Pathologe* **2022**, *43*, 135–139. [CrossRef] [PubMed]
9. Barcelos, I.D.E.S.; Penna, I.A.D.A.; Soligo, A.D.G.; Costa, Z.B.; Martins, W.P. Vertical Transmission of SARS-CoV-2: A Systematic Review. *Rev. Bras. Ginecol. Obstet.* **2021**, *43*, 207–215. Available online: https://pubmed.ncbi.nlm.nih.gov/33860504/ (accessed on 29 August 2022). [CrossRef] [PubMed]
10. Schwartz, D.A.; Hyg, M. An Analysis of 38 Pregnant Women with COVID-19, Their Newborn Infants, and Maternal-Fetal Transmission of SARS-CoV-2: Maternal Coronavirus Infections and Pregnancy Outcomes. *Arch. Pathol. Lab. Med.* **2020**, *144*, 799–805. Available online: https://meridian.allenpress.com/aplm/article/144/7/799/441923/An-Analysis-of-38-Pregnant-Women-With-COVID-19 (accessed on 29 August 2022). [CrossRef]
11. Karimi-Zarchi, M.; Neamatzadeh, H.; Dastgheib, S.A.; Abbasi, H.; Mirjalili, S.R.; Behforouz, A.; Ferdosian, F.; Bahrami, R. Vertical Transmission of Coronavirus Disease 19 (COVID-19) from Infected Pregnant Mothers to Neonates: A Review. *Fetal Pediatr. Pathol.* **2020**, *39*, 246–250. [CrossRef]
12. Liu, P.; Zheng, J.; Yang, P.; Wang, X.; Wei, C.; Zhang, S.; Feng, S.; Lan, J.; He, B.; Zhao, D.; et al. The immunologic status of newborns born to SARS-CoV-2-infected mothers in Wuhan, China. *J. Allergy Clin. Immunol.* **2020**, *146*, 101–109.e1. Available online: https://pubmed.ncbi.nlm.nih.gov/32437740/ (accessed on 29 August 2022). [CrossRef] [PubMed]
13. Lazarus, J.V.; Ratzan, S.C.; Palayew, A.; Gostin, L.O.; Larson, H.J.; Rabin, K.; Kimball, S.; El-Mohandes, A. A global survey of potential acceptance of a COVID-19 vaccine. *Nat. Med.* **2021**, *27*, 225–228. Available online: https://pubmed.ncbi.nlm.nih.gov/33082575/ (accessed on 29 August 2022). [CrossRef]
14. Bardají, A.; Sevene, E.; Cutland, C.; Menéndez, C.; Omer, S.B.; Aguado, T.; Muñoz, F.M. The need for a global COVID-19 maternal immunisation research plan. *Lancet* **2021**, *397*, e17–e18. Available online: https://pubmed.ncbi.nlm.nih.gov/33508228/ (accessed on 29 August 2022). [CrossRef]
15. PRISMA. PRISMA for Systematic Review Protocols. Available online: https://prisma-statement.org/Extensions/Protocols (accessed on 29 May 2022).
16. Fernández-Pérez, G.C.; Miranda, M.O.; Fernández-Rodríguez, P.; Casares, M.V.; Calle, M.C.; de la López, Á.F.; Blanco, M.D.; Cuchat, J.M.O. SARS-CoV-2: What it is, how it acts, and how it manifests in imaging studies. *Radiologia* **2021**, *63*, 115. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7862877/ (accessed on 29 August 2022). [CrossRef] [PubMed]
17. Yang, R.; Mei, H.; Zheng, T.; Fu, Q.; Zhang, Y.; Buka, S.; Yao, X.; Tang, Z.; Zhang, X.; Qiu, L.; et al. Pregnant women with COVID-19 and risk of adverse birth outcomes and maternal-fetal vertical transmission: A population-based cohort study in Wuhan, China. *BMC Med.* **2020**, *18*, 330. Available online: https://bmcmedicine.biomedcentral.com/articles/10.1186/s12916-020-01798-1 (accessed on 29 August 2022). [CrossRef] [PubMed]
18. Ghema, K.; Lehlimi, M.; Toumi, H.; Badre, A.; Chemsi, M.; Habzi, A.; Benomar, S. Outcomes of newborns to mothers with COVID-19. *Infect. Dis. Now* **2021**, *51*, 435–439. [CrossRef]
19. Resta, L.; Vimercati, A.; Cazzato, G.; Mazzia, G.; Cicinelli, E.; Colagrande, A.; Fanelli, M.; Scarcella, S.V.; Ceci, O.; Rossi, R. SARS-CoV-2 and Placenta: New Insights and Perspectives. *Viruses* **2021**, *13*, 723. Available online: https://www.mdpi.com/1999-4915/13/5/723/htm (accessed on 29 August 2022). [CrossRef]

20. Saroyo, Y.B.; Rumondang, A.; Febriana, I.S.; Harzif, A.K.; Irwinda, R. Remdesivir Treatment for COVID 19 in Pregnant Patients with Moderate to Severe Symptoms: Serial Case Report. *Infect. Dis. Rep.* **2021**, *13*, 437–443. Available online: https://pubmed.ncbi.nlm.nih.gov/34067822/ (accessed on 29 August 2022). [CrossRef] [PubMed]
21. Wang, C.L.; Liu, Y.Y.; Wu, C.H.; Wang, C.Y.; Wang, C.H.; Long, C.Y. Impact of COVID-19 on Pregnancy. *Int. J. Med. Sci.* **2021**, *18*, 763. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7797535/ (accessed on 29 August 2022). [CrossRef] [PubMed]
22. Khedmat, L.; Mohaghegh, P.; Veysizadeh, M.; Hosseinkhani, A.; Fayazi, S.; Mirzadeh, M. Pregnant women and infants against the infection risk of COVID-19: A review of prenatal and postnatal symptoms, clinical diagnosis, adverse maternal and neonatal outcomes, and available treatments. *Arch. Gynecol. Obstet.* **2021**, *306*, 323–335. Available online: https://link.springer.com/article/10.1007/s00404-021-06325-y (accessed on 29 August 2022). [CrossRef] [PubMed]
23. Robaina-Castellanos, G.R.; de la Caridad Riesgo-Rodríguez, S. Congenital and intrapartum SARS-CoV-2 infection in neonates: Hypotheses, evidence and perspectives. *MEDICC Rev.* **2021**, *23*, 72–83.
24. Kazemi, S.N.; Hajikhani, B.; Didar, H.; Hosseini, S.S.; Haddadi, S.; Khalili, F.; Mirsaeidi, M.; Nasiri, M.J. COVID-19 and cause of pregnancy loss during the pandemic: A systematic review. *PLoS ONE* **2021**, *16*, e0255994. Available online: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0255994 (accessed on 29 August 2022). [CrossRef]
25. Leal, C.R.V.; Maciel, R.A.M.; Corrêa Júnior, M.D. SARS-CoV-2 Infection and Placental Pathology. *Rev. Bras. Ginecol. Obstet.* **2021**, *43*, 474–479. Available online: https://pubmed.ncbi.nlm.nih.gov/34077991/ (accessed on 29 August 2022). [CrossRef]
26. Aghaamoo, S.; Ghods, K.; Rahmanian, M. Pregnant women with COVID-19: The placental involvement and consequences. *J. Mol. Histol.* **2021**, *52*, 427–435. Available online: https://link.springer.com/article/10.1007/s10735-021-09970-4 (accessed on 29 August 2022). [CrossRef]
27. Dhir, S.K.; Kumar, J.; Meena, J.; Kumar, P. Clinical Features and Outcome of SARS-CoV-2 Infection in Neonates: A Systematic Review. *J. Trop. Pediatr.* **2021**, *67*, fmaa059. Available online: https://academic.oup.com/tropej/article/67/3/fmaa059/5898408 (accessed on 29 August 2022). [CrossRef]
28. Ferrer-Oliveras, R.; Mendoza, M.; Capote, S.; Pratcorona, L.; Esteve-Valverde, E.; Cabero-Roura, L.; Alijotas-Reig, J. Immunological and physiopathological approach of COVID-19 in pregnancy. *Arch. Gynecol. Obstet.* **2021**, *304*, 39–57. Available online: https://link.springer.com/article/10.1007/s00404-021-06061-3 (accessed on 29 August 2022). [CrossRef]
29. Auriti, C.; de Rose, D.U.; Santisi, A.; Martini, L.; Piersigilli, F.; Bersani, I.; Ronchetti, M.P.; Caforio, L. Pregnancy and viral infections: Mechanisms of fetal damage, diagnosis and prevention of neonatal adverse outcomes from cytomegalovirus to SARS-CoV-2 and Zika virus. *Biochim. Biophys. Acta (BBA) Mol. Basis Dis.* **2021**, *1867*, 166198. [CrossRef]
30. Cavalcante, M.B.; de Melo Bezerra Cavalcante, C.T.; Sarno, M.; Barini, R.; Kwak-Kim, J. Maternal immune responses and obstetrical outcomes of pregnant women with COVID-19 and possible health risks of offspring. *J. Reprod. Immunol.* **2021**, *143*, 103250. [CrossRef]
31. Jamieson, D.J.; Rasmussen, S.A. An update on COVID-19 and pregnancy. *Am. J. Obstet. Gynecol.* **2022**, *226*, 177–186. Available online: http://www.ajog.org/article/S0002937821009911/fulltext (accessed on 29 August 2022). [CrossRef]
32. Yoon, S.H.; Kang, J.M.; Ahn, J.G. Clinical outcomes of 201 neonates born to mothers with COVID-19: A systematic review. *Eur. Rev. Med. Pharmacol. Sci.* **2020**, *24*, 7804–7815. Available online: https://pubmed.ncbi.nlm.nih.gov/32744708/ (accessed on 29 August 2022).
33. Wiese, M.D.; Berry, M.J.; Hissaria, P.; Darby, J.R.T.; Morrison, J.L. COVID-19: Can we treat the mother without harming her baby? *J. Dev. Orig. Health Dis.* **2022**, *13*, 9–19. Available online: https://www.cambridge.org/core/journals/journal-of-developmental-origins-of-health-and-disease/article/covid19-can-we-treat-the-mother-without-harming-her-baby/E8DBFD52AEA0A1A38B6B288ADE740892 (accessed on 29 August 2022). [CrossRef]
34. Ryan, L.; Plötz, F.B.; van den Hoogen, A.; Latour, J.M.; Degtyareva, M.; Keuning, M.; Klingenberg, C.; Reiss, I.K.M.; Giannoni, E.; Roehr, C.; et al. Neonates and COVID-19: State of the art: Neonatal Sepsis series. *Pediatr. Res.* **2022**, *91*, 432–439. [CrossRef] [PubMed]
35. Crovetto, F.; Crispi, F.; Llurba, E.; Pascal, R.; Larroya, M.; Trilla, C.; Camacho, M.; Medina, C.; Dobaño, C.; Gomez-Roig, M.D.; et al. Impact of Severe Acute Respiratory Syndrome Coronavirus 2 Infection on Pregnancy Outcomes: A Population-based Study. *Clin. Infect. Dis.* **2021**, *73*, 1768–1775. Available online: https://academic.oup.com/cid/article/73/10/1768/6131375 (accessed on 29 August 2022). [CrossRef] [PubMed]
36. Vazquez-Alejo, E.; Tarancon-Diez, L.; Carrasco, I.; Vigil-Vázquez, S.; Muñoz-Chapuli, M.; Rincón-López, E.; Saavedra-Lozano, J.; Santos-Sebastián, M.; Aguilera-Alonso, D.; Hernanz-Lobo, A.; et al. SARS-CoV2 Infection during Pregnancy Causes Persistent Immune Abnormalities in Women without Affecting the Newborns. *Front. Immunol.* **2022**, *13*, 947549. Available online: http://www.ncbi.nlm.nih.gov/pubmed/35911743 (accessed on 29 August 2022). [CrossRef]
37. Kitano, T.; Kitano, M.; Krueger, C.; Jamal, H.; Al Rawahi, H.; Lee-Krueger, R.; Sun, R.D.; Isabel, S.; García-Ascaso, M.T.; Hibino, H.; et al. The differential impact of pediatric COVID-19 between high-income countries and low- and middle-income countries: A systematic review of fatality and ICU admission in children worldwide. *PLoS ONE* **2021**, *16*, e0246326. Available online: https://pubmed.ncbi.nlm.nih.gov/33513204/ (accessed on 29 August 2022). [CrossRef] [PubMed]
38. Sterlin, D.; Mathian, A.; Miyara, M.; Mohr, A.; Anna, F.; Claër, L.; Quentric, P.; Fadlallah, J.; Devilliers, H.; Ghillani, P.; et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2. *Sci. Transl. Med.* **2021**, *13*, eabd2223. [CrossRef]

39. Di Mascio, D.; Khalil, A.; Saccone, G.; Rizzo, G.; Buca, D.; Liberati, M.; Vecchiet, J.; Nappi, L.; Scambia, G.; Berghella, V.; et al. Outcome of coronavirus spectrum infections (SARS, MERS, COVID-19) during pregnancy: A systematic review and meta-analysis. *Am. J. Obstet. Gynecol. MFM* **2020**, *2*, 100107. Available online: http://www.ajogmfm.org/article/S2589933320300379/fulltext (accessed on 29 August 2022). [CrossRef]
40. Saccone, G.; Carbone, F.I.; Zullo, F. The novel coronavirus (2019-nCoV) in pregnancy: What we need to know. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2020**, *249*, 92–93. Available online: http://www.ejog.org/article/S0301211520301743/fulltext (accessed on 29 August 2022). [CrossRef]
41. Marshall, M. How COVID-19 can damage the brain. *Nature* **2020**, *585*, 342–343. [CrossRef]
42. Wang, Z.; Lorenzi, J.C.C.; Muecksch, F.; Finkin, S.; Viant, C.; Gaebler, C.; Cipolla, M.; Hoffmann, H.H.; Oliveira, T.Y.; Oren, D.A.; et al. Enhanced SARS-CoV-2 neutralization by dimeric IgA. *Sci. Transl. Med.* **2021**, *13*, eabf1555. Available online: https://pubmed.ncbi.nlm.nih.gov/33288661/ (accessed on 29 August 2022). [CrossRef]
43. SEGO. SEGO|Sociedad Española de Ginecología y Obstetricia. Available online: https://sego.es/info_covid-19 (accessed on 15 June 2022).
44. Schwartz, D.A. Clinical Trials and Administration of Zika Virus Vaccine in Pregnant Women: Lessons (that Should Have Been) Learned from Excluding Immunization with the Ebola Vaccine during Pregnancy and Lactation. *Vaccines* **2018**, *6*, 81. Available online: https://www.mdpi.com/2076-393X/6/4/81/htm (accessed on 29 August 2022). [CrossRef]
45. Skjefte, M.; Ngirbabul, M.; Akeju, O.; Escudero, D.; Hernandez-Diaz, S.; Wyszynski, D.F.; Wu, J.W. COVID-19 vaccine acceptance among pregnant women and mothers of young children: Results of a survey in 16 countries. *Eur. J. Epidemiol.* **2021**, *36*, 197–211. Available online: https://pubmed.ncbi.nlm.nih.gov/33649879/ (accessed on 29 August 2022). [CrossRef]
46. Skirrow, H.; Barnett, S.; Bell, S.; Riaposova, L.; Mounier-Jack, S.; Kampmann, B.; Holder, B. Women's views on accepting COVID-19 vaccination during and after pregnancy, and for their babies: A multi-methods study in the UK. *BMC Pregnancy Childbirth* **2022**, *22*, 33. [CrossRef]
47. Yigit, M.; Ozkaya-Parlakay, A.; Senel, E. Evaluation of COVID-19 Vaccine Refusal in Parents. *Pediatr. Infect. Dis. J.* **2021**, *40*, e134–e136. [CrossRef] [PubMed]
48. Citu, I.M.; Citu, C.; Gorun, F.; Sas, I.; Bratosin, F.; Motoc, A.; Burlea, B.; Rosca, O.; Malita, D.; Gorun, O.M. The Risk of Spontaneous Abortion Does Not Increase Following First Trimester mRNA COVID-19 Vaccination. *J. Clin. Med.* **2022**, *11*, 1698. Available online: https://www.mdpi.com/2077-0383/11/6/1698/htm (accessed on 21 September 2022). [CrossRef] [PubMed]
49. Farrell, R.; Michie, M.; Pope, R. Pregnant Women in Trials of Covid-19: A Critical Time to Consider Ethical Frameworks of Inclusion in Clinical Trials. *Ethics Hum. Res.* **2020**, *42*, 17–23. [CrossRef]
50. Joubert, E.; Kekeh, A.C.; Amin, C.N. COVID-19 and novel mRNA vaccines in pregnancy: An updated literature review. *BJOG Int. J. Obstet. Gynaecol.* **2022**, *129*, 21–28. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/1471-0528.16973 (accessed on 21 September 2022). [CrossRef] [PubMed]
51. Woodworth, K.R.; Olsen, E.O.; Neelam, V.; Lewis, E.L.; Galang, R.R.; Oduyebo, T.; Aveni, K.; Yazdy, M.M.; Harvey, E.; Longcore, N.D.; et al. Birth and Infant Outcomes Following Laboratory-Confirmed SARS-CoV-2 Infection in Pregnancy—SET-NET, 16 Jurisdictions, March 29–October 14, 2020. *MMWR Morb. Mortal. Wkly. Rep.* **2020**, *69*, 1635–1640. [CrossRef] [PubMed]
52. Hajissa, K.; Mussa, A. Positive aspects of the mRNA platform for SARS-CoV-2 vaccines. *Hum. Vaccin. Immunother.* **2021**, *17*, 2445–2447. [CrossRef]
53. Zambrano, L.D.; Ellington, S.; Strid, P.; Galang, R.R.; Oduyebo, T.; Tong, V.T.; Woodworth, K.R.; Nahabedian, J.F.; Azziz-Baumgartner, E.; Gilboa, S.M.; et al. Update: Characteristics of Symptomatic Women of Reproductive Age with Laboratory-Confirmed SARS-CoV-2 Infection by Pregnancy Status—United States, January 22–October 3, 2020. *MMWR Morb. Mortal. Wkly. Rep.* **2020**, *69*, 1641–1647. [CrossRef]

biomedicines

MDPI

Review

Phenotyping Post-COVID Pain as a Nociceptive, Neuropathic, or Nociplastic Pain Condition

César Fernández-de-las-Peñas [1,2,*], Jo Nijs [3,4,5], Randy Neblett [6], Andrea Polli [3,7,8], Maarten Moens [3,9,10,11,12], Lisa Goudman [3,7,9,10,11], Madhura Shekhar Patil [8], Roger D. Knaggs [13], Gisele Pickering [14,15] and Lars Arendt-Nielsen [2,16]

1 Department of Physical Therapy, Occupational Therapy, Rehabilitation and Physical Medicine, Universidad Rey Juan Carlos, 28922 Alcorcón, Spain
2 Center for Neuroplasticity and Pain (CNAP), SMI, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, 9220 Aalborg, Denmark
3 Pain in Motion Research Group (PAIN), Department of Physiotherapy, Human Physiology and Anatomy, Faculty of Physical Education & Physiotherapy, Vrije Universiteit Brussel, 1050 Brussels, Belgium
4 Chronic Pain Rehabilitation, Department of Physical Medicine and Physiotherapy, University Hospital Brussels, 1050 Brussels, Belgium
5 Unit of Physiotherapy, Department of Health and Rehabilitation, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, 41390 Gotëborg, Sweden
6 PRIDE Research Foundation, Dallas, TX 75235, USA
7 Research Foundation–Flanders (FWO), 1000 Brussels, Belgium
8 Laboratory of Clinical Epigenetics, Department of Public Health and Primary Care, Centre for Environment & Health, KU Leuven, 3000 Leuven, Belgium
9 Department of Neurosurgery, Universitair Ziekenhuis Brussel, 1090 Brussels, Belgium
10 STIMULUS Research Group (reSearch and TeachIng neuroModULation Uz bruSsel), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium
11 Center for Neurosciences (C4N), Vrije Universiteit Brussel, 1050 Brussels, Belgium
12 Department of Radiology, Universitair Ziekenhuis Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium
13 Clinical Pharmacy Practice, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, UK
14 Plateforme d'Investigation Clinique, University Hospital Clermont-Ferrand, Inserm CIC 1405, F-63000 Clermont-Ferrand, France
15 Department of Medicine and Clinical Pharmacology, University Clermont Auvergne, Inserm 1107, F-63000 Clermont-Ferrand, France
16 Department of Medical Gastroenterology, Mech-Sense, Aalborg University Hospital, 9000 Aalborg, Denmark
* Correspondence: cesar.fernandez@urjc.es

Citation: Fernández-de-las-Peñas, C.; Nijs, J.; Neblett, R.; Polli, A.; Moens, M.; Goudman, L.; Shekhar Patil, M.; Knaggs, R.D.; Pickering, G.; Arendt-Nielsen, L. Phenotyping Post-COVID Pain as a Nociceptive, Neuropathic, or Nociplastic Pain Condition. *Biomedicines* **2022**, *10*, 2562. https://doi.org/10.3390/biomedicines10102562

Academic Editor: Rosanna Di Paola

Received: 10 September 2022
Accepted: 11 October 2022
Published: 13 October 2022

Abstract: Pain after an acute Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and coronavirus disease 2019 (COVID-19) condition (post-COVID pain) is becoming a new healthcare emergency. Precision medicine refers to an evidence-based method of grouping patients based on their diagnostic/symptom presentation and then tailoring specific treatments accordingly. Evidence suggests that post-COVID pain can be categorized as nociceptive (i.e., pain attributable to the activation of the peripheral receptive terminals of primary afferent neurons in response to noxious chemical, mechanical, or thermal stimuli), neuropathic (i.e., pain associated with a lesion or disease of the somatosensory nervous system and limited to a "neuroanatomically plausible" distribution of the system), nociplastic (i.e., pain arising from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain), or mixed type (when two pain phenotypes co-exist). Each of these pain phenotypes may require a different treatment approach to maximize treatment effectiveness. Accordingly, the ability to classify post-COVID pain patients into one of these phenotypes would likely be critical for producing successful treatment outcomes. The 2021 International Association for the Study of Pain (IASP) clinical criteria and grading system provide a framework for classifying pain within a precision pain medicine approach. Here we present data supporting the possibility of grouping patients with post-COVID pain into pain phenotypes, using the 2021 IASP classification criteria, with a specific focus on nociplastic pain, which is probably the primary mechanism involved in post-COVID pain. Nociplastic pain, which is usually associated with comorbid symptomology (e.g., poor sleep quality, fatigue, cognitive–emotional disturbances, etc.)

and is considered to be more difficult to treat than other pain types, may require a more nuanced multimodal treatment approach to achieve better treatment outcomes.

Keywords: COVID-19; post-COVID; nociplastic pain; neuropathic pain; musculoskeletal pain; precision medicine; peripheral sensitization; central sensitization; nociceptive

1. Introduction

The worldwide outbreak induced by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus responsible for the coronavirus disease 2019 (COVID-19), has dramatically changed healthcare systems over the last few years. After millions of infections, healthcare professionals are now confronted with another associated crisis—the development or persistence of symptoms after the acute phase of SARS-CoV-2 infection, a condition called long COVID [1] or post-COVID-19 [2]. More than 100 symptoms have been described, affecting multiple systems, e.g., cardiovascular, neurological, respiratory, and musculoskeletal [3]. In fact, several meta-analyses have observed that almost 50% of COVID-19 survivors exhibit a plethora of lingering symptoms lasting for weeks or months [4–6] and up to one year after infection [7–9]. A recent systematic review investigating multiple post-COVID symptoms identified that 20% of COVID-19 survivors reported post-COVID pain at different follow-up periods during the first year after infection [10]. Other studies, which have specifically investigated post-COVID pain symptoms, found prevalence rates of up to 60% of COVID-19 survivors [11–14]. Accordingly, post-COVID pain could be underestimated and undertreated if not properly identified and classified.

Precision medicine refers to an evidence-based method of subgrouping patients, based on diagnostic and symptom presentation, and then tailoring specific treatments to individual patient phenotypes based on the prognosis for positive treatment outcomes and susceptibility to negative outcomes [15]. Three major pain phenotypes have been identified (i.e., nociceptive, neuropathic, and nociplastic pain). Accordingly, subgrouping patients with post-COVID pain could result in the most successful treatment outcomes. However, discrimination between these phenotypes can be challenging, since patients can fit into more than one phenotype (e.g., mixed-type); since identifying one type (i.e., neuropathic) does not exclude another (i.e., nociplastic) [16].

Nociplastic pain has been defined by the International Association for the Study of Pain (IASP) as "pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain"; it was introduced as a third mechanistic pain descriptor in addition to nociceptive and neuropathic pain [17]. Though it has become well-established in recently published literature, this definition of nociplastic pain has also raised questions [18]. One challenge with this definition is that it relies on a determination of altered pain processing (e.g., pain hypersensitivity). Currently, no gold standard exists for determining whether an individual patient is experiencing a normal or heightened pain response. In 2021, the IASP released the first set of clinical criteria and a grading system for identifying nociplastic pain [19]. These criteria are comprehensive, robust, properly developed, and have a high potential to be useful for clinicians [20]. Although primarily developed for patients with chronic pain of musculoskeletal origin, the IASP nociplastic criteria can be also applied to individuals with post-COVID pain.

It should be noted that some individuals who were infected with COVID-19 had a previous history of chronic pain. It stands to reason that they might respond to the virus differently and may be more susceptible to long COVID pain than individuals without previous chronic pain conditions. In such a scenario, premorbid pain could lead to a worse prognosis of post-COVID pain and represents a risk factor for future development of the nociplastic pain phenotype. The millions of individuals infected by SARS-COV-2 may provide a unique opportunity to investigate pain after a viral infection, as the pain features

may be similar to other previous viral infections (not yet investigated as COVID-19) and, therefore, knowledge can be transferred between conditions.

Being able to identify individuals with a nociplastic pain phenotype has the potential to improve precision pain medicine practices in musculoskeletal pain conditions [21]. In the current narrative review, an international group of experts in chronic pain propose a clinical rationale for the application of the 2021 IASP clinical criteria to identify nociplastic pain in the growing population of COVID-19 survivors with post-COVID pain so that the most effective treatment approaches can be provided. Proper distinction among pain phenotypes is important because neuropathic and nociplastic pain are considered to be more difficult to treat than pure nociceptive (e.g., musculoskeletal) pain. In addition, some treatment approaches for nociceptive pain disorders, which have a high probability for success with this phenotype, could be ineffective or even exacerbate symptoms in patients with the other phenotypes, especially those with nociplastic pain. This paper will help clinicians to potentially classify individuals with post-COVID pain symptoms into one pain phenotype and will also propose the clinical reasoning for the treatment of post-COVID pain patients according to the identified pain phenotype.

2. Phenotyping Post-COVID Pain

2.1. Nociceptive Pain

Nociceptive pain is defined as pain attributable to the activation of the peripheral receptive terminals of primary afferent neurons in response to noxious chemical, mechanical, or thermal stimuli [22]. Clinically, the term nociceptive pain can be used when a pain response is proportional to the nociceptive input [23]. Current theories propose that SARS-CoV-2 cytokine- and interleukin-associated storms may lead to the sensitization of pain pathways [24,25]. Accordingly, it is possible that patients with post-COVID pain can exhibit nociceptive pain features.

D'Souza et al. observed that the most common type of post-COVID pain described by patients themselves on social media resembles a musculoskeletal/nociceptive pain phenotype [26]. In fact, a large cohort study reported that post-COVID pain in previously hospitalized COVID-19 survivors was of musculoskeletal origin in 45% of subjects at eight [27] and twelve [28] months after hospitalization. These authors stated that 30% of COVID-19 survivors with post-COVID pain reported the presence of symptoms solely in localized body areas (e.g., neck, shoulder, elbow, knee, or hip), another 30% exhibited pain in the extremities, and 20% in the spine, whereas the remaining 20% had widespread symptoms [27,28]; however, these authors did not differentiate between pain from muscular or articular origin.

The presence of post-COVID joint pain has reportedly ranged from 8% to 55% [29]. The most frequently involved joints are the knee (38%), followed by hand (25%) and shoulder (19%) [30]. Post-COVID articular pain can be treated with non-steroidal anti-inflammatory drugs (NSAIDs) and local steroids with good results [31], suggesting that these localized, post-COVID arthritic pain symptoms exhibit nociceptive features. Nevertheless, it is important to consider that a large proportion of subjects with post-COVID joint or muscle pain exhibit generalized/widespread symptoms [32]. This generalized pain pattern may be related to the hypothesis that local connective tissue damage caused by SARS-CoV-2 in patients with joint hypermobility can lead to widespread symptomatology [33]. In fact, a widespread pain pattern is indicative of nociplastic pain, which could be present in a subgroup of joint and musculoskeletal pain patients.

2.2. Neuropathic Pain

The IASP has proposed the following definition of neuropathic pain: 1. a lesion or disease of the somatosensory nervous system (i.e., central or peripheral nervous system) is identifiable; 2. pain is limited to a "neuroanatomically plausible" distribution of the system; and 3. supported by clinical examination findings as well as imaging and/or laboratory findings [34]. The neuro-invasive potential associated with SARS-CoV-2 infection, related

to the high expression of angiotensin-converting enzyme 2 (ACE2) receptors detected in nervous system cells, including neurons and microglia within the spinal dorsal horn [35], could explain the development of neuropathic pain in COVID-19 survivors. However, the exact role of ACE2 receptors in peripheral small-fiber sensory neurons is still unknown [36].

The presence of neuropathic pain has been well-documented in some individuals with long COVID, e.g., by developing post-herpetic neuralgia, trigeminal neuralgia, or brachial plexopathy [37]. These types of neuropathic pain sequelae have also been seen in other viruses such as Epstein–Barr virus, cytomegalovirus, influenza A virus, or Zika [38]. A cohort study found that 25% of patients with post-COVID pain self-reported neuropathic pain symptoms [39]. Using the Self-Report Leeds Assessment of Neuropathic Symptoms (S-LANSS) [40], Herrero-Montes et al. found that 20% of patients with post-COVID pain fulfilled the criteria (S-LANSS $\geq$ 12 points) for susceptible neuropathic features [41]. The use of the S-LANSS and PainDETECT to determine the prevalence of neuropathic features has produced slightly different results—using the S-LANSS cutoff score of $\geq$12 points, 26% of COVID-19 survivors with post-COVID pain exhibited neuropathic features, whereas using the PainDETECT cutoff score of $\geq$18 points, just 12% of COVID-19 survivors with post-COVID pain had neuropathic features [42]. Still, it should be stressed that neuropathic pain cannot be diagnosed by using self-reported tools only. Instead, per definition, diagnosing or excluding neuropathic pain requires review of the medical record, history taking, and clinical examination (and possibly additional diagnostic examination such as imaging).

Several groups have aimed to identify potential serological findings associated with the presence of neuropathic features in long-COVID patients. Magdy et al. reported higher serum levels of neurofilament light chain in individuals with persistent neuropathic pain symptoms after COVID-19 [43]. On the contrary, no serological biomarker at hospital admission has been associated with development of persistent neuropathic pain after the acute infection [44]. It is hypothesized that long-lasting increased levels of pro-inflammatory biomarkers could facilitate the development of neuropathic pain [43] in agreement with current theories of the SARS-CoV-2 virus [24,25].

It should be noted that none of the abovementioned studies have used objective tests (e.g., electromyography, imaging, or tissue biopsies) for identifying the presence of a neuropathic origin of the pain. A recent case series, including seventeen patients with long COVID, reported that 59% were positive on $\geq$1 test (e.g., skin biopsy 63%, electrodiagnostic findings 17%, and autonomic function test 50%), confirming neuropathy [45]. Accordingly, the real prevalence of pain of neuropathic origin confirmed with objective measures in individuals with long COVID is still unknown.

2.3. Nociplastic Pain

Central sensitization, defined by the IASP as an increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input [46], is thought to be the main underlying mechanism of nociplastic pain [21]. Other central nervous system-derived symptoms related to neuro-immune alteration, such as fatigue, sleep problems, memory loss, concentration problems, or psychological disturbances, are also typical of nociplastic pain conditions [47] and are often present in individuals with long COVID [4–9].

Emerging evidence suggests the presence of central sensitization-associated symptoms in a subgroup of COVID-19 survivors with post-COVID pain. Oguz-Akarsu et al. found that almost 60% of COVID-19 survivors reported multiple pain sites and more than two types of pain symptoms [39]. Ursini et al. observed, through a web-based survey, that 30% of post-COVID pain patients self-reported symptoms compatible with fibromyalgia syndrome [48]. Goudman et al. showed that 70% of subjects with long COVID exhibited sensitization-associated symptoms measured by the Central Sensitization Inventory (CSI) [49,50] (total score $\geq$ 40/100 points) [51]. Fernández-de-las-Peñas et al. reported a 34% prevalence rate of sensitization-associated symptoms (CSI $\geq$ 40 points) in another group of patients with post-COVID pain [52].

However, current evidence supporting the presence of central sensitization in post-COVID pain is based on self-reported data only. No study has included semi-objective measures of central sensitization, such as quantitative sensory testing or psychophysical testing (e.g., pain thresholds, temporal summation, or conditioned pain modulation testing assessing the functioning of the endogenous pain modulation system). Identification of people with post-COVID pain who exhibit pressure or temperature pain hypersensitivity, impaired temporal summation, or conditioning pain modulation would help to determine the presence of central sensitization in this population. Still, impaired temporal summation or conditioning pain modulation are not specific for patients with nociplastic pain, as they tend to be impaired in those with neuropathic pain too. However, they potentially discriminate between nociceptive and nociplastic pain.

A primary feature of nociplastic pain is the presence of regional or widespread pain symptoms [19]. The presence of regional, including widespread, pain can reach up to 70% of COVID-19 survivors [27,28]. Generalized pain symptoms, combined with central sensitization-associated symptoms (e.g., higher CSI scores) could help identify nociplastic pain in the IASP-established criteria. A recent analysis proposed for phenotyping post-COVID pain supports a model where regional/widespread pain, psychological/emotional disturbance, and other central sensitization-associated symptoms are interconnected, reflecting a nociplastic condition in a subgroup of people with post-COVID pain [53].

3. Clinical Criteria/Grading System for Nociplastic Pain in Post-COVID Pain

This section describes the IASP criteria and clinical reasoning process for determining a nociplastic pain phenotype in individuals with post-COVID pain [19] and how to differentiate nociplastic pain from the nociceptive, neuropathic, or mixed phenotypes. Because one patient can fulfill criteria for more than one pain phenotype, it may be most productive to first determine whether nociceptive pain is the predominant pain type. Then, if a nociceptive pain pattern is rejected, additional criteria can be used to differentiate between neuropathic and nociplastic pain.

3.1. Step 1—Duration of Pain

An initial requirement for nociplastic pain, according to IASP clinical criteria, is for the patient to report pain symptoms for at least 3 months. It should be noted that the proposed definition for post-COVID-19 syndrome includes the presence of symptoms for at least 2 months post-infection: "Post-COVID-19 condition occurs in people with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis" [2]. In this first step, two demographic features such as age and sex should be considered. For instance, fibromyalgia syndrome, a nociplastic pain condition, has a female predominance. Similarly, female sex has been clearly identified as a risk factor for the development of long COVID symptomatology [54] and also specifically for post-COVID pain [27,28]. The role of age as a risk factor for long COVID is not yet clear [54].

3.2. Step 2—Distribution of Pain

To clinically classify nociplastic pain, patients must report a generalized or widespread pain pattern. Secondary to injury, the nociplastic pain extends beyond a specific area of the damaged structure [19]. In contrast, a nociceptive pain pattern is discrete and localized, makes neuroanatomical sense, and can usually be exacerbated with clearly defined pain triggers (specific movements and activities). Accordingly, a careful assessment and interpretation of the patient's pain distribution is needed. Pain drawings can be used to standardize and optimize the assessment of the individual's pain distribution in a reliable and valid way [55]. A recent study using pain drawings illustrated that widespread symptoms were present in 20% of COVID-19 survivors exhibiting post-COVID pain [56].

3.3. Step 3—Determine Whether Nociceptive Pain Is Present

Another mandatory criterion is that pain cannot be entirely explained by nociceptive mechanisms [19]. Hence, if imaging (such as ultrasonography, X-rays, magnetic resonance imaging (MRI) or computerized tomography (CT) scans) has identified a specific pain generator (e.g., tissue damage) capable of producing nociceptive input that coincides with the patient's self-reported pain pattern, nociplastic pain can be ruled out as a primary phenotype. If a potential source of nociception is identified that seems likely to be responsible for the post-COVID pain symptoms, the pain should be classified as primarily nociceptive. For instance, as previously stated, COVID-19 survivors can develop localized arthralgias reflecting a potential nociceptive pain mechanism [29–31]. It is important to note that identification of a source of nociception in post-COVID pain does not exclude the possibility of concomitant nociplastic or neuropathic pain. This is especially true if pain persists after the source of nociception (e.g., tissue damage) resolves (e.g., tissue damage has healed, but pain remains).

3.4. Step 4—Determine Whether Neuropathic Pain Is Present

Similar to nociceptive pain, another mandatory criterion for nociplastic pain states that symptoms cannot entirely be explained by neuropathic pain mechanisms [19]. This includes either diagnosing or refuting neuropathic origin [57] as the dominant post-COVID pain phenotype. According to the IASP definition of neuropathic pain, procedures confirming a lesion or disease of the somatosensory nervous system are mandatory for diagnosis. As previously noted, no study has systematically found evidence of somatosensory nervous system damage in people with post-COVID pain. Development of clinical guidelines for identifying neuropathic pain in this population is clearly needed.

When a neuropathic mechanism is considered to be primarily responsible for post-COVID pain, the pain phenotype should be classified as neuropathic. However, there is a great deal of overlap between neuropathic and nociplastic pain phenotypes, which can make the determination of primary neuropathic pain difficult. Indeed, sustained neuropathic pain can result in increased hyperexcitability of peripheral and central nervous system pain pathways over time [58]. The relationship between neuropathic pain and nervous system sensitization can provide one explanation for the spreading of the pain beyond the innervation territory of the lesioned nervous structure (as with carpal tunnel syndrome) [59], which is consistent with a nociplastic pain phenotype. Thus, it is possible that post-COVID pain patients can exhibit both neuropathic and nociceptive pain patterns (as well as nociplastic pain).

3.5. Step 5—Elucidate the Presence of Pain Hypersensitivity

Step 5 involves screening for signs of pain hypersensitivity [19]. This step entails the clinical examination of hyperalgesic (defined as an exaggerated pain response to painful stimuli) and allodynic (defined as pain in response to stimuli that normally do not elicit pain) sensitivity. Nociplastic-related hyperalgesia and allodynia can occur both within the painful region and outside the painful region. Indeed, in patients with nociplastic pain, hyperalgesia and allodynia are often widespread. Clinicians can determine signs of hyperalgesia and/or allodynia with manual palpation or with quantitative sensory testing methods, including pain responses to static and dynamic mechanical or thermal stimuli [60,61]. According to the IASP clinical criteria, if the first five steps are positive for nociplastic pain, a patient can be classified as having "possible nociplastic pain" [19]. If the patient meets criteria in step 6, then pain can be considered "probably nociplastic pain".

3.6. Step 6—Check for History of Pain Hypersensitivity

Step 6 involves examining whether the patient with post-COVID pain reports symptoms of pain hypersensitivity after the infection, which can be assessed by questioning patients about their level of sensitivity to different stimuli. Symptoms of hypersensitivity include pain: when clothing, belts, or jewelry touch or bind one's skin; a breeze blows on

exposed skin; the skin is exposed to water during a bath or shower; a handbag hangs on one's shoulder; due to pressure on the buttocks while sitting; and during basic activities of daily living.

3.7. Step 7—Determine Whether Comorbidities Are Present

The final step involves screening for sensitivity to other stimuli, including sensitivity to sound (phonophobia), light (photophobia), or odors, and the presence of other comorbid symptoms, including poor sleep quality, fatigue, and cognitive problems [19]. As previously documented, all these symptoms are frequently present in individuals with long COVID [4–9] and those without post-COVID pain. The CSI can be useful for assessing comorbid symptoms [49,50]. More recently, Tran et al. have developed and published a set of disease-specific PROMs which assess a wide array of post-COVID symptoms that have been reported by long COVID patients [62].

If all seven criteria are met, post-COVID pain can be classified as "probable nociplastic pain" [19]. Figure 1 provides a clinical decision-making tree for clinicians who wish to use the IASP clinical criteria for assessing nociplastic pain in people with long COVID. It is important to stress that more research is needed to examine the reliability and validity of the 2021 IASP clinical criteria for nociplastic pain [19] in people with post-COVID pain and other chronic conditions. In fact, it is possible that some patients with post-COVID pain will likely be outside of this phenotype classification, as they will not fall into any of the three categories. In addition, more research is needed to determine the prognostic value and responsiveness of the IASP clinical criteria for nociplastic pain on key treatment outcomes such as pain, function, and health-related quality of life. The treatment responsiveness of the IASP nociplastic criteria [19] in post-COVID pain patients, specifically targeting underlying mechanisms of nociplastic pain such as central sensitization, should be examined in randomized clinical trials. No studies have investigated these proposals.

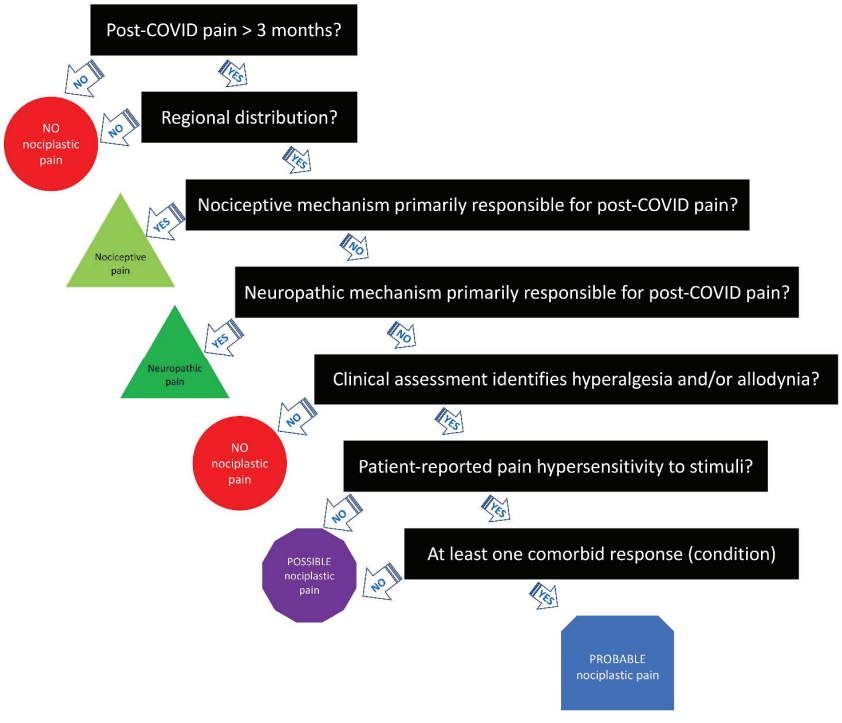


Figure 1. Clinical decision-making tree of the IASP clinical criteria for nociplastic pain applied to post-COVID pain.

4. Toward Precision Pain Medicine for Post-COVID Pain?

Current knowledge regarding central nervous system sensitization, arguably the main underlying mechanism of nociplastic pain, has resulted in a paradigm shift in the understanding and management of chronic pain conditions [21] and should be directly applied to post-COVID pain. The IASP has provided the first set of clinical criteria, with a grading system linked to nociplastic pain as the third mechanistic pain descriptor (in addition to nociceptive and neuropathic pain) [19]. The application of IASP clinical criteria in long COVID patients will allow clinicians to provide specific treatments according to the pain phenotype. Interestingly, a "musculoskeletal pain cycle" has been recently proposed as a model for guiding therapeutic interventions in chronic musculoskeletal pain conditions [63]. Identification of the predominant pain phenotype in individuals with post-COVID pain will permit the development of a "post-COVID pain" model.

A potential pitfall of this process is that clinicians might neglect the individual variability in one particular pain phenotype. For instance, exercise is a therapeutic strategy recommended for chronic pain and is being proposed as beneficial for individuals with long COVID [64]. In fact, evidence supports that programs combining resistance and aerobic exercises may improve the functional capacity and quality of life (reduce stress or mental disorders) in patients with post-COVID-19 symptoms [65]. However, underlying pain mechanisms must be considered in order to optimize the exercise prescription, especially in people with a nociplastic pain phenotype [66].

This topic is of particular relevance in patients with long COVID, since almost 60% of patients with long COVID report post-exertional malaise (PEM) similar to patients with myalgic encephalomyelitis [67]. In such cases, exercise should be provided with caution, and pacing or other cognitive approaches can be proposed (either in isolation or in combination with exercise therapy). Further, treatment of comorbid symptoms that can perpetuate and interact with pain (e.g., sleep disturbances, fatigue, dyspnea, or autonomic disturbances), especially those with a nociplastic pain phenotype, are essential for optimizing treatment outcomes [68]. In fact, successful outcomes are less likely if treatment is solely focused on improving underlying pain mechanisms (i.e., decreasing central sensitization in the nociplastic post-COVID pain phenotype) without managing associated factors.

It is noted that all known upcoming and current rehabilitation programs for long COVID are focused on aerobic exercise and endurance strategies [69]. Identifying patients with a nociplastic pain phenotype can steer clinicians toward additional treatment approaches, such as pain neuroscience education, cognitive behavioral techniques, or self-regulation/mindfulness strategies, in synergy with exercise. In agreement with this proposal, Bodes-Pardo et al. demonstrated that combining pain neurophysiology education with therapeutic exercise is more effective than application of therapeutic exercise alone in another nociplastic pain condition, chronic lower back pain [70]. Accordingly, multimodal/multifactorial treatment approaches using a biopsychosocial model, which address relevant comorbidities and lifestyle factors for each patient, might amplify the rehabilitation effects for long COVID patients with a nociplastic pain phenotype and produce the most successful treatment outcomes.

5. Conclusions

Post-COVID pain remains underestimated and most likely undertreated due to lack of recognition of the phenomenon. Available evidence suggests that nociplastic pain is present in a subgroup of these patients. Applying the global move towards precision medicine to post-COVID pain, and the identification of specific pain phenotypes using the 2021 IASP clinical criteria and grading system [19], could help guide clinical decision making and aid in the most effective treatment planning. The ability of clinicians to phenotype patients with post-COVID pain into nociceptive, neuropathic, nociplastic, or mixed type is important for the following four reasons: (1) proper classification of the pain phenotypes can help clinicians choose proper therapeutic interventions; (2) neuropathic and

nociplastic post-COVID pain phenotypes are considered to be more difficult to manage than the nociceptive pain phenotype; (3) to achieve the best treatment outcomes, long COVID patients with nociplastic pain could likely respond best to multimodal treatment approaches to address comorbid symptoms; and (4) the application of mechanism-based treatments may have better clinical outcomes in future clinical trials. Studies examining the clinimetric properties of the 2021 IASP clinical criteria and grading system for nociplastic pain in long COVID patients are needed. Finally, this paper proposes that treatment strategies to be applied to patients with post-COVID pain should be based on pain phenotype and that multimodal approaches should be encouraged. Future trials investigating potential treatment approaches based on the proposed clinical reasoning are now needed.

Author Contributions: Conceptualization, all authors; methodology, C.F.-d.-l.-P., J.N. and L.A.-N. validation, all authors; investigation, all authors; resources, J.N. and L.A.-N.; writing—original draft preparation, all authors; writing—review and editing, all authors; visualization, all authors; supervision, J.N. and L.A.-N.; funding acquisition, C.F.-d.-l.-P. and L.A.-N. All authors have read and agreed to the published version of the manuscript.

Funding: C. Fernández-de-las-Peñas is supported by a grant associated with the Fondo Europeo de Desarrollo Regional-Recursos REACT-UE del Programa Operativo de Madrid 2014–2020, en la línea de actuación de proyectos de I+D+i en materia de respuesta a COVID 19 (LONG-COVID-EXP-CM). C. Fernández-de-las-Peñas and L. Arendt-Nielsen are supported by a grant from the Novo Nordisk Foundation 0067235 (Denmark).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The Center for Neuroplasticity and Pain (CNAP) is supported by the Danish National Research Foundation (DNRF121) and the Novo Nordisk Foundation (0067235).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Fernández-de-las-Peñas, C. Long COVID: Current definition. *Infection* **2022**, *50*, 285–286. [CrossRef] [PubMed]
2. Soriano, J.B.; Murthy, S.; Marshall, J.C.; Relan, P.; Diaz, J.V.; WHO Clinical Case Definition Working Group on Post-COVID-19 Condition. A clinical case definition of post-COVID-19 condition by a Delphi consensus. *Lancet Infect. Dis.* **2022**, *22*, e102–e107. [CrossRef]
3. Hayes, L.D.; Ingram, J.; Sculthorpe, N.F. More Than 100 Persistent Symptoms of SARS-CoV-2 (Long COVID): A scoping review. *Front Med.* **2021**, *8*, 750378. [CrossRef]
4. Michelen, M.; Manoharan, L.; Elkheir, N.; Cheng, V.; Dagens, A.; Hastie, C.; O'Hara, M.; Suett, J.; Dahmash, D.; Bugaeva, P.; et al. Characterising long COVID: A living systematic review. *BMJ Glob. Health* **2021**, *6*, e005427. [CrossRef] [PubMed]
5. Fernández-de-las-Peñas, C.; Palacios-Ceña, D.; Gómez-Mayordomo, V.; Florencio, L.L.; Cuadrado, M.L.; Plaza-Manzano, G.; Navarro-Santana, M. Prevalence of post-COVID-19 symptoms in hospitalized and non-hospitalized COVID-19 survivors: A systematic review and meta-analysis. *Eur. J. Inter. Med.* **2021**, *92*, 55–70. [CrossRef] [PubMed]
6. Chen, C.; Haupert, S.R.; Zimmermann, L.; Shi, X.; Fritsche, L.G.; Mukherjee, B. Global prevalence of post COVID-19 condition or long COVID: A meta-analysis and systematic review. *J. Infect. Dis.* **2022**, jiac136. [CrossRef] [PubMed]
7. Alkodaymi, M.S.; Omrani, O.A.; Fawzy, N.A.; Shaar, B.A.; Almamlouk, R.; Riaz, M.; Obeidat, M.; Obeidat, Y.; Gerberi, D.; Taha, R.M.; et al. Prevalence of post-acute COVID-19 syndrome symptoms at different follow-up periods: A systematic review and meta-analysis. *Clin. Microbiol. Infect.* **2022**, *28*, 657–666. [CrossRef]
8. Han, Q.; Zheng, B.; Daines, L.; Sheikh, A. Long-Term Sequelae of COVID-19: A Systematic Review and Meta-Analysis of One-Year Follow-Up Studies on Post-COVID Symptoms. *Pathogens* **2022**, *11*, 269. [CrossRef] [PubMed]
9. Yang, T.; Yan, M.Z.; Li, X.; Lau, E.H.Y. Sequelae of COVID-19 among previously hospitalized patients up to 1 year after discharge: A systematic review and meta-analysis. *Infection* **2022**, *50*, 1067–1109. [CrossRef]
10. Fernández-de-las-Peñas, C.; Navarro-Santana, M.; Plaza-Manzano, G.; Palacios-Ceña, D.; Arendt-Nielsen, L. Time course prevalence of Post-COVID pain symptoms of musculoskeletal origin in patients who had survived to SARS-CoV-2 infection: A systematic review and meta-analysis. *Pain* **2022**, *163*, 1220–1231. [CrossRef]
11. Bakılan, F.; Gökmen, İ.G.; Ortanca, B.; Uçan, A.; Eker Güvenç, Ş.; Şahin Mutlu, F.; Gökmen, H.M.; Ekim, A. Musculoskeletal symptoms and related factors in postacute COVID-19 patients. *Int. J. Clin. Pract.* **2021**, *75*, e14734. [CrossRef] [PubMed]

12. Karaarslan, F.; Demircioğlu, G.F.; Kardeş, S. Post-discharge rheumatic and musculoskeletal symptoms following hospitalization for COVID-19: Prospective follow-up by phone interviews. *Rheumatol. Int.* **2021**, *41*, 1263–1271. [CrossRef] [PubMed]
13. Soares, F.H.C.; Kubota, G.T.; Fernandes, A.M.; Hojo, B.; Couras, C.; Costa, B.V.; Lapa, J.D.D.S.; Braga, L.M.; Almeida, M.M.; Cunha, P.H.M.D.; et al. "Pain in the Pandemic Initiative Collaborators". Prevalence and characteristics of new-onset pain in COVID-19 survivours, a controlled study. *Eur. J. Pain* **2021**, *25*, 1342–1354. [CrossRef] [PubMed]
14. Bileviciute-Ljungar, I.; Norrefalk, J.R.; Borg, K. Pain burden in post-COVID-19 syndrome following mild COVID-19 infection. *J. Clin. Med.* **2022**, *11*, 771. [CrossRef]
15. National Research Council Committee on AFfDaNToD. The National Academies Collection: Reports Funded by National Institutes of Health. In *Toward Precision Medicine: Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease*; National Academies Press: Washington, DC, USA, 2011.
16. Shraim, M.A.; Massé-Alarie, H.; Hodges, P.W. Methods to discriminate between mechanism-based categories of pain experienced in the musculoskeletal system: A systematic review. *Pain* **2021**, *162*, 1007–1037. [CrossRef]
17. Kosek, E.; Cohen, M.; Baron, R.; Gebhart, G.F.; Mico, J.A.; Rice, A.S.; Rief, W.; Sluka, A.K. Do we need a third mechanistic descriptor for chronic pain states? *Pain* **2016**, *157*, 1382–1386. [CrossRef]
18. Aydede, M.; Shriver, A. Recently introduced definition of nociplastic pain by the International Association for the Study of Pain needs better formulation. *Pain* **2018**, *159*, 1176–1177. [CrossRef]
19. Kosek, E.; Clauw, D.; Nijs, J.; Baron, R.; Gilron, I.; Harris, R.E.; Mico, J.A.; Rice, A.S.C.; Sterling, M. Chronic nociplastic pain affecting the musculoskeletal system: Clinical criteria and grading system. *Pain* **2021**, *162*, 2629–2634. [CrossRef]
20. Nijs, J.; Lahousse, A.; Kapreli, E.; Bilika, P.; Saraçoğlu, İ.; Malfliet, A.; Coppieters, I.; De Baets, L.; Leysen, L.; Roose, E.; et al. Nociplastic pain criteria or recognition of central sensitization? Pain phenotyping in the past, present and future. *J. Clin. Med.* **2021**, *10*, 3203.
21. Nijs, J.; George, S.Z.; Clauw, D.J.; Fernández-de-las-Peñas, C.; Kosek, E.; Ickmans, K.; Fernández-Carnero, J.; Polli, A.; Kapreli, E.; Huysmans, E.; et al. Central sensitisation in chronic pain conditions: Latest discoveries and their potential for precision medicine. *Lancet Rheumatol.* **2021**, *3*, e383–e392. [CrossRef]
22. Smart, K.M.; Blake, C.; Staines, A.; Doody, C. Clinical indicators of "nociceptive", "peripheral neuropathic" and "central" mechanisms of musculoskeletal pain. A Delphi survey of expert clinicians. *Man. Ther.* **2010**, *15*, 80–87. [CrossRef] [PubMed]
23. Nijs, J.; Torres-Cueco, R.; van Wilgen, C.P.; Girbes, E.L.; Struyf, F.; Roussel, N.; van Oosterwijck, J.; Daenen, L.; Kuppens, K.; Vanwerweeen, L.; et al. Applying modern pain neuroscience in clinical practice: Criteria for the classification of central sensitization pain. *Pain Phys.* **2014**, *17*, 447–457. [CrossRef]
24. Mulchandani, R.; Lyngdoh, T.; Kakkar, A.K. Deciphering the COVID-19 cytokine storm: Systematic review and meta-analysis. *Eur. J. Clin. Investig.* **2021**, *51*, e13429. [CrossRef] [PubMed]
25. Coomes, E.A.; Haghbayan, H. Interleukin-6 in Covid-19: A systematic review and meta-analysis. *Rev. Med. Virol.* **2020**, *30*, 1–9. [CrossRef] [PubMed]
26. D'Souza, R.S.; Kilgore, A.E.; D'Souza, S. Manifestations of pain during the COVID-19 pandemic portrayed on social media: A cross-sectional study. *Pain Med.* **2021**, *23*, 229–233. [CrossRef] [PubMed]
27. Fernández-de-las-Peñas, C.; de-la-Llave-Rincón, A.I.; Ortega-Santiago, R.; Ambite-Quesada, S.; Gómez-Mayordomo, V.; Cuadrado, M.L.; Arias-Navalón, J.A.; Hernández-Barrera, V.; Martín-Guerrero, J.D.; Pellicer-Valero, O.J.; et al. Prevalence and risk factors of musculoskeletal pain symptoms as long-term post-COVID sequelae in hospitalized COVID-19 survivors: A multicenter study. *Pain* **2022**, *163*, e989–e996. [CrossRef] [PubMed]
28. Fernández-de-las-Peñas, C.; Cancela-Cilleruelo, I.; Rodríguez-Jiménez, J.; Moro-López-Menchero, P.; Pellicer-Valero, O.J.; Martín-Guerrero, J.D.; Arendt-Nielsen, L. Exploring the trajectory curve of long-term musculoskeletal post-COVID pain symptoms in hospitalized COVID-19 survivors: A multicenter study. *Pain* **2022**. [CrossRef] [PubMed]
29. Nguyen, N.N.; Hoang, V.T.; Dao, T.L.; Dudouet, P.; Eldin, C.; Gautret, P. Clinical patterns of somatic symptoms in patients suffering from post-acute long COVID: A systematic review. *Eur. J. Clin. Microbiol. Infect. Dis.* **2022**, *41*, 515–545. [CrossRef]
30. Cui, D.; Wang, Y.; Huang, L.; Gu, X.; Huang, Z.; Mu, S.; Wang, C.; Cao, B. Rheumatic symptoms following Coronavirus Disease 2019 (COVID-19): A chronic post-COVID-19 condition. *Open Forum Infect. Dis.* **2022**, *9*, ofac170. [CrossRef]
31. Pal, A.; Roongta, R.; Mondal, S.; Sinha, D.; Sinhamahapatra, P.; Ghosh, A.; Chattopadhyay, A. Does post-COVID reactive arthritis exist? Experience of a tertiary care centre with a review of the literature. *Reumatol. Clin.* **2022**. [CrossRef]
32. Khoja, O.; Silva Passadouro, B.; Mulvey, M.; Delis, I.; Astill, S.; Tan, A.L.; Sivan, M. Clinical characteristics and mechanisms of musculoskeletal pain in long COVID. *J. Pain Res.* **2022**, *15*, 1729–1748. [CrossRef] [PubMed]
33. Gavrilova, N.; Soprun, L.; Lukashenko, M.; Ryabkova, V.; Fedotkina, T.V.; Churilov, L.P.; Shoenfeld, Y. New clinical phenotype of the post-covid syndrome: Fibromyalgia and joint hypermobility condition. *Pathophysiology* **2022**, *29*, 24–29. [PubMed]
34. Scholz, J.; Finnerup, N.B.; Attal, N.; Aziz, Q.; Baron, R.; Bennett, M.I.; Benoliel, R.; Cohen, M.; Cruccu, G.; Davis, K.D.; et al. Classification Committee of the Neuropathic Pain Special Interest Group (NeuPSIG). The IASP classification of chronic pain for ICD-11: Chronic neuropathic pain. *Pain* **2019**, *160*, 53–59. [CrossRef] [PubMed]
35. Torices, S.; Cabrera, R.; Stangis, M.; Naranjo, O.; Fattakhov, N.; Teglas, T.; Adesse, D.; Toborek, M. Expression of SARS-CoV-2-related receptors in cells of the neurovascular unit: Implications for HIV-1 infection. *J. Neuroinflamm.* **2021**, *18*, 167. [CrossRef]

36. Oaklander, A.L. Clinical significance of angiotensin-converting enzyme 2 receptors for severe acute respiratory syndrome coronavirus 2 (COVID-19) on peripheral small-fiber sensory neurons is unknown today. *Pain* **2020**, *161*, 2431–2433. [CrossRef] [PubMed]
37. Joshi, D.; Gyanpuri, V.; Pathak, A.; Chaurasia, R.N.; Mishra, V.N.; Kumar, A.; Singh, V.K.; Dhiman, N.R. Neuropathic pain associated with COVID-19: A systematic review of case reports. *Curr. Pain Headache Rep.* **2022**, *22*, 1–9. [CrossRef]
38. Rodríguez, Y.; Vatti, N.; Ramirez-Santana, C.; Chang, C.; Mancera-Páez, O.; Gershwin, M.E.; Anaya, J.M. Chronic inflammatory demyelinating polyneuropathy as an autoimmune disease. *J. Autoimmun.* **2019**, *102*, 8–37. [CrossRef] [PubMed]
39. Oguz-Akarsu, E.; Gullu, G.; Kilic, E.; Dinç, Y.; Ursavas, A.; Yilmaz, E.; Zarifoglu, M.; Karli, N. Pandemic Study Team. Insight into pain syndromes in acute phase of mild-to-moderate COVID-19: Frequency, clinical characteristics, and associated factors. *Eur. J. Pain* **2021**, *26*, 492–504. [CrossRef] [PubMed]
40. Attal, N.; Bouhassira, D.; Baron, R. Diagnosis and assessment of neuropathic pain through questionnaires. *Lancet Neurol.* **2018**, *17*, 456–466. [CrossRef]
41. Herrero-Montes, M.; Fernández-de-las-Peñas, C.; Ferrer-Pargada, D.; Tello-Mena, S.; Cancela-Cilleruelo, I.; Rodríguez-Jiménez, J.; Palacios-Ceña, D.; Parás-Bravo, P. Prevalence of neuropathic component in post-COVID pain symptoms in previously hospitalized COVID-19 survivors. *Int. J. Clin. Pract.* **2022**, *2022*, 3532917. [CrossRef] [PubMed]
42. Fernández-de-las-Peñas, C.; Valera-Calero, J.A.; Herrero-Montes, M.; Del-Valle-Loarte, P.; Rodríguez-Rosado, R.; Ferrer-Pargada, D.; Arendt-Nielsen, L.; Parás-Bravo, P. The Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) and PainDETECT questionnaires in COVID-19 survivors with post-COVID pain. *Viruses* **2022**, *14*, 1486. [CrossRef] [PubMed]
43. Magdy, R.; Eid, R.A.; Fathy, W.; Abdel-Aziz, M.M.; Ibrahim, R.E.; Yehia, A.; Sheemy, M.S.; Hussein, M. Characteristics and risk factors of persistent neuropathic pain in recovered COVID-19 patients. *Pain Med.* **2022**, *23*, 774–781. [CrossRef]
44. Fernández-de-las-Peñas, C.; Herrero-Montes, M.; Ferrer-Pargada, D.; Izquierdo-Cuervo, S.; Palacios-Ceña, D.; Arendt-Nielsen, L.; Torres-Macho, J.; Parás-Bravo, P. Development of neuropathic post-COVID pain symptoms is not associated with serological biomarkers at hospital admission in COVID-19 survivors: A secondary analysis. *Pain Med.* **2022**, pnac086. [CrossRef] [PubMed]
45. Oaklander, A.L.; Mills, A.J.; Kelley, M.; Toran, L.S.; Smith, B.; Dalakas, M.C.; Nath, A. Peripheral neuropathy evaluations of patients with prolonged long COVID. *Neurol. Neuroimmunol. Neuroinflamm.* **2022**, *9*, e1146. [CrossRef]
46. Loeser, J.D.; Treede, R.D. The Kyoto protocol of IASP basic pain terminology. *Pain* **2008**, *137*, 473–477. [CrossRef] [PubMed]
47. Fitzcharles, M.A.; Cohen, S.P.; Clauw, D.J.; Littlejohn, G.; Usui, C.; Häuser, W. Nociplastic pain: Towards an understanding of prevalent pain conditions. *Lancet* **2021**, *397*, 2098–2110. [CrossRef]
48. Ursini, F.; Ciaffi, J.; Mancarella, L.; Lisi, L.; Brusi, V.; Cavallari, C.; D'Onghia, M.; Mari, A.; Borlandelli, E.; Faranda Cordella, J.; et al. Fibromyalgia: A new facet of the post-COVID-19 syndrome spectrum? Results from a web-based survey. *RMD Open* **2021**, *7*, e001735. [CrossRef]
49. Neblett, R.; Cohen, H.; Choi, Y.; Hartzell, M.M.; Williams, M.; Mayer, T.G.; Gatchel, R.J. The Central Sensitization Inventory (CSI): Establishing clinically significant values for identifying central sensitivity syndromes in an outpatient chronic pain sample. *J. Pain* **2013**, *14*, 438–445. [CrossRef]
50. Mayer, T.G.; Neblett, R.; Cohen, H.; Howard, K.J.; Choi, Y.H.; Williams, M.J.; Perez, Y.; Gatchel, R.J. The development and psychometric validation of the central sensitization inventory. *Pain Pract.* **2012**, *12*, 276–285. [CrossRef]
51. Goudman, L.; De Smedt, A.; Noppen, M.; Moens, M. Is central sensitisation the missing link of persisting symptoms after COVID-19 infection? *J. Clin. Med.* **2021**, *10*, 5594. [CrossRef]
52. Fernández-de-las-Peñas, C.; Parás-Bravo, P.; Ferrer-Pargada, D.; Cancela-Cilleruelo, I.; Rodríguez-Jiménez, J.; Nijs, J.; Arendt-Nielsen, L.; Herrero-Montes, M. Sensitization symptoms are associated with psychological and cognitive variables in COVID-19 survivors exhibiting post-COVID pain. *Pain Pract.* **2022**. [CrossRef] [PubMed]
53. Fernández-de-las-Peñas, C.; Herrero-Montes, M.; Cancela-Cilleruelo, I.; Rodríguez-Jiménez, J.; Parás-Bravo, P.; Varol, U.; Del-Valle-Loarte, P.; Flox-Benítez, G.; Arendt-Nielsen, L.; Valera-Calero, J.A. Understanding sensitization, cognitive and neuropathic associated mechanisms behind post-COVID pain: A Network Analysis. *Diagnostics* **2022**, *12*, 1538. [CrossRef]
54. Maglietta, G.; Diodati, F.; Puntoni, M.; Lazzarelli, S.; Marcomini, B.; Patrizi, L.; Caminiti, C. Prognostic Factors for Post-COVID-19 Syndrome: A Systematic Review and Meta-Analysis. *J. Clin. Med.* **2022**, *11*, 1541. [CrossRef]
55. Barbero, M.; Navarro-Santana, M.J.; Palacios-Ceña, M.; Ortega-Santiago, R.; Cescon, C.; Falla, D.; Fernández-de-las-Peñas, C. Clinical significance and diagnostic value of pain extent extracted from pain drawings: A scoping review. *Diagnostics* **2020**, *10*, 604. [CrossRef] [PubMed]
56. Fernández-de-las-Peñas, C.; Fuensalida-Novo, S.; Ortega-Santiago, S.; Valera-Calero, J.A.; Cescon, C.; Derboni, M.; Giuffrida, V.; Barbero, M. Pain extent is not associated with sensory-associated symptoms, cognitive and psychological variables in COVID-19 survivors suffering from post-COVID pain. *J. Clin. Med.* **2022**, *11*, 4633. [CrossRef]
57. Finnerup, N.B.; Haroutounian, S.; Kamerman, P.; Baron, R.; Bennett, D.L.H.; Bouhassira, D.; Cruccu, G.; Freeman, R.; Hansson, P.; Nurmikko, T.; et al. Neuropathic pain: An updated grading system for research and clinical practice. *Pain* **2016**, *157*, 1599–1606. [CrossRef] [PubMed]
58. Boadas-Vaello, P.; Castany, S.; Homs, J.; Álvarez-Pérez, B.; Deulofeu, M.; Verdú, E. Neuroplasticity of ascending and descending pathways after somatosensory system injury: Reviewing knowledge to identify neuropathic pain therapeutic targets. *Spinal Cord* **2016**, *54*, 330–340. [CrossRef] [PubMed]

59. Fernández-de-las-Peñas, C.; Plaza-Manzano, G. Carpal tunnel syndrome: Just a peripheral neuropathy? *Pain Manag.* **2018**, *8*, 209–216. [CrossRef]
60. Uddin, Z.; MacDermid, J.C. Quantitative sensory testing in chronic musculoskeletal pain. *Pain Med.* **2016**, *17*, 1694–1703. [CrossRef]
61. Walk, D.; Sehgal, N.; Moeller-Bertram, T.; Edwards, R.R.; Wasan, A.; Wallace, M.; Irving, G.; Argoff, C.; Backonja, M.M. Quantitative sensory testing and mapping: A review of nonautomated quantitative methods for examination of the patient with neuropathic pain. *Clin. J. Pain* **2009**, *25*, 632–640. [CrossRef] [PubMed]
62. Tran, V.T.; Riveros, C.; Clepier, B.; Desvarieux, M.; Collet, C.; Yordanov, Y.; Ravaud, P. Development and validation of the Long Coronavirus Disease (COVID) Symptom and Impact Tools: A Set of Patient-Reported Instruments Constructed from patients' lived experience. *Clin. Infect. Dis.* **2022**, *74*, 278–287. [CrossRef]
63. Bonanni, R.; Cariati, I.; Tancredi, V.; Iundusi, R.; Gasbarra, E.; Tarantino, U. Chronic pain in musculoskeletal diseases: Do you know your enemy? *J. Clin. Med.* **2022**, *11*, 2609. [CrossRef] [PubMed]
64. Jimeno-Almazán, A.; Pallarés, J.G.; Buendía-Romero, Á.; Martínez-Cava, A.; Franco-López, F.; Sánchez-Alcaraz Martínez, B.J.; Bernal-Morel, E.; Courel-Ibáñez, J. Post-COVID-19 syndrome and the potential benefits of exercise. *Int. J. Environ. Res. Public Health* **2021**, *18*, 5329. [CrossRef]
65. Ahmadi Hekmatikar, A.H.; Ferreira Júnior, J.B.; Shahrbanian, S.; Suzuki, K. Functional and psychological changes after exercise training in post-COVID-19 patients discharged from the hospital: A PRISMA-compliant systematic review. *Int. J. Environ. Res. Public Health* **2022**, *19*, 2290. [CrossRef] [PubMed]
66. Ferro Moura Franco, K.; Lenoir, D.; Dos Santos Franco, Y.R.; Jandre Reis, F.J.; Nunes Cabral, C.M.; Meeus, M. Prescription of exercises for the treatment of chronic pain along the continuum of nociplastic pain: A systematic review with meta-analysis. *Eur. J. Pain* **2021**, *25*, 51–70. [CrossRef]
67. Twomey, R.; DeMars, J.; Franklin, K.; Culos-Reed, S.N.; Weatherald, J.; Wrightson, J.G. Chronic fatigue and postexertional malaise in people living with long COVID: An observational study. *Phys. Ther.* **2022**, *102*, pzac005. [CrossRef]
68. Cattadori, G.; Di Marco, S.; Baravelli, M.; Picozzi, A.; Ambrosio, G. Exercise training in post-COVID-19 patients: The need for a multifactorial protocol for a multifactorial pathophysiology. *J. Clin. Med.* **2022**, *11*, 2228. [CrossRef] [PubMed]
69. Bailly, M.; Pélissier, L.; Coudeyre, E.; Evrard, B.; Bingula, R.; Rochette, C.; Mériade, L.; Blavignac, C.; Fournier, A.C.; Bignon, Y.J.; et al. Systematic review of COVID-19-related physical activity-based rehabilitations: Benefits to be confirmed by more robust methodological approaches. *Int. J. Environ. Res. Public Health* **2022**, *19*, 9025. [CrossRef] [PubMed]
70. Bodes-Pardo, G.; Lluch-Girbés, E.; Roussel, N.A.; Gallego-Izquierdo, T.; Jiménez-Penick, V.; Pecos-Martín, D. Pain neurophysiology education and therapeutic exercise for patients with chronic low back pain: A single-blind randomized controlled trial. *Arch. Phys. Med. Rehabil.* **2018**, *99*, 338–347. [CrossRef]

biomedicines

MDPI

Article

Growth Differentiation Factor 15 (GDF-15) Levels Associate with Lower Survival in Chronic Kidney Disease Patients with COVID-19

Andrea Galassi [1,†], Paola Ciceri [1,†], Valeria Bono [2], Lorenza Magagnoli [1], Matteo Sala [2], Luisa Artioli [1], Roberta Rovito [2], Mohamad Hadla [2], Vaibhav Yellenki [2], Antonella D'Arminio Monforte [2], Camilla Tincati [2], Mario Cozzolino [1,*] and Giulia Marchetti [2]

[1] Department of Health Sciences, Renal Division, University of Milan, ASST Santi Paolo e Carlo, 20142 Milan, Italy
[2] Department of Health Sciences, Clinic of Infectious Diseases, University of Milan, ASST Santi Paolo e Carlo, 20142 Milan, Italy
* Correspondence: mario.cozzolino@unimi.it; Tel.: +39-02-81844215
† These authors contributed equally to this work.

Abstract: A cytokine storm drives the pathogenesis of severe COVID-19 infection and several biomarkers have been linked to mortality. Chronic kidney disease (CKD) emerged as a risk factor for severe COVID-19. We investigated the association between selected biomarkers and mortality in 77 patients hospitalized for COVID-19, and whether they differ in patients with eGFR higher and lower than 45 mL/min. The association between patients' characteristics, plasma biomarkers and mortality was conducted by univariate logistic regression models and independent predictors of mortality were then used to create a multivariate prediction model through Cox regression. Patients with lower eGFR had a significant increase of GDF-15, CD-25 and RAGE, with higher plasma levels in non-survivors and in patients who needed ventilation. At univariate analysis, low and mid-low GDF-15 quartiles (<4.45 ng/mL) were associated with lower mortality risk, while mid-high and high quartiles (>4.45 ng/mL) were associated with higher mortality risk. Independent association between GDF-15 quartiles and mortality risk was confirmed in the Cox model and adjusted for eGFR, age, fever and dyspnea (HR 2.28, CI 1.53–3.39, $p < 0.0001$). The strength of the association between GDF-15 quartiles and mortality risk increased in patients with lower compared to higher eGFR (HR 2.53, CI 1.34–4.79 versus HR 1.99, CI 1.17–3.39). Our findings may suggest a further investigation of the effect of GDF-15 signaling pathway inhibition in CKD.

Keywords: CKD; COVID-19; GDF-15; mortality

Citation: Galassi, A.; Ciceri, P.; Bono, V.; Magagnoli, L.; Sala, M.; Artioli, L.; Rovito, R.; Hadla, M.; Yellenki, V.; D'Arminio Monforte, A.; et al. Growth Differentiation Factor 15 (GDF-15) Levels Associate with Lower Survival in Chronic Kidney Disease Patients with COVID-19. *Biomedicines* **2022**, *10*, 3251. https://doi.org/10.3390/biomedicines10123251

Academic Editor: César Fernández De Las Peñas

Received: 22 November 2022
Accepted: 10 December 2022
Published: 14 December 2022

1. Introduction

By October 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing the coronavirus disease 2019 (COVID-19) pandemic, had infected millions of people, causing millions of deaths [1]. A key unmet clinical need is the earlier and more precise identification of subjects at a higher risk of severe disease, exploring the need to investigate the disease-associated factors to individuate patients with COVID-19 poor prognosis. Besides the disease per se, several comorbidities are associated with the severity of COVID-19 infection, also implying a need to investigate the potential impact of medications commonly used [2,3].

Recently, chronic kidney disease (CKD) emerged as one of the strongest risk factors for severe COVID-19 [4–7]. Indeed, patients with advanced CKD are at an increased risk of mortality from several causes, led by cardiovascular disease (CVD) and infections [8,9]. It is very important to underline the relevancy of CKD to the course of COVID-19 disease since the impairment of renal function is often missed by physicians.

COVID-19 is characterized by a cytokine storm that contributes to the development of endothelial vascular dysfunction, which can lead to acute respiratory distress syndrome,

multi-organ failure and finally, death [10]. Several biomarkers are deregulated in the disease course. An emerging factor is the growth and differentiation factor 15 (GDF-15), which belongs to the transforming growth factor-beta (TGF-beta) superfamily of proteins. It has been demonstrated that GDF-15 has a pivotal role in the development and progression of diseases such as CKD [11], congestive heart failure (CHF) [12] and chronic pulmonary obstructive disease (COPD) [13] because of its role as a metabolic regulator [14]. In COVID-19, GDF-15 activity represents a strong predictor of poor outcomes in critically ill patients acting as a central mediator of inflammation [15]. Due to the role of CKD as a major risk factor for severe COVID-19, the research interest on the inflammatory response to SARS-CoV-2 in CKD patients is continuously growing in order to provide clues on the pathogenesis of COVID-19 and on successful treatments in CKD patients [16]. Furthermore, the association between GDF-15 and COVID-19 in CKD patients has been poorly investigated.

The present study explores the association between GDF-15 and in-hospital mortality among CKD patients hospitalized for COVID-19.

2. Methods and Materials

2.1. Study Design and Population

This was a retrospective observational study conducted on patients hospitalized due to COVID-19 infection and admitted in the ward of Tropical and Infectious Diseases at San Paolo Hospital in Milan (Italy) from February to September 2020.

2.2. Data Collection

Data on demographics, medical history and clinical status were taken from electronic clinical charts and recorded on the online database application REDCap; the data, therefore, was collected from it for the purpose of the present research. Estimated glomerular filtration rate (eGFR) was assessed by a CKD-EPI formula at hospital admission and patients were stratified in two groups (eGFR $\geq$ 45 mL/min/1.73 m^2 or eGFR < 45 mL/min/1.73 m^2). Data on mortality and on the length of stay were collected as part of the study protocol.

2.3. Plasma Cytokine Quantification

Peripheral blood samples collected at admission were centrifuged for 15 min at 2.500 rpm. Plasma was then harvested and stored at -80 °C. Plasmatic levels of the following 20 biomarkers were thereafter assessed by Luminex technology and ELISA assay, according to the manufacturer's instructions: GDF-15, CD-25, receptor for advanced glycation end products (RAGE), interleukine-6 (IL-6), interleukin-7 (IL-7), interleukin-18 (IL-18), interleukine-6 receptor (IL-6R), tumor necrosis factor alpha TNFa, tumor necrosis factor receptor 1 (TNFR I), tumor necrosis factor receptor 2 (TNFR II), leukemia inhibitory factor (LIF), Fas, YKL-40, pentraxin-3 (PTX-3), platelet derived growth factor—AA (PDGF-AA)—vascular endothelial growth factor (VEGF), a-1-acid glycoprotein (a1-AGP), TNF-related apoptosis-inducing ligand (TRAIL), kynurenine and tryptophane.

2.4. Statistical Analysis

Categorical variables were reported as rates (%). Continuous variables were reported as mean $\pm$ standard deviation and median [interquartile range], according to the normality of distribution assessed by the Shapiro-Wilk test. Comparisons between categorical and continuous variables were performed by Fisher's exact test and Mann–Whitney U test, respectively. Demographic, clinical and biochemical characteristics at baseline were stratified according to eGFR (higher-equal vs lower than 45 mL/min/1.73 m^2). Plasma biomarkers were also stratified according to survival and the need of non-invasive ventilation (NIV). Linear correlation between biomarkers and eGFR was assessed by a Pearson correlation test.

The association between patients' characteristics, plasma biomarkers and mortality was first conducted by univariate logistic regression models. Independent predictors of in-hospital mortality ($p < 0.05$) were then investigated by Cox proportional hazard models selected by stepwise procedure. Predictors included at the first step were arbitrarily

selected based on the significance of their univariate association with mortality (for clinical characteristics) and with eGFR, NIV and mortality (for biochemical markers). Independency from basal renal function was tested by a forced inclusion of eGFR into the first step of model selection. The proportional hazards assumption was checked by Schoenfeld residuals test for both the single covariates and the whole model. The association between single GDF-15 quartiles and mortality was investigated by univariate logistic regression models. Survival curves for GDF-15 quartiles were plotted by the Kaplan-Meier method. Nonlinear association between GDF-15 and survival was modelled by polynomial splines in all the patients and in eGFR subgroups. A sensitivity analysis was performed by excluding outliers for GDF-15. Furthermore, as reduced eGFR at admission might be due to either CKD (known or unrecognized) or acute kidney injury, the association between GDF-15 and mortality risk was also assessed in the subset of patients with eGFR < 45 mL/min/1.73 m^2 and known CKD reported in their medical history as a sensitivity analysis. The *p*-value for significance was set at <0.05.

Analysis was conducted by R package version 4.1.1.

3. Results

Seventy-seven patients aged 79 (70–86) years were enrolled. Patients' characteristics are presented in Table 1A,B. In the overall population median, eGFR was 48.4 mL/min/1.73 m^2. Twenty (26%) patients reported a history of CKD with 4 (5%) patients receiving maintenance hemodialysis. Thirty-three (43%) patients presented eGFR < 45 mL/min/1.73 m^2. A history of cardiovascular disease (CVD) was highly prevalent (49%), including patients affected by CHF (14%), myocardial infarction (22%) and arrhythmias (19%). Diabetes and COPD were reported in 25 (32%) and 9 (12%) patients, respectively. The most frequent symptoms at admission were fever (69%), cough (34%) and dyspnea (56%). Pneumonia and acute respiratory distress syndrome were documented in 67 (87%) and 36 (47%) patients, respectively. Forty-two (55%) patients required NIV, while only 2 (3%) were admitted to an intensive care unit (ICU). The specific treatments most frequently prescribed included heparin (75%), hydroxychloroquine (74%) and steroids (22%). The median time from symptom onset to hospitalization was 4 days (IQR 2–8). A 45% in-hospital mortality rate was observed. Median time from admission to death or dis-charge was 18 (11–35) days. Patients with eGFR < 45 mL/min/1.73 m^2 presented higher prevalence of CKD, diabetes and higher neutrophil count. Lower eGFR was not associated with any other significant difference in baseline characteristics, clinical severity and in-hospital mortality rate.

GDF-15, CD-25 and RAGE resulted in the unique plasma biomarkers significantly associated with eGFR, a need of NIV and mortality out of the 20 tested molecules (Table S1). Plasma levels of these biomarkers were negatively associated with basal eGFR (Figure 1). Plasma concentrations of GDF-15, CD-25 and RAGE were significantly higher in deceased patients and in those receiving NIV (Figure 1).

Patients with an age > 75 years ($p = 0.005$), fever ($p = 0.005$), dyspnea ($p = 0.003$) and P/F < 300 ($p = 0.034$) were significantly associated with mortality at univariate analysis. Survival curves stratified according to GDF-15 quartiles are presented in Figure 2. In a multivariate Cox regression model, each increase in GDF-15 quartiles was associated with a 128% increased mortality risk [HR 2.28 (1.53–3.39, 95% CI), $p < 0.001$] independent from basal eGFR and the aforementioned predictors (Table 2). CD-25 and RAGE were excluded from the model by a stepwise selection procedure. Stronger association between GDF-15 and mortality was descriptively observed among patients with eGFR < 45 mL/min/1.73 m^2 [HR 2.54 (1.34–4.79), 95% CI] compared with a higher eGFR strata [HR 1.99 (1.17–3.39, 95% CI)]. Results were unchanged in the sensitivity analysis after the exclusion of 8 outlying observations for GDF-15 (Table S2) and dialysis patients (Table S3).

Table 1. (**A**) Patients characteristics. Demographics and comorbidities. (**B**) Patients' characteristics. Clinical findings.

(A)				
Characteristic	**All Patients**	**eGFR ≥ 45 mL/min**	**eGFR < 45 mL/min**	***p***
	(*n* = 77)	**(*n* = 44)**	**(*n* = 33)**	
Sex. male	40 (52)	25 (57)	15 (46)	0.36
Age (years)	79 {70–86}	78 {69–86}	79 {73–86}	0.45
Ethnicity				
Caucasian	72 (94)	42 (96)	30 (91)	0.65
Middle east	1 (1)	0 (0)	1 (3)	0.43
Latin American	3 (4)	2 (5)	1 (3)	1.00
East Asian	1 (1)	0 (0)	1 (3)	0.43
BMI (Kg/m^2)	25.9 ± 5.1	26.9 ± 5.3	23.9 ± 4.3	0.33
Medical history				
Hypertension	51 (66)	25 (57)	26 (79)	0.05
Cardio-vascular disease	38 (49)	20 (46)	18 (55)	0.49
Myocardial infarction	17 (22)	9 (21)	8 (24)	0.78
Heart failure	11 (14)	6 (14)	5 (15)	1.00
Arrythmias	15 (19)	9 (21)	6 (18)	1.00
Valvulopathies	4 (5)	3 (7)	1 (3)	0.63
Vascular disease	16 (21)	6 (14)	10 (30)	0.09
Cerebrovascular disease	8 (10)	7 (16)	1 (3)	0.13
Dementia	17 (22)	8 (18)	9 (27)	0.41
COPD	9 (12)	6 (14)	3 (9)	0.72
Asthma	1 (1)	1 (2)	0 (0)	1.00
Cancer	9 (12)	5 (11)	4 (12)	1.00
CKD	20 (26)	2 (5)	18 (55)	<0.001
Maintenance hemodialysis	4 (5)	0 (0)	4 (12)	0.03
Rheumatologic disease	2 (3)	1 (2)	1 (3)	1.00
Diabetes mellitus	25 (32)	10 (23)	15 (46)	0.05
Chronic liver disease	4 (5)	2 (5)	2 (6)	1.00
Age-adjusted CCI	3 {3–4}	3 {2–4}	3 {3–4}	0.58
(B)				
Characteristic	**All Patients**	**eGFR ≥ 45 mL/min**	**eGFR < 45 mL/min**	***p***
	(*n* = 77)	**(*n* = 44)**	**(*n* = 33)**	
Laboratory findings				
Hemoglobin (g/dL)	12.0 {11.2–13.3}	12.5 {11.4–13.8}	11.6 {10.7–12.6}	0.07
White blood cells ($\times 10^3$/uL)	7.00 {5.42–9.71}	6.69 {4.93–9.03}	7.58 {6.01–10.22}	0.13
Neutrophils ($\times 10^3$/uL)	4.93 {3.93–7.51}	4.67 {3.60–6.35}	5.87 {4.15–9.14}	0.05
Lymphocytes ($\times 10^3$/uL)	1.04 {0.64–1.34}	1.05 {0.64–1.49}	1.01 {0.67–1.21}	0.61
N/L ratio	5.16 {3.12–9.81}	4.28 {2.89–8.20}	6.73 {3.77–11.47}	0.07
Platelets ($\times 10^3$/uL)	204 {162–304}	222 {170–299}	192 {151–306}	0.52
C reactive protein (mg/L)	69 {27–99}	68 {27–102}	73 {27–98}	0.94
Procalcitonin (ng/mL)	0.13 {0.07–1.21}	0.11 {0.05–0.61}	0.21 {0.08–4.00}	0.315
Creatinine (mg/dL)	1.3 {1.0–2.2}	1.1 {0.7–1.2}	2.3 {1.6–3.8}	<0.001
Symptoms				
Fever	53 (69)	31 (71)	22 (67)	0.81
Anosmia/Disgeusia	3 (4)	2 (5)	1 (3)	1.00
Arthromyalgias	2 (3)	2 (5)	0 (0)	0.50
Cough	26 (34)	13 (30)	13 (39)	0.47
Dyspnoea	43 (56)	22 (50)	21 (64)	0.26
Abdominal pain	4 (5)	2 (5)	2 (6)	1.00
Nausea/vomiting	2 (3)	2 (5)	0 (0)	0.50
Diarrhea	3 (4)	2 (5)	1 (3)	1.00

Table 1. *Cont.*

Pneumonia on X-ray	67 (87)	38 (86)	29 (88)	1.00
P/F at admission	302 ± 99	297 ± 114	309 ± 77	0.96
SpO_2 at admission	96 {91–97}	96 {90–97}	95 {93–97}	0.79
ARDS	36 (47)	19 (43)	17 (52)	0.50
Time (days)				
Symptoms ⟶ admission	4 {2–8}	4 {2–9}	5 {3–7}	0.88
Symptoms ⟶ Discharge/death	18 {11–35}	18 {11–29}	17 {11–52}	0.51
Admission ⟶ Discharge/death	12 {6–25}	10 {6–20}	16 {7–35}	0.07
Therapy				
Lopinavir/Ritonavir	10 (13)	6 (14)	4 (12)	1.00
Hydroxychloroquine	57 (74)	32 (73)	25 (76)	0.80
Remdesevir	1 (1)	1 (2)	0 (0)	1.00
Steroids	17 (22)	7 (16)	10 (30)	0.17
Heparin	58 (75)	33 (75)	25 (76)	1.00
Biological	10 (13)	4 (9)	6 (18)	0.31
Need for intensive care	2 (3)	1 (2)	1 (3)	1.00
Need for ventilation	42 (55)	25 (57)	17 (52)	0.65
Survivors	42 (55)	25 (57)	17 (52)	0.65

Abbreviations: BMI, body mass index; CCI, Charlson comorbidity index; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease. Abbreviations: ARDS, acute respiratory distress syndrome; N/L ratio, neutrophil to lymphocyte ratio; P/F, ratio of arterial oxygen partial pressure to fractional inspired oxygen; SpO_2, peripheral capillary oxygen saturation.

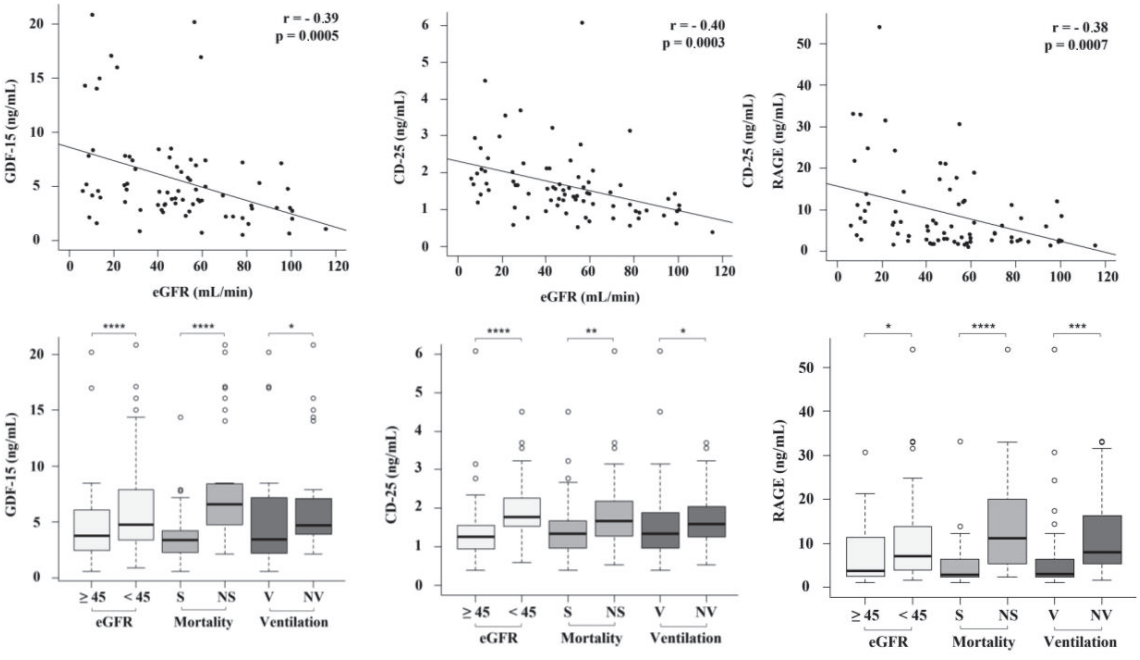


Figure 1. Association between GDF-15, CD-25 and RAGE with eGFR, ventilation and mortality. Abbreviations: eGFR, estimated glomerular filtration rate; GDF-15, growth and differentiation factor 15; RAGE, receptor for advanced glycation end products; S, survivors; NS, non survivors; NV, non-ventilated; V, ventilated; <45, eGFR < 45 mL/min/1.73 m^2; >45 mL/min, eGFR > 45 mL/min/1.73 m^2; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Table 2. Multivariate Cox regression model for survival in all patients, stratified by eGFR. Abbreviations: eGFR, estimated glomerular filtration rate; GDF-15, growth and differentiation factor 15; P/F, ratio of arterial oxygen partial pressure to fractional inspired oxygen.

Variable	All Patients		eGFR ≥ 45 mL/min		eGFR < 45 mL/min	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
eGFR < 45 mL/min	0.58 (0.28–1.19)	0.14	-	-	-	-
Age ≥ 75 years	2.79 (1.19–6.59)	0.02	2.31 (0.75–7.09)	0.14	3.61 (0.95–13.79)	0.06
Fever	3.75 (1.38–10.17)	0.009	2.83 (0.63–12.74)	0.18	4.78 (1.17–19.56)	0.03
Dyspnea	1.78 (0.81–3.94)	0.15	2.22 (0.76–6.51)	0.15	1.21 (0.37–3.95)	0.75
P/F < 300	1.67 (0.82–3.41)	0.16	1.82 (0.65–5.09)	0.25	1.64 (0.56–4.77)	0.36
GDF-15. quartiles	2.28 (1.53–3.39)	<0.001	1.99 (1.17–3.39)	0.01	2.53 (1.34–4.79)	0.004

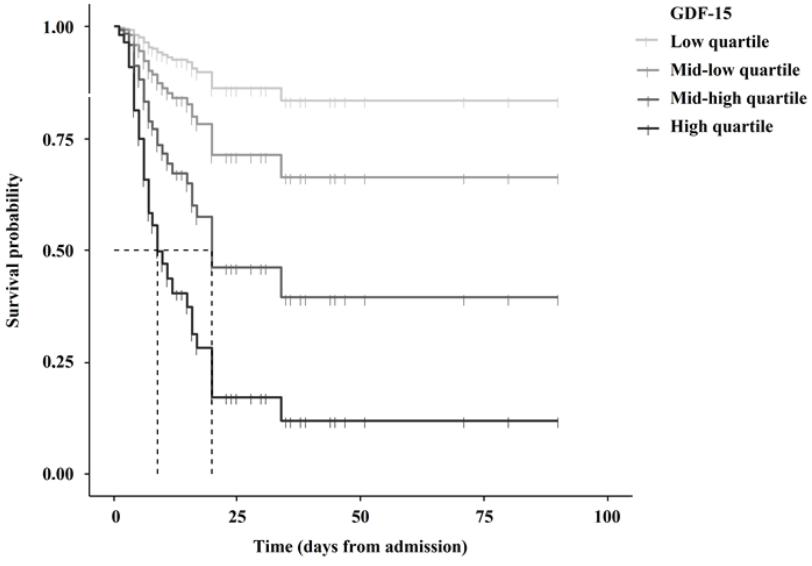
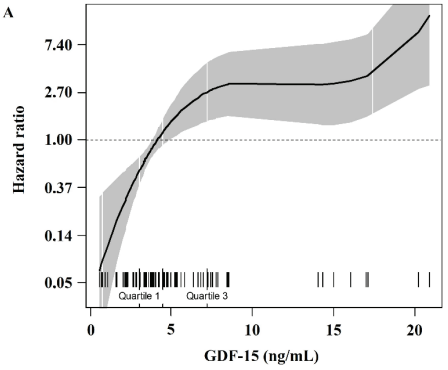


Figure 2. Association between GDF-15 quartiles and survival.

Univariate polynomial splines revealed a nonlinear association between GDF-15 and survival (Figure 3A). At univariate analysis, first and second GDF-15 quartiles were singularly associated with a lower mortality risk [HR 0.33 (0.12–0.95, 95% CI) and HR 0.14 (0.03–0.57, 95% CI), respectively] (Figure 3B). On the other hand, third and fourth GDF-15 quartiles were singularly associated with an increased mortality risk [HR 2.13 (1.09–4.31, 95% CI) and 3.4 (1.74–6.64, 95% CI), respectively]. Protective and harmful associations between GDF-15 and mortality were observed for circulating levels below and beyond the median (4.45 ng/mL), respectively, after adjustment for age, fever, dyspnea, P/F and eGFR (Figure 4A). Both the protective and the harmful associations between GDF-15 and mortality were descriptively more pronounced among patients with eGFR < 45 mL/min/1.73 m^2 (Figure 4B). The trend was confirmed after the exclusion of GDF-15 outliers (Figure 4C,D). In the subset of patients with eGFR < 45 mL/min/1.73 m^2 and known CKD reported in their medical history (18 patients, 10 of whom died), we found a linear and positive association between GDF-15 and mortality risk in the univariate Cox regression [HR 1.13 (1.01–1.27, 95% CI) for every 1 ng/mL increase in GDF-15 level], although the small sample size and low number of events did not allow us to investigate this association in multivariate models.



B

Mortality risk		
GDF-15 (ng/mL)	**HR (95% CI)**	***p***
< 3.02 (low)	0.33 (0.12-0.95)	0.039
3.02 – 4.45 (mid-low)	0.14 (0.03-0.57)	0.006
4.45 – 7.18 (mid-high)	2.17 (1.09-4.31)	0.028
> 7.18 (high)	3.40 (1.74-6.64)	<0.001

Figure 3. Univariate association between GDF-15 and mortality risk. (**A**) Cubic spline analysis revealing nonlinear association between GDF-15 and mortality risk: the solid line represents the HR according to GDF-15 level, the gray area represents the 95% CI, ticks in the lower part of the figure represent each observation. (**B**) Univariate association between GDF-15 quartiles and mortality risk; each quartile was compared with 3 other quartiles as a whole comparator.

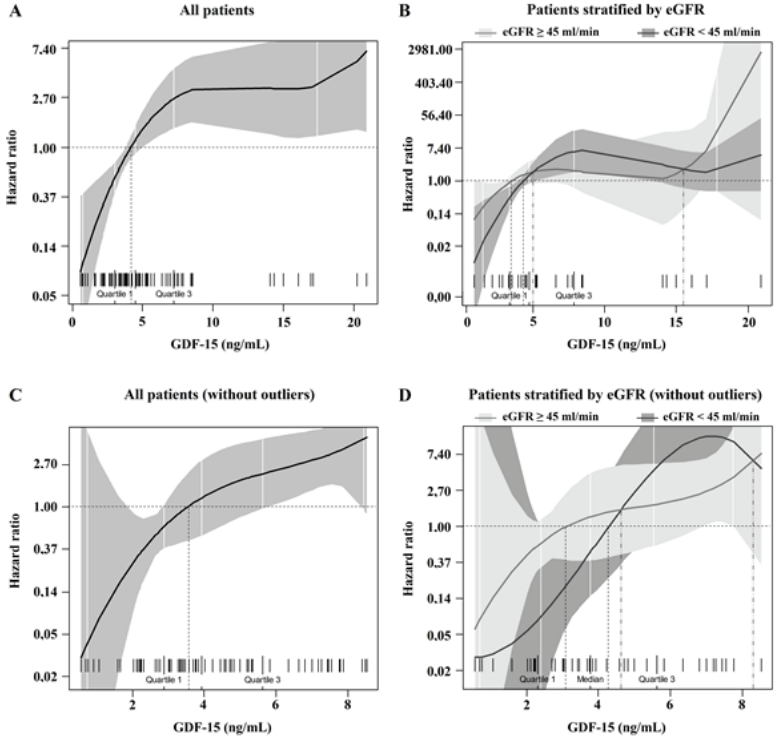


Figure 4. Non-linear association between continuous GDF-15 and mortality risk, adjusted for eGFR, age, fever, dyspnea and P/F. The solid line represents HR according to GDF-15 level. The colored area represents 95% CI, ticks in the lower part of the figure represent each observation. (**A**) All patients of the whole study cohort. (**B**) Patients stratified according to eGFR strata. (**C**) All patients, excluding outliers for GDF-15. (**D**) Patients stratified by eGFR, excluding outliers for GDF-15.

4. Discussion

Since the initial description of COVID-19 at the end of 2019 [17,18], over 250 million confirmed cases of COVID-19 have been reported to date, with more than 6 million deaths worldwide (World Health Organization, April 2022). While the majority of patients develop mild to moderate COVID-19, severe disease has been shown to occur in about 10–15% of infected individuals with a critical disease [19].

Several clinical and epidemiological factors have been associated with the development of severe COVID-19 and include older age, obesity and dysmetabolic co-morbidity, hypertension and immune depression [1–3]; however, a detailed profile of the pathogenetic pathways associated with the worst disease outcome is still largely elusive.

Among clinical factors associated with disease severity, CKD retains a high impact on the poor outcomes of COVID–19, underlining the importance of identifying strategies to prevent SARS-CoV-2 infection in CKD [20]. Undoubtedly, infections, sepsis and bacteremia represent major causes of morbidity and mortality in renal patients [21]. Moreover, infections in CKD patients cause a longer duration of hospitalization and a higher mortality rate from pneumonia [22,23]. Therefore, the choice of renal replacement treatment in advanced CKD patients, with techniques able to efficiently remove uremic toxins and reduce infection risk [24], remains important.

Indeed, a condition known as cytokine release syndrome has been described as a hallmark of aggressive COVID-19 that consists of the uncontrolled release of both pro- and anti-inflammatory cytokines, and that in turn is associated with tissue damage and dysfunctional and delayed immune response [25]. In the early phase of the pandemic, several biomarkers of the importance in dictating COVID-19 severity were first identified in case series of patients hospitalized with COVID-19 [2–4]. Furthermore, numerous inflammatory and cardiovascular biomarkers were assessed in association with outcome and were identified as particularly strong prognostic markers [26].

Because CKD has been associated with COVID-19 severity, we hereby sought to investigate the clinical role of several biomarkers in COVID-19 outcomes in the setting of CKD patients hospitalized for COVID-19.

Our bio-bank study of unselected, consecutive patients hospitalized with COVID-19 provides important insights to these associations, given that our design alleviates the risk of selection bias. Interestingly, GDF-15 was the only cytokine to be retained in the regression model for predicting mortality risk in patients with eGFR < 45 mL/min/1.73 m^2.

GDF-15 is a member of the TGF-beta superfamily and patrols immunotolerance during pregnancy, as witnessed by its high placental expression [27]. GDF-15 is secreted as a 25 kDa dimer [28] in several other organs, including the kidney, lungs, heart, brain, lymph nodes, bladder and prostate [27,29], where it is endowed with the potential to mediate immune response, inflammation tissue tolerance, energy homeostasis and body weight regulation [30]. Notably, multiple cell lines participate in GDF-15 synthesis as macrophages, endothelial cells, epithelial cells, vascular smooth muscle cells, adipocytes and cardiomyocytes [15]. Although GDF-15 expression is mainly quiescent outside of reproductive organs, it increases in several conditions of tissue damage triggered by inflammatory and oxidative stimuli [31–33]. GDF-15 has been postulated to enhance the ability of tissues to control the inflammatory insult through metabolic adaptation [34] as well as control immune cell infiltration [27]. However, GDF-15 was also associated with the severity and progression of acute-as-chronic diseases involving renal [11], cardiovascular [35,36], respiratory [13] and immune systems [37] in humans.

Renal expression of GDF-15 was documented in tubular cells, where it is hypothesized to enhance the protective response against renal damage [11,38]. However, observational studies reported a direct association between GDF-15 and an increased risk of incident CKD [39] and CKD progression [11]. GDF-15 resulted in an independent predictor of mortality in stage-3 CKD [40,41], as well as in dialysis patients [42]. Furthermore, GDF-15 emerged as a promising risk factor in cardiorenal syndrome. GDF-15 was associated with the risk of CHF in renal patients and predicted mortality in CHF [40,43]. Interestingly,

although GDF-15 is expressed in cardiomyocytes, the majority of circulating GDF-15 in patients with CHF was postulated to be of renal origin, secondary to kidney injury induced by venous congestion. Nonetheless, pulmonary epithelial and endothelial cells express GDF-15 under stimulation by hypoxia [44], cigarette smoking [45] and shear stress [46]. In vitro and animal models recently identified GDF-15 as an amplifier of lung inflammation during viral infections [47], therefore representing a major pathogenetic mechanism of susceptibility and disease severity in patients with already damaged airways.

COVID-19 represents a peculiar condition of systemic inflammation with multi-organ involvement, including pulmonary, cardiac and renal damage, which is often responsible for life threatening implications [48]. GDF-15 integrates information on cellular oxygenation, inflammatory response and cardio-renal dysfunction, which are all key mechanisms in COVID-19 pathophysiology, suggesting GDF-15 as an ideal candidate as a prognostic marker in COVID-19.

GDF-15 has been associated with poorer respiratory function, disease severity and mortality among hospitalized patients hospitalized due to SARS-CoV-2 infection [30,49,50]. However, the association of GDF-15 with disease severity and mortality is mainly unexplored in renal patients. To date, a unique study by Gisby et al. identified GDF-15 as a relevant biomarker of COVID-19 severity among 55 dialysis patients out of 203 tested molecules [51].

To our knowledge, this is the first study designed to investigate GDF-15 prognostic value in non-dialysis renal patients hospitalized for COVID-19. In agreement with the aforementioned data, higher GDF-15 levels were associated with disease severity and mortality independently from traditional risk factors. Nonetheless, the present study first documented a trend toward a protective association between GDF-15 < 4.45 ng/mL and survival. Notably, the strength of association was descriptively more pronounced in patients with basal eGFR < 45 mL/min/1.72 m^2.

GDF-15 was herein inversely associated with renal function, being significantly higher in patients with eGFR < 45 mL/min/1.72 m^2. The reasons for increased levels in the presence of reduced eGFR are debated. The low molecular weight hampers the plausibility of reduced clearance, suggesting increased renal synthesis and/or altered half-life as mechanisms responsible for its higher circulating levels in CKD [38]. Notably, present data suggests that the predictability of mortality risk in COVID-19 patients by GDF-15 could be stronger in the presence of eGFR < 45 mL/min/1.73 m^2.

In the present study, GDF-15 emerged as the only biomarker independently associated with poor outcomes in non-dialysis renal patients affected by SARS-CoV-2 infection, out of the other 19 molecules responsive to inflammatory stimuli. Notably, the panel of cytokines, chemokines and uremic toxins that were investigated was built according to the literature review on the more promising biomarkers dysregulated in the course of COVID-19 and/or renal disease [52–59].

Present data needs to be taken cautiously due to several limitations: small sample size, the absence of pre-specified power calculation, the monocentric design and the advanced age of the enrolled population, which limits the generalizability of the results. Furthermore, a discrepancy between median values of eGFR in the whole cohort and the low prevalence of reported CKD make the baseline eGFR more susceptible to acute disease in addition to chronic renal damage. No data were available for discriminating contribution of renal, cardiac and pulmonary synthesis to GDF-15 circulating levels. The generalizability of the study deserves caution. The population enrolled had several differences compared with those usually reported in COVID-19 studies due to older age, lower BMI, absence of an invasive ventilation requirement and a low rate of steroid administration. Eventual, but not ascertained, limited life support might have influenced the value of prognostic markers in the present study.

Taken together, these findings show that along with significant changes in inflammatory and cardiovascular biomarkers during SARS-CoV-2 infection, GDF-15 may represent a clinically useful risk stratification tool that provides important pathophysiological insights

and prognostic information in CKD patients hospitalized with COVID-19. Specially designed studies are advocated to explore GDF-15 as the ideal candidate prognostic marker in the context of inflammatory diseases with pulmonary and cardio-renal involvement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10123251/s1, Table S1: Association between all biomarkers and eGFR strata, mortality and need for ventilation; Table S2: Multivariate Cox regression model for survival predictability in all patients and in subgroups stratified according to eGFR, excluding outliers for GDF-15; Table S3: Multivariate Cox regression model for survival predictability in all patients and in subgroups stratified according to eGFR, excluding maintenance hemodialysis patients.

Author Contributions: A.G., P.C., M.C. and G.M. conceived the study. V.B., L.M., M.S., L.A., R.R., M.H. and V.Y. undertook data collection. L.M. and A.G. performed statistical analyses. P.C. wrote the initial draft. A.D.M., C.T., A.G., M.C. and G.M. critically revised the article. All authors have read and agreed to the published version of the manuscript.

Funding: Part of this study was supported by "Piano di Sostegno alla Ricerca—Linea 2–Dotazione annuale", by Department of Health Sciences University of Milan.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of ASST Santi Paolo and Carlo (no. 2020/ST/049).

Informed Consent Statement: All enrolled patients provided written informed consent according to the Ethical Committee of our institution (no. 2020/ST/049).

Data Availability Statement: Data are available on reasonable request.

Conflicts of Interest: A. Galassi received speaking honoraria from Amgen, Shire and Vifor Pharma. A. d'Arminio Monforte received speaking honoraria from Gilead, VIIV, Jannsen. M. Cozzolino reports received research funding from Abbvie, Baxter, Keryx and Shire; reports receiving honoraria from Abbvie, Amgen, Baxter, Shire and Vifor-Pharma; reports serving as a scientific advisor or member of and reports speakers bureau for Abbvie, Amgen, Keryx, Shire and Vifor Pharma. G. Marchetti received speaking honoraria from Gilead, VIIV, Jannsen. All other authors do not have disclosures to declare.

References

1. Petersen, E.; Koopmans, M.; Go, U.; Hamer, D.H.; Petrosillo, N.; Castelli, F.; Storgaard, M.; al Khalili, S.; Simonsen, L. Comparing SARS-CoV-2 with SARS-CoV and influenza pandemics. *Lancet Infect. Dis.* **2020**, *20*, e238–e244. [CrossRef] [PubMed]
2. Singh, A.K.; Gillies, C.L.; Singh, R.; Singh, A.; Chudasama, Y.; Coles, B.; Seidu, S.; Zaccardi, F.; Davies, M.J.; Khunti, K. Prevalence of co-morbidities and their association with mortality in patients with COVID-19: A systematic review and meta-analysis. *Diabetes Obes. Metab.* **2020**, *22*, 1915–1924. [CrossRef]
3. Nandy, K.; Salunke, A.; Pathak, S.K.; Nandy, K.; Salunke, A.; Pathak, S.K.; Pandey, A.; Doctor, C.; Puj, K.; Sharma, M.; et al. Coronavirus disease (COVID-19): A systematic review and meta-analysis to evaluate the impact of various comorbidities on serious events. *Diabetes Metab. Syndr.* **2020**, *14*, 1017–1025. [CrossRef] [PubMed]
4. Williamson, E.J.; Walker, A.J.; Bhaskaran, K.; Bacon, S.; Bates, C.; Morton, C.E.; Curtis, H.J.; Mehrkar, A.; Evans, D.; Inglesby, P.; et al. Factors associated with COVID-19-related death using OpenSAFELY. *Nature* **2020**, *584*, 430–436. [CrossRef]
5. Hsu, C.M.; Weiner, D.E.; Aweh, G.; Miskulin, D.C.; Manley, H.J.; Stewart, C.; Ladik, V.; Hosford, J.; Lacson, E.C.; Johnson, D.S.; et al. COVID-19 Among US Dialysis Patients: Risk Factors and Outcomes From a National Dialysis Provider. *Am. J. Kidney Dis.* **2021**, *77*, 748–756.e741. [CrossRef]
6. Flythe, J.E.; Assimon, M.M.; Tugman, M.J.; Chang, E.H.; Gupta, S.; Shah, J.; Sosa, M.A.; Renaghan, A.D.; Melamed, M.L.; Wilson, F.P.; et al. Characteristics and Outcomes of Individuals with Pre-existing Kidney Disease and COVID-19 Admitted to Intensive Care Units in the United States. *Am. J. Kidney Dis.* **2021**, *77*, 190–203.e191. [CrossRef]
7. Gur, E.; Levy, D.; Topaz, G.; Naser, R.; Wand, O.; Kitay-Cohen, Y.; Benchetrit, S.; Sarel, E.; Cohen-Hagai, K. Disease severity and renal outcomes of patients with chronic kidney disease infected with COVID-19. *Clin. Exp. Nephrol.* **2022**, *26*, 445–452. [CrossRef]
8. Ortiz, P.A.; Covic, A.; Fliser, D.; Fouque, D.; Goldsmith, D.; Kanbay, M.; Mallamaci, F.; Massy, Z.A.; Rossignol, P.; Vanholder, R.; et al. Epidemiology, contributors to, and clinical trials of mortality risk in chronic kidney failure. *Lancet* **2014**, *383*, 1831–1843. [CrossRef]
9. Go, A.S.; Yang, J.; Ackerson, L.M.; Lepper, K.; Shlipak, M.G.; Robbins, S.; Massie, B.M. Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: The Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) Study. *Circulation* **2006**, *113*, 2713–2723. [CrossRef]

10. Karki, R.; Sharma, B.R.; Tuladhar, S.; Williams, E.P.; Zalduondo, L.; Samir, P.; Zheng, M.; Sundaram, B.; Banoth, B.; Malireddi, R.K.S.; et al. Synergism of TNF-α and IFN-γ Triggers Inflammatory Cell Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes. *Cell* **2021**, *184*, 149–168.e117. [CrossRef]
11. Nair, V.; Robinson-Cohen, C.; Smith, M.R.; Bellovich, K.A.; Bhat, Z.Y.; Bobadilla, M.; Brosius, F.; De Boer, I.H.; Essioux, L.; Formentini, I.; et al. Growth Differentiation Factor-15 and Risk of CKD Progression. *J. Am. Soc. Nephrol.* **2017**, *28*, 2233–2240. [CrossRef] [PubMed]
12. Kempf, T.; von Haehling, S.; Peter, T.; Allhoff, T.; Cicoira, M.; Doehner, W.; Ponikowski, P.; Filippatos, G.S.; Rozentryt, P.; Drexler, H.; et al. Prognostic utility of growth differentiation factor-15 in patients with chronic heart failure. *J. Am. Coll. Cardiol.* **2007**, *50*, 1054–1060. [CrossRef] [PubMed]
13. Eisner, M.D.; Anthonisen, N.; Coultas, D.; Kuenzli, N.; Perez-Padilla, R.; Postma, D.; Romieu, I.; Silverman, E.K.; Balmes, J.R. An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **2010**, *182*, 693–718. [CrossRef]
14. Rochette, L.; Zeller, M.; Cottin, Y.; Vergely, C. GDF15: An emerging modulator of immunity and a strategy in COVID-19 in association with iron metabolism. *Trends Endocrinol. Metab.* **2021**, *32*, 875–889. [CrossRef] [PubMed]
15. Ahmed, D.S.; Isnard, S.; Berini, C.; Lin, J.; Routy, J.-P.; Royston, L. Coping With Stress: The Mitokine GDF-15 as a Biomarker of COVID-19 Severity. *Front. Immunol.* **2022**, *13*, 820350. [CrossRef]
16. Council, E.-E.; Group, E.W. Chronic kidney disease is a key risk factor for severe COVID-19: A call to action by the ERA-EDTA. *Nephrol. Dial. Transplant.* **2021**, *36*, 87–94. [CrossRef] [PubMed]
17. Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **2020**, *395*, 497–506. [CrossRef] [PubMed]
18. Zhu, N.; Zhang, D.; Wang, W.; Li, X.; Yang, B.; Song, J.; Zhao, X.; Huang, B.; Shi, W.; Lu, R.; et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **2020**, *382*, 727–733. [CrossRef]
19. Wu, Z.; McGoogan, J.M. Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. *JAMA* **2020**, *323*, 1239–1242. [CrossRef]
20. Jdiaa, S.S.; Mansour, R.; El Alayli, A.; Gautam, A.; Thomas, P.; Mustafa, R.A. COVID-19 and chronic kidney disease: An updated overview of reviews. *J. Nephrol.* **2022**, *35*, 69–85. [CrossRef]
21. Sarnak, M.J.; Jaber, B.L. Mortality caused by sepsis in patients with end-stage renal disease compared with the general population. *Kidney. Int.* **2000**, *58*, 1758–1764. [CrossRef] [PubMed]
22. Naqvi, S.B.; Collins, A.J. Infectious complications in chronic kidney disease. *Adv. Chronic. Kidney Dis.* **2006**, *13*, 199–204. [CrossRef] [PubMed]
23. Sarnak, M.J.; Jaber, B.L. Pulmonary infectious mortality among patients with end-stage renal disease. *Chest* **2001**, *120*, 1883–1887. [CrossRef] [PubMed]
24. Cozzolino, M.; Magagnoli, L.; Ciceri, P.; Conte, F.; Galassi, A. Effects of a medium cut-off (Theranova®) dialyser on haemodialysis patients: A prospective, cross-over study. *Clin. Kidney J.* **2021**, *14*, 382–389. [CrossRef] [PubMed]
25. Tincati, C.; Cannizzo, E.S.; Giacomelli, M.; Badolato, R.; Monforte, A.d.; Marchetti, G. Heightened Circulating Interferon-Inducible Chemokines, and Activated Pro-Cytolytic Th1-Cell Phenotype Features COVID-19 Aggravation in the Second Week of Illness. *Front. Immunol.* **2020**, *11*, 580987. [CrossRef]
26. Melo, A.K.G.; Milby, K.M.; Caparroz, A.L.M.A.; Pinto, A.C.P.N.; Santos, R.R.P.; Rocha, A.P.; Ferreira, G.A.; Souza, V.A.; Valadares, L.D.A.; Vieira, R.M.R.A.; et al. Biomarkers of cytokine storm as red flags for severe and fatal COVID-19 cases: A living systematic review and meta-analysis. *PLoS ONE* **2021**, *16*, e0253894. [CrossRef]
27. Wischhusen, J.; Melero, I.; Fridman, W.H. Growth/Differentiation Factor-15 (GDF-15): From Biomarker to Novel Targetable Immune Checkpoint. *Front. Immunol.* **2020**, *11*, 951. [CrossRef]
28. Unsicker, K.; Spittau, B.; Krieglstein, K. The multiple facets of the TGF-β family cytokine growth/differentiation factor-15/macrophage inhibitory cytokine-1. *Cytokine Growth Factor Rev.* **2013**, *24*, 373–384. [CrossRef]
29. Johann, K.; Kleinert, M.; Klaus, S. The Role of GDF15 as a Myomitokine. *Cells* **2021**, *10*, 2990. [CrossRef]
30. Teng, X.; Zhang, J.; Shi, Y.; Liu, Y.; Yang, Y.; He, J.; Luo, S.; Huang, Y.; Liu, Y.; Liu, D.; et al. Comprehensive Profiling of Inflammatory Factors Revealed That Growth Differentiation Factor-15 Is an Indicator of Disease Severity in COVID-19 Patients. *Front. Immunol.* **2021**, *12*, 662465. [CrossRef]
31. Zimmers, T.A.; Jin, X.; Hsiao, E.C.; McGrath, S.A.; Esquela, A.F.; Koniaris, L.G. Growth differentiation factor-15/macrophage inhibitory cytokine-1 induction after kidney and lung injury. *Shock* **2005**, *23*, 543–548. [PubMed]
32. Hsiao, E.C.; Koniaris, L.G.; Zimmers-Koniaris, T.; Sebald, S.M.; Huynh, T.V.; Lee, S.J. Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. *Mol. Cell Biol.* **2000**, *20*, 3742–3751. [CrossRef] [PubMed]
33. Ago, T.; Sadoshima, J. GDF15, a cardioprotective TGF-beta superfamily protein. *Circ. Res.* **2006**, *98*, 294–297. [CrossRef] [PubMed]
34. Luan, H.H.; Wang, A.; Hilliard, B.K.; Carvalho, F.; Rosen, C.E.; Ahasic, A.M.; Herzog, E.L.; Kang, I.; Pisani, M.A.; Yu, S.; et al. GDF15 Is an Inflammation-Induced Central Mediator of Tissue Tolerance. *Cell* **2019**, *178*, 1231–1244.e1211. [CrossRef] [PubMed]

35. Wang, T.J.; Wollert, K.C.; Larson, M.G.; Coglianese, E.; McCabe, E.L.; Cheng, S.; Ho, J.E.; Fradley, M.G.; Ghorbani, A.; Xanthakis, V.; et al. Prognostic utility of novel biomarkers of cardiovascular stress: The Framingham Heart Study. *Circulation* **2012**, *126*, 1596–1604. [CrossRef]
36. Rohatgi, A.; Patel, P.; Das, S.R.; Ayers, C.R.; Khera, A.; Martinez-Rumayor, A.; Berry, J.D.; McGuire, D.K.; de Lemos, J. Association of growth differentiation factor-15 with coronary atherosclerosis and mortality in a young, multiethnic population: Observations from the Dallas Heart Study. *Clin. Chem.* **2012**, *58*, 172–182. [CrossRef]
37. Buendgens, L.; Yagmur, E.; Bruensing, J.; Herbers, U.; Baeck, C.; Trautwein, C.; Koch, A.; Tacke, F. Growth Differentiation Factor-15 Is a Predictor of Mortality in Critically Ill Patients with Sepsis. *Dis. Markers* **2017**, *2017*, 5271203. [CrossRef]
38. Perez-Gomez, M.V.; Pizarro-Sanchez, S.; Gracia-Iguacel, C.; Cano, S.; Cannata-Ortiz, P.; Sanchez-Rodriguez, J.; Sanz, A.B.; Sanchez-Niño, M.D.; Ortiz, A. Urinary Growth Differentiation Factor-15 (GDF15) levels as a biomarker of adverse outcomes and biopsy findings in chronic kidney disease. *J. Nephrol.* **2021**, *34*, 1819–1832. [CrossRef]
39. Bao, X.; Xu, B.; Borné, Y.; Orho-Melander, M.; Melander, O.; Nilsson, J.; Christensson, A.; Engström, G. Growth differentiation factor-15 and incident chronic kidney disease: A population-based cohort study. *BMC Nephrol.* **2021**, *22*, 351. [CrossRef]
40. Tuegel, C.; Katz, R.; Alam, M.; Bhat, Z.; Bellovich, K.; de Boer, I.; Brosius, F.; Gadegbeku, C.; Gipson, D.; Hawkins, J.; et al. GDF-15, Galectin 3, Soluble ST2, and Risk of Mortality and Cardiovascular Events in CKD. *Am. J. Kidney Dis.* **2018**, *72*, 519–528. [CrossRef]
41. Wang, K.; Zelnick, L.R.; Anderson, A.; Cohen, J.; Dobre, M.; Deo, R.; Feldman, H.; Go, A.; Hsu, J.; Jaar, B.; et al. Cardiac Biomarkers and Risk of Mortality in CKD (the CRIC Study). *Kidney Int. Rep.* **2020**, *5*, 2002–2012. [CrossRef] [PubMed]
42. Breit, S.N.; Carrero, J.J.; Tsai, V.W.; Yagoutifam, N.; Luo, W.; Kuffner, T.; Bauskin, A.R.; Wu, L.; Jiang, L.; Barany, P.; et al. Macrophage inhibitory cytokine-1 (MIC-1/GDF15) and mortality in end-stage renal disease. *Nephrol. Dial. Transpl.* **2012**, *27*, 70–75. [CrossRef] [PubMed]
43. Benes, J.; Kotrc, M.; Wohlfahrt, P.; Conrad, M.J.; Franekova, J.; Jabor, A.; Lupinek, P.; Kautzner, J.; Melenovsky, V.; Jarolim, P. The Role of GDF-15 in Heart Failure Patients with Chronic Kidney Disease. *Can. J. Cardiol.* **2019**, *35*, 462–470. [CrossRef] [PubMed]
44. Al-Mudares, F.; Reddick, S.; Ren, J.; Venkatesh, A.; Zhao, C.; Lingappan, K. Role of Growth Differentiation Factor 15 in Lung Disease and Senescence: Potential Role Across the Lifespan. *Front. Med.* **2020**, *7*, 594137. [CrossRef]
45. Verhamme, F.; Seys, L.J.M.; De Smet, E.; Provoost, S.; Janssens, W.; Elewaut, D.; Joos, G.F.; Brusselle, G.; Bracke, K. Elevated GDF-15 contributes to pulmonary inflammation upon cigarette smoke exposure. *Mucosal. Immunol.* **2017**, *10*, 1400–1411. [CrossRef]
46. Nickel, N.; Jonigk, D.; Kempf, T.; Bockmeyer, C.L.; Maegel, L.; Rische, J.; Laenger, F.; Lehmann, U.; Sauer, C.; Greer, M.; et al. GDF-15 is abundantly expressed in plexiform lesions in patients with pulmonary arterial hypertension and affects proliferation and apoptosis of pulmonary endothelial cells. *Respir. Res.* **2011**, *12*, 62. [CrossRef]
47. Wu, Q.; Jiang, D.; Schaefer, N.R.; Harmacek, L.; O'Connor, B.P.; Eling, T.E.; Eickelberg, O.; Chu, H.W. Overproduction of growth differentiation factor 15 promotes human rhinovirus infection and virus-induced inflammation in the lung. *Am. J. Physiol. Lung. Cell Mol. Physiol.* **2018**, *314*, L514–L527. [CrossRef]
48. Guven, G.; Ince, C.; Topeli, A.; Caliskan, K. Cardio-Pulmonary-Renal Consequences of Severe COVID-19. *Cardiorenal. Med.* **2021**, *11*, 133–139. [CrossRef]
49. Myhre, P.L.; Prebensen, C.; Strand, H.; Røysland, R.; Jonassen, C.M.; Rangberg, A.; Sørensen, V.; Søvik, S.; Røsjø, H.; Svensson, M.; et al. Growth Differentiation Factor 15 Provides Prognostic Information Superior to Established Cardiovascular and Inflammatory Biomarkers in Unselected Patients Hospitalized With COVID-19. *Circulation* **2020**, *142*, 2128–2137. [CrossRef]
50. Alserawan, L.; Peñacoba, P.; Orozco Echevarría, S.E.; Castillo, D.; Ortiz, E.; Martínez-Martínez, L.; Naranjo, E.M.; Domingo, P.; Castellví, I.; Juárez, C.; et al. Growth Differentiation Factor 15 (GDF-15): A Novel Biomarker Associated with Poorer Respiratory Function in COVID-19. *Diagnostics* **2021**, *11*, 1998. [CrossRef]
51. Gisby, J.; Clarke, C.L.; Medjeral-Thomas, N.; Malik, T.H.; Papadaki, A.; Mortimer, P.M.; Buang, N.B.; Lewis, S.; Pereira, M.; Toulza, F.; et al. Longitudinal proteomic profiling of dialysis patients with COVID-19 reveals markers of severity and predictors of death. *Elife* **2021**, *10*, e64827. [CrossRef] [PubMed]
52. Ciceri, P.; Bono, V.; Magagnoli, L.; Sala, M.; d'Arminio Monforte, A.; Galassi, A.; Barassi, A.; Marchetti, G.; Cozzolino, M. Cytokine and chemokine retention profile in COVID-19 patients with chronic kidney disease. *Toxins* **2022**, *14*, 673. [CrossRef] [PubMed]
53. Prietl, B.; Odler, B.; Kirsch, A.H.; Artinger, K.; Eigner, M.; Schmaldienst, S.; Pfeifer, V.; Stanzer, S.; Eberl, A.; Raml, R.; et al. Chronic inflammation might Protect hemodialysis patients from severe COVID-19. *Front. Immunol.* **2022**, *13*, 821818. [CrossRef] [PubMed]
54. Chiappalupi, S.; Salvadori, L.; Donato, R.; Riuzzi, F.; Sorci, G. Hyperactivated RAGE in comorbidities as a risk factor for Severe COVID-19-The role of RAGE-RAS crosstalk. *Biomolecules* **2021**, *11*, 876. [CrossRef]
55. Gohda, T.; Murakoshi, M.; Suzuki, Y.; Hiki, M.; Naito, T.; Takahashi, K.; Tabe, K. Circulating tumor necrosis factor receptors are associated with mortality and disease severity in COVID-19 patients. *PLoS ONE* **2022**, *17*, e0275745. [CrossRef] [PubMed]
56. Pine, A.B.; Meizlish, M.L.; Goshua, G.; Chang, C.H.; Zhang, H.; Bishai, J.; Bahel, P.; Patel, A.; Gbyli, R.; Kwan, J.M.; et al. Circulating markers of angiogenesis and endotheliopathy in COVID-19. *Pulm. Circ.* **2020**, *10*, 2045894020966547. [CrossRef]
57. Thomas, T.; Stefanoni, D.; Reisz, J.A.; Nemkov, T.; Bertolone, L.; Francis, R.O.; Hudson, K.E.; Zimring, J.C.; Hansen, K.C.; Hod, E.A.; et al. COVID-19 infection alters kynurenine and fatty acid metabolism, correlating with IL-6 levels and renal status. *JCI Insight* **2020**, *5*, e140327. [CrossRef]

58. Wang, K.; Long, Q.X.; Deng, H.J.; Hu, J.; Gao, Q.Z.; Zhang, G.J.; He, C.L.; Huang, L.Y.; Hu, J.L.; Chen, J.; et al. Longitudinal dynamics of the neutralizing antibody response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. *Clin. Infect. Dis.* **2021**, *73*, e531–e539. [CrossRef]
59. Takeshita, H.; Yamamoto, K. Tryptophan Metabolism and COVID-19-induced skeletal muscle damage: Is ACE2 a key regulator? *Front. Nutr.* **2022**, *9*, 868845. [CrossRef]

biomedicines

MDPI

Article

Severe COVID-19 Illness and α1-Antitrypsin Deficiency: COVID-AATD Study

Juan Luis Rodríguez Hermosa [1,2], Gianna Vargas Centanaro [1,2], María Estela González Castro [3], Marc Miravitlles [4], Lourdes Lázaro-Asegurado [5], Beatriz María Jiménez-Rodríguez [6], Rosanel Amaro Rodríguez [7], Rosaly Moreno Méndez [8], María Torres-Duran [9], José María Hernández-Pérez [10], Ana María Humanes-Navarro [11] and Myriam Calle Rubio [1,2,*]

1 Pulmonology Department, Research Institute of Hospital Clínico San Carlos (IdISSC), 28040 Madrid, Spain
2 Department of Medicine, Faculty of Medicine, Complutense University of Madrid, 28040 Madrid, Spain
3 Pneumology Department, Hospital Universitario Torrecardenas, 04009 Almería, Spain
4 Pneumology Department, Hospital Universitari Vall d'Hebron, Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron Barcelona Hospital Campus, 08035 Barcelona, Spain
5 Pneumology Department, Complejo Asistencial Universitario de Burgos, 09006 Burgos, Spain
6 Pneumology Department, Hospital Virgen de las Nieves, 18014 Granada, Spain
7 Pneumology Department, Hospital Clinic, 08036 Barcelona, Spain
8 Pneumology Department, Hospital La Plana, 12002 Castellon, Spain
9 Pneumology Department, Hospital Álvaro Cunqueiro, NeumoVigo I+i Research Group, IIS Galicia Sur, 36213 Vigo, Spain
10 Pneumology Department, Hospital Universitario Nuestra Señora de La Candelaria, 38010 Santa Cruz de Tenerife, Spain
11 Preventive Medicine Clinical Management Unit, Research Institute of Hospital Clínico San Carlos (IdISSC), 28040 Madrid, Spain
* Correspondence: mcallerubio@gmail.com

Citation: Rodríguez Hermosa, J.L.; Vargas Centanaro, G.; González Castro, M.E.; Miravitlles, M.; Lázaro-Asegurado, L.; Jiménez-Rodríguez, B.M.; Rodríguez, R.A.; Moreno Méndez, R.; Torres-Duran, M.; Hernández-Pérez, J.M.; et al. Severe COVID-19 Illness and α1-Antitrypsin Deficiency: COVID-AATD Study. *Biomedicines* **2023**, *11*, 516. https://doi.org/10.3390/biomedicines11020516

Academic Editor: César Fernández De Las Peñas

Received: 24 January 2023
Revised: 4 February 2023
Accepted: 8 February 2023
Published: 10 February 2023

Abstract: Background: Epidemiologic studies have reported that the geographical distribution of the prevalence of allelic variants of serine protein inhibitor-A1 (SERPINA1) and severe cases of COVID-19 were similar. Methods: A multicenter, cross-sectional, observational study to evaluate the frequency of alpha-1 antitrypsin deficiency (AATD) in patients with COVID-19 and whether it was associated with having suffered severe COVID-19. Results: 2022 patients who had laboratory-confirmed SARS-CoV-2 infection. Mutations associated with AATD were more frequent in severe COVID versus non-severe (23% vs. 18.8%, $p = 0.022$). The frequency of Pi*Z was 37.8/1000 in severe COVID versus 17.5/1000 in non-severe, $p = 0.001$. Having an A1AT level below 116 was more frequent in severe COVID versus non-severe (29.5% vs. 23.1, $p = 0.003$). Factors associated with a higher likelihood of severe COVID-19 were being male, older, smoking, age-associated comorbidities, and having an A1AT level below 116 mg/dL [OR 1.398, $p = 0.003$], and a variant of the SERPINA1 gene that could affect A1AT protein [OR 1.294, $p = 0.022$]. Conclusions: These observations suggest that patients with AATD should be considered at a higher risk of developing severe COVID-19. Further studies are needed on the role of A1AT in the prognosis of SARS-CoV-2 infection and its possible therapeutic role.

Keywords: SARS-CoV-2 infection; severe COVID-19; alpha-1 antitrypsin deficiency; genetic mutations

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a pandemic, with more than 6 million deaths [1]. There are remarkably different infection and mortality rates for SARS-CoV-2 between different countries [2]. Moreover, there are remarkably interindividual differences in the clinical severity of coronavirus disease 2019 (COVID-19) that cannot be completely explained by environmental factors, comorbidities, and age-related fragility [3]. On the basis of these observations and the susceptibility

of hosts, it could be argued that genetic differences among populations, ethnicities, and individuals may contribute to the different epidemiological and clinical manifestations of COVID-19 [4]. Recent studies have investigated genetic susceptibility to SARS-CoV-2 and reported that approximately 20% of life-threatening COVID-19 cases are associated with genetic errors and gene loci, most of which are involved in immune signaling pathways [5].

In-hospital COVID-19 patient, studies have described a proinflammatory syndrome with a disproportionately high rate of progression to acute respiratory distress syndrome [2]. Recent data indicates that the COVID-19 cytokinemia is distinct in critical care presentations, showing marked differences in the balance between proinflammatory and anti-inflammatory cytokines and a blunted alpha-1 antitrypsin (A1AT) acute phase response. Cytokine ratios, such as high IL-6:A1AT levels, are related to worse prognosis in COVID-19 patients [2].

Alpha-1 antitrypsin deficiency (AATD) is the most common inherited disorder in adults; it is often under-diagnosed [6] and characterized by reduced plasma levels or the abnormal functioning of A1AT, a human blood serine protease inhibitor, which is encoded by the serine protein inhibitor-A1 (SERPINA1) gene. Recent studies confirmed a correlation between the COVID-19 pandemic and the prevalence of AATD in the same geographical areas [7].

A1AT is a tissue protector, as well as an antiviral and anti-inflammatory molecule. Indeed, A1AT has several biological functions that may antagonize SARS-CoV-2 infection and pathophysiologic processes resulting in cellular entry. Recent studies have demonstrated that A1AT is an inhibitor of SARS-CoV-2 infection and two of the most important proteases in the pathophysiology of COVID-19: transmembrane serine protease 2 and the disintegrin and metalloproteinase 17, as was well as an inhibitor of inflammatory molecules, such as IL-8, TNF-α, and neutrophil elastase [8,9]. Other potential A1AT protective mechanisms of action are the inhibitory effect on thrombin and delayed thrombus formation [10] and decreased oxidative stress, inflammation, and cell wall deterioration [11].

Therefore, we focused on the possible role of AATD as a risk factor for severe COVID-19 progression. A poor prognosis for COVID-19 patients may be related to A1AT levels. In our study, we examined the presence of genetic mutations associated with AATD and A1AT levels in patients who had suffered a SARS-CoV-2 infection in order to assess whether AATD was associated with having suffered severe COVID-19.

2. Materials and Methods

COVID-AATD is a multicenter, cross-sectional, observational study conducted from 1 May 2021 to 1 September 2022. The sample population was adults who had laboratory confirmed SARS-CoV-2 infection and were treated by a pneumology department. Participants were enrolled consecutively at 9 centers in the inpatient ward or in follow-up consultation after discharge. There were no exclusion criteria, except for patients' or families' explicit refusal to participate. The study was performed according to the Declaration of Helsinki and its amendments. All patients gave written informed consent. The study was approved by the Research Ethics Committee at Hospital Clínico San Carlos, Madrid, Spain (internal code 20/809-E). The personal data of the patients was kept under strict confidentiality in compliance with the provisions of Spanish Organic Law 3/2018, of December 5, on the Protection of Personal Data and Guarantee of Digital Rights (LOPDGDD) and its development regulations, and in accordance with the provisions of Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016, regarding the protection of natural persons with regard to the processing of personal data and the free circulation of these data.

A retrospective review was performed through the analysis of electronic medical records where SARS-CoV-2 infection clinical data were collected. Patients were defined as suffering severe COVID-19 if they had been treated with high-flow nasal cannula (HFNC) oxygenation, non-invasive ventilation therapy, or were admitted to the intensive care unit

at any stage of the disease according to the WHO Clinical Progression Scale [12], or if they died as a result of COVID-19.

The data collected during the only visit were concurrent. The information collected was clinical data (demographic data, smoking status, comorbidities). Allele-specific genotyping testing was carried out in all patients using the Progenika A1AT Genotyping Test. The test allows the identification of the 14 most frequent deficiency variants of the SERPINA1 gene: PI*S, PI*Z, PI*I, PI*Mprocida, PI*Mmalton, PI*Siiyama, PI*Q0granite falls, PI*Q0west, PI*Q0bellingham, PI*F, PI*Plowell, PI*Q0mattawa, PI*Q0clayton, and PI*Mheerlen. SERPINA1 gene sequencing was performed in the cases where none of the 14 mutations were found and the A1AT serum level was <60 mg/dL. The test is CE marked and United States Food and Drug Administration approved. The test is intended for use with genomic DNA extracted from human whole blood samples collected in K3-ethylenediaminetetraacetic acid (EDTA) tubes or as dried blood spots (DBS), or from human buccal swab samples [13]. The biological samples related to the study were numbered with a code to guarantee the confidentiality of the sample and the associated clinical data. There were no data in the database that could be used to identify patients. The patients signed a written informed consent authorizing the genetic study to be carried out according to Spanish legislation.

In the clinical stability phase, serum A1AT levels were analyzed using nephelometric and C-reactive protein (CRP) in plasma as a potential confounder by the immunonephelometry method. Although the lower limit of normal A1AT by nephelometry is 90 mg/dL, the use of a higher than normal cut-off value was established as a threshold value to study the possible presence of a deficient allele. The variability of A1AT levels has been described for different AATD genotypes and how it may be influenced by increased systemic inflammation [14].

Statistical Analysis

Descriptive statistics are reported as mean (standard deviation [SD]) or median (interquartile range [IQR]). Differences between the non-severe and severe COVID-19 groups were analyzed for statistical significance using the chi-square or Fisher's exact test for categorical variables and the two-sample *t*-test or Wilcoxon rank sum test for continuous variables, as applicable. Adjustment variables (patient characteristics, genotyping test, and serum A1AT levels) with a p-value < 0.05 in the univariate analysis were included in the simple logistic regression analysis. Statistical significance was assumed as $p < 0.05$. All analyses were performed with Stata software version 17 (Stata Corp LLC, College Station, TX, USA). The study size was determined by the number of patients referred to the follow-up clinic in a pneumology department during the enrolment period.

3. Results

3.1. Characteristics of the Study Population

In total, 2022 patients were included in the analysis. Table 1 describes the sociodemographic and clinical characteristics of the enrolled patients. An amount of 43.2% had severe COVID-19 infection and six (0.3%) deaths occurred. The mean (SD) age of the overall COVID-19 cohort was 60.3 ± 14 years; 59.9% were men and 45.6% of patients were current or former smokers. Comorbidities were common in the study population.

A1AT serum levels were available in 1691 (83.6%) cases, with a mean value of 132.1 (28.8) mg/dL. There were 390 (19.9%) carrying frequent mutations (S or Z), and 14 (0.7%) carrying rare alleles. In total, 67 samples were not processed due to the poor quality of the sample or due to errors recording the identification code on the web. The prevalence of the frequent allele combinations in this selected population was as follows: MS 16.3%, MZ 2.1%, SS 1.1%, SZ 0.3%, and ZZ 0.2%. Considered globally, 2.5% were Z carriers and 17.6% S carriers.

Table 1. Characteristics of the study population.

Enrolled Patients	**n = 2022**
Age, years, median (IQR)	61.2 (51–71)
Gender (male), n %	59.9
▪ Smoking status, (%)	
Current smoker	5.9
Former smoker	39.7
Never smoked	54.4
▪ IPA, median (IQR)	25 (12–40)
Body mass index, kg/m^2, median (IQR)	29.2 (26.1–33)
Pulmonary comorbidity, n (%)	427 (21.1)
▪ COPD, n (%)	122 (6)
▪ Asthma, n (%)	186 (9.2)
▪ AOS, n (%)	151 (7.5)
▪ ILD, n (%)	23 (1.1)
Home oxygen therapy, n (%)	25 (1.2)
Comorbidities	
▪ Diabetes mellitus, n (%)	385 (19.1)
▪ Hypertension, n (%)	795 (39.4)
▪ Dyslipemia, n (%)	677 (33.5)
▪ Coronary artery disease, n (%)	226 (11.2)
▪ Nephropatia, n (%)	90 (4.5)
▪ Hepatopathy, n (%)	80 (4)
▪ Immunosuppression, n (%)	63 (3.1)
▪ History of cancer, n (%)	147 (7.3)
Bilateral pneumonia, n (%)	1533 (76.3)
Inpatient, n (%)	1592 (78.8)
Hospitalization day, median (IQR)	9 (3–17)
ICU/UCIR, n (%)	507 (25.1)
High flow oxygen or NIV/CPAP, n (%)	872 (43.2)
Deaths, n (%)	6 (0.3)
A1AT mg/dL, median (IQR)	129 (116–148)
▪ $\geq$116, (%)	74
▪ <116 y $\geq$ 60, (%)	25.6
▪ <60, (%)	0.4
CRP level, m (SD)	0.90 (0.29–4.42)
Genotyping Test, n (%)	
▪ Absence mutations	1551 (79.3)
▪ MI	6 (0.3)
▪ MS	318 (16.3)
▪ MZ	41 (2.1)
▪ MM malton	2 (0.1)
▪ MP lowell	5 (0.3)
▪ MM procida	1 (0.1)
▪ SS	22 (1.1)
▪ SZ	5 (0.3)
▪ ZZ	4 (0.2)

Abbreviations: IPA: Pack-years; COPD: chronic obstructive pulmonary disease, ILD: diffuse interstitial lung disease, AOS: sleep apnea syndrome, ICU: intensive care unit, UCRI: intermediate respiratory care unit; NIV/CPAP: non-invasive ventilation/continuous positive airway pressure; A1AT: alpha-1 antitrypsin; CRP: C-reactive protein.

3.2. Characteristics According to the Presence of Genetic Mutations Associated with AATD

Patients with variants of the SERPINA1 gene that could affect A1AT protein activity or expression were older than patients without mutations (mean [SD] age: 61.8 [14.1] versus 60 [13.9] years; $p = 0.021$) and current smokers were more prevalent (7.9% versus 5.3%; $p = 0.004$). There were no differences in respiratory or non-respiratory comorbidities, Table 2. The frequency of severe COVID was also higher in patients positive for A1AT genotyping testing (48.8% vs. 42.4%; $p = 0.022$). A1AT serum levels were significantly lower in patients with mutations associated with AATD (106.3 [24] versus 138.8 [25.8]; $p < 0.001$).

Table 2. Characteristics of enrolled patients according A1AT genotyping test.

Enrolled Patients with Genotyping Test n = 1955	**Absence Mutations n = 1551 (79.3%)**	**Presence Mutations n = 404 (20.7%)**	***p***
Age, years, m (SD)	60 (13.9)	61.8 (14.1)	0.021
Gender (male), n %	921 (59.4)	255 (63.1)	0.176
▪ Smoking status, n (%)			
Current smoker	82 (5.3)	32 (7.9)	0.044
Former smoker	596 (38.7)	173 (43.3)	0.099
▪ IPA, median (IQR)	22.7 (10–40)	25 (15–40)	0.039
Body mass index, kg/m^2, median (IQR)	29.2 (26.1–33)	29.39 (26–33.3)	0.618
▪ Pulmonary comorbidity, n (%)	333 (21.5)	84 (20.8)	0.785
COPD, n (%)	91 (5.9)	27 (6.7)	0.534
Asthma, n (%)	144 (9.3)	35 (8.7)	0.702
ILD, n (%)	17 (1.1)	4 (1)	1.000
Home oxygen therapy, n (%)	16 (1)	9 (2.2)	0.056
▪ Comorbidities, n (%)			
Diabetes mellitus	291 (18.8)	84 (20.9)	0.333
Hypertension	611 (39.4)	165 (40.9)	0.578
Dyslipemia	495 (32)	159 (39.5)	0.005
Coronary artery disease	179 (11.6)	45 (11.2)	0.839
Nephropatia	69 (4.5)	19 (4.7)	0.789
Hepatopathy	66 (4.3)	14 (3.5)	0.461
Immunosuppression	41 (2.6)	18 (4.5)	0.057
History of cancer	105 (6.8)	36 (8.9)	0.136
Bilateral pneumonia, n (%)	1165 (75.6)	328 (81.8)	0.009
Inpatient, n (%)	1211 (78,1)	342 (84,9)	0.003
Hospitalization day, m (SD)	8 (3–16)	11 (4–20)	<0.001
ICU/UCIR, n (%)	368 (23.7)	120 (29.8)	0.013
High flow oxygen or NIV/CPAP, n (%)	649 (41.9)	193 (48)	0.028
Severe COVID-19, n (%)	656 (42.4)	196 (48.8)	0.022
Deaths, n (%)	4 (0.3)	2 (0.5)	0.610
A1AT mg/dL, m (SD)	138.8 (25.8)	106.3 (24)	<0.001
▪ ≥116, n (%)	1153 (86.2)	91 (26.6)	<0.001
▪ <116, n (%)	184 (13.8)	251 (73.4)	

Abbreviations: IPA: pack-years; COPD: chronic obstructive pulmonary disease, ILD: diffuse interstitial lung disease, AOS: sleep apnea syndrome, ICU: intensive care unit, UCRI: intermediate respiratory care unit; NIV/CPAP: non-invasive ventilation/continuous positive airway pressure; A1AT: alpha-1 antitrypsin; CRP: C-reactive protein.

3.3. Characteristics According to A1AT Levels

There were 440 (26%) patients with A1AT serum levels below 116 mg/dL, Table 3. The frequency of severe COVID was higher in patients with A1AT serum levels below 116 mg/dL compared with those above or equal to 116 mg/dL (51.9% versus 43.9%, $p = 0.003$), Figure 1.

Table 3. Characteristics of patients, according A1AT level (≥116 mg/dL versus <116 mg/dL).

Enrolled Patients with A1AT Level n = 1691	**A1AT ≥ 116 mg/dL n = 1251 (74%)**	**A1AT < 116 mg/dL n = 440 (26%)**	*p*
Age, years, m (SD)	61 (14)	60.2 (13.2)	0.288
Gender (male), n %	744 (59.5)	279 (63.4)	0.151
▪ Smoking status, n (%)			
Current smoker	62 (5)	33 (7.5)	0.047
Former smoker	488 (39.2)	185 (42)	0.289
▪ IPA, median (IQR)	25 (11.4–40)	25 (15–40)	0.464
Body mass index, kg/m^2, median (IQR)	29.3 (26.2–33.2)	29.6 (26.2–33.2)	0.455
Pulmonary comorbidity, n (%)	268 (21.4)	91 (20.7)	0.744
▪ COPD, n (%)	78 (6.2)	30 (6.8)	0.670
▪ Asthma, n (%)	117 (9.4)	35 (8)	0.380
▪ ILD, n (%)	10 (0.8)	6 (1.4)	0.389
Home oxygen therapy, n (%)	15 (1.2)	10 (2.3)	0.109
Comorbidities, n (%)			
▪ Diabetes_mellitus	251 (20.1)	85 (19.3)	0.731
▪ Hypertension	535 (42.8)	158 (35.9)	0.011
▪ Dyslipemia	435 (34.9)	160 (36.4)	0.569
▪ Coronary artery disease	159 (12.7)	38 (8.7)	0.022
▪ Nephropatia	56 (4.5)	16 (3.6)	0.449
▪ Hepatopathy	55 (4.1)	17 (3.9)	0.622
▪ Immunosuppression	28 (2.2)	12 (2.7)	0.561
▪ History of cancer	91 (7.3)	27 (6.1)	0.418
Bilateral pneumonia, n (%)	926 (74.4)	362 (83)	<0.001
Inpatient, n (%)	973 (77.8)	372 (84.5)	0.003
Hospitalization day, m (SD)	8 (3–16.2)	11 (5–19.7)	<0.001
ICU/UCIR, n (%)	296 (23.7)	135 (30.7)	0.004
High flow oxygen or NIV/CPAP, n (%)	539 (43.2)	224 (51)	0.005
Deaths, n (%)	4 (0.3)	2 (0.5)	0.654
Genotyping Test, n (%)			
▪ Absence mutations	1153 (92.7)	184 (42.3)	
▪ MI	2 (0.2)	4 (0.9)	
▪ MS	87 (7)	78 (40.9)	
▪ MZ	0	37 (8.5)	
▪ MM malton	0	2 (0.5)	
▪ MP lowell	2 (0.2)	3 (0.7)	
▪ MM procida	0	1 (0.2)	
▪ SS	0	18 (4.1)	
▪ SZ	0	4 (0.9)	
▪ ZZ	0	4 (0.9)	
A1AT mg/dL, m (SD)	143.4 (23.6)	99.8 (13.8)	<0.001
CRP_nivel, m (SD)	0.95 (0.29–5)	1 (0.20–6.25)	0.436

Abbreviations: IPA: pack-years; COPD: chronic obstructive pulmonary disease, ILD: diffuse interstitial lung disease, AOS: sleep apnea syndrome, ICU: intensive care unit, UCRI: intermediate respiratory care unit; NIV/CPAP: non-invasive ventilation/continuous positive airway pressure; A1AT: alpha-1 antitrypsin; CRP: C-reactive protein.

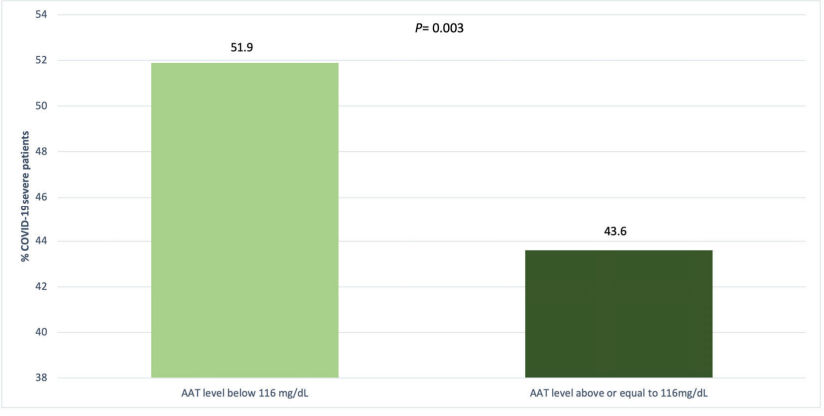


Figure 1. Distribution of COVID-19 severity by serum AAT levels.

3.4. Characteristics According to COVID-19 Severity

There were 872 (43.2) patients defined as suffering severe COVID-19. Cases with severe COVID were older than patients with non-severe COVID (mean [SD] age: 62.8 [12.8] versus 58.9 [14.7] years; $p < 0.001$) and being male was more frequent (67.4% versus 54.3%, $p < 0.001$), Table 4. Having A1AT levels below 116 was more frequent in cases with severe COVID versus non-severe COVID (29.5% versus 23.1, $p = 0.003$). Cases carrying mutations associated with AATD were more frequent in severe COVID versus non-severe COVID (23% versus 18.8%, $p = 0.022$), Figure 2.

Table 4. Characteristics of patients, according to severity of COVID-19.

n = 2217	**Non-Severe COVID-19 n = 1145 (56.8)**	**Severe COVID-19 n = 872 (43.2)**	***p*-Value**
Age, years, m (SD)	58.9 (14.7)	62.2 (12.8)	<0.001
Gender (male), n %	54.3	67.4	<0.001
Smoking status, n (%)			
▪ Current smoker	83 (7.3)	37 (4.3)	0.005
▪ Former smoker	393 (34.7)	400 (46.1)	<0.001
IPA, median (IQR)	20 (10–40)	25 (15-40)	<0.001
Body mass index, kg/m^2, m (SD)	29 (25.7–33.3)	29.4 (26.7–32.6)	0.140
Pulmonary comorbidity, n (%)	243 (21.2)	183 (21)	0.897
▪ COPD, n (%)	65 (5.7)	57 (6.5)	0.418
▪ Asthma, n (%)	117 (10.2)	69 (7.9)	0.076
▪ ILD	13 (1.1)	10 (1.2)	0.978
Home oxygen therapy, n (%)	9 (0.8)	15 (1.7)	0.055
▪ Comorbidities, n (%)			
Diabetes_mellitus	174 (15.2)	210 (24.1)	<0.001
Hypertension	402 (35.1)	391 (44.8)	<0.001
Dyslipemia	301 (26.3)	374 (42.9)	<0.001
Coronary artery disease	98 (8.6)	127 (14.6)	<0.001
Nephropathy	45 (3.9)	45 (5.2)	0.188
Hepatopathy	39 (3.4)	41 (4.8)	0.135
Immunosuppression	34 (3)	29 (3.3)	0.649
History of cancer	72 (6.3)	75 (8.6)	0.048

Table 4. *Cont.*

n = 2217	Non-Severe COVID-19 n = 1145 (56.8)	Severe COVID-19 n = 872 (43.2)	*p*-Value
AAT mg/dL, m (SD)	131.9 (31.1)	132.2 (26.6)	
▪ ≥116, n (%)	704 (76.9)	544 (70.5)	0.859
▪ <116 y ≥ 60, n (%)	209 (22.8)	223 (28.9)	0.006
▪ <60, n (%)	2 (0.2)	5 (0.6)	
A1AT mg/dL, %			
▪ Level ≥ 116, n (%)	704 (76.9)	544 (70.5)	0.003
▪ Level < 116, n (%)	211 (18.4)	228 (29.5)	
A1AT genotyping test, n (%)			
▪ Absence mutations	892 (81.2)	656 (77)	
▪ Presence mutations (%)	206 (18.8)	196 (23)	
MI, n (%)	3 (0.3)	3 (0.4)	
MS, n (%)	166 (15.1)	150 (17.6)	
MZ, n (%)	14 (1.3)	27 (3.2)	0.022
MM malton	2 (0.2)	0	
MP lowell	4 (0.4)	1 (0.1)	
MM procida	1 (0.1)	0	
SS	13 (1.2)	9 (1.1)	
SZ	2 (0.2)	3 (0.4)	
ZZ	1 (0.1)	3 (0.4)	

Abbreviations: IPA: pack-years; COPD: chronic obstructive pulmonary disease, ILD: diffuse interstitial lung disease, AOS: sleep apnea syndrome, ICU: intensive care unit, UCRI: intermediate respiratory care unit; NIV/CPAP: non-invasive ventilation/continuous positive airway pressure; A1AT: alpha-1 antitrypsin; CRP: C-reactive protein.

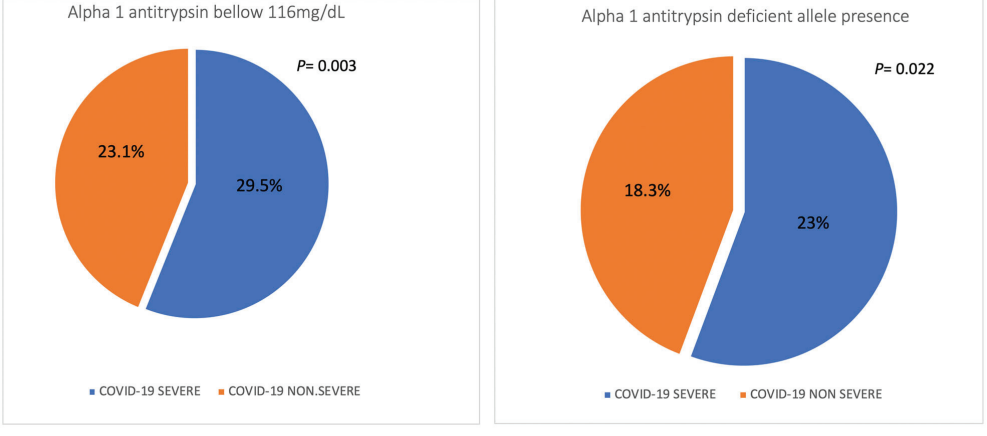


Figure 2. Distribution of the severity of COVID-19 in population with AAT levels < 116 mg/dL and in population with deficiency-related mutations.

3.5. Factors Related to COVID-19 Severity

In the simple logistic regression analysis, the factors associated with a greater likelihood of having suffered severe COVID-19 were being older, being a male, being a former smoker, having cardiovascular comorbidities and a history of cancer, having A1AT levels below 116 mg/dL [OR 1.398 (CI95%: 1.124–1.739), p = 0.003], and having a mutation associated with AATD [OR 1.294 (CI95%: 1.038–1.612), p = 0.022], Table 5.

Table 5. Clinical associations with a severe COVID-19 disease.

	OR (95% CI)	*p*-Value
Age	1.017 (1.011–1.024)	<0.001
Gender (female)	0.573 (0.477–0.689)	<0.001
Diabetes_mellitus	1.773 (1.417–2.218)	<0.001
Hypertension	1.500 (1.253–1.797)	<0.001
Dyslipemia	2.105 (1.744–2.540)	<0.001
Coronary artery disease	1.820 (1.376–2.408)	<0.001
History of cancer	1.401 (1.001–1.961)	0.049
Current smoker	0.568 (0.381–0.846)	0.005
Former smoker	1.611 (1.344–1.930)	<0.001
A1AT level < 116 mg/dL	1.398 (1.124–1.739)	0.003
Presence mutations	1.294 (1.038–1.612)	0.022

4. Discussion

This multicenter observational study investigates the association between AATD and the severity of COVID-19 in patients with a SARS-CoV-2 infection that were treated by pneumology departments in Spain. This analysis demonstrates that, having mutations, variants of the SERPINA1 gene that could affect A1AT protein activity or expression and that having decreased A1AT levels was significantly associated with a higher likelihood of suffering from a severe COVID-19 case. This is consistent with data that suggested that AATD might explain the high COVID-19 mortality in countries with a high AATD prevalence. During the COVID-19 pandemic, several epidemiologic studies have reported that the geographical distributions of the prevalence of SERPINA1 allelic variants and severe cases of COVID-19 were similar, although confounding factors should be considered in these analyses, such as the different control measures established by governments, SARS-CoV-2 vaccination, socioeconomic status, and population health [3,7,15]. Other observational studies in patients with AATD also found a higher frequency of SARS-CoV-2 infection and a higher risk for symptomatic SARS-CoV-2 infection in patients with severe AATD with lung disease [16,17]. Recently, an EARCO ERS Clinical Research Collaboration analysis that investigated the impact of COVID-19 on patients with severe AATD (PiZZ, PiSZ, or rare variants with an equivalent serum A1AT level < 60 mg/dL) [18] showed that while a poor outcome was more frequent in PiZZ compared with PiSZ, this did not reach statistical significance; non-respiratory comorbidities were more strongly associated with a poor outcome than genotype, baseline FEV1, or oxygen saturation. However, it should be noted that in this cohort of patients with AATD, although 88% were diagnosed with COVID-19 with a positive PCR, only 31% required hospitalization. In addition, an analysis of a community-based cohort with > 500,000 participants that assessed the association between AATD and COVID-19 in the United Kingdom Biobank showed that the most common and mild AATD genotypes were not associated with increased SARS-CoV-2 infection rates or increased SARS-CoV-2 fatalities, although it must be noted that there were very few cases of severe AATD in this study [19]. In our population of patients with SARS-CoV-2 pneumonia, the frequency of Pi*S was 176/1000 and Pi*Z 25/1000. These figures are high in relation to the estimated prevalence in Spain [20], with a mean SZ prevalence of 278/1000, Pi*Z 17/1000, and Pi*S 104/1000. This higher frequency of mutations related to severe impairment (ZZ, SZ) found in our cohort could support our hypothesis that a poor prognosis for COVID-19 patients may be related to the presence of genetic mutations associated with AATD. A large proportion of patients in our cohort required supportive therapies and intensive care for COVID-19, which could be explained by the fact that patients with more severe COVID-19 are usually referred to the pneumology follow-up clinic because they are at higher risk of developing complications [21]. Indeed, our data showed that cases carrying mutations associated with AATD were more frequent in severe COVID versus non-severe COVID (23% vs. 18.8%, $p = 0.022$). The frequency of Pi*Z was 37.8/1000 in severe COVID versus 17.5/1000 in non-severe COVID, $p = 0.001$. The presence

of genetic mutations associated with AATD was found to be a predictor factor associated with a higher likelihood of suffering a severe COVID-19 case [OR 1.294 (CI95%: 1.038–1.612), $p = 0.022$], which was consistent with studies that confirmed a correlation between the frequency of Pi*Z and Pi*S alleles and mortality rates due to COVID-19 [7]. Furthermore, recent studies have investigated genetic susceptibility to SARS-CoV-2 and reported that approximately 20% of life-threatening COVID-19 cases were associated with genetic errors and gene loci, most of which are involved in two immune signaling pathways [5,22]. Thus, we could hypothesize that upon exposure to the same virus, while some individuals show asymptomatic or mild illness, plausibly due to effective immune reactions, severe COVID-19 patients may reflect dysfunctional immune reactions that lead to increased lung injury.

Regarding information on risk factors for the development of SARS-CoV-2 pneumonia and a severe disease course, our study supports many of the findings from previous reports indicating that the epidemiology of COVID-19 shows a diverse pattern across people who are different in age, sex, ethnicity, and particularly among those with pre-existing medical conditions [3,23–25]. In our cohort, being male, being older, having a history of smoking, and having age-associated comorbidities significantly contributes to the severity of acute COVID-19. However, it should be noted that in our analysis, patients with the presence of genetic mutations associated with AATD or A1AT levels below 116 mg/dL do not have a higher prevalence of hypertension, diabetes, heart disease, chronic kidney disease, or chronic obstructive pulmonary disease. However, there are other potential factors, such as the dominant COVID strain at the time of infection, as this is not an assessment that is performed in daily clinical practice, or vaccination status against SARS-CoV-2; however, vaccination coverage in adults in Spain was very high with more than 85% are vaccinated.

Several studies have focused on the possibility of shared pathogenic pathways between AATD and SARS-CoV-2 infection. Indeed, A1AT has several biological functions that may antagonize SARS-CoV-2 infection and pathophysiological processes. Alpha-1-antitrypsin is a tissue protector with antiviral and anti-inflammatory properties [26,27]. The main function of A1AT is inactivating proteolytic enzymes [28], which are released in pulmonary tissue. Furthermore, a protective role for A1AT has been described for several viral infections. A1AT levels may be relevant to the development of viral diseases as rhinovirus infection, human immunodeficiency virus, hepatitis B and C, and complications [29–31].

The relationship between a worse prognosis of SARS-CoV-2 infection and lower levels of AAT could be explained by the possible protective role of A1AT against COVID-19. A1AT reduces transmembrane serine protease 2 activity [27], protection against acute lung injury [32], and strong anti-inflammatory properties [10]. In relation to the potential A1AT protective mechanisms of action, our analysis showed that having A1AT levels below 116 was more frequent in cases with severe COVID versus non-severe COVID (29.5% versus 23.1, $p = 0.003$), and the presence of A1AT levels below 116mg/dL was identified as a predictor factor associated with a higher likelihood of suffering a severe COVID-19 case, which was consistent with studies that demonstrated the COVID-19 cytokinemia is distinct from that of other types of pneumonia. In these studies, the production and sialylation of A1AT are increased in COVID-19, but this anti-inflammatory response is overwhelmed in severe illness, with the IL-6: A1AT ratio being markedly higher in patients requiring ICU admission. In critically unwell patients with COVID-19, increases in IL-6: A1AT predicted a prolonged ICU stay and mortality, whereas improvement in IL-6:A1AT was associated with clinical resolution [2]. In this regard, supplementation of the acute A1AT response with exogenous A1AT may merit consideration, as it has been shown to modulate the production and activity of the key proinflammatory cytokines described in [28,33] while preserving the production of IL-10 [34]. Indeed, it has recently been reported that abrupt cessation of A1AT augmentation therapy for patients with AATD resulted in marked increases in levels of these specific proinflammatory cytokines, a loss of IL-10, and subsequent progression to respiratory failure [35].

Our study has several limitations. First, COVID-AATD is a cross-sectional, observational study in patients treated by pneumology departments and this may carry some bias.

Patients with more severe COVID-19 are controlled mainly by pulmonologists, which may result in overestimates since it is more likely the most unwell patients are selected. On the other hand, most cases were included at the follow-up consultation after discharge, and very few were during hospitalization for COVID-19, which results in underestimated fatal COVID cases, despite the fact that the sample size is quite large and stratified by COVID infection severity. Second, the multiplex system studies the 14 most frequent mutations that include more than 99% of the deficient variants observed in the world. Therefore, the identification of Pi*M is achieved through exclusion, since the absence of any of these 14 alleles suggests with more than 99% probability that it is an M. However, when none of the 14 mutations were found and the A1AT serum level was <60 mg/dL, SERPINA1 gene sequencing was performed, which did not occur in our study. Third, although the lower limit of normal A1AT by nephelometry is 90 mg/dL, we established an above-normal cut-off value in our analysis on the basis that deficient mutations can be detected above this level and may also be influenced by increased systemic inflammation [36]. Consequently, the use of an above-normal cut-off value could be argued as a threshold value to screen for the possible presence of a deficient allele. Fourth, in the logistic regression analysis, other variables are not considered such as vaccines and specific therapies that could impact association estimates.

5. Conclusions

Our study identifies the presence of mutations associated with A1AT and that have A1AT levels below 116 as predictors associated with an increased likelihood of severe COVID-19. These observations suggest that patients with AATD should be considered at a higher risk of developing severe COVID-19. These findings highlight the need for further studies on the role of the A1AT in the pathogenesis and prognosis of SARS-CoV-2 infection and a potential therapeutic role.

Author Contributions: Conceptualization, M.C.R. and J.L.R.H.; Methodology, M.C.R. and J.L.R.H.; M.C.R. and J.L.R.H. wrote the manuscript. Investigation and writing—review and editing, M.C.R., J.L.R.H., G.V.C., M.E.G.C., M.M., L.L.-A., B.M.J.-R., R.A.R., R.M.M., M.T.-D. and J.M.H.-P.; A.M.H.-N. did the statistical analysis. All authors have read and agreed to the published version of the manuscript.

Funding: This study was promoted by the Madrid Society of Pneumology and Thoracic Surgery (Neumomadrid). We thank Grifols for its financial support to carry out the study. The financing entities did not participate in the design of the study, data collection, analysis, publication, or preparation of this manuscript. The participation of the Vall d'Hebron University Hospital in this study has been funded by a research grant from the Fundació Catalana de Pneumologia (FUCAP) 2021.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Research Ethics Committee at Hospital Clínico San Carlos, Madrid, Spain (internal code 20/809-E).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments: Lucía Gómez Martín-Caro, Jorge García Aragón, Íñigo García Peñuelas (Hospital Clínico San Carlos, Madrid), Alexa Núñez, Gerard Orriols, Georgina Farago (Hospital Universitari Vall d'Hebron, Barcelona), Teresa Peña Miguel, Rosario González Campo, Elsa Merino García (Complejo Asistencial Universitario de Burgos), Marta Núñez-Fernández y Cristina Ramos-Hernández (Hospital Álvaro Cunqueiro, Vigo), Ester Sánchez Guerra, Jin-Seok Yo (Hospital Clinic, Barcelona), Ana Bustamante Ruiz (Hospital de Torrelavega, Santander), Marta Arroyo Cózar, Pilar Alba (Hospital Infanta Cristina, Madrid), Olga Rajas (Hospital de La Princesa, Madrid), for their participation in the collection of data.

Conflicts of Interest: J.L.R.H. has received speaker fees from Bial, Boehringer Ingelheim, CSL Behring, GlaxoSmithKline, Grifols, and Zambon, and consulting fees from Bial. G.V.C. has received speaker

fees from GlaxoSmithKline, and Grifols. Marc Miravitlles has received speaker fees from AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, Menarini, Rovi, Bial, Kamada, Takeda, Sandoz, Zambon, CSL Behring, Specialty Therapeutics, Janssen, Grifols, and Novartis, consulting fees from AstraZeneca, Atriva Therapeutics, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Bial, Gebro Pharma, CSL Behring, Inhibrx, Laboratorios Esteve, Ferrer, Menarini, Mereo Biopharma, Verona Pharma, Spin Therapeutics, ONO Pharma, pH Pharma, Palobiofarma SL, Takeda, Novartis, Sanofi, and Grifols and research grants from Grifols. L.L.A. has received speaker fees from AstraZeneca, Boehringer Ingelheim, Chiesi, CSL Bering, GlaxoSmithKline, Gebro Pharma, Grifols, Novartis, and Pfizer, and consulting feels from CSL Bering. M.C.R. has received speaker fees from AstraZeneca, Bial, Chiesi, CSL Behring, GlaxoSmithKline, Menarini, and Grifols, and consulting fees from GlaxoSmithKline, and Bial. The other authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

AATD = alpha-1 antitrypsin deficiency; A1AT = alpha-1 antitrypsin; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; COVID-19 = coronavirus disease 2019; IPA: Pack-years; COPD: chronic obstructive pulmonary disease, ILD: diffuse interstitial lung disease, AOS: sleep apnea syndrome, ICU: intensive care unit, UCRI: intermediate respiratory care unit; HFNC = high-flow nasal cannula; NIV/CPAP: non-invasive ventilation/continuous positive airway pressure; CRP: C-reactive protein. IQR = interquartile range.

References

1. World Health Organization. WHO Coronavirus (COVID-19) Dashboard. Available online: https://covid19.who.int/ (accessed on 28 September 2022).
2. McElvaney, O.J.; McEvoy, N.L.; McElvaney, O.F.; Carroll, T.P.; Murphy, M.P.; Dunlea, D.M.; Choileáin, O.N.; Clarke, J.; O'Connor, E.; Hogan, G.; et al. Characterization of the Inflammatory Response to Severe COVID-19 Illness. *Am. J. Respir. Crit. Care Med.* **2020**, *202*, 812–821. [CrossRef] [PubMed]
3. Yamamoto, N.; Yamamoto, R.; Ariumi, Y.; Mizokami, M.; Shimotohno, K.; Yoshikura, H. Does Genetic Predisposition Contribute to the Exacerbation of COVID-19 Symptoms in Individuals with Comorbidities and Explain the Huge Mortality Disparity between the East and the West? *Int. J. Mol. Sci.* **2021**, *22*, 5000. [CrossRef] [PubMed]
4. van der Made, C.I.; Simons, A.; Schuurs-Hoeijmakers, J.; van den Heuvel, G.; Mantere, T.; Kersten, S.; van Deuren, R.C.; Steehouwer, M.; van Reijmersdal, S.V.; Jaeger, M.; et al. Presence of Genetic Variants Among Young Men with Severe COVID-19. *Jama* **2020**, *324*, 663–673. [CrossRef]
5. McCoy, K.; Peterson, A.; Tian, Y.; Sang, Y. Immunogenetic Association Underlying Severe COVID-19. *Vaccines* **2020**, *8*, 700. [CrossRef] [PubMed]
6. Calle Rubio, M.; Soriano, J.B.; López-Campos, J.L.; Soler-Cataluña, J.J.; Alcázar Navarrete, B.; Rodríguez González-Moro, J.M.; Miravitlles, M.; Barrecheguren, M.; Ferrer, M.E.F.; Hermosa, J.L.R.; et al. Testing for alpha-1 antitrypsin in COPD in outpatient respiratory clinics in Spain: A multilevel, cross-sectional analysis of the EPOCONSUL study. *PLoS ONE* **2018**, *13*, e0198777. [CrossRef]
7. Shapira, G.; Shomron, N.; Gurwitz, D. Ethnic differences in alpha-1 antitrypsin deficiency allele frequencies may partially explain national differences in COVID-19 fatality rates. *FASEB J.* **2020**, *34*, 14160–14165. [CrossRef]
8. Wettstein, L.; Weil, T.; Conzelmann, C.; Müller, J.A.; Groß, R.; Hirschenberger, M.; Seidel, A.; Klute, S.; Zech, F.; Bozzo, C.P.; et al. Alpha-1 antitrypsin inhibits TMPRSS2 protease activity and SARS-CoV-2 infection. *Nat. Commun.* **2021**, *12*, 1726. [CrossRef]
9. Azouz, N.P.; Klingler, A.M.; Callahan, V.; Akhrymuk, I.V.; Elez, K.; Raich, L.; Henry, B.M.; Benoit, J.L.; Benoit, S.W.; Noé, F.; et al. Alpha 1 Antitrypsin is an Inhibitor of the SARS-CoV-2-Priming Protease TMPRSS2. *Pathog. Immun.* **2021**, *6*, 55–74. [CrossRef]
10. Wang, J.Z.; Zhang, R.Y.; Bai, J. An anti-oxidative therapy for ameliorating cardiac injuries of critically ill COVID-19-infected patients. *Int. J. Cardiol.* **2020**, *312*, 137–138. [CrossRef]
11. Petrache, I.; Fijalkowska, I.; Medler, T.R.; Skirball, J.; Cruz, P.; Zhen, L.; Petrache, H.I.; Flotte, T.R.; Tuder, R.M. Alpha-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am. J. Pathol.* **2006**, *169*, 1155–1166. [CrossRef]
12. WHO Working Group on the Clinical Characterisation; Management of COVID-19 infection. A minimal common outcome measure set for COVID-19 clinical research. *Lancet Infect. Dis.* **2020**, *20*, e192–e197. [CrossRef]
13. López-Campos, J.L.; Casas-Maldonado, F.; Torres-Duran, M.; Medina-Gonzálvez, A.; Rodriguez-Fidalgo, M.L.; Carrascosa, I.; Calle, M.; Osaba, L.; Rapun, N.; Drobnic, E.; et al. Results of a diagnostic procedure based on multiplex technology on dried blood spots and buccal swabs for subjects with suspected alpha1 antitrypsin deficiency. *Arch. Bronconeumol.* **2021**, *57*, 42–50. [CrossRef]
14. Janciauskiene, S.; DeLuca, D.S.; Barrecheguren, M.; Welte, T.; Miravitlles, M. Serum Levels of Alpha1-antitrypsin and their relationship with COPD in the General Spanish Population. *Arch. Bronconeumol.* **2020**, *56*, 76–83. [CrossRef]

15. Yoshikura, H. Epidemiological correlation between COVID-19 epidemic and prevalence of α-1 antitrypsin deficiency in the world. *Glob. Health Med.* **2021**, *3*, 73–81. [CrossRef]
16. Ferrarotti, I.; Ottaviani, S.; Balderacchi, A.M.; Barzon, V.; De Silvestri, A.; Piloni, D.; Mariani, F.; Corsico, A. COVID-19 infection in severe Alpha 1-antitrypsin deficiency: Looking for a rationale. *Respir. Med.* **2021**, *183*, 106440. [CrossRef]
17. Faria, N.; Inês Costa, M.; Gomes, J.; Sucena, M. Alpha-1 antitrypsin deficiency severity and the risk of COVID-19: A Portuguese cohort. *Respir. Med.* **2021**, *181*, 106387. [CrossRef]
18. Parr, D.G.; Chorostowska-Wynimko, J.; Corsico, A.; Esquinas, C.; McElvaney, G.N.; Sark, A.D.; Sucena, M.; Tanash, H.; Turner, A.M.; Miravitlles, M. Impact of COVID-19 in Patients with Severe Alpha-1 Antitrypsin Deficiency: The IMCA1 Study of the EARCO Clinical Research Collaboration. *Arch. Bronconeumol.* **2022**, *58*, 840–842, Epub ahead of print. [CrossRef]
19. Schneider, C.V.; Strnad, P. SARS-CoV-2 infection in alpha1-antitrypsin deficiency. *Respir. Med.* **2021**, *184*, 106466. [CrossRef]
20. Blanco, I.; Bueno, P.; Diego, I.; Pérez-Holanda, S.; Lara, B.; Casas-Maldonado, F.; Esquinas, C.; Miravitlles, M. Alpha-1 antitrypsin Pi*SZ genotype: Estimated prevalence and number of SZ subjects worldwide. *Int. J. Chronic Obstr. Pulm. Dis.* **2017**, *12*, 1683–1694. [CrossRef]
21. Vargas Centanaro, G.; Calle Rubio, M.; Álvarez-Sala Walther, J.L.; Martinez-Sagasti, F.; Albuja Hidalgo, A.; Herranz Hernández, R.; Rodríguez Hermosa, J.L. Long-term Outcomes and Recovery of Patients who Survived COVID-19: LUNG INJURY COVID-19 Study. *Open Forum Infect. Dis.* **2022**, *9*, ofac098. [CrossRef]
22. López-Rodríguez, R.; Del Pozo-Valero, M.; Corton, M.; Minguez, P.; Ruiz-Hornillos, J.; Pérez-Tomás, M.E.; Barreda-Sánchez, M.; Mancebo, E.; Villaverde, C.; Núñez-Moreno, G.; et al. Presence of rare potential pathogenic variants in subjects under 65 years old with very severe or fatal COVID-19. *Sci. Rep.* **2022**, *12*, 10369. [CrossRef] [PubMed]
23. von der Thüsen, J.; van der Eerden, M. Histopathology and genetic susceptibility in COVID-19 pneumonia. *Eur. J. Clin. Investig.* **2020**, *50*, e13259. [CrossRef] [PubMed]
24. Zhou, F.; Yu, T.; Du, R.; Fan, G.; Liu, Y.; Liu, Z.; Xiang, J.; Wang, Y.; Song, B.; Gu, X.; et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. *Lancet* **2020**, *395*, 1054–1062. [CrossRef] [PubMed]
25. Guan, W.J.; Liang, W.H.; Zhao, Y.; Liang, H.R.; Chen, Z.S.; Li, Y.M.; Liu, X.Q.; Chen, R.C.; Tang, C.L.; Wang, T.; et al. China Medical Treatment Expert Group for COVID-19. Comorbidity and its impact on 1590 patients with COVID-19 in China: A nationwide analysis. *Eur. Respir. J.* **2020**, *55*, 2000547. [CrossRef] [PubMed]
26. Gassen, N.C.; Niemeyer, D.; Muth, D.; Corman, V.M.; Martinelli, S.; Gassen, A.; Hafner, K.; Papies, J.; Mösbauer, K.; Zellner, A.; et al. SKP2 attenuates autophagy through Beclin1-ubiquitination and its inhibition reduces MERS-Coronavirus infection. *Nat. Commun.* **2019**, *10*, 5770. [CrossRef]
27. Oguntuyo, K.Y.; Stevens, C.S.; Siddiquey, M.N.; Schilke, R.M.; Woolard, M.D.; Zhang, H.; Acklin, J.A.; Ikegame, S.; Huang, C.-T.; Lim, J.K.; et al. In plain sight: The role of alpha-1-antitrypsin in COVID-19 pathogenesis and therapeutics. *bioRxiv* **2020**, Preprint. [CrossRef]
28. Bergin, D.A.; Reeves, E.P.; Hurley, K.; Wolfe, R.; Jameel, R.; Fitzgerald, S.; McElvaney, N.G. The circulating proteinase inhibitor α-1 antitrypsin regulates neutrophil degranulation and autoimmunity. *Sci. Transl. Med.* **2014**, *6*, 217ra1. [CrossRef]
29. Berman, R.; Jiang, D.; Wu, Q.; Chu, H.W. alpha1-Antitrypsin reduces rinovirus infection in primary human airway epithelial cells exposed to cigarette smoke. *Int. J. Chronic Obstr. Pulm. Dis.* **2016**, *11*, 1279. [CrossRef]
30. Stephenson, S.E.; Wilson, C.L.; Crothers, K.; Attia, E.F.; Wongtrakool, C.; Petrache, I.; Schnapp, L.M. Impact of HIV infection on α1-antitrypsin in the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2017**, *314*, L583–L592. [CrossRef]
31. Hashemi, M.; Alavian, S.M.; Ghavami, S.; de Serres, F.J.; Salehi, M.; Doroudi, T.; Fard, A.H.M.; Mehrabifar, H.; Milani, B.; Shahri, S.J.S. High prevalence of Alpha 1 antitrypsin phenotypes in viral hepatitis B infected patients in Iran. *Hepatol. Res.* **2005**, *33*, 292–297. [CrossRef]
32. Ishii, T.; Doi, K.; Okamoto, K.; Imamura, M.; Dohi, M.; Yamamoto, K.; Fujita, T.; Noiri, E. Neutrophil elastase contributes to acute lung injury induced by bilateral nephrectomy. *Am. J. Pathol.* **2010**, *177*, 1665–1673. [CrossRef]
33. McCarthy, C.; Dunlea, D.M.; Saldova, R.; Henry, M.; Meleady, P.; McElvaney, O.J.; Marsh, B.; Rudd, P.M.; Reeves, E.P.; McElvaney, N.G. Glycosylation Repurposes Alpha-1 Antitrypsin for Resolution of Community-acquired Pneumonia. *Am. J. Respir. Crit. Care Med.* **2018**, *197*, 1346–1349. [CrossRef]
34. Janciauskiene, S.M.; Nita, I.M.; Stevens, T. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. *J. Biol. Chem.* **2007**, *282*, 8573–8582. [CrossRef]
35. McElvaney, O.J.; Carroll, T.P.; Franciosi, A.N.; Sweeney, J.; Hobbs, B.D.; Kowlessar, V.; Gunaratnam, C.; Reeves, E.P.; McElvaney, N.G. Consequences of Abrupt Cessation of Alpha1-Antitrypsin Replacement Therapy. *N. Engl. J. Med.* **2020**, *382*, 1478–1480. [CrossRef]
36. Sanders, C.L.; Ponte, A.; Kueppers, F. The Effects of Inflammation on Alpha 1 Antitrypsin Levels in a National Screening Cohort. *COPD* **2018**, *15*, 10–16. [CrossRef]

biomedicines

MDPI

Brief Report

Clinical Variables Related to Functional Capacity and Exertional Desaturation in Patients with COVID-19

Santiago Larrateguy [1,2], Julian Vinagre [1], Federico Londero [1], Johana Dabin [1], Evangelina Ricciardi [1], Santiago Jeanpaul [1], Rodrigo Torres-Castro [3,4,5], Rodrigo Núñez-Cortés [3,6], Diana Sánchez-Ramírez [7], Elena Gimeno-Santos [4,5,8,9,*] and Isabel Blanco [4,5,8]

1 Servicio de Kinesiología y Fisioterapia, Hospital de la Baxada "Dra. Teresa Ratto", Paraná 3100, Argentina; santilarra@gmail.com (S.L.); julianvinagre10@gmail.com (J.V.); londero2@hotmail.com (F.L.); jdabindittler@gmail.com (J.D.); mericciardi85@gmail.com (E.R.); santiagojeanpaul@gmail.com (S.J.)
2 Facultad de Ciencias de la Salud, Universidad Adventista del Plata, Libertador San Martin 3103, Argentina
3 Department of Physical Therapy, Faculty of Medicine, University of Chile, Santiago 8380453, Chile; rodritorres@uchile.cl (R.T.-C.); r_nunez@uchile.cl (R.N.-C.)
4 Department of Pulmonary Medicine, Hospital Clínic, University of Barcelona, 08036 Barcelona, Spain; iblanco2@clinic.cat
5 Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain
6 Physiotherapy in Motion Multispeciality Research Group (PTinMOTION), Department of Physiotherapy, University of Valencia, 46003 Valencia, Spain
7 Department of Respiratory Therapy, College of Rehabilitation Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada; diana.sanchez-ramirez@umanitoba.ca
8 Biomedical Research Networking Center on Respiratory Diseases (CIBERES), 30627 Madrid, Spain
9 Barcelona Institute for Global Health (ISGlobal), 08036 Barcelona, Spain
* Correspondence: elenagimeno@gmail.com

Abstract: Impaired functional capacity is one of the most commonly reported consequences among post-COVID-19 patients. This study aimed to analyse the clinical variables related to functional capacity and exertional desaturation in post-COVID-19 patients at the time of hospital discharge. A cross-sectional study was conducted on patients recovering from COVID-19 pneumonia. The main outcomes measures were functional capacity, assessed using the 1 min sit-to-stand test (1 min STST), and exertional desaturation, defined as a drop of ≥4% in the arterial oxygen saturation. Factors used to characterise the participant outcomes included the use of a high-flow nasal cannula (HFNC), prolonged hospitalisation, occurrence of pulmonary embolism during hospitalisation, and underlying comorbidities. A total of 381 participants (mean age = 53.7 ± 13.2 years, 65.6% men) were included. Participants completed a mean of 16.9 ± 6.2 repetitions in the 1 min STST. Exertional desaturation was observed in 51% of the patients. Higher odds of exertional desaturation were found in the participants who used a HFNC (OR = 3.6; 95%CI: 1.6 to 7.8), were admitted in the hospital >10 days (OR = 4.2; 95%CI: 2.6 to 6.8), and had a pulmonary embolism (OR = 3.5; 95%CI: 2.2. to 5.3). Use of a HFNC (β = −3.4; 95%CI: −5.3 to −1.44), a hospital stay >10 days (β = −2.2; 95%CI: −3.4 to −0.9), and a history of pulmonary embolism (β = −1.4; 95%CI: −2.6 to −0.2) were also negatively associated with the 1 min STST. Most post-COVID-19 patients exhibited reduced functional capacity at the time of hospital discharge, and approximately half had exertional desaturation after the 1 min STST. The use of a HFNC, prolonged hospitalisation and pulmonary embolism were the main clinical variables associated with worse a 1 min STST performance and a higher likelihood of exertional desaturation.

Keywords: COVID-19; SARS-CoV-2; coronavirus disease 2019; exercise capacity; rehabilitation

Citation: Larrateguy, S.; Vinagre, J.; Londero, F.; Dabin, J.; Ricciardi, E.; Jeanpaul, S.; Torres-Castro, R.; Núñez-Cortés, R.; Sánchez-Ramírez, D.; Gimeno-Santos, E.; et al. Clinical Variables Related to Functional Capacity and Exertional Desaturation in Patients with COVID-19. *Biomedicines* **2023**, *11*, 2051. https://doi.org/10.3390/biomedicines11072051

Academic Editor: César Fernández De Las Peñas

Received: 30 May 2023
Revised: 1 July 2023
Accepted: 7 July 2023
Published: 20 July 2023

1. Introduction

The coronavirus disease (COVID-19) has been a challenge for health systems across the world, affecting more than 670 million people, with more than 6.8 million deaths by May 2023 [1]. Although the majority of people infected by the severe acute respiratory

syndrome coronavirus 2 (SARS-CoV-2) developed asymptomatic or mild disease, about 20% developed severe disease requiring hospitalisation, and close to 6% required critical care in an intensive care unit [2]. Among the severe cases, pulmonary embolism is a frequent complication associated with the clinical worsening of COVID-19 [3]. In addition, some cases may require respiratory support (e.g., a high-flow nasal cannula (HFNC)) for the treatment of acute hypoxemic respiratory failure [4]. Thus, prolonged hospitalisation due to COVID-19 complications may lead to worse outcomes at discharge. Although COVID-19 is primarily a respiratory disease, it can affect multiple systems, such as the cardiovascular or neurological, leaving a vast number of sequelae that impact the patient's quality of life and the ability to return to work [5–8]. Among the most reported sequelae are fatigue, dyspnoea, and impairment of functional capacity [6,9].

Due to the functional limitations that COVID-19 generates in a significant part of the population, different national health systems have developed follow-up programmes focused on imaging, lung function, symptoms, and functional capacity [10–12]. One of the pillars of the follow-up and intervention programmes is the evaluation of functional capacity [13], which can be assessed with laboratory tests, such as the cardiopulmonary exercise test (CPET) or field tests, such as the six-minute walk test (6MWT) or the 1 min sit-to-stand test (1 min STST) [14–18].

The 6MWT is the most commonly used test for respiratory, cardiological, metabolic, or neurological diseases [14]. This test has been widely demonstrated to be helpful in assessing functional capacity and can be performed in low-resource contexts [14]. However, to provide specific information about functional or exercise capacity, a test should be chosen according to the characteristics of each subject, the setting, and the physiologically expected answer [19]. The 6MWT requires a 30 m corridor (at least 20 m), which is often unavailable in hospitals or rehabilitation centers and even less at home [14]. The 1 min STST has the advantage of requiring a small space compared to the 6MWT, and less sophisticated equipment as compared with tests using treadmills or cycle ergometers; as such it may be an alternative to evaluate functional capacity when the 6MWT cannot be performed [16]. The 1 min STST has significantly correlated with the 6MWT in patients with different diseases, including post-COVID-19, chronic obstructive pulmonary disease (COPD), and pulmonary hypertension (PH) patients [16,20,21].

Functional capacity assessments are widely used in intervention programmes such as pulmonary rehabilitation [22–24]. Due to the high number of patients left with sequelae and the recommendation to evaluate functional capacity according to the guidelines recommendations [12,25], it is necessary to determine which clinical variables may affect the results of functional evaluations and to identify the people with an increased risk of having a poor result. Therefore, our objective was to analyse the clinical variables related to functional capacity and exertional desaturation in post-COVID-19 patients at hospital discharge.

2. Materials and Methods

2.1. Design and Participants

We conducted a cross-sectional study in patients recovering from COVID-19 pneumonia once they were discharged from the Hospital de la Baxada between April 2021 and March 2022. Ethics committee approval was obtained, and all patients signed the informed consent. This study followed the recommendations of the STrengthening the Reporting of OBservational studies in Epidemiology guidelines (STROBE) [26].

The inclusion criteria were as follows: patients older than 18 years, and a diagnostic of COVID-19 by positive PCR assay findings for nasal and pharyngeal swab specimens. In addition, the exclusion criteria were the presence of locomotor or cognitive impairment before the infection, refusal to participate, and any pre-existing condition, such as orthopaedic or neurological conditions, that limited the ability to perform the 1 min STST.

2.2. Measurements

Demographic characteristics, medical history, exposure history, and underlying comorbidities were collected at discharge. The main outcome measure was functional capacity, assessed through the 1 min STST at hospital discharge. All tests were conducted in the same room, with only the presence of the evaluator and the patient, to avoid distractions.

The 1 min STST was performed with a standard height chair (46 cm) without armrests, positioned against a wall. Participants were not allowed to use their hands/arms to push the chair's seat or their body. Participants were instructed to complete as many sit-and-stand cycles as possible in 60 s at a self-paced speed [25]. We used the reference values based on the healthy adult population previously reported by Strassmann et al. [27].

A finger oximeter was used to record the oxygen saturation (SpO_2) and heart rate (HR). A drop of $\geq$4% in the arterial oxygen saturation was considered clinically significant [28]. The evaluator had previous experience (5 years) in performing field tests to assess physical capacity, including the 6MWT and 1 min STST. The 6MWT was performed following the recommendations of the European Respiratory Society/American Thoracic Society (ERS/ATS) clinical guidelines [14]. The 1 min STST was performed only once to avoid a learning effect [29].

The clinical variables were as follows: (1) Use of a HFNC during hospitalisation; (2) prolonged hospitalisation. Based on previous studies in patients with COVID-19, length of stay >10 days was established as the cut-off point for defining a prolonged hospital stay [18,30]; (3) pulmonary embolism during hospitalisation; (4) history of underlying comorbidities (diabetes, hypertension, or chronic respiratory disease) at the time of hospitalization; (5) obesity (i.e., body mass index $\geq$ 30 kg/m^2)

2.3. Statistics

All statistical analyses were performed with SPSS software (v. 22.00 for Windows, Chicago, IL, USA). The normality of the data distribution was assessed using the Shapiro–Wilk test. Data were described as the mean $\pm$ standard deviation, frequency, and percentages. To evaluate the associations of each of the clinical variables with the exertional desaturation after the 1 min STST (outcome), binary logistic regression analysis adjusted for sex and age (covariates) was performed. Data were presented as the odds ratio (OR) with 95% confidence intervals (95%CI). The effect sizes of the OR were characterised as small, moderate, or large and were established by an OR of 1.68, 3.47, and 6.71, respectively [31]. The sample size was pragmatic and depended on the ability of the clinical staff to recruit the participants continuously and to collect the data.

To evaluate the associations of each of the clinical variables with the number of repetitions in the 1 min STST (outcome), a simple linear regression analysis adjusted for sex and age (covariates) was performed. Data were presented as the regression coefficient (β), with 95%CI. The level of statistical significance was set at $p < 0.05$.

3. Results

A total of 381 participants (mean age = 53.7 $\pm$ 13.2 years, 65.6% men) were included in the study (Table 1). The mean number of repetitions in the 1 min STST was 16.9 $\pm$ 6.2. Moreover, 78.4% of the cases obtained results below the lower limit of normality (percentile 2.5), according to the reference values. A total of 51.1% of the patients presented exertional desaturation after the 1 min STST. Table 2 shows the adjusted models for the association between the clinical variables and exertional desaturation after the 1 min STST. Participants with a history of HFNC (OR = 3.6; 95%CI = 1.6 to 7.8), hospital stay >10 days (OR = 4.2; 95%CI = 2.6 to 6.8), and pulmonary embolism (OR = 3.5; 95%CI = 2.2. to 5.3) had a significantly higher risk of exertional desaturation, with a moderate effect size. Table 3 shows the adjusted models for the association between the clinical variables and functional capacity. Use of a HFNC (β = $-$3.4; 95%CI = $-$5.3 to $-$1.44]), hospital stay >10 days (β = $-$2.2; 95%CI = $-$3.4 to $-$0.9) and a history of pulmonary embolism (β = $-$1.4;

95%CI = −2.6 to −0.2) were negatively associated with the number of repetitions in the 1 min STST.

Table 1. Clinical characteristics of patients (*n* = 381).

Characteristics	Value
Age (years)	53.7 ± 13.2
Sex male *n* (%)	250 (65.6)
BMI (kg/m^2)	31.3 ± 6.3
HFNC therapy, *n* (%)	39 (10.3)
Hospital stay (days)	9.5 ± 6.6
Hospital stay >10 days, *n* (%)	124 (32.5)
Pulmonary embolism, *n* (%)	161 (42.2)
Diabetes, *n* (%)	90 (23.6)
Hypertension, *n* (%)	141 (37.0)
Chronic respiratory disease, *n* (%)	21 (5.5)
Obesity, *n* (%)	200 (52.4)
1 min STST (repetitions)	16.9 ± 6.2
Repetitions < 2.5th percentile, *n* (%)	299 (78.4)
Exertional desaturation, *n* (%)	195 (51.1)

Data are presented as mean ± standard deviation or *n* (%). Abbreviations: BMI = Body mass index; HFNC = High-flow nasal cannula; 1 min STST = 1 min sit-to-stand test.

Table 2. Associations between clinical variables and exertional desaturation after 1 min STST in post-COVID-19 patients.

Clinical Variables	Category	Adjusted OR [95%CI] [a]
HFNC therapy	Yes vs. No	**3.6 [1.6 to 7.8]**
Hospital stay > 10 days	Yes vs. No	**4.2 [2.6 to 6.8]**
Pulmonary embolism	Yes vs. No	**3.5 [2.2. to 5.3]**
Diabetes	Yes vs. No	1.0 [0.6 to 1.6]
Hypertension	Yes vs. No	1.1 [0.7 to 1.8]
Chronic respiratory disease	Yes vs. No	0.9 [0.4 to 2.1]
Obesity	Yes vs. No	**1.6 [1.1 to 2.4]**

Abbreviations: CI = confidence interval; HFNC = High-flow nasal cannula. [a] Adjusted for sex and age.

Table 3. Associations between clinical variables and number of repetitions in the 1 min STST in post-COVID-19 patients.

Clinical Variables	Category	Adjusted β [95%CI] [a]
HFNC therapy	Yes vs. No	**−3.4 [−5.3 to −1.44]**
Hospital stay > 10 days	Yes vs. No	**−2.2 [−3.4 to −0.9]**
Pulmonary embolism	Yes vs. No	**−1.4 [−2.6 to −0.2]**
Diabetes	Yes vs. No	−0.3 [−1.7 to 1.2]
Hypertension	Yes vs. No	−0.4 [−1.7 to 0.9]
Chronic respiratory disease	Yes vs. No	−1.7 [−4.3 to 0.9]
Obesity	Yes vs. No	0.4 [−0.9 to 1.6]

Abbreviations: CI = confidence interval; HFNC = High-flow nasal cannula. [a] Adjusted for sex and age.

4. Discussion

At hospital discharge, most patients that had recovered from acute COVID-19 infection had decreased functional capacity, and approximately half had exertional desaturation. The patients with a history of HFNC, prolonged hospitalisation, and pulmonary embolism, had worse 1 min STST performance and a higher likelihood of exertional desaturation. We found that almost 80% of the patients had a functional capacity lower than the 2.5th percentile of the reference values used. Our results were in line with other reports that showed a great affectation in the functional capacity of post-COVID-19 patients [18,24].

Our findings showed that hospitalisation for more than ten days and using respiratory support (through the HFNC) increased the risk of exertional desaturation. These were not unexpected since the most severe patients require ventilatory support and consequently spend more days hospitalised in critical units [32,33]. Nevertheless, the literature has shown that impaired functional capacity is not necessarily related to the severity of the disease [34]; damage caused by the virus or generated by ventilatory dependence must also be considered together with the harmful effects of prolonged rest [33].

The patients who had a pulmonary embolism during hospitalisation had an OR of 3.5 to develop exertional desaturation. This aligned with a recent study that found that 29% (24/84) of patients with COVID-19 had a pulmonary embolism; the authors reported a lower level of peripheral oxygen saturation (86.8% vs. 88.6% $p = 0.016$) and longer time of hospitalisation ($p < 0.01$) in patients with a pulmonary embolism compared with the no-pulmonary embolism cases [35]. These findings were related to structural damage, as in 87% of patients, the pulmonary embolism was found in the lung parenchyma affected by COVID-19 pneumonia, with a worse chest tomography severity score and a greater number of lung lobar involvement compared with the non-pulmonary embolism patients [35].

The pathogenesis of COVID-19 associated with pulmonary embolism is unclear [35]. However, it has been reported that some patients with COVID-19 showed pulmonary vascular compromise [36]. On the other hand, some studies have reported vascular compromise in areas of pulmonary opacities, which could indicate an inflammatory response with vascular involvement leading to thrombosis [37,38].

The use of the 1 min STST proved to be a good tool to assess functional capacity and exertional desaturation in the post-COVID-19 patients, which, given its advantages in terms of low space and equipment requirements, could be applied in different settings (e.g., in the office or in telehealth) to identify cases with major functional limitations, and to guide rehabilitation teams in decision making (e.g., exercise prescription) [39]. In fact, the 30 s sit-stand test, as a variant of the sit-to-stand test, has been shown to be a viable and safe option for telehealth assessment and is associated with persistent post-COVID-19 sequelae (e.g., fatigue, dyspnoea, and pain) in non-hospitalised patients [40]. Although there are no protocols comparing the 1 min STST or the 30s STST in patients with COVID-19, there are studies that compare them in COPD showing that the 1 min STST was even better associated with important clinical outcomes such as functional exercise capacity, functional status, and physical activity in daily life [41]. Consequently, healthcare professionals may use this method (as well as the 1 min STST) when face-to-face assessment of physical COVID-19 sequelae is not feasible due to geographical and socio-economic constraints.

On the other hand, this assessment should be complemented by evaluating other relevant health indicators in post-COVID-19 patients, such as pulmonary function [8], social factors [42], comorbidity burden [43], performance in activities of daily living [44], and persistent symptoms such as dyspnea and fatigue [40]. In addition, it should be noted that there were several factors, both from the patients' and the testing centre's perspective, that influenced the patients' physical capacity. For example, advanced age and frailty were strongly associated with reduced functional capacity in COVID-19 survivors [45,46]. These factors can affect muscle strength (older people take longer to regain muscle strength), mobility, endurance, and balance, which may affect independence and quality of life [47]. Therefore, physical function tests such as the short physical performance battery are recommended for this population [48]. Also, cognitive status, which may be impaired in

post-ICU patients due to medication use and/or the presence of delirium [13], may have limited the assessment of physical capacity by using a test that required the following of instructions [14]. Therefore, the characteristics of each individual must be considered when selecting the appropriate test.

Our assessment was mainly based on evaluating physical capacity. However, the long-term effects on physical capacity are closely associated with lung damage, which can be measured through the lung function test and imaging evaluations. The existing literature indicates that approximately 50% of patients exhibit residual lung function abnormalities three months after hospital discharge [49], which is higher in the post-ICU patients [50]. We did not perform lung function evaluation, since clinical guidelines suggest an evaluation between 8- and 12-weeks post discharge [12], since the lung function would reflect the exaggerated inflammatory response of the host to viral pneumonia with severe gas exchange impairment and excessive stress and strain on the lung parenchyma [50,51] more than the actual lung function of the patients.

Strengths and Limitations

The strengths of our study included the relatively large sample size and the use of a validated tool to assess the main outcome. In contrast, this study had some limitations. First, due to the nature of the cross-sectional analysis, we could not establish a causal relationship between the clinical variables and outcomes. Therefore, the results should be interpreted with caution. In addition, we could not rule out selection bias due to the nature of convenience sampling. Also, the absence of radiological images as well as the underreporting of comorbidities in each patient's medical history may have underestimated the true strength of the association of these variables with the outcomes. On the other hand, information on the level of physical activity prior to hospitalisation was unavailable. Therefore, residual confounding bias was possible due to a lack of adjustment for physical activity variables. Finally, the results were compared with international reference values since they are unavailable for our country.

5. Conclusions

Most post-COVID-19 patients experienced a decrease in functional capacity at the time of hospital discharge, and approximately half of them exhibited exertional desaturation after the 1 min STS. The use of a HFNC, prolonged hospitalization, and the occurrence of a pulmonary embolism during hospitalisation were the main clinical variables associated with poorer performance in the 1 min STST performance and with a higher likelihood of exertional desaturation. Future studies should assess the energy expenditure and oxygen consumption to determine if, from a metabolic standpoint, the 1 min STST behaves similarly to the 6MWT in post-COVID-19 patients. Clinical guidelines should incorporate this type of field test to facilitate the evaluation of physical capacity and exercise-induced desaturation, with a focus on patients with a history of HFNC use, prolonged hospitalisation, and pulmonary embolism.

Author Contributions: Conceptualization, S.L., J.V., F.L., J.D., E.R., S.J. and R.T.-C.; methodology, S.L., R.T.-C. and R.N.-C.; software, R.T.-C. and R.N.-C.; validation, S.L., D.S.-R., E.G.-S. and I.B.; formal analysis, S.L., R.T.-C. and R.N.-C.; investigation, S.L., J.V., F.L., J.D., E.R. and S.J.; resources, S.L.; data curation, S.L., J.V., F.L., J.D., E.R. and S.J.; writing—original draft preparation, S.L., R.T.-C., R.N.-C., D.S.-R., E.G.-S. and I.B.; writing—review and editing, S.L., R.T.-C., R.N.-C., D.S.-R., E.G.-S. and I.B.; supervision, S.L.; project administration, S.L. and R.T.-C.; funding acquisition, S.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Comité Independiente de Ética en Investigación Entrerios (protocol code CE000344 and 29 November 2022).

Informed Consent Statement: Patient consent was waived because an anonymized database of a retrospective nature was used.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. John Hopkins University. COVID-19 Dashboard by Centre for Systems Science and Engineering at the John Hopkins University. 2023. Available online: https://coronavirus.jhu.edu/map.html (accessed on 12 May 2023).
2. Berlin, D.A.; Gulick, R.M.; Martinez, F.J. Severe Covid-19. *N. Engl. J. Med.* **2020**, *383*, 2451–2460. [CrossRef] [PubMed]
3. Suh, Y.J.; Hong, H.; Ohana, M.; Bompard, F.; Revel, M.-P.; Valle, C.; Gervaise, A.; Poissy, J.; Susen, S.; Hékimian, G.; et al. Pulmonary Embolism and Deep Vein Thrombosis in COVID-19: A Systematic Review and Meta-Analysis. *Radiology* **2021**, *298*, E70–E80. [CrossRef] [PubMed]
4. Crimi, C.; Pierucci, P.; Renda, T.; Pisani, L.; Carlucci, A. High-Flow Nasal Cannula and COVID-19: A Clinical Review. *Respir. Care* **2022**, *67*, 227–240. [CrossRef]
5. Ahmad, S.J.; Feigen, C.M.; Vazquez, J.P.; Kobets, A.J.; Altschul, D.J. Neurological Sequelae of COVID-19. *J. Integr. Neurosci.* **2022**, *21*, 77. [CrossRef] [PubMed]
6. Sanchez-Ramirez, D.C.; Normand, K.; Zhaoyun, Y.; Torres-Castro, R. Long-Term Impact of COVID-19: A Systematic Review of the Literature and Meta-Analysis. *Biomedicines* **2021**, *9*, 900. [CrossRef]
7. Willi, S.; Lüthold, R.; Hunt, A.; Hänggi, N.V.; Sejdiu, D.; Scaff, C.; Bender, N.; Staub, K.; Schlagenhauf, P. COVID-19 sequelae in adults aged less than 50 years: A systematic review. *Travel Med. Infect. Dis.* **2021**, *40*, 101995. [CrossRef]
8. Torres-Castro, R.; Vasconcello-Castillo, L.; Alsina-Restoy, X.; Solis-Navarro, L.; Burgos, F.; Puppo, H.; Vilaró, J. Respiratory function in patients post-infection by COVID-19: A systematic review and meta-analysis. *Pulmonology* **2021**, *27*, 328–337. [CrossRef]
9. Cares-Marambio, K.; Montenegro-Jiménez, Y.; Torres-Castro, R.; Vera-Uribe, R.; Torralba, Y.; Alsina-Restoy, X.; Vasconcello-Castillo, L.; Vilaró, J. Prevalence of potential respiratory symptoms in survivors of hospital admission after coronavirus disease 2019 (COVID-19): A systematic review and meta-analysis. *Chronic Respir. Dis.* **2021**, *18*, 14799731211002240. [CrossRef]
10. Antoniou, K.M.; Vasarmidi, E.; Russell, A.-M.; Andrejak, C.; Crestani, B.; Delcroix, M.; Dinh-Xuan, A.T.; Poletti, V.; Sverzellati, N.; Vitacca, M.; et al. European Respiratory Society Statement on Long COVID-19 Follow-Up. *Eur. Respir. J.* **2022**, *60*, 2102174. [CrossRef]
11. Sisó-Almirall, A.; Brito-Zerón, P.; Conangla Ferrín, L.; Kostov, B.; Moragas Moreno, A.; Mestres, J.; Sellarès, J.; Galindo, G.; Morera, R.; Basora, J.; et al. Long Covid-19: Proposed Primary Care Clinical Guidelines for Diagnosis and Disease Management. *Int. J. Environ. Res. Public Health* **2021**, *18*, 4350. [CrossRef]
12. Sibila, O.; Molina-Molina, M.; Valenzuela, C.; Ríos-Cortés, A.; Arbillaga-Etxarri, A.; Torralba García, Y.; Díaz-Pérez, D.; Landete, P.; Mediano, O.; Tomás López, L.; et al. Documento de consenso de la Sociedad Española de Neumología y Cirugía Torácica (SEPAR) para el seguimiento clínico post-COVID-19. *Open Respir. Arch.* **2020**, *2*, 278–283. [CrossRef]
13. Torres-Castro, R.; Solis-Navarro, L.; Sitjà-Rabert, M.; Vilaró, J. Functional limitations post-COVID-19: A comprehensive assessment strategy. *Arch. Bronconeumol.* **2021**, *57*, 7–8. [CrossRef] [PubMed]
14. Holland, A.E.; Spruit, M.A.; Troosters, T.; Puhan, M.A.; Pepin, V.; Saey, D.; McCormack, M.C.; Carlin, B.W.; Sciurba, F.C.; Pitta, F.; et al. An official European respiratory society/American thoracic society technical standard: Field walking tests in chronic respiratory disease. *Eur. Respir. J.* **2014**, *44*, 1428–1446. [CrossRef] [PubMed]
15. Bohannon, R.W.; Crouch, R. 1-Minute Sit-To-Stand Test: Systematic review of procedures, performance, and clinimetric properties. *J. Cardiopulm. Rehabil. Prev.* **2019**, *39*, 2–8. [CrossRef] [PubMed]
16. Peroy-Badal, R.; Sevillano-Castaño, A.; Torres-Castro, R.; García-Fernández, P.; Maté-Muñoz, J.L.; Dumitrana, C.; Sánchez Rodriguez, E.; de Frutos Lobo, M.J.; Vilaró, J. Comparison of different field tests to assess the physical capacity of post-COVID-19 patients. *Pulmonology* **2022**. [CrossRef]
17. Simonelli, C.; Paneroni, M.; Vitacca, M.; Ambrosino, N. Measures of physical performance in COVID-19 patients: A mapping review. *Pulmonology* **2021**, *27*, 518–528. [CrossRef]
18. Núñez-Cortés, R.; Rivera-Lillo, G.; Arias-Campoverde, M.; Soto-García, D.; García-Palomera, R.; Torres-Castro, R. Use of sit-to-stand test to assess the physical capacity and exertional desaturation in patients post COVID-19. *Chronic Respir. Dis.* **2021**, *18*, 1479973121999205. [CrossRef]
19. Torres-Castro, R.; Gimeno-Santos, E.; Blanco, I. Use of six-minute walking test to predict peak oxygen consumption in pulmonary vascular disease. *Pulm. Circ.* **2022**, *12*, e12129. [CrossRef]
20. Reychler, G.; Boucard, E.; Peran, L.; Pichon, R.; Le Ber-Moy, C.; Ouksel, H.; Liistro, G.; Chambellan, A.; Beaumont, M. One minute sit-to-stand test is an alternative to 6MWT to measure functional exercise performance in COPD patients. *Clin. Respir. J.* **2018**, *12*, 1247–1256. [CrossRef]
21. Kronberger, C.; Mousavi, R.A.; Öztürk, B.; Willixhofer, R.; Dachs, T.M.; Rettl, R.; Camuz-Ligios, L.; Rassoulpour, N.; Krall, C.; Litschauer, B.; et al. Functional capacity testing in patients with pulmonary hypertension (PH) using the one-minute sit-to-stand test (1-min STST). *PLoS ONE* **2023**, *18*, e0282697. [CrossRef]

22. Spruit, M.A.; Singh, S.J.; Garvey, C.; Zu Wallack, R.; Nici, L.; Rochester, C.; Hill, K.; Holland, A.E.; Lareau, S.C.; Man, W.D.; et al. An official American thoracic society/European respiratory society statement: Key concepts and advances in pulmonary rehabilitation. *Am. J. Respir. Crit. Care Med.* **2013**, *188*, e13–e64. [CrossRef]
23. Benavides-Cordoba, V.; Barros-Poblete, M.; Vieira, R.P.; Mazzucco, G.; Fregonezi, G.; Torres-Castro, R. Provision of pulmonary rehabilitation in Latin America 18 months after the COVID-19 pandemic: A survey of the Latin American Thoracic Association. *Chronic Respir. Dis.* **2022**, *19*, 147997312211041. [CrossRef] [PubMed]
24. Dalbosco-Salas, M.; Torres-Castro, R.; Rojas Leyton, A.; Morales Zapata, F.; Henríquez Salazar, E.; Espinoza Bastías, G.; Beltrán Díaz, M.E.; Tapia Allers, K.; Mornhinweg Fonseca, D.; Vilaró, J. Effectiveness of a Primary Care Telerehabilitation Program for Post-COVID-19 Patients: A Feasibility Study. *J. Clin. Med.* **2021**, *10*, 4428. [CrossRef]
25. Vitacca, M.; Carone, M.; Clini, E.M.; Paneroni, M.; Lazzeri, M.; Lanza, A.; Privitera, E.; Pasqua, F.; Gigliotti, F.; Castellana, G.; et al. Joint Statement on the Role of Respiratory Rehabilitation in the COVID-19 Crisis: The Italian Position Paper. *Respiration* **2020**, *99*, 493–499. [CrossRef] [PubMed]
26. von Elm, E.; Altman, D.G.; Egger, M.; Pocock, S.J.; Gøtzsche, P.C.; Vandenbroucke, J.P. The strengthening the reporting of observational studies in epidemiology (STROBE) statement: Guidelines for reporting observational studies. *Int. J. Surg.* **2014**, *12*, 1495–1499. [CrossRef]
27. Strassmann, A.; Steurer-Stey, C.; Lana, K.D.; Zoller, M.; Turk, A.J.; Suter, P.; Puhan, M.A. Population-based reference values for the 1-min sit-to-stand test. *Int. J. Public Health* **2013**, *58*, 949–953. [CrossRef] [PubMed]
28. Wedzicha, J.A. Domiciliary oxygen therapy services: Clinical guidelines and advice for prescribers. Summary of a report of the Royal College of Physicians. *J. R. Coll. Physicians Lond.* **1999**, *33*, 445–447. [PubMed]
29. Sevillano-Castaño, A.; Peroy-Badal, R.; Torres-Castro, R.; Gimeno-Santos, E.; García Fernández, P.; Garcia Vila, C.; Ariza Alfaro, A.; De Dios Álvarez, R.; Vilaró, J.; Blanco, I. Is there a learning effect on 1-min sit-to-stand test in post-COVID-19 patients? *ERJ Open Res.* **2022**, *8*, 3. [CrossRef]
30. Yue, H.; Yu, Q.; Liu, C.; Huang, Y.; Jiang, Z.; Shao, C.; Zhang, H.; Ma, B.; Wang, Y.; Xie, G.; et al. Machine learning-based CT radiomics method for predicting hospital stay in patients with pneumonia associated with SARS-CoV-2 infection: A multicenter study. *Ann. Transl. Med.* **2020**, *8*, 859. [CrossRef]
31. Chen, H.; Cohen, P.; Chen, S. How Big is a Big Odds Ratio? Interpreting the Magnitudes of Odds Ratios in Epidemiological Studies. *Commun. Stat. Simul. Comput.* **2010**, *39*, 860–864. [CrossRef]
32. Cronin, J.N.; Camporota, L.; Formenti, F. Mechanical ventilation in COVID-19: A physiological perspective. *Exp. Physiol.* **2022**, *107*, 683–693. [CrossRef]
33. Pescaru, C.C.; Marițescu, A.; Costin, E.O.; Trăilă, D.; Marc, M.S.; Trușculescu, A.A.; Pescaru, A.; Oancea, C.I. The Effects of COVID-19 on Skeletal Muscles, Muscle Fatigue and Rehabilitation Programs Outcomes. *Medicina* **2022**, *58*, 1199. [CrossRef] [PubMed]
34. Townsend, L.; Dowds, J.; O'Brien, K.; Sheill, G.; Dyer, A.H.; O'Kelly, B.; Hynes, J.P.; Mooney, A.; Dunne, J.; Ni Cheallaigh, C.; et al. Persistent Poor Health after COVID-19 Is Not Associated with Respiratory Complications or Initial Disease Severity. *Ann. Am. Thorac. Soc.* **2021**, *18*, 997–1003. [CrossRef] [PubMed]
35. Cau, R.; Pacielli, A.; Fatemeh, H.; Vaudano, P.; Arru, C.; Crivelli, P.; Stranieri, G.; Suri, J.S.; Mannelli, L.; Conti, M.; et al. Complications in COVID-19 patients: Characteristics of pulmonary embolism. *Clin. Imaging* **2021**, *77*, 244–249. [CrossRef]
36. Connors, J.M.; Levy, J.H. COVID-19 and its implications for thrombosis and anticoagulation. *Blood* **2020**, *135*, 2033–2040. [CrossRef] [PubMed]
37. Saba, L.; Sverzellati, N. Is COVID Evolution Due to Occurrence of Pulmonary Vascular Thrombosis? *J. Thorac. Imaging* **2020**, *35*, 344–345. [CrossRef]
38. Gando, S.; Wada, T. Pathomechanisms Underlying Hypoxemia in Two COVID-19-Associated Acute Respiratory Distress Syndrome Phenotypes: Insights From Thrombosis and Hemostasis. *Shock* **2022**, *57*, 1. [CrossRef]
39. Holland, A.E.; Malaguti, C.; Hoffman, M.; Lahham, A.; Burge, A.T.; Dowman, L.; May, A.K.; Bondarenko, J.; Graco, M.; Tikellis, G.; et al. Home-based or remote exercise testing in chronic respiratory disease, during the COVID-19 pandemic and beyond: A rapid review. *Chronic Respir. Dis.* **2020**, *17*, 147997312095241. [CrossRef]
40. Núñez-Cortés, R.; Flor-Rufino, C.; Martínez-Arnau, F.M.; Arnal-Gómez, A.; Espinoza-Bravo, C.; Hernández-Guillén, D.; Cortés-Amador, S. Feasibility of the 30 s Sit-to-Stand Test in the Telehealth Setting and Its Relationship to Persistent Symptoms in Non-Hospitalized Patients with Long COVID. *Diagnostics* **2022**, *13*, 24. [CrossRef]
41. Morita, A.A.; Bisca, G.W.; Machado, F.V.C.; Hernandes, N.A.; Pitta, F.; Probst, V.S. Best Protocol for the Sit-to-Stand Test in Subjects With COPD. *Respir. Care* **2018**, *63*, 1040–1049. [CrossRef]
42. Núñez-Cortés, R.; Ortega-Palavecinos, M.; Soto-Carmona, C.; Torres-Gangas, P.; Concha-Rivero, M.P.; Torres-Castro, R. Social determinants of health associated with severity and mortality in patients with COVID-19. *Gac. Med. Mex.* **2021**, *157*, 263–270. [CrossRef]
43. Núñez-Cortés, R.; Malhue-Vidal, C.; Gath, F.; Valdivia-Lobos, G.; Torres-Castro, R.; Cruz-Montecinos, C.; Martinez-Arnau, F.M.; Pérez-Alenda, S.; López-Bueno, R.; Calatayud, J. The Impact of Charlson Comorbidity Index on the Functional Capacity of COVID-19 Survivors: A Prospective Cohort Study with One-Year Follow-Up. *Int. J. Environ. Res. Public Health* **2022**, *19*, 7473. [CrossRef]

44. Pizarro-Pennarolli, C.; Sánchez-Rojas, C.; Torres-Castro, R.; Vera-Uribe, R.; Sanchez-Ramirez, D.C.; Vasconcello-Castillo, L.; Solís-Navarro, L.; Rivera-Lillo, G. Assessment of activities of daily living in patients post COVID-19: A systematic review. *PeerJ* **2021**, *9*, e11026. [CrossRef] [PubMed]
45. Núñez-Cortés, R.; Leyton-Quezada, F.; Pino, M.B.; Costa-Costa, M.; Torres-Castro, R. Physical and emotional sequelae after hospitalization for COVID-19. *Rev. Med. Chile* **2021**, *149*, 1031–1035. [CrossRef] [PubMed]
46. Prampart, S.; Le Gentil, S.; Bureau, M.L.; Macchi, C.; Leroux, C.; Chapelet, G.; de Decker, L.; Rouaud, A.; Boureau, A.S. Functional decline, long term symptoms and course of frailty at 3-months follow-up in COVID-19 older survivors, a prospective observational cohort study. *BMC Geriatr.* **2022**, *22*, 542. [CrossRef] [PubMed]
47. Amarante do Nascimento, M.; Nunes, J.P.; Pina, F.L.C.; Ribeiro, A.S.; Carneiro, N.H.; Venturini, D.; Barbosa, D.S.; Mayhew, J.L.; Cyrino, E.S. Comparison of 2 Weekly Frequencies of Resistance Training on Muscular Strength, Body Composition, and Metabolic Biomarkers in Resistance-Trained Older Women: Effects of Detraining and Retraining. *J. Strength Cond. Res.* **2022**, *36*, 1437–1444. [CrossRef]
48. Torres-Castro, R.; Núñez-Cortés, R.; Larrateguy, S.; Alsina-Restoy, X.; Barberà, J.A.; Gimeno-Santos, E.; García, A.R.; Sibila, O.; Blanco, I. Assessment of Exercise Capacity in Post-COVID-19 Patients: How Is the Appropriate Test Chosen? *Life* **2023**, *13*, 621. [CrossRef]
49. Orzes, N.; Pini, L.; Levi, G.; Uccelli, S.; Cettolo, F.; Tantucci, C. A prospective evaluation of lung function at three and six months in patients with previous SARS-COV-2 pneumonia. *Respir. Med.* **2021**, *186*, 106541. [CrossRef]
50. Pini, L.; Montori, R.; Giordani, J.; Guerini, M.; Orzes, N.; Ciarfaglia, M.; Arici, M.; Cappelli, C.; Piva, S.; Latronico, N.; et al. Assessment of respiratory function and exercise tolerance at 4-6 months after COVID-19 infection in patients with pneumonia of different severity. *Intern. Med. J.* **2023**, *53*, 202–208. [CrossRef]
51. Batah, S.S.; Fabro, A.T. Pulmonary pathology of ARDS in COVID-19: A pathological review for clinicians. *Respir. Med.* **2021**, *176*, 106239. [CrossRef]

biomedicines

MDPI

Review

Efficacy of Palmitoylethanolamide and Luteolin Association on Post-Covid Olfactory Dysfunction: A Systematic Review and Meta-Analysis of Clinical Studies

Anna Paola Capra [1], Alessio Ardizzone [1], Lelio Crupi [1], Fabrizio Calapai [1,2], Michela Campolo [1], Salvatore Cuzzocrea [1] and Emanuela Esposito [1,*]

[1] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98166 Messina, Italy; annapaola.capra@unime.it (A.P.C.); aleardizzone@unime.it (A.A.); lelio.crupi@student.unime.it (L.C.); fabrizio.calapai@unime.it (F.C.); campolom@unime.it (M.C.); salvator@unime.it (S.C.)

[2] Department of Clinical and Experimental Medicine, University of Messina, 98125 Messina, Italy

* Correspondence: eesposito@unime.it; Tel.: +39-090-6765208

Abstract: Post-Covid Olfactory Dysfunction (PCOD) is characterized by olfactory abnormalities, hyposmia, and anosmia, which are among the most often enduring symptoms in individuals who have recovered from SARS-CoV-2 infection. This disorder has been reported to persist in subsets of patients well after 12 months following infection, significantly affecting their quality of life. Despite the high prevalence of PCOD among patients who suffered from SARS-CoV-2 infection, specific therapeutic strategies are still limited. Among these, emerging evidence seems to indicate the administration of CoUltraPEALut, a combination of micronized Palmitoylethanolamide (PEA), an endogenous fatty acid amide, and Luteolin, a natural antioxidant flavonoid, as a viable therapy, especially when given as an adjuvant to olfactory training. Based on the above, a systematic review and a meta-analysis of the literature were conducted, with the aim of evaluating the efficacy of CoUltraPEALut as an addition to olfactory training (OT), in treating PCOD symptoms. Pubmed (MEDLINE), Embase (OVID), and Web of Science scientific databases were screened from the inception until 31 May 2023, and a total of 407 articles were recovered; only five of these studies (441 total patients between treated and control groups) were included in the systematic review. CoUltraPEALut demonstrated significant efficacy in the overall recovery of the olfactory function, compared to the conventional therapy, suggesting that it could represent a possible future adjuvant treatment for PCOD.

Keywords: palmitoylethanolamide (PEA); Luteolin; CoUltraPEALut; SARS-CoV-2; COVID-19; Post-Covid Olfactory Dysfunction (PCOD); respiratory disorders

Citation: Capra, A.P.; Ardizzone, A.; Crupi, L.; Calapai, F.; Campolo, M.; Cuzzocrea, S.; Esposito, E. Efficacy of Palmitoylethanolamide and Luteolin Association on Post-Covid Olfactory Dysfunction: A Systematic Review and Meta-Analysis of Clinical Studies. *Biomedicines* **2023**, *11*, 2189. https://doi.org/10.3390/biomedicines11082189

Academic Editor: César Fernández De Las Peñas

Received: 19 June 2023
Revised: 27 July 2023
Accepted: 2 August 2023
Published: 3 August 2023

1. Introduction

N-acetylethanolamines (NAEs) are a family of endogenous lipid molecules that include palmitoylethanolamide (PEA) [1,2].

PEA is produced "on demand" by our body, in response to stressful conditions or inflammatory stimuli, thus denoting its key role in maintaining cellular homeostasis [3]. Even though it is speculated that PEA can interact via binding to certain nuclear receptors like PPAR, and in particular to PPAR-α, as well as to a cannabinoid-type receptor GPR55, the mechanism of action of PEA is not yet completely understood [4]. Although PEA has been shown to have powerful protective activities by Autacoid Local Injury Antagonism (ALIA) mechanism [5], one of its main problems is its poor bioavailability [2]. Concerning this, pharmaceutical micronization processes have proven to be very advantageous for dissolution enhancement of poorly water-soluble drugs [6]. PEA-um, as a new formulation of PEA, has been shown to be effective in reducing inflammatory processes [2].

The conjugation of PEA with antioxidant molecules may increase its efficacy and provide stronger pharmacological effects. In fact, PEA lacks a direct antioxidant ability for preventing the generation of free radicals and mitigating the damage that free radicals cause to DNA, lipids, and proteins.

In recent years, scientific interest in flavonoids has increased enormously; in fact, considering their biological effects, these precious natural molecules can be a valuable support against several diseases, providing beneficial effects for human health. In particular, Luteolin occurs naturally in many vegetables and fruits, and its therapeutic effects on various pathologies were demonstrated by much scientific evidence. Due to its biological characteristics, it has potent anti-inflammatory, anti-diabetic, antioxidant, and anticancer effects, which improve patient clinical outcomes in many pathological settings.

The pharmaceutical formulation combining PEA with Luteolin, known as CoUltraPEALut, has been shown to have neuroprotective and neuroregenerative properties following traumatic brain injury (TBI) or MPTP-induced Parkinson's disease [7].

These beneficial effects on human health would suggest the use of CoUltraPEALut also in different clinical pictures.

Over the past three years, most clinical efforts have been directed toward the COVID-19 pandemic, which has constituted one of the worst global public health emergencies.

Although the rapid development of vaccines has mitigated the impact of COVID-19 on severe clinical outcomes and mortality rate, the search for pharmacological strategies effective in moderating the long COVID-19 syndrome remains an unsolved clinical challenge. The most typical symptoms of post-COVID-19 syndrome include fatigue, headache, dyspnea, hoarseness of voice, and myalgia, and the presence of comorbidities may worsen long-term illnesses [8]. In this scenario, many COVID-19 patients exhibited olfactory dysfunction, now identified as Post-Covid Olfactory Dysfunction (PCOD), which is a major concern following infection [9]. Both peripheral and central mechanisms have been suggested as possibly involved in the occurrence of PCOD [10]. As far as a peripheral mechanism is concerned, SARS-CoV-2 is known to have a marked tropism for respiratory epithelial cells, particularly for sustentacular cells, because of the high expression rate on these cells of ACE-2 receptors [11]. On the other hand, there is evidence showing that SARS-CoV-2 has only marginal affinity for olfactory sensory neurons, namely the mediators of the olfactory perceptions [12]. However, it has been proposed that an extensive damage of the respiratory epithelium may result in long-term reduction of the olfactory functionality. Recent evidence seems to support this hypothesis, in that a primary damage of sustentacular cells may disrupt homeostasis of nearby olfactory sensory neurons, thus inducing a marked and sustained alteration in the gene expression of olfactory sensory neurons [13].

On the other hand, it has been suggested that several neurological signs in SARS-CoV-2 patients may be due to the diffusion of the infectious virion, or of inflammatory mediators, from the cribriform plate up to the olfactory bulb via paracellular or transcellular pathways [10]. However, only fragmentary information is presently available on the involvement of the olfactory bulbs during SARS-CoV-2 infection. Evidence from COVID-19 autopsy reports has been provided, showing marked inflammation in the olfactory bulbs, along with significantly increased SARS-CoV-2 RNA levels in the olfactory bulbs compared to other cerebral regions [14,15]. Furthermore, some studies, using MRI techniques, discovered morphological changes (mainly a decreased volume) in the olfactory bulb and in the related cortical areas [16,17].

Considering these assumptions, our review question focuses on whether CoUltraPEALut could improve olfactory loss following COVID-19 infection.

Therefore, by investigating PubMed (MEDLINE), Embase (OVID), and Web of Science scientific databases, this systematic review and meta-analysis aimed to assess the efficacy of CoUltraPEALut in improving olfactory dysfunction-related outcomes in long COVID-19 patients.

2. Methods

2.1. Search Strategy

We performed the literature search by using PubMed (MEDLINE), Embase (OVID), and Web of Science bibliographic databases. We used the Preferred Reporting Items for Systematic Review and Meta-analysis Protocols (PRISMA-P) guidelines in order to report a detailed search strategy of the articles. Based on the qualifying requirements listed in Table 1 and taking into account only English-language literature, APC and AA conducted the bibliographic search.

Table 1. Description of inclusion and exclusion criteria employed for the literature search.

Inclusion Criteria	Exclusion Criteria
Clinical Trials or Randomized Controlled Trials evaluating CoUltraPEALut formulation (Glialia®, Epitech Group SpA, Saccolongo, Italy) in long COVID-19 olfactory dysfuction.	Observational studies, case-control studies, case reports, cross-sectional studies, cohort studies, editorials, letters, reviews, guidelines, abstracts and paper conferences, systematic reviews and meta-analyses, and ongoing studies. Articles not written in English.

The search strategy was developed, and the study was supervised by two content experts (MC and EE).

We searched for a period comprising from 2020 to 2023, no geographic exclusion criteria were imposed. Terms related to CoUltraPEALut in the context of long COVID-19 were explored in PubMed (MEDLINE), Embase (OVID), and Web of Science databases by using specific keywords summarized in Table 2.

Table 2. Keyword combinations used during the search strategy.

CoUltra PEALut	Long COVID-19
Palmitoylethanolamide, PEA, Luteolin, CoUltra PEALut, Co-Ultra PEALut, um-PEA-LUT, PEA-LUT, Glialia.	COVID-19, COVID19, COVID-19 Virus, COVID-19 Viruses, COVID-2019, SARS-CoV-2, SARS-CoV-2 Infection, Coronavirus, Coronaviruses, long COVID-19, long COVID-19 syndrome, long COVID-19 syndromes, post COVID-19, post COVID-19 syndrome, post COVID-19 syndromes, COVID-19 syndrome, COVID-19 syndromes, COVID-19 olfactory loss, COVID-19 olfactory dysfunction, COVID-19 olfaction, long-haul COVID syndrome, post-acute sequelae of SARS-CoV-2 infection (PASC).

2.2. Study Selection

We first performed our search using PubMed (MEDLINE), Embase (OVID), and Web of Science databases and then we excluded duplicates. After this step, the titles and abstracts of all findings found were then separately examined by the two review authors (APC and AA) to weed out any records that were not pertinent. After that, we carefully examined the full-text articles to select those that met the requirements for eligibility. The involvement of a third review author (EE) helped to reconcile differing viewpoints.

The included studies' data were extracted by two authors (APC and AA). We collected the following information from the five included studies: title, author(s), publication year, study catchment area (i.e., geographic zone), study participants, and associated clinical outcomes.

2.3. Assessment of Risk of Bias

Using the Cochrane Risk of Bias 2.0 (RoB2) tool, two reviewers (APC and AA) independently evaluated the quality of the eligible records.

Specifically, they evaluated the risk of bias on five domains: randomization process (D1), deviations from the intended interventions (D2), missing outcome data (D3), measurement of the outcome (D4), and selection of the reported result (D5).

This assessment led to the classification of the study's value as low, medium, or high. A third review author (EE), who assisted in attaining consensus, was brought in to help settle differences in score allocations. None of the papers were deemed to be at a high risk of bias after the authors' evaluations.

2.4. Data Synthesis Methods for Meta-Analysis

For statistical analysis in the meta-analysis, we employed an odds ratio (OR) measure and the random-effects model with the Mantel–Haenszel approach. We successfully combined estimates of the variant effect (OR) and its corresponding 95% confidence interval (CI). The forest plots were graphically examined to determine the heterogeneity, which was then measured using the I^2 statistic [18,19]. The meta-analysis of the pooled data was carried out using Review Manager (RevMan Version 5.4., The Nordic Cochrane Centre, The Cochrane Collaboration: Copenhagen, Denmark, 2014).

3. Results and Discussion

3.1. Findings from Systematic Search

Using the PRISMA-P flowchart, we show the entire screening procedure in Figure 1. By combining the search terms listed in Table 2, we were able to find 407 records in the PubMed (MEDLINE), Embase (OVID), and Web of Science databases. After removing the duplicates, we had 273 records left, which we next examined for eligibility considering the title and abstract. Because their title and abstract were not pertinent to our review topic, we disregarded 250 publications in this phase.

After checking 23 articles' entire texts for eligibility, we eliminated 18 records since they did not meet the inclusion and exclusion requirements. As illustrated by the PRISMA Flowchart presented in Figure 1, we finally included five studies in our systematic review that assessed the effectiveness of CoUltraPEALut in PCOD. We also performed a meta-analysis to determine if CoUltraPEALut was successful in treating PCOD patients.

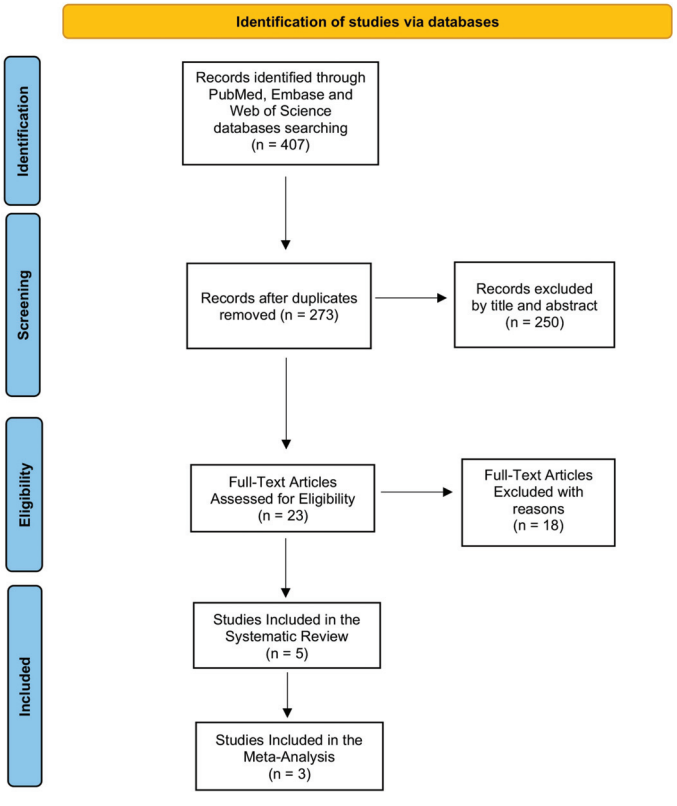


Figure 1. PRISMA flow diagram. The picture outlines each phase of the search strategy and screening procedure, which was carried out in accordance with PRISMA-P guidelines.

Only three records that examined the effects of CoUltraPEALut on olfactory dysfunction (measured by Sniffin' Sticks and reported as TDI score) compared to a control group were chosen for this purpose from the five studies that were included in the systematic review.

3.2. Evaluation of Included Studies in the Systematic Review

Olfactory dysfunction was evaluated in each included trial using the Sniffin' Stick score (TDI score) between the control group and treatment group both at T0 (baseline) and T1 (endpoint). Overall, in each study (summarized in Table 3), CoUltraPEALut treatment plus olfactory training (OT) considerably increased the TDI score values compared to the control group improvement, indicating a stronger recovery of olfactory function.

Twelve people, ranging in age from 18 to 90, were included in the study of D'Ascanio et al. Patients had a documented history of COVID-19 and anosmia or hyposmia that persisted for at least 90 days following a negative COVID-19 nasopharyngeal swab result. Subjective reports at T0, the beginning of the trial, indicated persistent smell problems. The evaluation at T1 (30 days following the trial's completion), which included OT sessions and adherence to the treatment group's supplement regimen, was conducted on all study participants [20]. Treatment combining olfactory rehabilitation with oral PEA and luteolin supplementation has been associated with increased olfactory function recovery (evaluated by Sniffin' Sticks), particularly pronounced in individuals with chronic olfactory impairment [20].

De Luca et al. included 69 patients with a verified history of COVID-19, including 43 women and 26 men, ages 18 to 80, with anosmia/hyposmia that persisted for at least 180 days (six months) following a negative COVID-19 nasopharyngeal swab. They were assigned to three groups: (1) individuals with prior OT received a daily PEA-LUT oral supplement and continued OT; (2) Training-Naïve 1 (PEA-LUT plus OT) patients consumed one sublingual sachet of PEA-LUT per day and performed OT three times a day; and (3) patients consumed one sublingual sachet of PEA-LUT per day and underwent no additional intervention [21].

Overall, the treatment using oral PEA-LUT and olfactory training improved olfactory dysfunction in individuals with protracted COVID and chronic olfactory loss [21] as evidenced by improvement in TDI score and an improved perception of smells through the administration of a questionnaire that contained 52 different odors.

With 185 patients, ranging in age from 18 to 80, who had verified COVID-19 histories and anosmia or hyposmia that persisted for more than 180 days (six months) after a later negative COVID-19 nasopharyngeal swab, Di Stadio et al. conducted a multicenter double-blinded randomized clinical study. These are the two study groups' definitions: (2) Conventional therapy (control group): daily treatment with placebo and OT; (1) Intervention therapy (intervention group): daily treatment with PEA-LUT oral supplement [22].

In a different study, Di Stadio and colleagues recruited a different group of patients with the same long covid condition, aged 18 to 60 years with a confirmed history of COVID-19 and anosmia/hyposmia persisting $\geq$180 days after a negative COVID-19 nasopharyngeal swab. The COVID-19 group subjects were treated using um-PEA-LUT plus OT [23].

In addition, the most recent work of Stadio et al. evaluated once again the efficacy of CoUltraPEALut and OT compared to OT alone for the treatment of smell disorders in another Italian region, thus confirming in another set of analyses the advantages of PEA and Lut combination on the quality smell disorders in the post-COVID population [24]. The research comprised a total of 130 patients; 94 patients (49 women and 45 men, average age 36.7 $\pm$ 11.8) were placed in the treatment group, while 36 patients (21 women and 15 men, average age 50.5 $\pm$ 12.7) were placed in the control group. Patients in the therapy group had smell alteration lasting 8.8 $\pm$ 3.7 months on average, compared to 8.5 $\pm$ 2 months for patients in the control group [24]. Nevertheless, the authors cautioned that their findings on parosmia are constrained and that the difference in average ages between the groups has affected their interpretation of the data. This discovery was verified by the identification of age and sniffing score at T0 as components that influenced the parosmia resolution. In fact, the control group comprised younger patients than the treatment group, who might have

benefited from spontaneous recovery, which is less likely in patients over 40. Therefore, it was noted that more research comparing patients of the same age is required to determine whether the PEA-Lut combination is effective in treating qualitative smell changes.

Table 3. Summary of the studies included in the systematic review that assessed the effectiveness of CoUltraPEALut for olfactory dysfunction.

First Author and Year of Publication	Number of Patients Included	CoUltraPEALut Dosage Regimen	Main Results	Reference
D'Ascanio et al., 2021	Total *n*: 12 patients with PCOD - Treatment group (OT + umPEA-LUT), *n*: 7 - Control group (OT + Placebo), *n*: 5	umPEA-LUT (PEA 700 mg and Luteolin 70 mg) once a day, for 30 consecutive days	Patients which received umPEA-LUT in combination to OT had a significant improvement in TDI scores, evaluated with the Sniffin' Sticks identification test, compared to the Control group.	[20]
De Luca et al., 2022	Total *n*: 69 patients with PCOD - Previously trained group: individuals previously exposed to OT (OT + umPEA-LUT), *n*: 10 - Training-Naïve 1: individuals not previously exposed to OT (OT + umPEA-LUT) *n*: 43 - Training-Naïve 2: individuals not previously exposed to OT (umPEA-LUT without OT), *n*: 16	umPEA-LUT (PEA 700 mg and Luteolin 70 mg) once a day, for 90 consecutive days	Treatment with umPEA-LUT was associated with an improvement in PCOD and mental clouding symptoms. The effects were more pronounced when combining PEA-LUT and OT.	[21]
Di Stadio et al., 2022	Total *n*: 185 patients with PCOD - Treatment group (OT + umPEA-LUT), *n*: 130 - Control group (OT + Placebo), *n*: 55	umPEA-LUT (PEA 700 mg and Luteolin 70 mg) once a day, for 90 consecutive days	Patients receiving umPEA-LUT showed a significantly greater improvement in TDI scores in comparison to patients in the control group.	[22]
Di Stadio et al., 2023	Total *n*: 130 patients with PCOD reporting parosmia as a symptom - Treatment group (OT + umPEA-LUT), *n*: 94 - Control group (OT + Placebo), *n*: 36	umPEA-LUT (PEA 700 mg and Luteolin 70 mg) once a day, for 90 consecutive days	umPEA-LUT in combination with OT shows significant efficacy compared to OT alone, in treating quantitative olfactory alterations, measured as TDI scores. No significant effects on qualitative alterations (parosmia) were observed between the groups.	[24]
Di Stadio et al., 2023	Total *n*: 45 with PCOD - PCOD group (OT + umPEA-LUT), *n*: 45	(umPEA-LUT) PEA 700 mg and Luteolin 70 mg once a day, for 90 consecutive days	PCOD patients at the end of the umPEA-LUT treatment period showed significantly higher TDI scores compared to the baseline scores.	[23]

3.3. Meta-Analysis

The inclusion criteria (quantitative comparison of TDI score between treated patients and controls) were only met by three records from the screening method used to identify trials eligible for meta-analysis.

Although there was significant heterogeneity (I^2 = 89%), the total OR was 3.07 (95% CI: 2.22–3.92) and the test for overall effect was $p < 0.00001$.

In addition, as can be seen in the forest plot (Figure 2), the ORs of each single study vary from 1.80 to 7.90. This outcome had a strong statistical significance, showing the significant increased rate of olfactory recovery after CoUltraPEALut administration associated with OT, compared to control subjected only to OT.

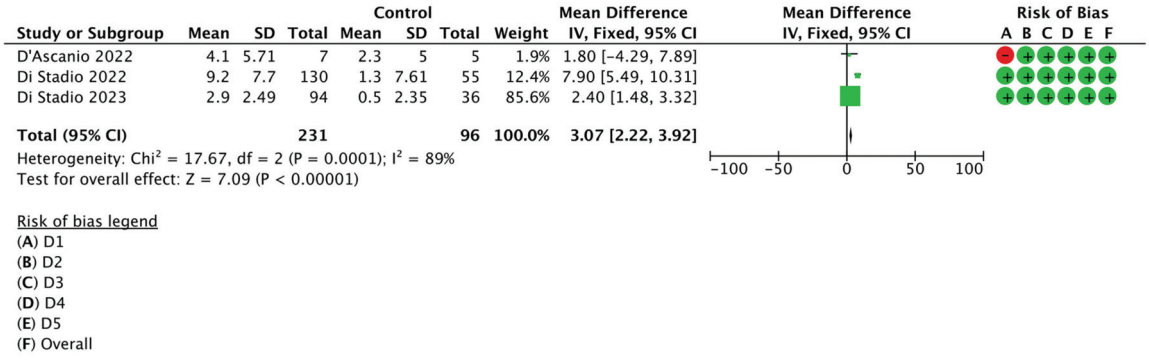


Figure 2. Forest plot of the studies analyzed in the quantitative synthesis [20,22,24]. By subtracting the value at endpoint with the value at baseline and calculating the combined SD, the forest plot shows how the TDI score improved in treated patients and control individuals. The impact estimate (ORs) is displayed as squares, with the size of each green square reflecting the weight assigned to each research in the meta-analysis. The 95% confidence intervals (CIs) for each effect estimate are shown as horizontal lines. The black diamond's width, which indicates the total 95% CI, represents the overall effect of intervention. A measurement of heterogeneity is the I^2 statistic. Effect size OR: 3.07 [2.22, 3.92]; $p < 0.00001$.

Furthermore, to emphasize how different statistical approaches impact the outcome, we choose to present the same data using the random-effect model due to the significant degree of heterogeneity detected in the previous analysis (Figure 3).

The employment of the random-effect model revealed the same degree of heterogeneity ($I^2 = 89\%$), while the total OR was greater, reaching a value of 4.28 although with an extended 95% CI: −0.04, 8.60 while the test for overall effect was less significant showing a $p = 0.05$.

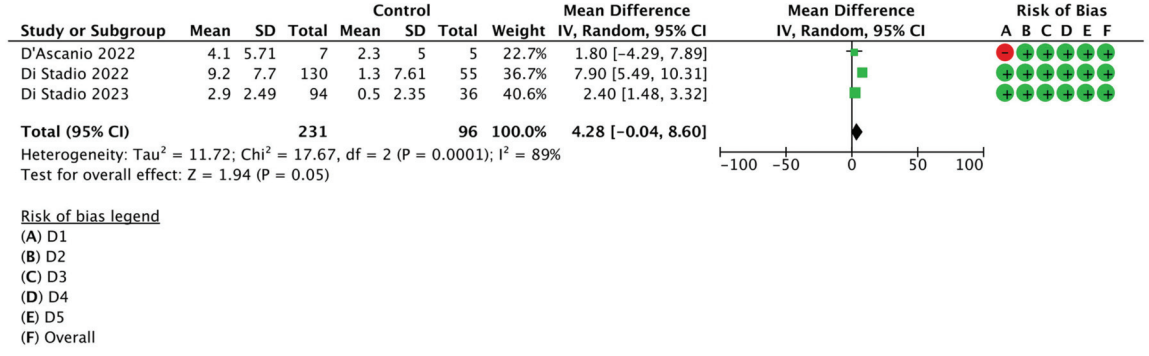


Figure 3. Forest plot of the studies analyzed in the quantitative synthesis [20,22,24]. By subtracting the value at endpoint with the value at baseline and calculating the combined SD, the forest plot shows how the TDI score improved in treated patients and control individuals. The impact estimate (ORs) is displayed as squares, with the size of each green square reflecting the weight assigned to each research in the meta-analysis. The 95% confidence intervals (CIs) for each effect estimate are shown as horizontal lines. The black diamond's width, which indicates the total 95% CI, represents the overall effect of intervention. A measurement of heterogeneity is the I^2 statistic. Effect size OR: 4.28 [−0.04, 8.60]; $p = 0.05$.

3.4. Discussion

Olfactory impairment or loss is one of the most common chemosensory dysfunctions associated with COVID-19 infection [25,26]. Its prevalence during the acute phase of the disease varies considerably among series, according to whether any degree of smell impairment is considered (i.e., hyposmia or only true anosmia), olfactory impairment is selectively evaluated, and not least the method used for olfactory loss detection [27]. Studies on the olfactory system show that SARS-CoV-2 can spread to the olfactory bulb and other parts of the central nervous system after entering the olfactory neuroepithelium [28]. SARS-CoV-2 is capable of persisting in patients' olfactory bulbs even after they have recovered from an acute infection, leading to persistent olfactory impairments [29].

According to research, brain inflammation may be a frequent mediator of symptoms in patients with altered smell, who may also experience headache or brain fog [30,31]. Additionally, in the long COVID-19 research field, studies using MRI have shown that SARS-CoV-2 infection caused inflammatory changes to the olfactory bulbs [16].

Here, we focused on the improvement of clinical outcomes in PCOD patients following the administration of CoUltraPEALut, through a systematic search of different scientific databases, with the aim of investigating its beneficial effects in long COVID-19 patients.

By lessening the degree of neuroinflammation brought on by SARS-CoV-2, CoUltraPEALut treatment could enhance regeneration during olfactory training [32].

In fact, it was widely recognized that PEA has been shown to shift microglia's polarization to a protective M2 phenotype, boosting brain regeneration and potentially favoring smell restoration [33]. Likewise, by preventing pro-inflammatory microglia from polarizing, luteolin prevents the deterioration of brain cells [34].

In accordance, our review including five studies and a total of 441 subjects (matching treated and control groups) showed persisting abnormalities of their sense of smell (hyposmia or anosmia) post COVID-19 infection. To solve olfactory dysfunction, in all these recent studies, including clinical trials and longitudinal study, the treatments with CoUltraPEALut, OT alone, or their combination, were compared.

In every systematically searched clinical study, olfactory recovery was better when oral CoUltraPEALut supplementation was paired with OT than OT alone. In particular, the goal of this multimodal strategy was to lessen neuroinflammation in the olfactory system and foster a regenerative environment that could promote olfactory healing.

Taken as a whole, these clinical trials imply that CoUltraPEALut should be taken for at least 30 days to demonstrate statistically meaningful benefit, and extended follow-ups of at least 60–90 days seem to show even greater consistency.

Moreover, the combination of three studies in meta-analysis resulted in a total of 327 patients. The meta-analysis carried out statically confirmed the significant increase in olfactory recovery in CoUltraPEALut plus OT treated patients compared to OT patients (OR: 3.07; 95% CI: 2.22–3.92; test for overall effect $p < 0.00001$). Even if with a less statical significance when the random-effect model was employed (OR: 4.28; 95% CI: −0.04, 8.60; test for overall effect $p = 0.05$).

Our findings are consistent with current research trends in the PCOD field. Indeed, the combination of PEA-LUT and OT was noted in a 2022 Cochrane review on treatments for smell disorders caused by SARS-CoV-2 infection [35], although no statistical validation was performed.

Nevertheless, despite the high PCOD prevalence, therapeutic chances are presently limited. As stated, the most employed therapeutic approach is OT, a non-pharmacological procedure based on the repeated exposure of the affected subject to known odorants, usually twice a day for 12 weeks [36]. This approach proved effective in reverting olfactory dysfunctions consequent to viral infections and, more recently, in COVID-19 infection [37,38]. In this regard, a trial conducted by Denis et al. on 548 patients showed how OT, conducted for a mean period of 27.7 days, induced a significant improvement in 64.2% of patients (352/548) [39]. Unfortunately, there are still existing limitations to the use OT for the therapy of PCOD, the

main ones being the high patient compliance needed and the length of the training periods needed in order to produce significant therapeutic results.

Therefore, combined approaches of OT and pharmacological treatments have been proposed. To date, only a limited number of randomized clinical trials have demonstrated promising therapeutic strategies. This lack of specific medications for the treatment of PCOD likely reflects the gaps in the knowledge concerning the pathogenesis of long-term olfactory damage in COVID-19 patients.

Both oral and intranasal corticosteroids have been among the first substances tested as a potential therapy for PCOD, with some evidence, to date, supporting their efficacy.

As an example, a pilot study by Le Bon et al. showed that OT in combination with short-term oral steroids (10 days) was associated with a greater improvement in the olfactory score than OT alone [40]. Different molecules have been included as potential candidates in PCOD therapeutic regimen like Omega-3 supplements, topical Vitamin A, Alpha-lipoic acid, and theophylline, among others; however, evidence available is mostly limited and from non-randomised studies [41].

Thus, among the potential compounds studied, CoUltraPEALut is one of the few that has demonstrated promising efficacy.

To date, based on the most recent estimates of the World Health Organization (WHO) on the global spread of the COVID-19 pandemic, the above rates translate into tens of millions of subjects who either experienced or are presently experiencing different degrees of smell impairments and related quality of life deterioration [42,43].

In particular, PCOD has been reported at 12–24 months after COVID-19 remission in 3–25% of patients initially complaining of olfactory symptoms [44], thus constituting a real clinical challenge. As stated, from a clinical perspective, patients affected from PCOD complain of both quantitative (hyposmia and anosmia) and qualitative olfactory alterations. The latter includes parosmia, i.e., a distortion in smell perception, and phantosmia, i.e., olfactory hallucinations, among the main ones.

In addition, it is important to underline that olfactory disorders may cause several daily life problems, such as altered social relations, decreased capacity for danger avoidance, abnormalities in food intake (either increased or decreased), and reduced working efficiency [45]. Moreover, chemosensory dysfunctions have been reported to be related to higher rates of depression and mood disorders [45].

Therefore, the validation of CoUltraPEALut in large-scale studies, as well as the future search of new care options represent an essential need to facilitate the full recovery of PCOD patients.

Despite that our work collected all the evidence published to date about the topic, several limitations persist. The small sample size, which is subject to underpowered analysis, is the main drawback. In addition, the baseline features of individuals can also vary, with noticeable variations in the degree and length of olfactory impairment at baseline. Additionally, we did not take into consideration co-factors like smoking history, other comorbidities like hypertension, cardiovascular diseases, obesity, diabetes, thyroid disorders, allergy, and psychiatric conditions, that can have a negative impact on the health condition and indirectly affect the physiological function of nasal mucosa. Furthermore, the pharmacological effect of CoUltraPEALut when administered in the presence of more complex clinical pictures that include chronic conditions and related symptoms together with PCOD is not known.

Moreover, the recovery of olfactory functions may require more time in the treatment group, despite improvements, and it is unclear whether adherence to the regimen and recovery would be sustained with a longer course of therapy due to the study's limited follow-up, which ranges from 30 to 90 days.

Finally, the absence of placebo control can determine a different interpretation of conclusive findings and the reliability and validity of olfactory assessments can affect results.

Furthermore, the five studies included analyzed the efficacy of CoUltraPEALut in different Italian populations. The data, therefore, related to individuals who are probably of Italian nationality, remain a contributing factor limiting the geographical heterogeneity.

More research in larger populations is required to corroborate our early findings, determining the best time and dose regimes, and assessing the potential anti-inflammatory impact of CoUltraPEALut studying its pharmacokinetics, or its synergic activity with other therapies.

4. Conclusions and Future Perspectives

Overall, this systematic review and meta-analysis highlighted the significant benefits on olfactory dysfunction from COVID-19 following the administration of CoUltraPEALut, which are particularly advantageous in increasing the Sniffin' Sticks test score. As far as we know, this is the first meta-analysis that probed the efficacy of CoUltraPEALut in long COVID-19 symptoms. However, despite the promising evidence, it is important to underline that there is a need for further large-scale clinical trials to further confirm these positive outcomes, and to better understand the mechanism of action of CoUltraPEALut underlying olfactory recovery.

Certainly, future well-designed clinical trials will be able to answer these questions, providing more details regarding the possible action of CoUltraPEALut on peripheral and central mechanisms involved in the occurrence of PCOD as well as on the modulation of inflammatory or neuroinflammatory pathways impacting olfactory bulbs. The collected data will be able to support not only the use of CoUltraPEALut in this peculiar picture of persistent symptoms linked to the previous SARS-CoV-2 infection but also in different clinical settings.

Author Contributions: A.P.C. and A.A. data extraction and drafted the manuscript; L.C. and F.C. performed the literature search; supervision M.C.; S.C. and E.E. conceptualization; A.P.C. and A.A. conducted the statistical analysis. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Since this is a systematic review of published articles patient consent was previously obtained in every single study.

Data Availability Statement: Data of this study are available to the corresponding author's address.

Acknowledgments: We wish to thank Giuseppe Arena, College Librarian, University of Messina for the guidance on literature searching.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Casili, G.; Lanza, M.; Campolo, M.; Siracusa, R.; Paterniti, I.; Ardizzone, A.; Scuderi, S.A.; Cuzzocrea, S.; Esposito, E. Synergic Therapeutic Potential of PEA-Um Treatment and NAAA Enzyme Silencing In the Management of Neuroinflammation. *Int. J. Mol. Sci.* **2020**, *21*, 7486. [CrossRef]
2. Ardizzone, A.; Fusco, R.; Casili, G.; Lanza, M.; Impellizzeri, D.; Esposito, E.; Cuzzocrea, S. Effect of Ultra-Micronized-Palmitoylethanolamide and Acetyl-l-Carnitine on Experimental Model of Inflammatory Pain. *Int. J. Mol. Sci.* **2021**, *22*, 1967. [CrossRef]
3. Skaper, S.D.; Facci, L.; Fusco, M.; Della Valle, M.F.; Zusso, M.; Costa, B.; Giusti, P. Palmitoylethanolamide, a naturally occurring disease-modifying agent in neuropathic pain. *Inflammopharmacology* **2014**, *22*, 79–94. [CrossRef]
4. Rankin, L.; Fowler, C.J. The Basal Pharmacology of Palmitoylethanolamide. *Int. J. Mol. Sci.* **2020**, *21*, 7942. [CrossRef]
5. Gugliandolo, E.; Peritore, A.F.; Piras, C.; Cuzzocrea, S.; Crupi, R. Palmitoylethanolamide and Related ALIAmides: Prohomeostatic Lipid Compounds for Animal Health and Wellbeing. *Vet. Sci.* **2020**, *7*, 78. [CrossRef]
6. Impellizzeri, D.; Bruschetta, G.; Cordaro, M.; Crupi, R.; Siracusa, R.; Esposito, E.; Cuzzocrea, S. Micronized/ultramicronized palmitoylethanolamide displays superior oral efficacy compared to nonmicronized palmitoylethanolamide in a rat model of inflammatory pain. *J. Neuroinflamm.* **2014**, *11*, 136. [CrossRef]

7. Campolo, M.; Crupi, R.; Cordaro, M.; Cardali, S.M.; Ardizzone, A.; Casili, G.; Scuderi, S.A.; Siracusa, R.; Esposito, E.; Conti, A.; et al. Co-Ultra PEALut Enhances Endogenous Repair Response Following Moderate Traumatic Brain Injury. *Int. J. Mol. Sci.* **2021**, *22*, 8717. [CrossRef]
8. Raveendran, A.V.; Jayadevan, R.; Sashidharan, S. Long COVID: An overview. *Diabetes Metab. Syndr.* **2021**, *15*, 869–875. [CrossRef]
9. Hintschich, C.A.; Dietz, M.; Haehner, A.; Hummel, T. Topical Administration of Mometasone Is Not Helpful in Post-COVID-19 Olfactory Dysfunction. *Life* **2022**, *12*, 1483. [CrossRef]
10. Xydakis, M.S.; Albers, M.W.; Holbrook, E.H.; Lyon, D.M.; Shih, R.Y.; Frasnelli, J.A.; Pagenstecher, A.; Kupke, A.; Enquist, L.W.; Perlman, S. Post-viral effects of COVID-19 in the olfactory system and their implications. *Lancet Neurol.* **2021**, *20*, 753–761. [CrossRef]
11. Chen, M.; Shen, W.; Rowan, N.R.; Kulaga, H.; Hillel, A.; Ramanathan, M., Jr.; Lane, A.P. Elevated ACE-2 expression in the olfactory neuroepithelium: Implications for anosmia and upper respiratory SARS-CoV-2 entry and replication. *Eur. Respir. J.* **2020**, *56*, 2001948. [CrossRef]
12. Kay, L.M. COVID-19 and olfactory dysfunction: A looming wave of dementia? *J. Neurophysiol.* **2022**, *128*, 436–444. [CrossRef]
13. Zazhytska, M.; Kodra, A.; Hoagland, D.A.; Frere, J.; Fullard, J.F.; Shayya, H.; McArthur, N.G.; Moeller, R.; Uhl, S.; Omer, A.D.; et al. Non-cell-autonomous disruption of nuclear architecture as a potential cause of COVID-19-induced anosmia. *Cell* **2022**, *185*, 1052–1064 e1012. [CrossRef]
14. Schurink, B.; Roos, E.; Radonic, T.; Barbe, E.; Bouman, C.S.C.; de Boer, H.H.; de Bree, G.J.; Bulle, E.B.; Aronica, E.M.; Florquin, S.; et al. Viral presence and immunopathology in patients with lethal COVID-19: A prospective autopsy cohort study. *Lancet Microbe* **2020**, *1*, e290–e299. [CrossRef]
15. Menter, T.; Haslbauer, J.D.; Nienhold, R.; Savic, S.; Hopfer, H.; Deigendesch, N.; Frank, S.; Turek, D.; Willi, N.; Pargger, H.; et al. Postmortem examination of COVID-19 patients reveals diffuse alveolar damage with severe capillary congestion and variegated findings in lungs and other organs suggesting vascular dysfunction. *Histopathology* **2020**, *77*, 198–209. [CrossRef]
16. Kandemirli, S.G.; Altundag, A.; Yildirim, D.; Tekcan Sanli, D.E.; Saatci, O. Olfactory Bulb MRI and Paranasal Sinus CT Findings in Persistent COVID-19 Anosmia. *Acad. Radiol.* **2021**, *28*, 28–35. [CrossRef]
17. Chiu, A.; Fischbein, N.; Wintermark, M.; Zaharchuk, G.; Yun, P.T.; Zeineh, M. COVID-19-induced anosmia associated with olfactory bulb atrophy. *Neuroradiology* **2021**, *63*, 147–148. [CrossRef]
18. Ardizzone, A.; Capra, A.P.; Mondello, S.; Briuglia, S.; La Rosa, M.A.; Campolo, M.; Esposito, E. H1299R Variant in Factor V and Recurrent Pregnancy Loss: A Systematic Review and Meta-Analysis Protocol. *Genes* **2022**, *13*, 1019. [CrossRef]
19. Capra, A.P.; Ardizzone, A.; Briuglia, S.; La Rosa, M.A.; Mondello, S.; Campolo, M.; Esposito, E. A Systematic Review and Meta-Analysis of the Association between the FV H1299R Variant and the Risk of Recurrent Pregnancy Loss. *Biology* **2022**, *11*, 1608. [CrossRef]
20. D'Ascanio, L.; Vitelli, F.; Cingolani, C.; Maranzano, M.; Brenner, M.J.; Di Stadio, A. Randomized clinical trial "olfactory dysfunction after COVID-19: Olfactory rehabilitation therapy vs. intervention treatment with Palmitoylethanolamide and Luteolin": Preliminary results. *Eur. Rev. Med. Pharmacol. Sci.* **2021**, *25*, 4156–4162. [CrossRef]
21. De Luca, P.; Camaioni, A.; Marra, P.; Salzano, G.; Carriere, G.; Ricciardi, L.; Pucci, R.; Montemurro, N.; Brenner, M.J.; Di Stadio, A. Effect of Ultra-Micronized Palmitoylethanolamide and Luteolin on Olfaction and Memory in Patients with Long COVID: Results of a Longitudinal Study. *Cells* **2022**, *11*, 2552. [CrossRef] [PubMed]
22. Di Stadio, A.; D'Ascanio, L.; Vaira, L.A.; Cantone, E.; De Luca, P.; Cingolani, C.; Motta, G.; De Riu, G.; Vitelli, F.; Spriano, G.; et al. Ultramicronized Palmitoylethanolamide and Luteolin Supplement Combined with Olfactory Training to Treat Post-COVID-19 Olfactory Impairment: A Multi-Center Double-Blinded Randomized Placebo- Controlled Clinical Trial. *Curr. Neuropharmacol.* **2022**, *20*, 2001–2012. [CrossRef]
23. Di Stadio, A.; Bernitsas, E.; La Mantia, I.; Brenner, M.J.; Ralli, M.; Vaira, L.A.; Colizza, A.; Cavaliere, C.; Laudani, M.; Frohman, T.C.; et al. Targeting Neuroinflammation to Alleviate Chronic Olfactory Dysfunction in Long COVID: A Role for Investigating Disease-Modifying Therapy (DMT)? *Life* **2023**, *13*, 226. [CrossRef] [PubMed]
24. Di Stadio, A.; Cantone, E.; De Luca, P.; Di Nola, C.; Massimilla, E.A.; Motta, G.; La Mantia, I.; Motta, G. Parosmia COVID-19 Related Treated by a Combination of Olfactory Training and Ultramicronized PEA-LUT: A Prospective Randomized Controlled Trial. *Biomedicines* **2023**, *11*, 1109. [CrossRef] [PubMed]
25. Gerkin, R.C.; Ohla, K.; Veldhuizen, M.G.; Joseph, P.V.; Kelly, C.E.; Bakke, A.J.; Steele, K.E.; Farruggia, M.C.; Pellegrino, R.; Pepino, M.Y.; et al. Recent Smell Loss Is the Best Predictor of COVID-19 Among Individuals With Recent Respiratory Symptoms. *Chem. Senses* **2021**, *46*, bjaa081. [CrossRef]
26. Whitcroft, K.L.; Hummel, T. Olfactory Dysfunction in COVID-19: Diagnosis and Management. *JAMA* **2020**, *323*, 2512–2514. [CrossRef]
27. Hannum, M.E.; Ramirez, V.A.; Lipson, S.J.; Herriman, R.D.; Toskala, A.K.; Lin, C.; Joseph, P.V.; Reed, D.R. Objective Sensory Testing Methods Reveal a Higher Prevalence of Olfactory Loss in COVID-19-Positive Patients Compared to Subjective Methods: A Systematic Review and Meta-Analysis. *Chem. Senses* **2020**, *45*, 865–874. [CrossRef]
28. Meinhardt, J.; Radke, J.; Dittmayer, C.; Franz, J.; Thomas, C.; Mothes, R.; Laue, M.; Schneider, J.; Brunink, S.; Greuel, S.; et al. Olfactory transmucosal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19. *Nat. Neurosci.* **2021**, *24*, 168–175. [CrossRef]

29. De Melo, G.D.; Lazarini, F.; Levallois, S.; Hautefort, C.; Michel, V.; Larrous, F.; Verillaud, B.; Aparicio, C.; Wagner, S.; Gheusi, G.; et al. COVID-19-related anosmia is associated with viral persistence and inflammation in human olfactory epithelium and brain infection in hamsters. *Sci. Transl. Med.* **2021**, *13*, abf8396. [CrossRef]
30. Kanberg, N.; Ashton, N.J.; Andersson, L.M.; Yilmaz, A.; Lindh, M.; Nilsson, S.; Price, R.W.; Blennow, K.; Zetterberg, H.; Gisslen, M. Neurochemical evidence of astrocytic and neuronal injury commonly found in COVID-19. *Neurology* **2020**, *95*, e1754–e1759. [CrossRef]
31. Cocco, A.; Amami, P.; Desai, A.; Voza, A.; Ferreli, F.; Albanese, A. Neurological features in SARS-CoV-2-infected patients with smell and taste disorder. *J. Neurol.* **2021**, *268*, 1570–1572. [CrossRef] [PubMed]
32. De Luca, P.; Scarpa, A.; Ralli, M.; Tassone, D.; Simone, M.; De Campora, L.; Cassandro, C.; Di Stadio, A. Auditory Disturbances and SARS-CoV-2 Infection: Brain Inflammation or Cochlear Affection? Systematic Review and Discussion of Potential Pathogenesis. *Front. Neurol.* **2021**, *12*, 707207. [CrossRef] [PubMed]
33. Guida, F.; Luongo, L.; Boccella, S.; Giordano, M.E.; Romano, R.; Bellini, G.; Manzo, I.; Furiano, A.; Rizzo, A.; Imperatore, R.; et al. Palmitoylethanolamide induces microglia changes associated with increased migration and phagocytic activity: Involvement of the CB2 receptor. *Sci. Rep.* **2017**, *7*, 375. [CrossRef]
34. Kempuraj, D.; Thangavel, R.; Kempuraj, D.D.; Ahmed, M.E.; Selvakumar, G.P.; Raikwar, S.P.; Zaheer, S.A.; Iyer, S.S.; Govindarajan, R.; Chandrasekaran, P.N.; et al. Neuroprotective effects of flavone luteolin in neuroinflammation and neurotrauma. *Biofactors* **2021**, *47*, 190–197. [CrossRef]
35. O'Byrne, L.; Webster, K.E.; MacKeith, S.; Philpott, C.; Hopkins, C.; Burton, M.J. Interventions for the treatment of persistent post-COVID-19 olfactory dysfunction. *Cochrane Database Syst. Rev.* **2022**, *9*, CD013876. [CrossRef]
36. Wu, T.J.; Yu, A.C.; Lee, J.T. Management of post-COVID-19 olfactory dysfunction. *Curr. Treat. Options Allergy* **2022**, *9*, 1–18. [CrossRef]
37. Kattar, N.; Do, T.M.; Unis, G.D.; Migneron, M.R.; Thomas, A.J.; McCoul, E.D. Olfactory Training for Postviral Olfactory Dysfunction: Systematic Review and Meta-analysis. *Otolaryngol. Head Neck Surg.* **2021**, *164*, 244–254. [CrossRef]
38. Pires, I.A.T.; Steffens, S.T.; Mocelin, A.G.; Shibukawa, D.E.; Leahy, L.; Saito, F.L.; Amadeu, N.T.; Lopes, N.M.D.; Garcia, E.C.D.; Albanese, M.L.; et al. Intensive Olfactory Training in Post-COVID-19 Patients: A Multicenter Randomized Clinical Trial. *Am. J. Rhinol. Allergy* **2022**, *36*, 780–787. [CrossRef] [PubMed]
39. Denis, F.; Septans, A.L.; Periers, L.; Maillard, J.M.; Legoff, F.; Gurden, H.; Moriniere, S. Olfactory Training and Visual Stimulation Assisted by a Web Application for Patients With Persistent Olfactory Dysfunction After SARS-CoV-2 Infection: Observational Study. *J. Med. Internet. Res.* **2021**, *23*, e29583. [CrossRef]
40. Le Bon, S.D.; Konopnicki, D.; Pisarski, N.; Prunier, L.; Lechien, J.R.; Horoi, M. Efficacy and safety of oral corticosteroids and olfactory training in the management of COVID-19-related loss of smell. *Eur. Arch. Otorhinolaryngol.* **2021**, *278*, 3113–3117. [CrossRef]
41. Karamali, K.; Elliott, M.; Hopkins, C. COVID-19 related olfactory dysfunction. *Curr. Opin. Otolaryngol. Head Neck Surg.* **2022**, *30*, 19–25. [CrossRef] [PubMed]
42. Ohla, K.; Veldhuizen, M.G.; Green, T.; Hannum, M.E.; Bakke, A.J.; Moein, S.T.; Tognetti, A.; Postma, E.M.; Pellegrino, R.; Hwang, D.L.D.; et al. A follow-up on quantitative and qualitative olfactory dysfunction and other symptoms in patients recovering from COVID-19 smell loss. *Rhinology* **2022**, *60*, 207–217. [CrossRef] [PubMed]
43. Burges Watson, D.L.; Campbell, M.; Hopkins, C.; Smith, B.; Kelly, C.; Deary, V. Altered smell and taste: Anosmia, parosmia and the impact of long Covid-19. *PLoS ONE* **2021**, *16*, e0256998. [CrossRef] [PubMed]
44. Liao, B.; Deng, Y.K.; Zeng, M.; Liu, Z. Long-term Consequences of COVID-19: Chemosensory Disorders. *Curr. Allergy Asthma Rep.* **2023**, *23*, 111–119. [CrossRef] [PubMed]
45. Croy, I.; Nordin, S.; Hummel, T. Olfactory disorders and quality of life--an updated review. *Chem. Senses* **2014**, *39*, 185–194. [CrossRef]

biomedicines

MDPI

Review

Efficacy of Pulmonary Rehabilitation in Post-COVID-19: A Systematic Review and Meta-Analysis

Erika Meléndez-Oliva [1,2,3], Oliver Martínez-Pozas [2,3,4,*], Juan Nicolás Cuenca-Zaldívar [2,5,6,7,*], Jorge Hugo Villafañe [8], Laura Jiménez-Ortega [9,10] and Eleuterio A. Sánchez-Romero [2,3,11,*]

1 Department of Physiotherapy, Faculty of Sport Sciences, European University of Valencia, Pg. de l'Albereda, 7, 46010 Valencia, Spain; erikamelendezoliva@gmail.com
2 Interdisciplinary Group on Musculoskeletal Disorders, Faculty of Sport Sciences, Universidad Europea de Madrid, 28670 Villaviciosa de Odón, Spain
3 Physiotherapy and Orofacial Pain Working Group, Sociedad Española de Disfunción Craneomandibular y Dolor Orofacial (SEDCYDO), 28009 Madrid, Spain
4 Department of Physical Therapy, Occupational Therapy, Rehabilitation and Physical Medicine, Escuela Internacional de Doctorado, Universidad Rey Juan Carlos, 28933 Alcorcón, Spain
5 Grupo de Investigación en Fisioterapia y Dolor, Departamento de Enfermería y Fisioterapia, Facultad de Medicina y Ciencias de la Salud, Universidad de Alcalá, 28801 Alcalá de Henares, Spain
6 Research Group in Nursing and Health Care, Puerta de Hierro Health Research Institute-Segovia de Arana (IDIPHISA), 28222 Majadahonda, Spain
7 Physical Therapy Unit, Primary Health Care Center "El Abajón", 28231 Madrid, Spain
8 IRCCS Fondazione Don Carlo Gnocchi, 20148 Milan, Italy; mail@villafane.it
9 Department of Psychobiology and Behavioral Sciences Methods, Faculty of Odontology, Complutense University of Madrid, 28040 Madrid, Spain; laurajim@ucm.es
10 Center of Human Evolution and Behavior, Carlos III Health Institute, Complutense University of Madrid, 28040 Madrid, Spain
11 Department of Physiotherapy, Faculty of Sport Sciences, European University of Madrid, Villaviciosa de Odón, 28670 Madrid, Spain
* Correspondence: oliver.martp@gmail.com (O.M.-P.); j.nicolas7251@gmail.com (J.N.C.-Z.); eleuterio.sanchez@universidadeuropea.es (E.A.S.-R.)

Citation: Meléndez-Oliva, E.; Martínez-Pozas, O.; Cuenca-Zaldívar, J.N.; Villafañe, J.H.; Jiménez-Ortega, L.; Sánchez-Romero, E.A. Efficacy of Pulmonary Rehabilitation in Post-COVID-19: A Systematic Review and Meta-Analysis. *Biomedicines* **2023**, *11*, 2213. https://doi.org/10.3390/biomedicines11082213

Academic Editor: Francesco B. Blasi

Received: 6 July 2023
Revised: 25 July 2023
Accepted: 26 July 2023
Published: 7 August 2023

Abstract: Background: This systematic review and meta-analysis examines how pulmonary rehabilitation impacts in patients suffering from subacute and long COVID-19 infections, gauging enhancements in of dyspnea, physical function, quality of life, psychological state (anxiety and depression), and fatigue. Methods: Three electronic databases (PubMed, Web of Science, Cochrane Library) were systematically searched for full-text articles published from inception to January 2023. Randomized, quasi-experimental, and observational studies were included, with adults diagnosed with subacute or long COVID-19 who received pulmonary rehabilitation as intervention. Outcomes related to dyspnea, physical function, quality of life, fatigue, and psychological status were included. Risk of bias was assessed with Cochrane Risk of Bias Tool for Randomized Controlled Trials and Risk of bias in non-randomized studies of intervention. The review was registered before starting in PROSPERO (CRD: 42022373075). Results: Thirty-four studies were included, involving 1970 patients with subacute and long COVID-19. The meta-analysis demonstrated moderate to large effects on dyspnea, physical function, quality of life, and depressive symptoms compared to usual care intervention. No significant differences were found in fatigue compared to usual care, nor in anxiety levels after pulmonary rehabilitation intervention. Conclusions: Pulmonary rehabilitation has the potential to improve health outcomes in patients with subacute and long COVID-19. However, due to the high risk of bias of included studies, conclusions should be taken with caution.

Keywords: COVID-19; pulmonary rehabilitation; exercise

1. Introduction

COVID-19 is a disease caused by infection with the highly contagious SARS-CoV-2 virus, whose main condition falls on the respiratory system, with symptoms such as dyspnea, fibrosis, and pulmonary edema, but which also presents a wide variety of comorbidities such as musculoskeletal pain, fatigue and muscle weakness, cardiovascular and cerebrovascular diseases, and psychological conditions such as depression and anxiety, deteriorating seriously, in most cases, the quality of life [1,2].

Currently, it can be argued that the severity of COVID-19 has decreased in many countries, possibly due to the wide vaccination coverage carried out worldwide and effective treatment [3]. However, many patients affected by COVID-19 continue to experience symptoms after the acute phase, such as breathlessness, fatigue, neuropsychological symptoms, cough, and musculoskeletal pain [4,5]. This post-acute syndrome is known as long COVID (LC), and it is defined by WHO as "the continuation or development of new symptoms three months after the initial SARS-CoV-2 infection" [6].

Pulmonary rehabilitation (PR) is a very effective exercise-based therapeutic strategy to improve functional capacity, dyspnea, and health-related quality of life in patients with chronic obstructive disease [7].

PR has been shown to improve the physical and psychological well-being of patients with COVID-19 [8]. This is achieved through aerobic endurance and resistance training, which help increase muscle mass and strength, especially in peripheral muscles [9,10]. In addition, PR can incorporate thoracic mobility exercises to improve lung expansion.

Previous studies [11,12] have reported that supervised PR programs are safe and effective in improving exercise capacity, lung function, exertional dyspnea, psychological well-being, and quality of life in patients with COVID-19. In addition, PR has been shown to significantly reduce the frequency and duration of hospital stays in individuals with restrictive lung disease [13].

Furthermore, several systematic reviews have recently been published showing that also leads to enhancements in both physical and pulmonary capabilities among patients with acute and subacute COVID-19 [14–18].

The main objective of this systematic review with meta-analysis is to provide an update about the efficacy of PR in patients with subacute and long COVID-19 (LC), and its effects on dyspnea, physical function, quality of life, psychological outcomes, and fatigue.

2. Materials and Methods

This systematic review was conducted by the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [19] and was registered before starting in PROSPERO (CRD42022373075).

2.1. Search Strategy

The literature search strategy involved structured searches of PubMed, Web of Science and Cochrane Library for relevant articles published from inception to January 2023. Reference lists of studies were reviewed for potential additional references not identified in the primary search, and the authors were contacted for further information if necessary. No language filters were applied to retrieve all potentially eligible studies, as recommended by international criteria [20].

The search terms combined medical subject headings (MeSH terms) and non-MeSH terms, adding a Boolean operator (OR and/or AND) to combine them. MeSH terms used were some such as "Rehabilitation", "Exercise", or "COVID-19" among other non-MeSH term such as "Pulmonary Rehabilitation", "Long-Covid", or "Post-acute COVID-19". The complete search strategy can be found in Appendix A, which shows the PubMed search strategy, which was adjusted for other databases if necessary.

2.2. Eligibility Criteria

The study population includes studies with adults (18 years or older) diagnosed with sub-acute or long COVID-19 (LC), being patients with symptoms persisting for less than three months considered sub-acute and those with symptoms lasting more than three months considered LC [6]. Studies with acute patients, with positive testing, were excluded.

The intervention consisted of PR, defined as "interventions based on, but not limited to, exercise training, education and behavior change designed to improve physical and psychological conditions of people with respiratory diseases" [21]. Both strategies, face-to-face and telerehabilitation, were included as PR.

Outcomes related to dyspnea, physical function, quality of life, fatigue, and psychological status were included.

Randomized controlled trials (RCTs), non-randomized controlled trials (quasi-experimental studies), and observational studies were eligible for inclusion in the review if they met the inclusion criteria.

2.3. Inclusion Procedure

Titles and abstracts were screened manually and independently by two authors (Oliver Martínez-Pozas, Erika Meléndez-Oliva) and any disagreements between reviewers were resolved by consensus or, if needed, by a third member (Eleuterio A. Sánchez-Romero). Articles were excluded if they: included patients with positive COVID-19 (acute phase) or hospitalized patients, or included patients who did not receive PR. To minimize the risk of investigator bias, all investigators had to agree on whether each study met all the eligibility criteria. The inclusion procedure included a first phase based on the study's title, abstract, and keywords. Subsequently, the studies were evaluated in their entirety to assess their potential eligibility according to inclusion criteria. The data were extracted by two researchers (Oliver Martínez-Pozas and Erika Meléndez-Oliva) and the data described in the Results section were extracted using a structured protocol that ensured that the most relevant information was obtained from each [22].

2.4. Risk of Bias Assessment

Two independent reviewers assessed the risk of bias in included studies (Oliver Martínez-Pozas, Erika Meléndez-Oliva) and disagreements were resolved through consensus and/or mediation by a third reviewer (Eleuterio A. Sánchez-Romero). The risk of bias of RCTs was evaluated using the "Revised Cochrane Risk of Bias Tool for Randomized Controlled Trials (RoB 2.0)" which contains five domains: bias arising from the randomization process, bias due to deviation from intended interventions, bias due to missing outcome data, bias in the measurement of the outcome, and bias in the selection of the reported result [23]. Each domain was scored as "low risk", "some concerns", and "high risk", and each study was classified into one of three categories as "high risk of bias", "some concerns", or "low risk of bias" [23].

Risk of bias in non-randomized controlled trials was evaluated using the "Risk of bias in non-Randomized studies of intervention" (ROBINS-I) which evaluates domains such as confounding factors, selection of participants, classification of interventions, deviations from the intended interventions, missing data, measurements of outcomes, and selection of the reported results [24]. Each study was classified into five categories as "low risk of bias", "moderate risk of bias", "serious risk of bias", "critical risk of bias", or "no information" [24].

2.5. Data Analysis

For the statistical analysis, the program R version 4.1.3 (R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Welthandelsplatz 1, 1020 Vienna, Austria) was used. Meta-analysis was conducted using the metafor and meta r packages [25,26].

In the articles in which the results were shown with median and with maximum and minimum or interquartile range, these were transformed into mean and standard deviation using the appropriate formulas [27,28].

In the RCTs, a meta-analysis of pre-post-intervention change was performed, analyzing the level of significance between the treatment and control groups using the standardized mean difference (SMD). Since no study reported the pre–post intervention mean ± standard deviation of change, these were calculated using the following formulas [29]:

$$\text{Meanchange} = \text{Meanfinal} - \text{Meanbaseline} \quad (1)$$

$$SDchange = \sqrt{SD^2 baseline + SD^2 final - (2 \cdot r \cdot SDbaseline \cdot SDfinal)} \quad (2)$$

In the formulas, *SD* is the standard deviation and "*r*" is the pre–post intervention correlation coefficient which, since the standard deviation of the change was not reported, was assigned a value of 0.7 in order to obtain a conservative estimate, [30] as has been done in other works [31–35].

In the study by Jimeno-Almanzán 2022 [36] in which, for the quality-of-life outcome, three interventions are reported against the control, these were sequentially combined using the appropriate formulas [29].

In observational studies, a single group meta-analysis was performed with the mean change pre-post intervention in each study.

In both cases a random effects model was applied given the heterogeneity between the studies. Heterogeneity was tested by estimating the between-study variance τ^2 (calculated with the DerSimonian–Laird estimator with Hartung–Knapp correction) using the Cochran Q test as well as the I^2 estimator. The latter being defined as not important (<30%), moderate (30–50%), large (50–75%), and important (>75%) heterogeneity.

Effect sizes were calculated in RCTs with Hedge's g being defined as small (<0.2), moderate (0.2–0.8), and large (>0.8).

Heterogeneity was assessed by detecting those outlier studies with absolute values in the standardized residuals greater than 3. In addition, a sensitivity analysis was performed using the leave-one-out method. The contribution of individual studies to heterogeneity was assessed using a Baujat plot showing the contribution of each study to heterogeneity calculated with Cochran's Q test versus its influence on the overall effect of the meta-analysis [37]. Subgroup meta-analyses were also performed to explore the heterogeneity detected based on the type of test used in each of the outcome variables.

Finally, publication bias was analyzed using trim and fill funnel plots and a Begg and Egger's test [38].

3. Results

Database searching reported 3541 articles among different databases. After screening for title and abstract and removing duplicates, 51 studies were assessed for eligibility. Nine studies were excluded due to population (included patients with acute COVID-19, still testing positive in COVID-19 test), two were excluded due to intervention (robot devices, ventilation therapy), one study was excluded due to outcomes (salivary biomarkers), and five due to study design (congress abstract, protocol). Finally, 34 studies were included for qualitative analysis in the present review (20 RCTs and 14 observational studies) [36,39–71]. For quantitative analysis, 26 studies were included. The flowchart of included studies can be found in Figure 1. In addition, PRISMA Checklist can be found in Supplementary Materials (Table S1).

3.1. Characteristics of the Included Studies

The present systematic review included 34 studies, with a total of 1970 adults. All participants were adults diagnosed with sub-acute COVID-19 (*n* = 18 studies) or LC (*n* = 16 studies). The number of patients for studies varied from 23 to 150. The average age

of patients varied from 32 to 82 years. The characteristics of the included, including data from overall population analyzed, are shown in Table 1.

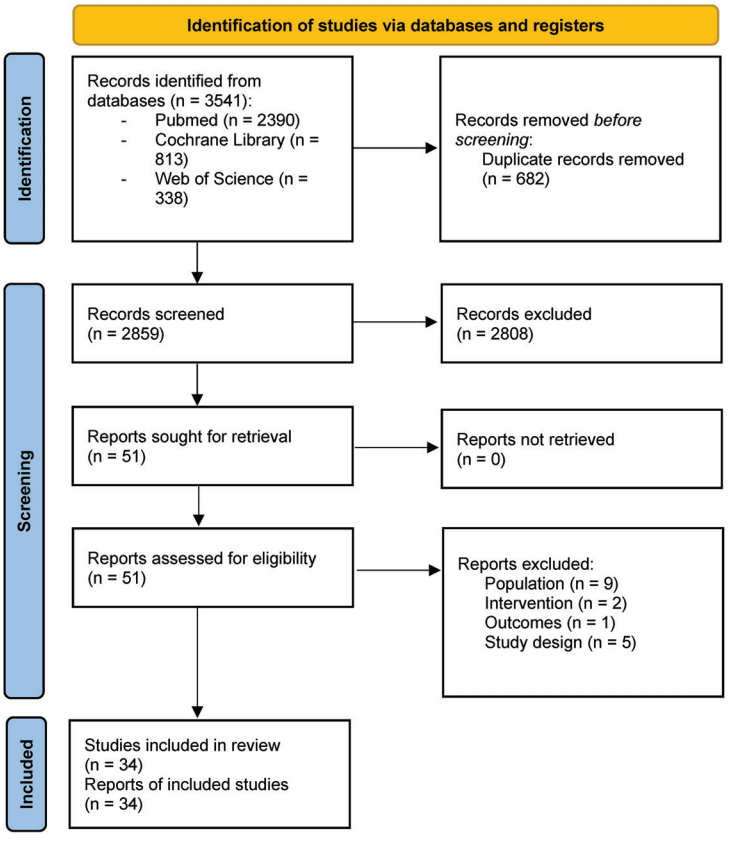


Figure 1. PRISMA 2020 Flowchart.

Table 1. Characteristics of included studies.

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Studies with Subacute COVID-19 Patients							
Abodonya et al. (2021) [39]	RCT	Adults with subacute COVID-19	$n = 42$ Int: $n = 21$ (19% F), Age: 48.3 ± 8.5 Con: $n = 21$ (23.8% F) Age: 47.8 ± 9.2	**Duration** 2 weeks. **Intervention** Breathing exercises.	Usual care	Dyspnea (DS-12) Quality of life (EQ-5D) Physical function (6MWT)	Intra-group analysis found statistically differences in intervention group in dyspnea ($p = 0.001$), quality of life ($p < 0.001$) and 6MWT ($p < 0.001$). Between group comparison, intervention group reported statistically significant differences compared to control in all outcomes with medium-large size effects.
Barhagi et al. (2021) [40]	RCT	Adults with subacute COVID-19	$n = 80$ (38.75% F) Int: $n = 40$, Age: 57.1 ± 18.7 Con: $n = 40$ Age: 58 ± 17.13	**Duration** Three days. **Intervention** Breathing exercises.	Usual care	Dyspnea (MBS)	After end of treatment, intervention group improved dyspnea with statistically differences compared to usual care ($p = 0.007$).

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Fereydounnia et al. (2022) [41]	RCT	Adults with subacute COVID-19	$n = 50$ (42% F) Int: $n = 25$, Age: 49.44 ± 14.78 Con: $n = 25$, Age: 45 ± 12.75	**Duration** 1 week. **Intervention** Myofascial release and breathing exercises.	Breathing exercises	Dyspnea (MBS) Physical function (6MWT) Fatigue (Borg)	Intervention group improved dyspnea with statistically differences at the end of the treatment compared to control ($p < 0.01$). No statistically differences were found in terms of physical function ($p = 0.033$) or fatigue ($p = 0.034$) improvement compared to control.
González-Gerez et al. (2021) [42]	RCT	Adults with subacute COVID-19	$n = 38$ Int: $n = 19$ (47.4% F), Age: 40.79 ± 9.84 Con: $n = 19$ (42.1% F), Age: 40.32 ± 12.53	**Duration** 1 week. **Intervention** Breathing exercises. Telerehabilitation.	Usual care	Physical function (6MWT; 30STS) Dyspnea (MD12; BS)	Statistically differences were found in terms of improving dyspnea ($p < 0.001$) and physical function ($p = 0.001$), in intervention, with no differences in control group. Between group analysis found statistically differences favoring intervention compared to control improving dyspnea ($p < 0.001$) and physical function ($p = 0.001$).
Hayden et al. (2021) [43]	Observational	Adults with subacute COVID-19	$n = 108$ (45.4% F) Age: 55.6 ± 10.1	**Duration** 3 weeks. **Intervention** Aerobic and strength training. Nutritional, psychological, and physical therapy support were included.	No control	Dyspnea (NRS/mMRC) Physical function (6MWT) Quality of life (EQ-5D) Fatigue (BFI) Depression and Anxiety (PHQ-9, GAD-7)	Dyspnea improved at rest ($p < 0.001$) and on exertion ($p < 0.001$) after treatment. Physical function improved after treatment ($p < 0.001$). Quality of life, fatigue, anxiety, and depression improved after treatment ($p < 0.001$)
Hockele et al. (2022) [44]	Observational	Adults with subacute COVID-19	$n = 29$ (51.7% F) Age: 54.4 ± 14.6	**Duration** 6–8 weeks. **Intervention** Aerobic and strength training.	No control	Physical function (6MWT, TUG) Dyspnea (mMRC)	Physical function improved after treatment with statistically significant differences compared to baseline in 6MWT ($p < 0.001$) and TUG ($p = 0.023$). Dyspnea improved after treatment with differences compared to baseline ($p = 0.003$).
Li et al. (2021) [45]	RCT	Adults with subacute COVID-19	$n = 119$ (55.46% F) Int: $n = 59$, Age: 49.17 ± 10.75 Con: $n = 60$, Age: 52.03 ± 11.10	**Duration** 6 weeks. 6 months follow-up. **Intervention** Aerobic, strength and breathing exercises. Telerehabilitation.	Usual care	Physical function (6 MWT) Dyspnea (mMRC) Quality of life (SF-12)	Intervention group improved physical function after treatment ($p < 0.001$) and at follow-up with statistically differences ($p < 0.001$). Perceived dyspnea improved after treatment with differences compared to control ($p = 0.001$) but without differences at follow-up ($p = 0.162$). Physical component of SF-12 improved with differences after treatment ($p = 0.004$) and at follow-up ($p = 0.045$). However, mental component found no differences at any point ($p = 0.116$; $p = 0.164$).

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Liu et al. (2020) [46]	RCT	Adults with subacute COVID-19	$n = 72$ Int: $n = 36$ (33.3% F), Age: 69.4 ± 8 Con: $n = 36$ (30.6% F) Age: 68.9 ± 7.6	**Duration** 6 weeks. **Intervention** Breathing exercises.	Usual care	Physical function (6MWT) Quality of life (SF-36) Anxiety and Depression (SDS, SAS)	Physical function improved with statistically differences in intervention group compared with baseline ($p < 0.05$), without statistically improvements in control group. Intervention group improved with statistically differences compared to control group ($p < 0.05$). Quality of life improved with statistically differences compared to baseline in intervention group ($p < 0.05$) and not on control group. Between group analysis found that intervention group improved with statistically differences in all items of SF-36 compared to control group ($p < 0.05$). Anxiety improved with statistically significant differences between groups favoring intervention ($p < 0.05$), but not depression.
Llurda-Almuzara et al. (2022) [47]	RCT	Adults with subacute COVID-19	$n = 70$ Int: $n = 35$, Age: 49.5 ± 13.7 Con: $n = 35$ Age: 55.1 ± 20.9	**Duration** 8 weeks. **Intervention** Aerobic, strength and breathing exercises. Telerehabilitation.	Usual care	Physical function (SPPB, 4MWT)	Physical function improved with moderate significant effects in intervention group compared to control.
Lobanov et al. (2022) [48]	RCT	Adults with subacute COVID-19	$n = 23$ Int: $n = 14$ Con: $n = 9$	**Duration** 2 weeks. **Intervention** Aerobic exercises in pool.	Exercise without pool.	Physical function (6MWT) Quality of life (EQ-5D) Dyspnea (BS)	Physical function improved with statistically significant differences compared to baseline ($p = 0.047$ both groups), with greater improvement in intervention group. Quality of life improved in anxiety/depression domain with statistically differences in control group ($p = 0.043$), but not in intervention group ($p = 0.69$). Dyspnea improved after treatment, but without statistical differences compared to baseline in any group.
Martín et al. (2021) [49]	Observational	Adults with subacute COVID-19	$n = 27$ Int: $n = 14$ (21.4% F), Age: 60.8 ± 10.4 Con: $n = 13$ (53.8% F), Age: 61.9 ± 10.7	**Duration** 6 weeks. **Intervention** Aerobic and strength exercises.	Usual Care	Physical function (1MSTST) Dyspnea (BS)	After treatment, statistically differences were found in 1min-STS favoring intervention group ($p = 0.004$). No differences were found in terms of dyspnea improvement ($p = 0.560$).
Nagy et al. (2022) [50]	RCT	Adults with subacute COVID-19	$n = 52$ Int: $n = 26$, Age: 40 ± 3.36 Con: $n = 26$, Age: 39.7 ± 3.55	**Duration** 6 weeks. **Intervention** Myofascial release and breathing exercises.	Breathing exercises	Dyspnea (mMRC) Physical function (6MWT) Fatigue (FSS)	Dyspnea, physical function, and fatigue improved with statistical differences compared to baseline in both groups ($p < 0.05$). Additionally, intervention group resulted in statistically significant differences compared to control ($p < 0.001$).
Nambi et al. (2022) [51]	RCT	Adults with subacute COVID-19	$n = 76$ Int: $n = 38$, Age: 63.2 ± 3.1 Con: $n = 38$ Age: 64.1 ± 3.2	**Duration** 8 weeks. **Intervention** Exercise at low intensity	Exercise at high intensity	Quality of life (SarQol)	Both groups improved quality of life after treatment with statistical differences compared to baseline ($p = 0.001$). However, patients allocated to low intensity group improved with better results in SarQol compared to baseline than those allocated to high intensity training.

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Pehlivan et al. (2022) [52]	RCT	Adults with subacute COVID-19	$n = 34$ Int: $n = 17$ (18% F), Age: 50.76 (32–82) Con: $n = 17$ (35% F), Age: 43.24 (23–71)	**Duration** 6 weeks. **Intervention** Aerobic, strength and breathing exercises. Telerehabilitation.	Usual care	Physical function (TUG/SPPB) Dyspnea (mMRC) Fatigue (VAS) Quality of life (SGRQ) Depression (BDI)	Although both groups improved outcomes, intra-group differences were only found mMRC ($p = 0.035$), TUG ($p = 0.005$) and SGRQ ($p = 0.002$) at intervention group, while not statistically differences were found in control group at the end of treatment. Between-groups analysis revealed statistically significant differences in terms of SGRQ improvement favor to intervention ($p = 0.042$). No significant changes were found after treatment in depression levels neither intra-group or between group comparison.
Puchner et al. (2021) [53]	Observational	Adults with subacute COVID-19	$n = 23$ (30% F) Age: 57 ± 10	**Duration** 3–4 weeks. **Intervention** Aerobic, strength and breathing exercises. Nutritional and psychological counseling.	No control	Physical function (6MWT)	Physical function improved after treatment with statistically differences compared to baseline ($p < 0.001$).
Rodríguez-Blanco et al. (2021) [54]	RCT	Adults with subacute COVID-19	$n = 36$ Int: $n = 18$ (50% F), Age: 39.39 ± 11.74 Con: $n = 18$ (55.5% F), Age: 41.33 ± 12.13	**Duration** 1 week. **Intervention** Strength exercises. Telerehabilitation.	Usual care	Physical function (6MWT/30STS) Dyspnea (BS)	Intervention group improved physical function after treatment with statistically differences compared to usual care ($p < 0.001$). However, although dyspnea improved in intervention group and did not improve in control group after treatment, differences were not significant ($p = 0.074$).
Rutkowski et al. (2022) [55]	RCT	Adults with subacute COVID-19	$n = 32$ (68% F) Age: 57.8 ± 4.9	**Duration** 3 weeks. **Intervention** Virtual reality exercise	Exercise without virtual reality	Depression and Anxiety (HADS) Quality of life (WHOQOL-BREF) Physical function (6MWT)	Intervention group ($p < 0.001$) and control group ($p < 0.05$) improved anxiety and depression after treatment compared to baseline levels. No significant changes were found in any group in terms of quality-of-life improvement after treatment. Physical function improved in both groups. However, patients in intervention group showed more improvements in walked distance after treatment than control group.
Teixeira do Amaral et al. (2022) [56]	RCT	Adults with subacute COVID-19	$n = 32$ Int: $n = 12$, Age: 51.9 ± 10.2 Con: $n = 20$, Age: 53.3 ± 11.6	**Duration** 12 weeks. **Intervention** Aerobic and strength exercises. Telerehabilitation.	Usual care	Physical function (6MWT, TUG, 5TSTS)	Both groups all physical function outcomes compared to baseline, but without statistically significant differences within-group or between groups.

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Studies with long COVID-19 patients							
Albu et al., 2022 [57]	Observational	Adults with long COVID-19	$n = 40$ (40% female) Mean Age: 52 ± 11.4 y/o	**Duration** 8 weeks **Intervention** Education Aerobic, strength and breathing exercises. Psychological counseling. **Intensity** Personalized according to patient status.	No control	Physical performance (SPPB) Fatigue (MFIS) Quality of life (WHOQOL-BREF)	After 8 weeks of rehabilitation, significant improvements in physical performance were found in SPPB compared to baseline with statistically differences ($p = 0.001$). Fatigue was improved after intervention with statistically differences for all measured domains ($p = 0.001$). Quality of life improved in physical, psychological, and environmental domains with statistical differences ($p = 0.001$), but not at social domain ($p = 0.15$).
Cahalan et al., 2022 [58]	Observational	Adults with long COVID-19	$n = 27$ (85% f) Mean age: 48.4 ± 10.1 y/o	**Duration** 10 weeks. **Intervention** Breathing exercises, psychological advice. Telerehabilitation. **Intensity** Not reported.	None	Dyspnea (C19YRS) Fatigue (C19YRS) Anxiety/Depression (C19YRS)	Statistical improvements were found after treatment in terms of dyspnea ($p < 0.001$), as well as in fatigue ($p = 0.03$). Although anxiety and depression improved after treatment, no significant differences were found ($p = 0.08$ for anxiety, $p = 0.337$ for depression).
Calvo-Paniagua 2022 [59]	Quasi-experimental	Adults with long COVID-19	$n = 68$ (61.8% f) Mean age: 48.5 ± 9.7 y/o	**Duration** 7 weeks. **Intervention** Aerobic, strength and breathing exercises. Telerehabilitation. **Intensity** Not reported.	None	Dyspnea (mMRC) Quality of life (SGRQ) Physical performance (6MWT)	Dyspnea improved significantly after intervention and at follow-up ($p < 0.001$). Quality of life improved significantly after intervention and at follow-up ($p < 0.001$). Physical performance improved with statistically differences after intervention and at follow-up ($p < 0.001$).
Compagno et al., 2022 [60]	Observational	Adults with long COVID-19	$n = 30$ (40% female) Mean Age: 58.37 ± 11.6 y/o	**Duration** 8–20 weeks **Intervention** Aerobic and strength exercises. Psychological counseling. **Intensity** Aerobic exercise at low and mid intensity. Strength at 30–50% 1RM.	No control	Quality of life (SF-36) Anxiety (SAS) Depression (SDS)	Quality of life improved after intervention with statistically differences ($p < 0.05$). Anxiety and depression improved with statistically differences after treatment (both $p < 0.05$).
Daynes et al., 2021 [61]	Observational	Adults with long COVID-19	$n = 30$ (48% female) Mean Age: 58 ± 16 y/o	**Duration** 6 weeks, with two supervised sessions per week. **Intervention** Aerobic and strength exercises. **Intensity** Not reported.	No control	Physical performance (ISWT) Fatigue (FACIT) Anxiety and depression (HADS) Quality of life (EQ-5D)	ISWT improved after treatment with statistically differences compared to baseline ($p < 0.01$). Fatigue improved with statistical differences at the end of treatment ($p < 0.01$), while anxiety and depression improved, but without statistically significant differences ($p = 0.5$ for anxiety and $p = 0.1$ for depression). Quality of life improved after treatment compared to baseline ($p = 0.05$).

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Del Corral 2022 [62]	RCT	Adults with long COVID-19	G1: n = 22, mean age: 48.9 ± 8.3 y/o; 77% f G2: n = 22, mean age: 45.3 ± 12.8 y/o; 73% f G3: n = 22, mean age: 46.5 ± 9.6 y/o, 64% f G4: n = 22, mean age: 45 ± 10.2 y/o, 73% f	**Duration** 8 weeks. **Intervention** Group 1: Inspiratory breathing exercises. Group 2: Inspiratory and expiratory breathing exercises. Telerehabilitation. **Intensity** 20–80% of maximal inspiratory pressure	Group 3: Sham inspiratory exercises. Group 4: Sham inspiratory and expiratory exercises. Sham procedures were with device without resistance	Quality of life (EQ-5D) Physical performance (1MSTST) Anxiety/Depression (HADS)	All groups improved quality of life after intervention compared to baseline ($p < 0.05$), except group 4. At 4 weeks follow-up, no statistical differences were found between groups improving quality of life. Physical performance improved with large effects in intervention groups compared with sham groups after intervention ($p < 0.01$), but without differences when comparing both intervention groups. Differences were not found between groups after 4 weeks follow-up in terms of physical performance improving. Although all groups improved psychological status, no statistical differences were found across groups.
Estébanez-Pérez 2022 [63]	Quasi-Experimental	Adults with long COVID-19	n = 32 (71.9% f) Mean age: 45.93 ± 10.65 y/o	**Duration** 4 weeks. **Intervention** Aerobic and strength training. Telerehabilitation. **Intensity** Aerobic exercises at low to moderate intensity. Strength training not reported.	None	Physical performance (SPPB, 1MSTST)	1mSTS and SPPB improved with statistically significant effects after treatment ($p < 0.05$).
Groenveld 2022 [64]	Observational	Adults with long COVID-19	n = 47 (68% f) Mean age: 54 (21–70)	**Duration** 6 weeks **Intervention** Virtual reality-based exercise. Telerehabilitation. **Intensity** Adjusted to patient.	None	Fatigue (BS) Physical performance (6MWT, TUG, 30CST) Quality of life (SF-12, PHQ) Anxiety/Depression (HADS)	Fatigue improved with clinical differences after treatment ($p = 0.03$). Significant differences were found in 6MWT ($p < 0.001$) and 30CST ($p = 0.02$) after intervention. Three patients performed TUG instead of 6MWT, with improvements after treatment. Statistical differences were found improving quality of life for physical sphere ($p < 0.049$) and mental sphere ($p < 0.01$) measured with SF-12, as well as with PHQ ($p = 0.04$) Symptoms measured with HADS decreased, but without statistical differences ($p = 0.08$).
Hasenoehrl et al., 2022 [65]	Quasi-experimental	Adults with long COVID-19	Group 1 (mild symptoms): n = 10 (60% female), mean age: 42.9 ± 12.4 y/o Group 2 (severe symptoms): n = 18 (89% female), mean age: 47.4 ± 10.1 y/o	**Duration** 8 weeks of supervised strength training, 2 times per week **Intervention** Aerobic and strength exercises. **Intensity** Strength exercises performed at 7–10 RPE. Aerobic exercises at moderate intensity.	No control	Physical performance (6 MWT/30 STST)	Both groups improved significantly 30 STST ($p < 0.001$) and 6 MWT ($p < 0.001$) after intervention.

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Jimeno-Almanzán et al., 2022 [36]	RCT	Adults with long COVID-19	$n = 80$ (69% female) Mean Age: 45.3 ± 8.0 y/o	**Duration** 8 weeks. **Intervention** G1: Strength and breathing exercises. G2: Strength exercises. G3: Breathing exercises. **Intensity** Strength at 50% 1 RM. Breathing exercises at 12–15 RPE.	G4: Usual care	Dyspnea (mMRC) Quality of life (SF-12) Anxiety and Depression (GAD-7/PHQ-9) Fatigue (FSS)	All outcomes improved in all study groups after intervention. After 8 weeks of intervention, no differences between groups were detected in mMRC, GAD-7 and SF-12. Fatigue and depression improved with differences in training groups (G1 and G2, $p = 0.007$). Breathing training group (G3) improved with differences in physical domain of SF-12 ($p < 0.05$). No relevant changes were observed in control group (G4) pre-post intervention.
Jimeno-Almanzán et al., 2022a [66]	RCT	Adults with long COVID-19	$n = 39$ (74.4% female) Mean Age: 45.2 ± 9.5 y/o	**Duration** 8 weeks. **Intervention** Strength exercises. **Intensity** 50% 1RM.	Usual care	Dyspnea (mMRC) Quality of life (SF-12) Anxiety and Depression (GAD-7/PHQ-9) Fatigue (FSS) Physical performance (5TSTST)	Intervention group resulted in statistically differences compared to control in physical domain of SF-12 ($p = 0.024$), fatigue ($p < 0.05$), depression symptoms ($p = 0.021$), and physical performance ($p = 0.009$). Although all studied outcomes improved in both groups, no statistical differences were found in other outcomes such as dyspnea improvement or anxiety.
Lloyd-Evans 2022 [67]	Observational	Adults with long COVID-19	$n = 110$ (68.1% f) Mean age: 46.3 ± 10.8	**Duration** 8–12 weeks **Intervention** Aerobic and strength exercises. Tel-erehabilitation. **Intensity** Not reported.	None	Quality of life (EQ-5D)	Statistically significant differences were found improving quality of life ($p < 0.01$).
McNarry 2022 [68]	RCT	Adults with long COVID-19	$n = 148$ (111 int, 86% f/37 con, 95% f) Mean age: 46.76 ± 12.03 (int)/ 46.13 ± 12.73 (con)	**Duration** 8 weeks, unsupervised. **Intervention** Breathing exercises. Tel-erehabilitation. **Intensity** 80% of sustained maximal inspiratory pressure.	Usual care	Quality of life (K-BILD) Dyspnea (TDI)	Although quality of life improved within-group, no statistically significant differences were found between groups. Dyspnea improved with statistical differences favoring intervention compared to control ($p = 0.005$).
Nopp et al., 2022 [69]	Observational	Adults with long COVID-19	$n = 58$ (43.1% female) Mean Age: 46.8 ± 12.6 y/o	**Duration** 6 weeks. **Intervention** Aerobic, strength and breathing exercises. **Intensity** Not reported.	No Control	Physical performance (6 MWT/1 MSTST) Dyspnea (mMRC) Quality of life (EQ-5D) Fatigue (FAS)	After intervention, patients improved 6 MWT and 1 MSTST with statistical differences ($p < 0.001$). Dyspnea improved with statistical differences compared to baseline ($p < 0.001$). Quality of life improved after treatment ($p < 0.001$). Fatigue improved after treatment with statistical differences ($p < 0.001$).

Table 1. *Cont.*

Author (Year)	Study Design	Population	Sample Size	Intervention	Control Group	Outcomes	Results
Okan 2022 [70]	RCT	Adults with long COVID-19	$n = 52$ (26 int, 42.3% f/26 con, 53.8% f) Mean age: 48.85 ± 10.85 (int)/ 52.19 ± 14.84 (con)	**Duration** 5 weeks, one session supervised. **Intervention** Aerobic and breathing exercises. Tel-erehabilitation. **Intensity** Aerobic exercises at moderate intensity. Breathing not reported.	Usual care	Dyspnea (mMRC) Physical performance (6 MWT) Quality of life (SGRQ)	Both groups improved dyspnea. However, it was significantly lower in intervention group than in control group ($p < 0.001$). Quality of life improved with statistical differences in intervention group compared to control after treatment ($p < 0.001$). Physical performance improved with statistically significant differences in intervention group compared to control ($p < 0.001$).
Philip 2022 [71]	RCT	Adults with long COVID-19	$n = 150$ (81% f) Mean age: 49 ± 12	**Duration** 6 weeks. **Intervention** Breathing exercises. Tel-erehabilitation. **Intensity** Not reported.	Usual care	Quality of life (SF-36) Dyspnea (DS-12) Anxiety (GAD-7)	Intervention group improved mental component of SF-36 with statistical differences compared to control ($p = 0.047$), while no differences in physical component ($p = 0.54$). Dyspnea improved in both groups compared to baseline, but without differences between groups ($p = 0.38$). Although anxiety improved in both groups, no statistical differences were found between group ($p = 0.085$).

Abbreviations: F (Female); DS-12 (Dyspnea Severity Index 12); EQ-5D (EuroQol 5D); 6MWT (6 Minute Walking Test); RCT (Randomized Controlled Trial); MBS (Modified Borg Scale); 30STS (30 s Sit-to-Stand Test); MD12 (Multidimensional Dyspnea 12); BS (Borg Scale); NRS (Numeric Rating Scale); mMRC (Modified Medical Research Council Scale); BFI (Brief Fatigue Inventory); PHQ-9 (Patient Health Questionnaire-9); GAD-7 (General Anxiety Disorders 7); TUG (Time up and go Test); SF-12 (Short Form 36); SDS (Self-Rating Depression Scale); SAS (Self-Rating Anxiety Scale); SPPB (Short Physical Performance Battery); 4MWT (4 min walking test); 1MSTST (1 min Sit to Stand Test); FSS (Fatigue Severity Scale); SarQol (Sarcopenia and Quality of life Questionnaire); VAS (Visual Analogue Scale); SGRQ (Saint George Respiratory Questionnaire); BDI (Beck Depression Inventory); HADS (Hospital Anxiety and Depression Scale); WHOQOL-BREF (World Health Organization Quality of Life Questionnaire); 5TSTS (5 Times Sit-to-stand); MFIS (Modified Fatigue Impact Scale); C19YRS (Covid 19 Yorkshire Rehabilitation Scale); SF-36 (Short Form 36); ISWT (Incremental Shuttle Walking Test); FACIT (Functional Assessment of Chronic Illness Therapy); FSS (Fatigue Severity Scale); K-BILD (King's Brief Interstitial Lung Disease Questionnaire); TDI (Transition Dyspnea Index); FAS (Fatigue Assessment Scale).

3.2. Intervention

Most of the included studies carried out performed PR programs based on exercise and breathing retraining as the main components of PR, varying in the number of sessions and intervention approaches employed.

Isolated breathing exercises were used in seven studies, five performed via telerehabilitation [42,58,62,68,71], and two face-to-face [39,40]. Breathing exercises were performed in addition to myofascial release in two studies [41,50]. Breathing exercises were performed with handheld devices, breathing control exercises or secretion mobilization exercises.

Exercise therapy in addition to breathing exercises was performed in ten studies [36,44–46,52,54,59,63,69,70]. Isolation exercise was used in ten studies [47,49,51,55,56,61,64–67]. One study used exercise therapy in addition to psychological therapy [60]. The different studies included a combination of aerobic and strength exercises, while two of them incorporated virtual reality as part of the exercise regimen [55,64].

In four studies, a multicomponent program with exercise, breathing training, psychological counseling, and nutritional advice was performed [43,48,53,57].

Regarding how PR was administered, 15 studies included telerehabilitation protocols, while the remaining were administered face-to-face.

3.3. Outcomes

The main outcomes measured were dyspnea, physical function, quality of life, psychological outcomes, and fatigue. However, high heterogeneity was found when measuring outcomes, with different scales measuring the same outcome.

The Modified Medical Council Research Scale was the most commonly used test for the assessment of dyspnea, followed by Borg Scale and Modified Borg Scale. Other scales used were the Dyspnea-12 questionnaire, Multidimensional Dyspnea 12, Visual Analogue Scale or Transition Dyspnea Index.

Regarding physical function, the 6-min walking test was the most used test to assess functional capacity. Other tests used were the 30-s sit-to-stand test, time up and go test, short physical performance battery, or 1-min sit-to-stand test.

Quality of life was analyzed with different scales, but Euroqol 5D was the most widely used, followed by SF-12 and SF-36. Other studies used the Saint George Respiratory Questionnaire, SarQol, WHOQOL-BREEF, and K-BILD scales.

Anxiety was usually analyzed with the Hospital Anxiety and Depression Scale and Generalized Anxiety Disorder-7 scale, and to a lesser extent with the Self-Rating Anxiety Scale and C19YRS Scale. Depressive symptoms were commonly evaluated with Hospital Anxiety and Depression Scale and with Patient Health Questionnaire-9, and to a lesser extent with the Self-Rating Depression Scale, Beck Depression Inventory, and C19YRS.

Finally, fatigue was the most heterogeneous outcome, as all studies reported different scales, such as Fatigue Assessment Scale, Borg Scale, Fatigue Severity Scale, or Brief Fatigue Inventory.

In addition, inverse scales were assessed among the included studies, which was considered when performing the meta-analysis. For example, when assessing physical function, higher scores on the 6MWT were related to higher physical function, while lower scores on 5-time sit-to-stand test were related to higher physical function.

3.4. Risk of Bias

The risk of bias of RCTs ranged from low to high, with ten studies with low risk of bias [42,45,47,50,51,54,62,66,70,71], eight with some concerns [36,39–41,46,52,55,68], and two with high risk [48,56]. Domains of bias due to the randomization process and bias due to deviation from the intended interventions were the domains with higher issues, while the domain related to the selection of the reported results was the domain with better scores. The quality of evidence of RCTs can be found in Figure 2.

Regarding observational studies, risk of bias ranged from low to serious risk of bias. Only two studies had a low risk of bias [59,60], while five studies had a moderate risk of bias [43,49,53,63,69] and seven had serious risk [44,57,58,61,64,65,67]. The domain of controlling for confounding factors was the most critical domain of all included studies, with only two studies controlling the main factors (such as ICU stay/length, and pre-existing comorbidities). Domain of missing data was another critical domain, with many studies reporting outcomes biased due to the high loss of patients. Quality of evidence of observational studies can be found in Figure 3.

3.5. Efficacy of Pulmonary Rehabilitation in COVID-19

In the RCTs a significant effect is observed with a higher pre-post intervention change in the treatment group in dyspnea with a large effect size (Hedge's g = −1.12 [−1.813, −0.427], Z =−3.656, $p = 0.005$), in physical function with a moderate effect size (Hedge's g = 0.771 [0.363, 1.178], Z = 4.276, $p = 0.002$), on quality of life with a large effect size (Hedge's g = 1.6 [0.266, 2.934], Z = 3.083, $p = 0.027$), and at the level of depression with a moderate effect size (Hedge's g = −0.295 [−0.446, −0.145], Z = −8.432, $p = 0.014$) without significant effects on the level of anxiety or fatigue perceived by the patients, although the effects in both occur in favor of the group of patients. The heterogeneity of the studies is important in dyspnea ($I^2 = 89\%$), fatigue ($I^2 = 92\%$), and quality of life ($I^2 = 94\%$); moderate physical function ($I^2 = 71\%$); and null in depression and anxiety ($I^2 = 0\%$) (Figure 4).

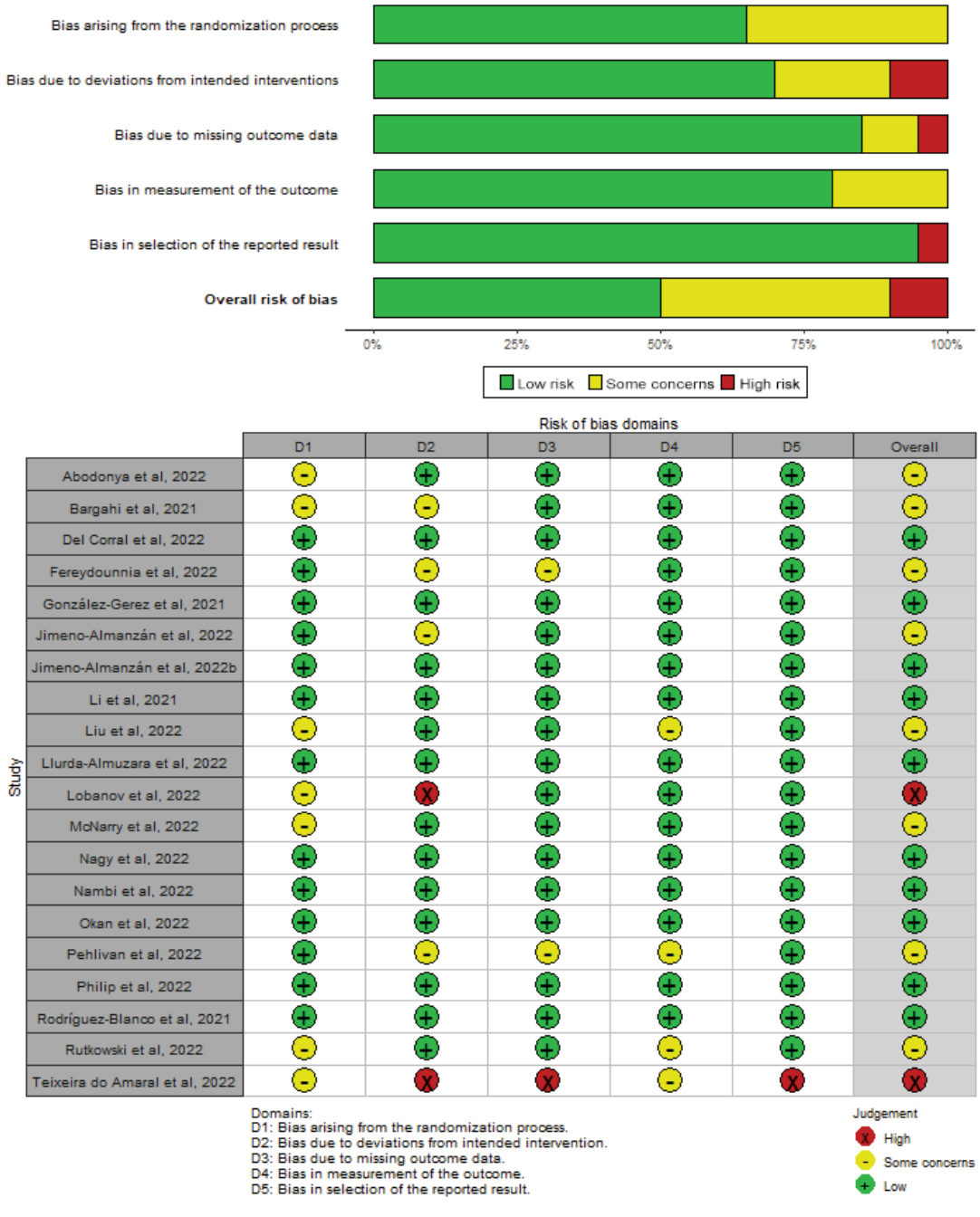


Figure 2. Risk of bias of randomized controlled trials (RoB 2.0) [36,39–42,45–48,50–52,54,55,62,66,68,70,71].

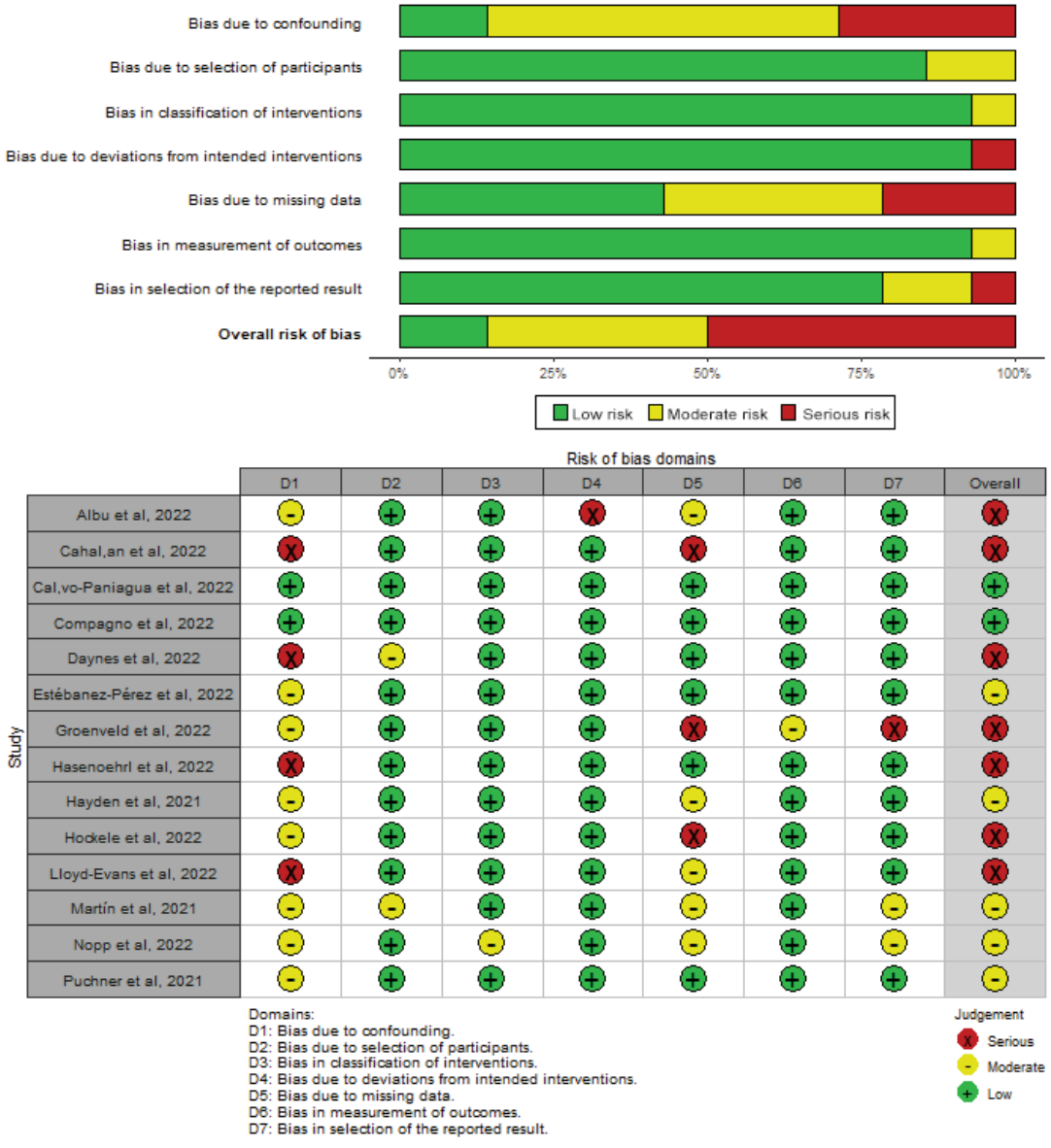


Figure 3. Risk of bias of nonrandomized controlled trials of intervention (ROBINS-I) [43,44,49,53,57–61,63–65,67,69].

In the case of observational studies, significant effects are observed in the pre-post-intervention change in quality of life with an improvement in quality of life (Mean = 12.916 [4.438, 21.395]) and on the level of perceived fatigue, with a decrease in it (Mean = −1.701 [−1.778, −1.624]), without producing significant changes in dyspnea, physical function, or in the level of anxiety and depression, although their effects show an improvement in the patients. The heterogeneity of the studies is important in dyspnea (I^2 = 99%), physical function (I^2 = 98%), quality of life (I^2 = 96%), anxiety (I^2 = 89%), and depression (I^2 = 91%), and null in the level of perceived fatigue (I^2 = 0%) (Figure 5).

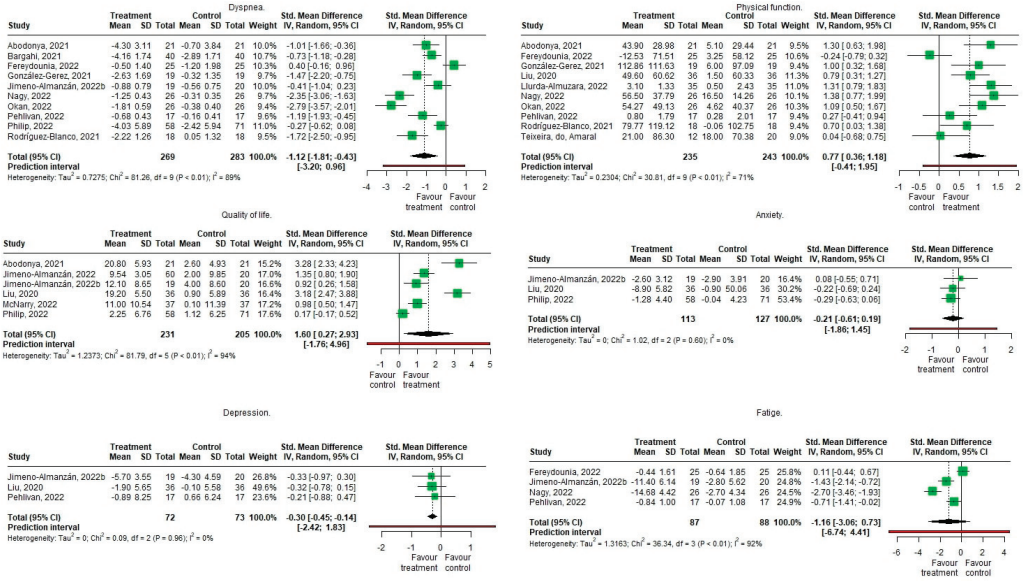


Figure 4. Meta-analysis (forest plot) of Randomized Controlled Trials on the effect of Pulmonary Rehabilitation in patients suffering from subacute and long COVID-19 infections, gauging enhancements in of dyspnea, physical function, quality of life, psychological state (anxiety and depression), and fatigue [36,39–42,45–48,50–52,54–56,62,66,68,70,71].

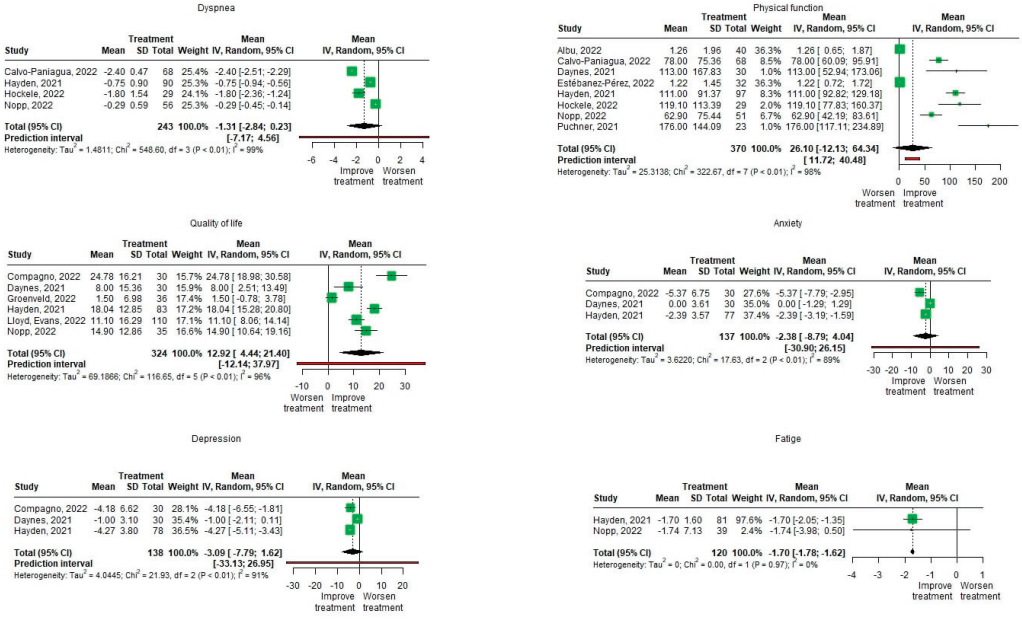


Figure 5. Meta-analysis (forest plot) of Observational Studies on the association of Pulmonary Rehabilitation in patients suffering from subacute and long COVID-19 infections, and improvements in dyspnea, physical function, quality of life, psychological state (anxiety and depression), and fatigue [43,44,49,53,57–61,63–65,67,69].

3.6. Subgroup Analysis

The subgroups meta-analysis in the RCTs (Supplementary Materials, Figure S1) showed that for dyspnea only, the studies using the Borg Scale (BS) scale show a significant and large decrease in dyspnea levels for the treatment group (Hedge's g = −1.59 [−3.161, −0.02], Z = −12.865, p =0.049) and with vanishing heterogeneity (I^2 reduced from 89% to 0%).

In physical function, studies using the 6 MWT are the one showing a significant and moderate effect in favor of the treatment group (Hedge's g = 0.756 [0.269, 1.242], Z = 3.673, p = 0.008) with an unchanging heterogeneity (I^2 of the 71% that persists in the 71%).

Regarding quality of life, only the study by Abodonya et al. [39] found a significant large reduction in the EQ−5D scale scores (Hedge's g = 3.276 [2.325, 4.228], Z = 6.751, p = 0), while, even without having a significant effect, studies using the SF-12 Physical scale were the ones presenting the smallest heterogeneity (I^2 from 94% which is reduced to 0%).

Finally, in the case of the level of depression, the three studies use different scales, so the impact on heterogeneity could not be evaluated based on the evaluation type.

The subgroups meta-analyses in the observational studies (Supplementary Materials, Figure S2), regarding the quality of life, a study by Compagno et al. [60] using SF-36 Physical scale shows a pre-post treatment significant increase in the scores for the patient's group (Mean = 24.78 [18.979, 30.581]). Similarly, in studies using the EQ-5D, significant increases in quality of life were observed (Mean = 13.304 [6.417, 20.19]), as well as a reduction in heterogeneity (I^2 from 96% to 82%).

Regarding fatigue, the study by Hayden et al. [43] uses the BFI scale observing pre-post treatment significant score decreases (Mean = −1.7 [−2.048, −1.352]).

In the variables without significant effects, however, a notable enhancement in physical function was observed in patients evaluated using the 6MWT (Mean = 101.188 [52.588, 149.788]) with a reduction in heterogeneity (I^2 of the 98% which reduces to the 83%); the study by Compagno et al. [60] assessed anxiety and depression levels with the SAS scale where significant reductions were observed for both scales (Mean = −5.37 [−7.785, −2.955]; Mean = −4.18 [−6.55, −1.81] for anxiety and depression, respectively).

3.7. Heterogeneity Analysis

Both in the RCT and in the observational studies with significant effects, no outlier study was detected (Supplementary Materials, Figures S3 and S4).

The leave-one-out analysis in the RCTs shows a stable line in dyspnea, indicating that all studies equally influenced the meta-analyses. Meanwhile, in the case of physical function, the Fereydounia et al. [41] study is the one that exerts the most influence on the results. Regarding quality of life, it is the studies by Abodonya et al. [39], Liu et al. [46], and Philip et al. [71] that most influence the results. Finally, in the level of depression, Pehlivan et al. [52] is the most influential, decreasing the level of total significance (Supplementary Materials, Figure S5). The analysis of the observational studies shows that Groenveld et al. [64] study is the most influential one in terms of quality of life, while neither of the two considered studies seems to influence the results of the meta-analysis regarding the level of fatigue (Supplementary Materials, Figure S6).

The Baujat plot in the RCTs shows how Fereydounia et al. [41] study on physical function, Philip et al. [71] study on quality of life, and Pehlivan et al. [52] study on the depression are the ones that contribute the most to the heterogeneity detected, while no article was found for dyspnea (Supplementary Materials, Figure S7). In the observational studies, the graphs show that for quality of life, Groenveld et al. [64] study is the principal contributor to the detected heterogeneity, while for fatigue levels there is no evidence of any article (Supplementary Materials, Figure S8).

3.8. Publication Bias

The Begg and Egger's tests are significant in the RCTs of dyspnea and in the case of the Egger test, also in the quality of life, while in the observational studies with significant

effects, there is no evidence of publication bias (Supplementary Materials, Table S2). The funnel plots show an asymmetric distribution of the RCTs with physical function and dyspnea (in the latter with a large number of studies outside the limits of significance), which corroborates the presence of publication bias in them (Supplementary Materials, Figure S9). In observational studies with quality of life, the funnel plot shows a symmetric distribution, which corroborates the absence of publication bias (Supplementary Materials, Figure S10).

4. Discussion

Based on the review, it has been found that PR has a positive impact on dyspnea, physical function, quality of life, and depressive symptoms when compared to usual care interventions. These improvements were of moderate to large magnitude. Furthermore, PR has been effective in reducing fatigue levels, although no significant differences were observed compared to usual care interventions. However, the review did not uncover any significant changes in anxiety levels resulting from PR.

The results of this systematic review are in line with other previously published systematic reviews [14–18,72,73]. However, while this review identified significant improvements in quality of life and depressive symptoms, others did not observe such effects [14,16,18]. Most systematic reviews included studies that assessed anxiety and depression using the Hospital Anxiety and Depression scale (HADS). While analyzing the questionary, Cosco et al. [74] discovered challenges in distinguishing between anxiety and depression. Consequently, the scale could still serve as a valuable total score indicator of emotional distress. On the other hand, Coyne and van Sonderen argue that additional research is unnecessary, advocating for the abandonment of the scale altogether [75]. It is worth noticing that in the present review, none of the included studies used the HADS for assessing depression. Additionally, recent studies have indicated a deterioration in quality of life and increases depressive symptoms over time in patients following COVID-19 infection [76,77]. In contrast to the aforementioned studies, this review encompassed patients with long COVID-19, including those with elevated levels of depression and poorer quality of life. This inclusion of patients with more severe symptoms may help explain the observed improvements in quality of life and depressive symptoms reported in our review. It is important to consider the unique characteristics and challenges faced by individuals with long COVID-19, as their experiences and outcomes may differ from those with subacute COVID-19. These distinctions could contribute to variations in the findings across different studies. Furthermore, future systematic reviews should deeply consider whether to include studies using HADS or include them contemplating HADS as a total score assessing simply emotional distress.

The findings of this study indicate that PR can contribute to improving the health status of patients following COVID-19 infection. However, it is important to acknowledge that the studies included in the review primarily focused on short-term outcomes and did not have long-term follow-up. Only one study examined long-term outcomes, revealing significant improvements in physical function after six months of follow-up in patients with subacute COVID-19 who underwent PR [45]. Thus, future research should aim to investigate the effects of PR on various long-term outcomes to gain a comprehensive understanding of its benefits in post-COVID-19 patients.

Despite the absence of a standardized protocol for training patients with COVID-19, exercise was consistently incorporated in all the studies analyzed, emphasizing its significance in managing post-COVID-19 conditions. This consistent inclusion of exercise highlights its role as a fundamental component in the overall treatment approach for patients recovering from COVID-19.

Exercise has demonstrated positive effects on the immune system, strength, fatigue, physical conditioning, and muscle dysfunctions associated with lung diseases, ultimately improving symptoms such as dyspnea [4]. Additionally, physical activity has been also associated with reduced levels of depression, anxiety, and mental well-being, regardless of age [78–80]. Furthermore, a recent review found that performing physical activity

during COVID-19 is associated with less depression and anxiety [81]. Therefore, exercise might have also helped to improve patients' mental well-being, resulting in the observed reduction in depression levels. However, considering that mental health disorders in patients with COVID-19 showed prevalence rates of 16% in terms of depression or 15% in terms of anxiety [82], and seeing that psychological therapies such as cognitive behavioral therapy had shown positive effects improving anxiety and depression in patients with COVID-19 compared to usual care [83], only a few studies included psychological therapy among their protocols [43,53,57,58,60]. Thus, future studies should incorporate broader multidisciplinary protocols that address both physical and mental health components.

PR plays a crucial role in the rehabilitation of patients who have experienced prolonged hospitalization in the intensive care unit and have undergone mechanical ventilation [84]. It offers significant potential in improving various aspects of post-COVID-19 syndrome, including dyspnea, fatigue, respiratory function, anxiety, depression, and overall quality of life. PR programs have shown promising results in enhancing these outcomes for patients with post-COVID-19 syndrome. However, despite these promising indications, the specific impact of PR programs on respiratory symptoms in patients with post-COVID-19 syndrome remains relatively limited and requires further exploration in the existing literature. More research is needed to investigate the optimal components and duration of PR interventions, as well as their long-term effects on respiratory symptoms and overall pulmonary function in this particular population. These studies will contribute to a deeper understanding of the role of PR in effectively addressing respiratory symptoms in patients recovering from COVID-19.

4.1. Strengths and Limitations

To our knowledge, this is the most comprehensive systematic review with meta-analysis and represents the most comprehensive evaluation of the efficacy of PR in patients with COVID-19, encompassing both subacute and LC patients. However, it is important to acknowledge certain limitations that arise from the following aspects. Firstly, only half of the RCTs included in this review were deemed to have a low risk of bias, and merely two out of the fourteen observational studies were classified as having a low risk of bias. Consequently, the results of this review may be prone to bias due to the high risk of bias exhibited in the included studies. Secondly, clinical heterogeneity was observed among the studies, characterized by variations in intervention protocols, duration, and intensity. This heterogeneity can complicate the synthesis and interpretation of the findings, thereby limiting the ability to draw definitive conclusions about the efficacy of PR in patients with COVID-19. Thirdly, heterogeneity was also evident in the assessment of outcomes, with different scales employed to measure the same outcome. For instance, fatigue was evaluated using eight distinct scales across the included studies, which introduces challenges in reaching robust conclusions regarding the effectiveness of PR in improving fatigue. Fourthly, the sample size of the included studies was generally low, and long-term effects of PR were only reported in a single study. Consequently, the limited sample sizes restrict the statistical power and generalizability of the findings, while the absence of long-term data impedes a comprehensive understanding of the sustained benefits of PR in this population.

4.2. Clinical Messages

Patients may develop persistent symptoms such as respiratory or physical function impairments, or psychological problems after COVID-19 infection.

Pulmonary rehabilitation has been shown to be effective improving symptoms after COVID-19, including dyspnea, physical function, quality of life, and depressive symptoms compared to usual care.

Future studies with improved methodology and long-term follow-up are needed to strengthen our conclusions.

5. Conclusions

In conclusion, the reviewed studies suggest that PR has the potential to improve various health outcomes in patients, including those recovering from COVID-19. PR has shown positive effects on dyspnea, physical function, quality of life, and depressive symptoms when compared to usual interventions. However, it is important to consider the limitations of the existing studies, such as methodological quality and small sizes, which call for mere comprehensive and well-designed research using valid assessment tools. Further investigation is needed to establish stronger evidence regarding the effectiveness of PR and its applicability to patients, particularly those with COVID-19.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines11082213/s1, Figure S1: Subgroup analysis of Randomized Controlled Trials; Figure S2: Subgroup analysis of Observational studies; Figure S3: Outlier studies analysis for RCT; Figure S4: Outlier studies analysis for observational studies; Figure S5: Leave-one-out analysis in RCT studies; Figure S6: Leave-one-out analysis in observational studies; Figure S7: Baujat plot in RCT studies; Figure S8: Baujat plot in observational studies; Figure S9: Funnel plot in RCT studies; Figure S10: Funnel plot in observational studies; Table S1: PRISMA 2020 Checklist; Table S2: Begg and Egger test for publication bias in included studies. Reference [19] is cited in Supplementary Materials.

Author Contributions: Conceptualization, O.M.-P., E.M.-O. and E.A.S.-R.; Methodology, O.M.-P., E.M.-O., J.N.C.-Z. and E.A.S.-R.; Software, J.N.C.-Z.; Validation, all authors; Investigation, all authors; Data curation, O.M.-P., E.M.-O. and J.N.C.-Z.; Writing—original draft preparation, O.M.-P., E.M.-O. and E.A.S.-R.; Writing—review and editing, E.M.-O., J.H.V., L.J.-O. and E.A.S.-R. Supervision, all authors. All authors have read and agreed to the published version of the manuscript.

Funding: The APCs have been sponsored by the European University of Madrid.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: J.H. Villafañe declares support and funding by the Italian Ministry of Health-Ricerca Corrente 2023.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Pubmed Search String

(Rehab* OR exercise OR training OR physical therapy OR breathing OR pulmonary rehabilitation OR rehabilitation OR pulmonary OR respiratory OR education) AND (post-coronavirus OR post-covid OR long covid OR post-covid-19 OR persistent covid OR long-covid-19 OR covid-19 symptoms OR post covid-19 OR post coronavirus OR post covid OR long-covid OR post-acute COVID-19 syndrome OR COVID-19 post-intensive care syndrome OR COVID-19 OR SARS-CoV-2 OR coronavirus OR post-acute COVID-19) AND (Quality of life [MeSH] OR health-related quality of life OR Functional status [MeSH] OR function OR functionally OR disability evaluation [MeSH] OR outcome assessment [MeSH] OR patient-reported outcome measure [MeSH] OR disability).

References

1. Klaser, K.; Thompson, E.J.; Nguyen, L.H.; Sudre, C.H.; Antonelli, M.; Murray, B.; Canas, L.S.; Molteni, E.; Graham, M.S.; Kerfoot, E.; et al. Anxiety and depression symptoms after COVID-19 infection: Results from the COVID Symptom Study app. *J. Neurol. Neurosurg. Psychiatry* **2021**, *92*, 1254–1258. [CrossRef]
2. Fiore, E.; Corbellini, C.; Acucella, L.; Gargano, S.; Sánchez Romero, E.A.; Cotella, D.; Villafañe, J.H. Dolor musculoesquelético en supervivientes del COVID-19 tras la hospitalización: Una breve revision (Musculoskeletal pain related to COVID-19 survivors after hospitalization: A short review). *Retos* **2022**, *44*, 789–795. [CrossRef]

3. El-Maradny, Y.A.; Rubio-Casillas, A.; Uversky, V.N.; Redwan, E.M. Intrinsic factors behind long-COVID: I. Prevalence of the extracellular vesicles. *J. Cell. Biochem.* **2023**, *124*, 656–673. [CrossRef]
4. Jimeno-Almazán, A.; Pallarés, J.G.; Buendía-Romero, Á.; Martínez-Cava, A.; Franco-López, F.; Martínez, B.J.S.-A.; Bernal-Morel, E.; Courel-Ibáñez, J. Post-COVID-19 Syndrome and the Potential Benefits of Exercise. *Int. J. Environ. Res. Public Health* **2021**, *18*, 5329. [CrossRef] [PubMed]
5. Fernández-de-Las-Peñas, C.; Palacios-Ceña, D.; Gómez-Mayordomo, V.; Palacios-Ceña, M.; Rodríguez-Jiménez, J.; de-la-Llave-Rincón, A.I.; Velasco-Arribas, M.; Fuensalida-Novo, S.; Ambite-Quesada, S.; Guijarro, C.; et al. Fatigue and Dyspnoea as Main Persistent Post-COVID-19 Symptoms in Previously Hospitalized Patients: Related Functional Limitations and Disability. *Respiration* **2022**, *101*, 132–141. [CrossRef] [PubMed]
6. Scurati, R.; Papini, N.; Giussani, P.; Alberti, G.; Tringali, C. The Challenge of Long COVID-19 Management: From Disease Molecular Hallmarks to the Proposal of Exercise as Therapy. *Int. J. Mol. Sci.* **2022**, *23*, 12311. [CrossRef]
7. Cheng, S.W.M.; McKeough, Z.J.; McNamara, R.J.; Alison, J.A. Pulmonary Rehabilitation Using Minimal Equipment for People with Chronic Obstructive Pulmonary Disease: A Systematic Review and Meta-Analysis. *Phys. Ther.* **2023**, *103*, pzad013. [CrossRef]
8. Sanchez-Ramirez, D.C.; Normand, K.; Zhaoyun, Y.; Torres-Castro, R. Long-Term Impact of COVID-19: A Systematic Review of the Literature and Meta-Analysis. *Biomedicines* **2021**, *9*, 900. [CrossRef]
9. Corbellini, C.; Villafane, J.; Gugliotta, E.; Tavella, S.; Zampese, S.; Pessina, P.; Monti, R.; Carnuccio, C.; Romero, E.A.S.; Meroni, R. Late Breaking Abstract—Pulmonary Rehabilitation in Post—COVID Subjects with Moderate Lung Restriction, A Case Series. *Eur. Respir. J.* **2021**, *58* (Suppl. 65), PA2003.
10. Sánchez Romero, E.A.; Alonso Pérez, J.L.; Vinuesa Suárez, I.; Corbellini, C.; Villafañe, J.H. Spanish experience on the efficacy of airways clearance techniques in SARS-CoV-2 (COVID-19) at intensive care unit: An editorial and case report. *SAGE Open Med. Case Rep.* **2022**, *10*, 2050313X221112507. [CrossRef]
11. Nicolás Cuenca-Zaldivar, J.; Acevedo, Á.M.; Fernández-Carnero, J.; Sánchez-Romero, E.A.; Villafañe, J.H.; Barragán Carballar, C. Effects of a Multicomponent Exercise Program on Improving Frailty in Post-COVID-19 Older Adults after Intensive Care Units:A Single-Group Retrospective Cohort Study. *Biology* **2022**, *11*, 1084. [CrossRef] [PubMed]
12. Martínez Rolando, L.; Villafañe, J.H.; Cercadillo García, S.; Sanz Argüello, A.; Villanueva Rosa, M.; Sánchez Romero, E.A. Multicomponent Exercise Program to Improve the Immediate Sequelae of COVID-19: A Prospective Study with a Brief Report of 2-Year Follow-Up. *Int. J. Environ. Res. Public Health* **2022**, *19*, 12396. [CrossRef]
13. Sánchez Romero, E.A.; Fernández Carnero, J.; Alonso Pérez, J.L.; Martínez Rolando, L.; Villafañe, J.H. Addressing post-COVID-19musculoskeletal symptoms through telemedicine: A study protocol. *F1000Research* **2022**, *11*, 898. [CrossRef]
14. Ahmed, I.; Mustafaoglu, R.; Yeldan, I.; Yasaci, Z.; Erhan, B. Effect of Pulmonary Rehabilitation Approaches on Dyspnea, Exercise Capacity, Fatigue, Lung Functions, and Quality of Life in Patients with COVID-19: A Systematic Review and Meta-analysis. *Arch. Phys. Med. Rehabil.* **2022**, *103*, 2051–2062. [CrossRef]
15. Reinert, G.; Müller, D.; Wagner, P.; Martínez-Pozas, O.; Cuenca-Záldivar, J.N.; Fernández-Carnero, J.; Sánchez Romero, E.A.; Corbellini, C. Pulmonary Rehabilitation in SARS-CoV-2: A Systematic Review and Meta-Analysis of Post-Acute Patients. *Diagnostics* **2022**, *12*, 3032. [CrossRef] [PubMed]
16. Al-Mhanna, S.B.; Mohamed, M.; Noor, N.M.; Afolabi, H.A.; Irekeola, A.A.; Bello, K.E.; Aldhahi, M.I.; Ghazali, W.S.W. Effectiveness of Pulmonary Rehabilitation among COVID-19 Patients: A Systematic Review and Meta-Analysis. *Healthcare* **2022**, *10*, 2130. [CrossRef]
17. Fugazzaro, S.; Contri, A.; Esseroukh, O.; Kaleci, S.; Croci, S.; Massari, M.; Facciolongo, N.C.; Besutti, G.; Iori, M.; Salvarani, C.; et al. Rehabilitation Interventions for Post-Acute COVID-19 Syndrome: A Systematic Review. *Int. J. Environ. Res. Public Health* **2022**, *19*, 5185. [CrossRef]
18. Da Silva Vieira, A.G.; Pinto, A.C.P.N.; Garcia, B.M.S.P.; Eid, R.A.C.; Mól, C.G.; Nawa, R.K. Telerehabilitation improves physical function and reduces dyspnoea in people with COVID-19 and post-COVID-19 conditions: A systematic review. *J. Physiother.* **2022**, *68*, 90–98. [CrossRef]
19. Page, M.J.; McKenzie, J.E.; Bossuyt, P.M.; Boutron, I.; Hoffmann, T.C.; Mulrow, C.D.; Shamseer, L.; Tetzlaff, J.M.; Akl, E.A.; Brennan, S.E.; et al. The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ* **2021**, *372*, n71. [CrossRef] [PubMed]
20. Shea, B.J.; Reeves, B.C.; Wells, G.; Thuku, M.; Hamel, C.; Moran, J.; Moher, D.; Tugwell, P.; Welch, V.; Kristjansson, E.; et al. AMSTAR 2: A critical appraisal tool for systematic reviews that include randomised or non-randomised studies of healthcare interventions, or both. *BMJ* **2017**, *358*, j4008. [CrossRef]
21. Spruit, M.A. Pulmonary rehabilitation. *Eur. Respir. Rev.* **2014**, *23*, 55–63. [CrossRef]
22. Barton, C.J.; Webster, K.E.; Menz, H.B. Evaluation of the scope and quality of systematic reviews on nonpharmacological conservative treatment for patellofemoral pain syndrome. *J. Orthop. Sports Phys. Ther.* **2008**, *38*, 529–541. [CrossRef]
23. Sterne, J.A.C.; Savović, J.; Page, M.J.; Elbers, R.G.; Blencowe, N.S.; Boutron, I.; Cates, C.J.; Cheng, H.Y.; Corbett, M.S.; Eldridge, S.M.; et al. RoB 2: A revised tool for assessing risk of bias in randomised trials. *BMJ* **2019**, *366*, l4898. [CrossRef] [PubMed]
24. Sterne, J.A.C.; Hernán, M.A.; Reeves, B.C.; Savović, J.; Berkman, N.D.; Viswanathan, M.; Henry, D.; Altman, D.G.; Ansari, M.T.; Boutron, I.; et al. ROBINS-I: A tool for assessing risk of bias in non-randomised studies of interventions. *BMJ* **2016**, *355*, i4919. [CrossRef] [PubMed]
25. Viechtbauer, W. Conducting meta-analyses in R with the metafor package. *J. Stat. Softw.* **2010**, *36*, 1–48. [CrossRef]

26. Balduzzi, S.; Rücker, G.; Schwarzer, G. How to perform a meta-analysis with R: A practical tutorial. *Evid Based Ment Health* **2019**, *22*, 153–160. [CrossRef]
27. Luo, D.; Wan, X.; Liu, J.; Tong, T. Optimally estimating the sample mean from the sample size, median, mid-range, and/or mid-quartile range. *Stat. Methods Med Res.* **2018**, *27*, 1785–1805. [CrossRef]
28. Shi, J.; Luo, D.; Weng, H.; Zeng, X.; Lin, L.; Chu, H.; Tong, T. Optimally estimating the sample standard deviation from the five-number summary. *Res. Synth. Methods* **2020**, *11*, 641–654. [CrossRef]
29. Higgins, J.P.T.; Thomas, J.; Chandler, J.; Cumpston, M.; Li, T.; Page, M.J.; Welch, V.A. (Eds.) Cochrane handbook for systematic reviews of interventions. In *Cochrane Handbook for Systematic Reviews of Interventions*; John Wiley & Sons: Chichester, UK, 2019; pp. 1–694.
30. Rosenthal, R. Meta-analytic procedures for social research. In *Meta-Analytic Procedures for Social Research*; SAGE Publications, Inc.: Thousand Oaks, CA, USA, 1991. [CrossRef]
31. Berlim, M.T.; McGirr, A.; Dos Santos, N.R.; Tremblay, S.; Martins, R. Efficacy of theta burst stimulation (TBS) for major depression: An exploratory meta-analysis of randomized and sham-controlled trials. *J. Psychiatr. Res.* **2017**, *90*, 102–109. [CrossRef]
32. Kallapiran, K.; Koo, S.; Kirubakaran, R.; Hancock, K. Review: Effectiveness of mindfulness in improving mental health symptoms of children and adolescents: A meta-analysis. *Child Adolesc. Ment. Health* **2015**, *20*, 182–194. [CrossRef]
33. McGirr, A.; Berlim, M.T.; Bond, D.J.; Neufeld, N.H.; Chan, P.Y.; Yatham, L.N.; Lam, R.W. A systematic review and meta-analysis of randomized controlled trials of adjunctive ketamine in electroconvulsive therapy: Efficacy and tolerability. *J. Psychiatr. Res.* **2015**, *62*, 23–30. [CrossRef] [PubMed]
34. Yagiz, G.; Akaras, E.; Kubis, H.-P.; Owen, J.A. The Effects of Resistance Training on Architecture and Volume of the Upper Extremity Muscles: A Systematic Review of Randomised Controlled Trials and Meta-Analyses. *Appl. Sci.* **2022**, *12*, 1593. [CrossRef]
35. Papadopoulos, V.P.; Apergis, N.; Filippou, D.K. Nocturia in CPAP-Treated Obstructive Sleep Apnea Patients: A Systematic Review and Meta-Analysis. *SN Compr. Clin. Med.* **2020**, *2*, 2799–2807. [CrossRef]
36. Jimeno-Almazán, A.; Buendía-Romero, Á.; Martínez-Cava, A.; Franco-López, F.; Sánchez-Alcaraz, B.J.; Courel-Ibáñez, J.; Pallarés, J.G. Effects of a concurrent training, respiratory muscle exercise, and self-management recommendations on recovery from post-COVID-19 conditions: The RECOVE trial. *J. Appl. Physiol.* **2023**, *134*, 95–104. [CrossRef]
37. Baujat, B.; Mahé, C.; Pignon, J.P.; Hill, C. A graphical method for exploring heterogeneity in meta-analyses: Application to a meta-analysis of 65 trials. *Stat. Med.* **2002**, *21*, 2641–2652. [CrossRef]
38. Shi, L.; Lin, L.; Omboni, S. The trim-and-fill method for publication bias: Practical guidelines and recommendations based on a large database of meta-analyses. *Medicine* **2019**, *98*, e15987. [CrossRef] [PubMed]
39. Abodonya, A.M.; Abdelbasset, W.K.; Awad, E.A.; Elalfy, I.E.; Salem, H.A.; Elsayed, S.H. Inspiratory muscle training for recovered COVID-19 patients after weaning from mechanical ventilation: A pilot control clinical study. *Medicine* **2021**, *100*, E25339. [CrossRef]
40. Bargahi, M.; Soltani, S.; Rastgoo, N.; Aryanejad, F.; Nemati, R.; Ghaebi, M.; Bajelan, A.; Esmaielzade, S. Effect of Balloon-Blowing on Dyspnea and Oxygenation in Noncritical Adult Covid19 Patients: A Pilot Study. *medRxiv* **2021**, preprint. [CrossRef]
41. Fereydounnia, S.; Shadmehr, A.; Tahmasbi, M.A.; Salehi, M.R.S. The Comparison of the Effectiveness of Respiratory Physiotherapy Plus Myofascial Release Therapy Versus Respiratory Physiotherapy Alone on Cardiorespiratory Parameters in Patients With COVID-19. *Int. J. Ther. Massage Bodywork Res. Educ. Pr.* **2022**, *15*, 4–14. [CrossRef]
42. Gonzalez-Gerez, J.J.; Saavedra-Hernandez, M.; Anarte-Lazo, E.; Bernal-Utrera, C.; Perez-Ale, M.; Rodriguez-Blanco, C. Short-Term Effects of a Respiratory Telerehabilitation Program in Confined COVID-19 Patients in the Acute Phase: A Pilot Study. *Int. J. Environ. Res. Public Health* **2021**, *18*, 7511. [CrossRef]
43. Hayden, M.C.; Limbach, M.; Schuler, M.; Merkl, S.; Schwarzl, G.; Jakab, K.; Nowak, D.; Schultz, K. Effectiveness of a Three-Week Inpatient Pulmonary Rehabilitation Program for Patients after COVID-19: A Prospective Observational Study. *Int. J. Environ. Res. Public Health* **2021**, *18*, 9001. [CrossRef]
44. Hockele, L.F.; Sachet Affonso, J.V.; Rossi, D.; Eibel, B. Pulmonary and Functional Rehabilitation Improves Functional Capacity, Pulmonary Function and Respiratory Muscle Strength in Post COVID-19 Patients: Pilot Clinical Trial. *Int. J. Environ. Res. Public Health* **2022**, *19*, 14899. [CrossRef] [PubMed]
45. Li, J.; Xia, W.; Zhan, C.; Liu, S.; Yin, Z.; Wang, J.; Chong, Y.; Zheng, C.; Fang, X.; Cheng, W.; et al. A telerehabilitation programme in post-discharge COVID-19 patients (TERECO): A randomised controlled trial. *Thorax* **2022**, *77*, 697–706. [CrossRef] [PubMed]
46. Liu, K.; Zhang, W.; Yang, Y.; Zhang, J.; Li, Y.; Chen, Y. Respiratory rehabilitation in elderly patients with COVID-19: A randomized controlled study. *Complement. Ther. Clin. Pract.* **2020**, *39*, 101166. [CrossRef]
47. Llurda-Almuzara, L.; Rodríguez-Sanz, J.; López-De-Celis, C.; Aiguadé-Aiguadé, R.; Arán-Jové, R.; Labata-Lezaun, N.; Fernández-De-Las-Peñas, C.; Bosch, J.; Pérez-Bellmunt, A. Effects of Adding an Online Exercise Program on Physical Function in Individuals Hospitalized by COVID-19: A Randomized Controlled Trial. *Int. J. Environ. Res. Public Health* **2022**, *19*, 16619. [CrossRef]
48. Lobanov, A.A.; Grishechkina, I.A.; Andronov, S.V.; Barashkov, G.N.; Popov, A.I.; Fesyun, A.D.; Ivanova, E.P.; Maccarone, M.C.; Masiero, S. Can aquatic exercises contribute to the improvement of the gait stereotype function in patients with Long COVID outcomes? *Eur. J. Transl. Myol.* **2022**, *32*. [CrossRef]

49. Martin, I.; Braem, F.; Baudet, L.; Poncin, W.; Fizaine, S.; Aboubakar, F.; Froidure, A.; Pilette, C.; Liistro, G.; De Greef, J.; et al. Follow-up of functional exercise capacity in patients with COVID-19: It is improved by telerehabilitation. *Respir. Med.* **2021**, *183*, 106438. [CrossRef]
50. Nagy, E.N.; Elimy, D.A.; Ali, A.Y.; Ezzelregal, H.G.; Elsayed, M.M. Influence of Manual Diaphragm Release Technique Combined with Inspiratory Muscle Training on Selected Persistent Symptoms in Men with Post-Covid-19 Syndrome: A Randomized Controlled Trial. *J. Rehabil. Med.* **2022**, *54*, jrm00330. [CrossRef] [PubMed]
51. Nambi, G.; Abdelbasset, W.K.; Alrawaili, S.M.; Elsayed, S.H.; Verma, A.; Vellaiyan, A.; Eid, M.M.; Aldhafian, O.R.; Nwihadh, N.B.; Saleh, A.K. Comparative effectiveness study of low versus high-intensity aerobic training with resistance training in community-dwelling older men with post-COVID 19 sarcopenia: A randomized controlled trial. *Clin. Rehabil.* **2022**, *36*, 59–68. [CrossRef]
52. Pehlivan, E.; Palalı, İ.; Atan, S.G.; Turan, D.; Çınarka, H.; Çetinkaya, E. The effectiveness of POST-DISCHARGE telerehabilitation practices in COVID-19 patients: Tele-COVID study-randomized controlled trial. *Ann. Thorac. Med.* **2022**, *17*, 110–117. [CrossRef]
53. Puchner, B.; Sahanic, S.; Kirchmair, R.; Pizzini, A.; Sonnweber, B.; Wöll, E.; Mühlbacher, A.; Garimorth, K.; Dareb, B.; Ehling, R.; et al. Beneficial effects of multi-disciplinary rehabilitation in postacute COVID-19: An observational cohort study. *Eur. J. Phys. Rehabilitation Med.* **2021**, *57*, 189–198. [CrossRef]
54. Rodriguez-Blanco, C.; Gonzalez-Gerez, J.J.; Bernal-Utrera, C.; Anarte-Lazo, E.; Perez-Ale, M.; Saavedra-Hernandez, M. Short-Term Effects of a Conditioning Telerehabilitation Program in Confined Patients Affected by COVID-19 in the Acute Phase. A Pilot Randomized Controlled Trial. *Medicina* **2021**, *57*, 684. [CrossRef]
55. Rutkowski, S.; Bogacz, K.; Rutkowska, A.; Szczegielniak, J.; Casaburi, R. Inpatient post-COVID-19 rehabilitation program featuring virtual reality—Preliminary results of randomized controlled trial. *Front. Public Health* **2023**, *11*, 1121554. [CrossRef] [PubMed]
56. Do Amaral, V.T.; Viana, A.A.; Heubel, A.D.; Linares, S.N.; Martinelli, B.; Witzler, P.H.C.; de Souza Zanini, G.; Mendes, R.G.; Ciolac, E.G. Published ahead of Print Cardiovascular, Respiratory, and Functional Effects of Home-based Exercise Training after COVID-19 Hospitalization. *Med. Sci. Sports Exerc.* **2022**, *54*, 1795–1803. [CrossRef] [PubMed]
57. Albu, S.; Zozaya, N.R.; Murillo, N.; García-Molina, A.; Chacón, C.A.F.; Kumru, H. Multidisciplinary outpatient rehabilitation of physical and neurological sequelae and persistent symptoms of covid-19: A prospective, observational cohort study. *Disabil. Rehabil.* **2021**, *44*, 6833–6840. [CrossRef]
58. Cahalan, R.M.; Meade, C.; Mockler, S. SingStrong-A singing and breathing retraining intervention for respiratory and other common symptoms of long COVID: A pilot study. *Can. J. Respir. Ther.* **2022**, *58*, 20–27. [CrossRef]
59. Calvo-Paniagua, J.; Díaz-Arribas, M.J.; Valera-Calero, J.A.; Gallardo-Vidal, M.I.; Fernández-De-Las-Peñas, C.; López-De-Uralde-Villanueva, I.; del Corral, T.; Plaza-Manzano, G. A tele-health primary care rehabilitation program improves self-perceived exertion in COVID-19 survivors experiencing Post-COVID fatigue and dyspnea: A quasi-experimental study. *PLoS ONE* **2022**, *17*, e0271802. [CrossRef]
60. Compagno, S.; Palermi, S.; Pescatore, V.; Brugin, E.; Sarto, M.; Marin, R.; Calzavara, V.; Nizzetto, M.; Scevola, M.; Aloi, A.; et al. Physical and psychological reconditioning in long COVID syndrome: Results of an out-of-hospital exercise and psychological-based rehabilitation program. *IJC Heart Vasc.* **2022**, *41*, 101080. [CrossRef]
61. Daynes, E.; Gerlis, C.; Chaplin, E.; Gardiner, N.; Singh, S.J. Early experiences of rehabilitation for individuals post-COVID to improve fatigue, breathlessness exercise capacity and cognition—A cohort study. *Chronic Respir. Dis.* **2021**, *18*. [CrossRef]
62. Del Corral, T.; Fabero-Garrido, R.; Plaza-Manzano, G.; Fernández-de-Las-Peñas, C.; Navarro-Santana, M.; López-de-Uralde-Villanueva, I. Home-based respiratory muscle training on quality of life and exercise tolerance in long-term post-COVID-19: Randomized controlled trial. *Ann. Phys. Rehabil. Med.* **2022**, *66*, 101709. [CrossRef] [PubMed]
63. Estebanez-Pérez, M.J.; Pastora-Bernal, J.M.; Martín-Valero, R. The Effectiveness of a Four-Week Digital Physiotherapy Intervention to Improve Functional Capacity and Adherence to Intervention in Patients with Long COVID-19. *Int. J. Environ. Res. Public Health* **2022**, *19*, 9566. [CrossRef]
64. Groenveld, T.; Achttien, R.; Smits, M.; de Vries, M.; van Heerde, R.; Staal, B.; van Goor, H. COVID Rehab Group Feasibility of Virtual Reality Exercises at Home for Post–COVID-19 Condition: Cohort Study. *JMIR Rehabilitation Assist. Technol.* **2022**, *9*, e36836. [CrossRef]
65. Hasenoehrl, T.; Palma, S.; Huber, D.F.-X.; Kastl, S.; Steiner, M.; Jordakieva, G.; Crevenna, R. Post-COVID: Effects of physical exercise on functional status and work ability in health care personnel. *Disabil. Rehabil.* **2022**. [CrossRef] [PubMed]
66. Jimeno-Almazán, A.; Franco-López, F.; Buendía-Romero, Á.; Martínez-Cava, A.; Sánchez-Agar, J.A.; Martínez, B.J.S.; Courel-Ibáñez, J.; Pallarés, J.G. Rehabilitation for post-COVID-19 condition through a supervised exercise intervention: A randomized controlled trial. *Scand. J. Med. Sci. Sports* **2022**, *32*, 1791–1801. [CrossRef] [PubMed]
67. Lloyd-Evans, P.H.I.; Baldwin, M.M.; Daynes, E.; Hong, A.; Mills, G.; Goddard, A.C.N.; Chaplin, E.; Gardiner, N.; Singh, S.J. Early experiences of the Your COVID Recovery® digital programme for individuals with long COVID. *BMJ Open Respir. Res.* **2022**, *9*, e001237. [CrossRef]
68. McNarry, M.A.; Berg, R.M.; Shelley, J.; Hudson, J.; Saynor, Z.L.; Duckers, J.; Lewis, K.; Davies, G.A.; Mackintosh, K.A. Inspiratory muscle training enhances recovery post-COVID-19: A randomised controlled trial. *Eur. Respir. J.* **2022**, *60*, 2103101. [CrossRef]

69. Nopp, S.; Moik, F.; Klok, F.A.; Gattinger, D.; Petrovic, M.; Vonbank, K.; Koczulla, A.R.; Ay, C.; Zwick, R.H. Outpatient Pulmonary Rehabilitation in Patients with Long COVID Improves Exercise Capacity, Functional Status, Dyspnea, Fatigue, and Quality of Life. *Respiration* **2022**, *101*, 593–601. [CrossRef] [PubMed]
70. Okan, F.; Okan, S.; Duran Yücesoy, F. Evaluating the Efficiency of Breathing Exercises via Telemedicine in Post-Covid-19 Patients: Randomized Controlled Study. *Clin. Nurs. Res.* **2022**, *31*, 771–781. [CrossRef]
71. Philip, K.E.J.; Owles, H.; McVey, S.; Pagnuco, T.; Bruce, K.; Brunjes, H.; Banya, W.; Mollica, J.; Lound, A.; Zumpe, S.; et al. An online breathing and wellbeing programme (ENO Breathe) for people with persistent symptoms following COVID-19: A parallel-group, single-blind, randomised controlled trial. *Lancet Respir. Med.* **2022**, *10*, 851–862. [CrossRef]
72. Chen, H.; Shi, H.; Liu, X.; Sun, T.; Wu, J.; Liu, Z. Effect of Pulmonary Rehabilitation for Patients with Post-COVID-19: A Systematic Review and Meta-Analysis. *Front. Med.* **2022**, *9*, 837420. [CrossRef]
73. Rahmati, M.; Shamsi, M.M.; Woo, W.; Koyanagi, A.; Lee, S.W.; Yon, D.K.; Shin, J.I.; Smith, L. Effects of physical rehabilitation interventions in COVID-19 patients following discharge from hospital: A systematic review. *J. Integr. Med.* **2023**, *21*, 149–158. [CrossRef] [PubMed]
74. Cosco, T.D.; Doyle, F.; Ward, M.; McGee, H. Latent structure of the Hospital Anxiety and Depression Scale: A 10-year systematic review. *J. Psychosom. Res.* **2012**, *72*, 180–184. [CrossRef] [PubMed]
75. Coyne, J.C.; van Sonderen, E. No further research needed: Abandoning the Hospital and Anxiety Depression Scale (HADS). *J. Psychosom. Res.* **2012**, *72*, 173–174. [CrossRef] [PubMed]
76. Menges, D.; Ballouz, T.; Anagnostopoulos, A.; Aschmann, H.E.; Domenghino, A.; Fehr, J.S.; Puhan, M.A. Burden of post-COVID-19 syndrome and implications for healthcare service planning: A population-based cohort study. *PLoS ONE* **2021**, *16*, e0254523. [CrossRef] [PubMed]
77. Premraj, L.; Kannapadi, N.V.; Briggs, J.; Seal, S.M.; Battaglini, D.; Fanning, J.; Suen, J.; Robba, C.; Fraser, J.; Cho, S.-M. Mid and long-term neurological and neuropsychiatric manifestations of post-COVID-19 syndrome: A meta-analysis. *J. Neurol. Sci.* **2022**, *434*, 120162. [CrossRef]
78. Johnston, S.A.; Roskowski, C.; He, Z.; Kong, L.; Chen, W. Effects of team sports on anxiety, depression, perceived stress, and sleep quality in college students. *J. Am. Coll. Health* **2020**, *69*, 791–797. [CrossRef]
79. Petruzzello, S.J.; Landers, D.M.; Hatfield, B.D.; Kubitz, K.A.; Salazar, W. A Meta-Analysis on the Anxiety-Reducing Effects of Acute and Chronic Exercise: Outcomes and Mechanisms. *Sports Med.* **1991**, *11*, 143–182. [CrossRef]
80. Dale, L.P.; Vanderloo, L.; Moore, S.; Faulkner, G. Physical activity and depression, anxiety, and self-esteem in children and youth: An umbrella systematic review. *Ment. Health Phys. Act.* **2019**, *16*, 66–79. [CrossRef]
81. Wolf, S.; Seiffer, B.; Zeibig, J.-M.; Welkerling, J.; Brokmeier, L.; Atrott, B.; Ehring, T.; Schuch, F.B. Is Physical Activity Associated with Less Depression and Anxiety During the COVID-19 Pandemic? A Rapid Systematic Review. *Sports Med.* **2021**, *51*, 1771–1783. [CrossRef]
82. Cénat, J.M.; Blais-Rochette, C.; Kokou-Kpolou, C.K.; Noorishad, P.-G.; Mukunzi, J.N.; McIntee, S.-E.; Dalexis, R.D.; Goulet, M.-A.; Labelle, P.R. Prevalence of symptoms of depression, anxiety, insomnia, posttraumatic stress disorder, and psychological distress among populations affected by the COVID-19 pandemic: A systematic review and meta-analysis. *Psychiatry Res.* **2021**, *295*, 113599. [CrossRef]
83. Liu, Z.; Qiao, D.; Xu, Y.; Zhao, W.; Yang, Y.; Wen, D.; Li, X.; Nie, X.; Dong, Y.; Tang, S.; et al. The Efficacy of Computerized Cognitive Behavioral Therapy for Depressive and Anxiety Symptoms in Patients with COVID-19: Randomized Controlled Trial. *J. Med. Internet Res.* **2021**, *23*, e26883. [CrossRef] [PubMed]
84. Pedersini, P.; Corbellini, C.; Villafañe, J.H. Italian Physical Therapists' Response to the Novel COVID-19 Emergency. *Phys. Ther.* **2020**, *100*, 1049–1051. [CrossRef] [PubMed]

biomedicines

MDPI

Review

Physical Therapies in the Treatment of Post-COVID Syndrome: A Systematic Review

Juan Carlos Sánchez-García [1,2], María Rentero Moreno [2], Beatriz Piqueras-Sola [1,3], Jonathan Cortés-Martín [1,2,*], Antonio Liñán-González [1,4], Elena Mellado-García [1,5] and Raquel Rodriguez-Blanque [1,2,6]

[1] Research Group CTS1068, Andalusia Research Plan, Junta de Andalucía, School of Nursing, Faculty of Health Sciences, University of Granada, 18016 Granada, Spain; jsangar@ugr.es (J.C.S.-G.); bpiquerassola@gmail.com (B.P.-S.); antoniolg@ugr.es (A.L.-G.); emg2684@gmail.com (E.M.-G.); rarobladoc@ugr.es (R.R.-B.)
[2] School of Nursing, Faculty of Health Sciences, University of Granada, 18071 Granada, Spain; mariiaren.mr@gmail.com
[3] University Hospital Virgen de las Nieves, 18014 Granada, Spain
[4] School of Nursing, Faculty of Health Sciences, Melilla Campus, University of Granada, 52005 Melilla, Spain
[5] Costa del Sol Health District, 29640 Fuengirola, Spain
[6] University Hospital San Cecilio, 18071 Granada, Spain
* Correspondence: jcortesmartin@ugr.es

Abstract: Introduction: Several days to months after diagnosis of SARS-CoV-2, 35% of patients have persistent symptoms in high incidence. This is referred to as post-COVID-19 Syndrome. There is a pressing need to find a way to help patients with the manifested symptoms. Objective: To show the different therapies that exist for post-COVID Syndrome and their efficacy. Methodology: A systematic review of the scientific literature was carried out. The data search was carried out in Scopus, PubMed, Cinahl, and Web of Science. Of the 106 articles found, 12 articles were obtained after applying the following eligibility criteria. Results: Interventions related to respiratory musculature and moderate intensity exercise both in supervised face-to-face sessions and in supervised home sessions led patients to a significant improvement in the symptoms presented. Conclusion: Physical therapies significantly reduce fatigue and dyspnea as well as other symptoms related to quality of life.

Keywords: physical exercise; post-COVID-19; fatigue; dyspnea; respiratory exercise

Citation: Sánchez-García, J.C.; Rentero Moreno, M.; Piqueras-Sola, B.; Cortés-Martín, J.; Liñán-González, A.; Mellado-García, E.; Rodriguez-Blanque, R. Physical Therapies in the Treatment of Post-COVID Syndrome: A Systematic Review. *Biomedicines* **2023**, *11*, 2253. https://doi.org/10.3390/biomedicines11082253

Academic Editor: César Fernández De Las Peñas

Received: 30 July 2023
Revised: 9 August 2023
Accepted: 10 August 2023
Published: 11 August 2023

1. Introduction

As is well known, in March 2020, due to the SARS-CoV-2 coronavirus, a pandemic called COVID-19 was declared. It is known that almost 50% of SARS-CoV-2 patients with COVID-19 pneumonia can recover spontaneously from a functional point of view at 3 months [1]; however, it has been possible to observe the persistence of symptoms (11.5 ± 5.7 days), and sometimes up to 10–35% of patients have persistent symptoms after several days or months. In the same way it can happen with people who have been mildly ill, undiagnosed, or who may have late or persistent symptoms [2,3].

This syndrome, which is appearing, attracts attention because it refers to the sum of very diverse symptoms that last until after the confirmation of SARS-CoV-2 infection. When we speak of a syndrome in health, we refer to a "coexistence of several symptoms" [4–6]. Therefore, this syndrome will continue to exist even after the acute phase has ended and several symptoms are still present.

Several names have been coined for this syndrome among patients, such as persistent COVID or long COVID [6], but the one recommended by the WHO [7] for use is the term post-COVID-19, since it does not allude to any kind of durability or causality [3].

The symptomatology of this syndrome can be very heterogeneous. The prevalent post-COVID symptoms encompass fatigue, difficulty breathing, impaired sense of smell

and taste, chest pain, muscle aches, as well as sleep and psychological disturbances [5]. This leads to a poor quality of life [2,3].

Studies, such as Simani et al. [8], have determined a prevalence rate of 5.8% to 43%. The symptoms of this syndrome related to physical and respiratory deterioration can affect the psychological health and, as a consequence, can condition the performance of physical activity [2]. All this affects the ability of individuals to achieve a full recovery, affecting the basic activities of daily living and even the return to work [9].

In order to find a correct approach to this syndrome, it is recommended to have a first consultation 4 weeks after the acute phase [10]. The assessment of each patient can be performed telematically or in person depending on the patient's data. The use of scales and/or questionnaires will also help us for the subsequent comparison of the state of health and the follow-up of the evaluation, and will also allow us to unify criteria with the health professionals.

There is a study, in particular, that talks about the symptoms associated with post-COVID syndrome. It shows that there is a high incidence of the syndrome in question, exposing the imperative need to find a way to effectively and efficiently help patients with the aforementioned symptoms [11].

For this reason, a review of the literature is proposed to show the different therapies that exist for patients with post-COVID syndrome and to evaluate their efficacy.

2. Materials and Methods

2.1. Review Protocol

The methodology used for this report was a systematic review of the scientific literature published on physical therapies for the treatment of post-COVID syndrome, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [12] review protocol, which consists of a 27-point checklist of the most representative parts of an original article, as well as the process of elaboration of these sections.

2.2. Eligibility Criteria

Articles with randomised clinical trial (RCT) methodology and articles with case study methodology were selected. The articles should be written after the COVID-19 pandemic was declared, January 2020, and should provide information on the modalities of therapies for the recovery of post-COVID syndrome in patients older than 18 years, without restriction in reference to the language of publication.

2.3. Sources of Information

This search was performed in the Scopus, PubMed, Cinahl, and Web of Science databases. In addition, a manual search was performed using reference lists of studies to find other relevant studies.

The structured language used was obtained by means of MeSH terms and health science descriptors (DeCS). The DeCs used were Post-Acute COVID-19 Syndrome and Physical Therapy Modalities, and the Boolean operators used were "OR" and "AND".

2.4. Search Strategy

The following table (Table 1) shows the search strategy used for this work, the source, filters, and the date on which the search was performed.

Table 1. Search strategy details: source, filters, and search date.

Source	Search String	Filters	Date of Search	Items
WEB OF SCIENCE	(TS = (Post-Acute COVID-19 Syndrome)) OR TS = ("COVID-19 Syndrome, Post-Acute" OR "Post-Acute COVID-19 Syndromes" OR "Long Haul COVID-19" OR "COVID-19, Long Haul" OR "Long Haul COVID 19" OR "Long Haul COVID-19s" OR "Post Acute COVID-19 Syndrome" OR "Post Acute COVID 19 Syndrome" OR "Long COVID" OR "Post-Acute Sequelae of SARS-CoV-2 Infection" OR "Post Acute Sequelae of SARS-CoV-2 Infection" OR "Post-COVID Conditions" OR "Post COVID Conditions" OR "Post-COVID Condition" OR "Long-Haul COVID" OR "COVID, Long-Haul" OR "Long Haul COVID" OR "Long-Haul COVIDs") AND (TS = (Physical Therapy Modalities)) OR TS = ("Modalities, Physical Therapy" OR "Modality, Physical Therapy" OR "Physical Therapy Modality" OR "Physiotherapy (Techniques)" OR "Physiotherapies (Techniques)" OR "Physical Therapy Techniques" OR "Physical Therapy Technique" OR "Techniques, Physical Therapy" OR "Group Physiotherapy" OR "Group Physiotherapies" OR "Physiotherapies, Group" OR "Physiotherapy, Group" OR "Physical Therapy" OR "Physical Therapies" OR "Physical" OR "Physical" OR "Neurological Physiotherapy" OR "Physiotherapy, Neurological" OR "Neurophysiotherapy")	Articles and article reviews	6 March 23	12 results
PUBMED	(("Post-Acute COVID-19 Syndrome" [MeSH Terms]) AND ("COVID-19 Syndrome, Post-Acute" OR "Post-Acute COVID-19 Syndromes" OR "Long Haul COVID-19" OR "COVID-19, Long Haul" OR "Long Haul COVID 19" OR "Long Haul COVID-19s" OR "Post Acute COVID-19 Syndrome" OR "Post Acute COVID 19 Syndrome" OR "Long COVID" OR "Post-Acute Sequelae of SARS-CoV-2 Infection" OR "Post Acute Sequelae of SARS-CoV-2 Infection" OR "Post-COVID Conditions" OR "Post COVID Conditions" OR "Post-COVID Condition" OR "Long-Haul COVID" OR "COVID, Long-Haul" OR "Long Haul COVID" OR "Long-Haul COVIDs" [Title/Abstract])) AND (("Physical Therapy Modalities" [MeSH Terms]) OR ("Modalities, Physical Therapy" OR "Modality, Physical Therapy" OR "Physical Therapy Modality" OR "Physiotherapy (Techniques)" OR "Physiotherapies (Techniques)" OR "Physical Therapy Techniques" OR "Physical Therapy Technique" OR "Techniques, Physical Therapy "OR" Group Physiotherapy" OR "Group Physiotherapies" OR "Physiotherapies, Group" OR "Physiotherapy, Group" OR "Physical Therapy" OR "Physical Therapies" OR "Therapy, Physical" OR "Neurological Physiotherapy" OR "Physiotherapy, Neurological" OR "Neurophysiotherapy" [Title/Abstract]))	Full text and in the last 5 years	6 March 23	65 results

Table 1. *Cont.*

Source	Search String	Filters	Date of Search	Items
SCOPUS	(((TITLE-ABS-KEY (“Post-Acute COVID-19 Syndrome”) OR TITLE-ABS-KEY (“COVID-19 Syndrome, Post-Acute” OR “Post-Acute COVID-19 Syndromes” OR “Long Haul COVID-19” OR “COVID-19, Long Haul” OR “Long Haul COVID 19” OR “Long Haul COVID-19s” OR “Post Acute COVID-19 Syndrome” OR “Post Acute COVID 19 Syndrome” OR “Long COVID” OR “Post-Acute Sequelae of SARS-CoV-2 Infection” OR “Post Acute Sequelae of SARS-CoV-2 Infection” OR “Post-COVID Conditions” OR “Post COVID Conditions” OR “Post-COVID Condition” OR “Long-Haul COVID” OR “COVID, Long-Haul” OR “Long Haul COVID” OR “Long-Haul COVIDs”))) AND (((TITLE-ABS-KEY (“Physical Therapy Modalities”) OR TITLE-ABS-KEY (“Modalities, Physical Therapy” OR “Modality, Physical Therapy” OR “Physical Therapy Modality” OR “Physiotherapy (Techniques)” OR “Physiotherapies (Techniques)” OR “Physical Therapy Techniques” OR “Physical Therapy Technique” OR “Techniques, Physical Therapy” OR “Group Physiotherapy” OR “Group Physiotherapies” OR “Physiotherapies, Group” OR “Physiotherapy, Group” OR “Physical Therapy” OR “Physical Therapies” OR “Therapy, Physical” OR “Neurological Physiotherapy” OR “Physiotherapy, Neurological” OR “Neurophysiotherapy”).))	Articles	6 March 23	17 results
CINHAL	(MH “Post-Acute COVID-19 Syndrome”) AND (MH “Physical Therapy+”)	Limiters: Refereed publications, expanders: Apply equivalent subjects and search modes: Boolean/Phrase	6 March 23	12 results

2.5. Data Extraction Process

After carrying out the search strategy, the articles found were transferred to the Mendeley web application using the Mendeley web importer tool. They were then structured by folders, according to the databases through which they had been obtained, and duplicates were later eliminated.

The included studies were randomised clinical trials (RCTs) and cohort studies with the objective of showing therapies in post-COVID syndrome patients and evaluating their efficacy. The studies were published between 2020 and 2023. The title, abstract and keywords of each study were examined, and the inclusion and exclusion criteria were applied.

2.6. Data Collection Process and Data Collected

The following data were extracted from each article: men and women over 18 years of age who have had the disease, number of participants, type of physical exercise performed, duration of exercise, intensity, and whether it was supervised by professionals.

Section 3 shows the selection process of the articles in more detail.

2.7. Risk of Bias in Individual Studies

To carry out the methodological evaluation of the articles selected for this study, we proceeded to analyse the design, methodology and type of study of each article, with the aim of selecting the most specific methodological evaluation scale for each case.

Of the 13 articles, 4 were case studies, 1 was a cohort study, 7 were RCTs, and 1 was a quasi-experimental study.

The articles whose design was a case study were evaluated using the Single-Case Experimental Design (SCED) [13]. The SCED was constructed including 11 items, of which 10 are used to evaluate methodological quality and one for the use of statistical analysis.

The following table (Table 2) shows the results obtained after the methodological evaluation using the SCED scale [13].

Table 2. Methodological evaluation results using SCED scale.

Author	Article	Numerical Score
Santos, et al. [9]	Musculoskeletal physiotherapy in physical sequelae of SARS-CoV-2 infection: A case report.	7/11
Wagner, et al. [14]	Successful application of pulsed electromagnetic fields in a patient with post-COVID-19 fatigue: A case report	4/11
Rausch, et al. [15]	The effects of Exercise Therapy Moderated by Sex in Rehabilitation of COVID-19	8/11
Daynes, et al. [16]	Early Experiences of Rehabilitation for individual sport-COVID to improve fatigue, breathlessness exercise capacity and cognition—A cohort Study	10/11
Zha, et al. [17]	Trigger point injections and dry needling can be effective in treating long COVID syndrome-related myalgia: a case report	6/11

For the articles whose methodology corresponded to a clinical trial, the scientific quality was evaluated using the PEDro scale [18]. This scale provides information on the clinical scientific evidence and scores it based on certain indicators, adding 1 point to each one if they are present and 0 points if they are not, giving a total score of 10 points. If the trial obtains a score between 9 and 10, it indicates that it is of very good quality; if it obtains between 6 and 8, it indicates good quality; if it is between 4 and 5, it indicates fair quality; and if it is less than 4, it indicates poor quality. In the case of the articles chosen for this systematic review, the values range between 6 and 9, receiving an average score of 8.30, which indicates that the average scientific quality is considered to be "good quality".

The following table (Table 3) shows the results obtained after carrying out the methodological evaluation using the PEDro scale [18].

Table 3. Assessment of methodology using the PEDro scale.

Author	Article	Numerical Score
Estebanez-Pérez, et al. [19]	The Effectiveness of a Four-Week Digital Physiotherapy Intervention to Improve Functional Capacity and Adherence to Intervention in Patients with Long COVID-19	6/10
Sharma, et al. [20]	Pulmonary Tele-Rehabilitation in Patients (Post COVID-19) With Respiratory Complications: A Randomized Controlled Trial	8/10
Jimeno-Almazán, et al. [21]	Rehabilitation for post-COVID-19 condition through a supervised exercise intervention: A randomized controlled trial	9/10
Sari, et al. [22]	Effects of Inspiratory Muscle Training in Patients with post-COVID-19	9/10
Okan, et al. [23]	Evaluating the Efficiency of Breathing Exercises via Telemedicine in Post-COVID-19 Patients: Randomized Controlled Study	9/10
McNarry, et al. [24]	Inspiratory muscle training enhances post-COVID-19 recovery: A Randomised controlled trial	8/10
Palau, et al. [25]	Effect of a home-based inspiratory muscle training programme on functional capacity in postdischarge patients with long COVID: The InsCOVID trial	9/10

3. Results

After applying the search strategy for articles in the different databases and applying the inclusion and exclusion criteria set out in the methodology, we identified 12 studies that we included in our review. Figure 1 shows the flow chart of the identified articles.

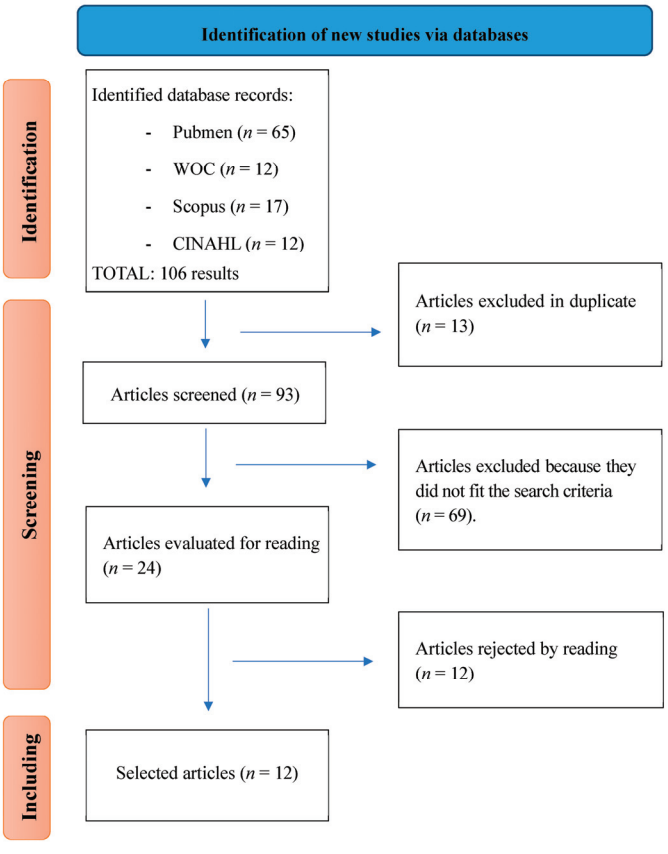


Figure 1. Flow diagram.

Taken together, the studies obtained highlight the efficacy of various therapeutic interventions to address the symptoms of prolonged COVID, encompassing physical and psychological well-being.

Overall, there were notable increases in physical function, with improvements in balance, muscle strength, and functional capacity, among others. Symptoms, such as fatigue and dyspnoea, decreased substantially in the intervention group compared to the control group. In addition, improvements in mental health and cardiovascular and pulmonary capacity were recorded. These results support the efficacy of exercise and rehabilitation strategies in the overall recovery of patients.

A summary of the results can be found in Table 4.

Table 4. Summary of the conclusions of the results obtained.

Author	Sample	Type of Therapy	Results
Estebanez-Perez, et al. (2022) [19]	n = 32 (23 women and 9 men) Average age = 45.93 years	Digital physiotherapy for 4 weeks, with individual evaluation. One daily session of 45–40 min, three to five times per week. Walking, jogging, or swimming (20–30 min) for 3 to 5 sessions/week. Increasing strength training, exercising 1–3 muscle groups with a load of 8 to 12. Recommendation of ventilatory techniques to improve ventilation and mobility of the thorax.	After 4 weeks of intervention, a significant improvement was shown ($p < 0.05$). In the SPPB test (balance, gait speed, and chair support test) an improvement of 1.21 points was found. In the 1-STS test, an improvement of 3.50 points was obtained. There was an improvement in functional capacity, with high adherence rate and MCID values.
Santos, et al. [9]	n = 1 Age = 60 years	Therapy consisted of the application of transcutaneous electrical nerve stimulation, Cyriax deep transverse massage, stretching exercises, balance, coordination, and manual therapy, with Maitland passive kinesitherapy. Three times per week for 5 weeks.	Muscle strength improved from 2/5 to 4/5 on the Daniels muscle range test. Walking balance increased along with more coordinated movements. Fatigue and weakness disappeared. Patient can perform BADLs and IADLs normally again.
Wagner, et al. (2022) [14]	n = 1 Age = 55 years	Having noticed no improvement in previous therapies, he decided to use an electromagnetic field therapy, an ionic induction. Ten sessions of 30 min each, twice a week for 5 weeks. Patient placed in the supine position, 6 min are administered on the abdominal area, 3 min on the sternum, 6 min on the dorsal area, 6 min on the soles of the feet, and 6 min on the pelvic floor. The frequency used was 2.5 Hz for the dorsal area and 1 Hz for the rest.	The patient improved markedly with increased energy and complete disappearance of fatigue. There were improvements in the dimensions of mood, work, relationships, and enjoyment of life. There were no side effects except for transient neck pain.
Sharma, et al. (2022) [20]	n = 30 Average age = 18–55 years	Pulmonary telerehabilitation. The control group received conventional care and the experimental group received a therapeutic treatment protocol 4 days a week for 6 weeks. Exercises to reduce fatigue and shortness of breath.	Significant improvement in both groups, and there was also a significant difference between CG and EG in MBDS ($p = 0.005$ and $p = 0.011$) and VAS-F ($p = 0.018$ and $p = 0.036$). Therefore, it is concluded that the experimental group recovered more quickly. Women were more fatigued than men.

Table 4. *Cont.*

Author	Sample	Type of Therapy	Results
Jimeno-Alamazán, et al. (2022) [21]	n = 39 CG = 20 EG = 19 Age = 45.2 years	Eight-week supervised, personalised multi-component exercise program. Two days resistance training (3 sets, 8 repetitions of squat, bench press, dead weight, and bench pull) combined with moderate intensity variable training and one day of light intensity continuous training (30–60 min) for the experimental group. For the control group, aerobic exercise was recommended for 20 to 30 min, 5 days a week, at a tolerable intensity together with strength exercises in 3 sessions a week.	STS test, HSQ 50% 1 RM, estimated VO_2max, and BP 50% 1 RM improved significantly in both groups. The most pronounced changes were dyspnoea (control vs. exercise: 83.3% vs. 5.4%, p = 0.003; V = 0.48) and fatigue (61.1% vs. 34.6%, p = 0.072; V = 0.30). In the exercise group, there was a progressive improvement in symptoms (94.7% vs. 72.2%, p = 0.063; V = 0.31), with patients being more likely to become asymptomatic (42.1% vs. 16.7%, p = 0.091; V = 0.28) than the control group. In cardiovascular parameters there was a loss in the main determinant of fitness in the control group (VO_2max, 5.7% vs. −0.8%, p = 0.01) and final HR (−50.0% vs. −13.3%, p = 0.01). Lower limbs recovered in both groups when measuring the STS test (−22.7% vs. −20.7%).
Rausch, et al. (2022) [15]	n = 233 Women = 94, mean age = 61.50 years Men = 139, mean age = 61.69 years	Moderate therapy exercise, duration of 3 weeks. The 6MWT and a pulmonary function test were performed. They followed a standardised program which consisted of respiratory muscle training (3 sets of 10 breaths and 1 min rest), strength exercises, endurance training, and relaxation exercises.	Men received more respiratory strength exercises than women. No significant correlations were found between the number of respiratory muscle training sessions and lung function parameters ($p > 0.05$). In the 6MWT test, both men and women had statistically significant results (T (232) = −16.67; $p < 0.001$; d = 0.48). Men showed a shorter distance run compared to women (T (231) = −3.04; $p < 0.01$; d = 0.41). The improvement in ICmax was significantly higher in men (F (1227.46) = 8.93; $p > 0.01$; $\omega_2 = 0.03$). Men showed higher FVC before and after. The same was true for FEV1. Women showed a smaller difference with respect to FEV1 improvement. Significant reduction in FVC.
Sari, et al. (2022) [22]	n = 24 TG = 13 CG = 11 Age = 18–65 years	Inspiratory muscle training. Diagrammatic respirations, together with thoracic expansion and exercises to increase thoracic distensibility. A total of 10 repetitions, 3 sets per day. Resistance training to strengthen the quadriceps (squats and bridge exercises) for 6 weeks every day with 10 repetitions and 3 sets per day.	The 6MWT distance and the 30 s standing test increased significantly in TG ($p < 0.001$) and CG ($p < 0.05$). mMCR dyspnea scale significantly decreased in TG, from 10 to 2 patients with dyspnea in TG and from 7 to 6 patients with dyspnea in CG. Muscular strength of hand pressure increased significantly in TG.

Table 4. *Cont.*

Author	Sample	Type of Therapy	Results
Okan, et al. (2022) [23]	n = 49 IG = 26 CG = 26 Age > 18 years	Breathing exercises. In IG, 10 breathing exercises for 3 sessions per day, every day for 5 weeks. Light walking 20–30 min, 5 times a week. The CG had the exercises explained through a handout plus a recommendation for light walking.	The FEV1 and FVC values after the test in IG were significantly higher (95% CI: 2.921–8.771, 95% CI: 2.619–7.381 $p_2 < 0.001$). Between IG and CG, the differences were not significant. In the MVV value, the IG had higher significance (97.54 ± 10.23). mMRC values were more significant in the CG. The 6MWT parameters were significantly higher in the IG.
Daynes, et al. (2021) [16]	n = 32	Rehabilitation for 6 weeks and 2 supervised days per week. Aerobic exercise, strength training of upper limbs and lower limbs. Educational meetings.	Thirty completed rehabilitations. All improved:ISWT by 112 m ($p < 0.01$) and 544 s ($p < 0.01$), FACIT by 6 points (pz 0.01), the EQ5D by 8 and MoCA by 2 ($p < 0.01$), and CAT by a score of 3 ($p < 0.05$). Anxiety and depression were not statistically significant.
McNarry, et al. (2022) [24]	n = 281 Age > 18 years	Inspiratory muscle training in 8 weeks. Intervention group and control group (standard care). Participants were trained to know how to use PrO_2. Three sessions per week unsupervised. A total of 6 blocks of 6 breaths, interspersed breaks decreasing from 40 to 10 s in a maximum time of 20 min.	KBILD (dyspnoea and activities and psychological) had a significant improvement in GI. The GI had a good reduction in dyspnoea. It also significantly increased inspiratory muscle strength in the IG. Physical fitness and functional capacity increased significantly in the IG with an increase in VO_2max.
Palau et al. (2022) [25]	n = 26 IG = 13 CG = 13 Age > 18 years old	Twelve weeks. IG training 2× week for 20 min each session with inspiratory muscle trainer applying a resistance of 25–30% of maximal inspiratory pressure. Diaphragmatic breathing will be instructed. The CG do not receive physiotherapy.	The IG mean VO_2max was higher than that of CG (22.2 mL/kg/min, 95% CI: 21.3 to 23.2). For VE/VCO2 there was no significant difference between IG and CG (Δ −1.92; 95% CI: −4.69 to 0.85; $p = 0.165$). Significant improvement in depression/anxiety in IG. Non-significant improvement in mobility, self-care, and pain in both groups. IG had a significant improvement in MIP.

Table 4. *Cont.*

Author	Sample	Type of Therapy	Results
Zha et al. (2022) [17]	$n = 1$ Age = 59 years	Dry and wet puncture. At 6 months after presenting pain, WN was performed with 1% lidocaine without epinephrine with a 25-gauge needle and 1.5 inches, with four in the neck and shoulders, and one on each side of the triceps. Four sessions were performed. At 12 months, DN was tried with a 21-gauge 1-inch needle at 10 sites (4 in the neck and upper back region, 1 in the posterior triceps, and 2 in each calf). She carried out two sessions.	After several punctures, the patient improved markedly as he remained pain free 18 months later.

SPPB, Short Physical Performance Battery. 1-STS, 1-min sit-to-stand test. MCID, Minimal Clinically Important Difference. BADL, Basic Activities of Daily Living. IADL, Instrumental Activities of Daily Living. CG, control group. EG, experimental group. MBDS, Minimum Basic Data Set. VAS-F, Visual Analog Scale—Fatigue. HSQ, half squat exercise. 1 RM, one repetition maximum. VO_2, volumen máximo de oxígeno. BP, bench press. HR, heat rate. 6MWT, 6 min walk test. ICmax, maximal inspiration capacity. FVC, forced vital capacity. FEV1, forced expiratory volume in the first second. TG, treatment group. mMRC, Modified Medical Research Council Dyspnea Scale. IG, intervention group. MVV, maximum voluntary ventilation. ISWT, Incremental Shuttle Walking Test. FACIT, Functional Assessment of Chronic Illness—Therapy Fatigue Scale. EQ5D, EuroQual 5 Domain. MoCA, Montreal Cognitive Assessment. CAT. COPD Assessment Test. PrO_2, PrO_2Fit Health (Device). KBILD, King's Brief Interstitial Lung Disease. MIP, maximal inspiratory pressure. WN, wet needling. DN, dry needling.

4. Discussion

The objective of this systematic review was to show the therapies that exist in patients with long-COVID and to evaluate their efficacy, and for this reason the study of the articles has been carried out.

This topic is closely related to the assessment of the systemic consequences of COVID-19, which is a broad field of research in which the assessment of respiratory function plays a key role. This was presented in the report by Pini et al. [26], where respiratory function was analysed 4–6 months after hospital discharge in these patients to study the negative consequences of COVID-19 pneumonia.

The results of this systematic review demonstrated that the exercise and rehabilitation strategies had a positive impact on multiple aspects of patients' health, from physical function to mental health. These findings support the efficacy of the interventions implemented and suggest a pathway to improved recovery and well-being in people facing similar health challenges.

Most of the articles selected in the elaboration have been published in the year 2022, since we are dealing with a recent disease, namely COVID-19, and, above all, our objective concerns therapies against post-COVID syndrome. After analysing them, we can conclude that the selected articles have a generally good methodological level. We have been able to answer the main objective, since we have found different therapies for persistent COVID, such as exercises of moderate intensity [19,21], exercises for the respiratory musculature [15,20,22–25], electromagnetic field therapy [15,20,22–25], application of cutaneous electromagnetic nerve stimulation [9], and trigger point injections [17].

In the clinical guideline for long-COVID care, they recommend for fatigue a type of progressive exercise therapy tailored to the individual patient [27], information that we have been finding offers good results after completion [19,21]. In relation to dyspnoea, the guideline recommends respiratory exercise [20]. However, we cannot determine the efficacy of all studies as these have been based on a single case [9,14,17].

Several studies mention the improvement in the 6MWT test. Thanks to the controlled exercise, it was observed that men run a shorter distance when compared to women, with a significant increase for both [15,22,23].

Another improvement observed with controlled exercise was dyspnoea, which was shown to decrease significantly, with a decrease of approximately 80% in the control groups [15,21,22,24].

Depression and anxiety are a more subjective issue, since some studies show that there is a significant improvement in the control groups [24,25] but there is another that does not show a significant difference [16]. Despite that, it is observed that controlled exercise improves depression and anxiety.

Regarding articles that discuss electromagnetic field therapy [14], namely the application of electromagnetic nerve stimulation [11], it is shown that both women improved the sensation of fatigue, pain disappeared completely, and quality of life improved. On the other hand, the patient who received the trigger injections only manifested a complete disappearance of the pain [17]. It is necessary to qualify this aspect, as it is interesting to relate dry and wet needling with evident improvements in pain control in patients with post-COVID symptomatology. As shown in the case of Zha et al. [17]. It is true that this relationship can only be seen in this specific patient, so it is proposed as a new line of research derived from this study to substantiate this possible new treatment pathway.

One of the limitations that have been found is the poor adherence of study participants to the interventions [15,19,21,22,24] and the very small samples used [9,14,17].

Although there are several studies that demonstrate the efficacy of physical therapies, it remains to be determined whether other types of therapies or treatment would be effective against physical and psychological symptoms. And, above all, it is necessary to provide psychological and emotional help to these patients.

In terms of the limitations observed, more studies are needed, as the limitations are evident and may compromise the validity and reliability of the results. These limitations

stem from sample sizes, the potential for bias, inadequate control of confounding variables and even the cross-sectional approach. Therefore, it is crucial to take these limitations into account when interpreting and applying the results of such studies to ensure accurate interpretation and appropriate use of their results in relation to physical therapies and prolonged COVID.

5. Conclusions

After searching the literature, we have found that moderate exercise and respiratory muscle exercises are beneficial for recovery from the most common symptoms of persistent COVID, namely fatigue and dyspnoea.

It can be concluded that, in cases where there was exercise control, patients have a considerable improvement in fatigue, depression and dyspnoea, among others.

However, there are still too few studies to be able to speak of the efficacy of certain therapies for the symptoms of long-COVID-19.

Author Contributions: Conceptualisation, J.C.-M., R.R.-B. and J.C.S.-G.; data curation, M.R.M., E.M.-G., R.R.-B. and J.C.S.-G.; formal analysis, J.C.-M., M.R.M., B.P.-S., A.L.-G. and R.R.-B.; investigation, R.R.-B. and E.M.-G.; methodology, R.R.-B., J.C.S.-G. and J.C.-M.; project administration, R.R.-B. and E.M.-G.; resources, R.R.-B. and A.L.-G.; supervision, J.C.S.-G.; validation, R.R.-B., J.C.-M., E.M.-G. and B.P.-S.; visualisation, R.R.-B. and J.C.-M.; writing—original draft, M.R.M., E.M.-G., J.C.-M., R.R.-B. and J.C.S.-G.; writing—review and editing, R.R.-B., M.R.M., B.P.-S., J.C.-M. and J.C.S.-G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This systematic review was carried out following a protocol, available on the web: http://www.crd.york.ac.uk/PROSPERO/ (accessed on 16 January 2023) and whose registration number is CRD42023391811.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to the Official College of Nursing of Granada (CODEGRA) for their help in the research support programme, and to the Chair of Research in Nursing Care of the University of Granada and the Official College of Nursing of Granada.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Orzes, N.; Pini, L.; Levi, G.; Uccelli, S.; Cettolo, F.; Tantucci, C. A prospective evaluation of lung function at three and six months in patients with previous SARS-COV-2 pneumonia. *Respir. Med.* **2021**, *186*, 106541. [CrossRef] [PubMed]
2. Gómez Conesa, A. ¿Cómo abordar desde la fisioterapia la salud mental en el COVID persistente? *Fisioterapia* **2022**, *44*, 1–5. [CrossRef]
3. López-Sampalo, A.; Bernal-López, M.R.; Gómez-Huelgas, R. Síndrome de COVID-19 persistente. Una revisión narrativa. *Rev. Clín. Esp.* **2022**, *222*, 241–250. [CrossRef] [PubMed]
4. Timewar, R.R.; Sumar, A.I.; Nitin, B.; Kohale; Suraj, B.; Rathod; Guarav, G. Manwar Post Acute Corona Virus (COVID-19) Syndrome. *Int. J. Adv. Res. Sci. Commun. Technol.* **2023**, *3*, 385–391. [CrossRef]
5. Maltezou, H.C.; Pavli, A.; Tsakris, A. Post-COVID Syndrome: An Insight on Its Pathogenesis. *Vaccines* **2021**, *9*, 497. [CrossRef] [PubMed]
6. Fernández-de-las-Peñas, C.; Palacios-Ceña, D.; Gómez-Mayordomo, V.; Cuadrado, M.L.; Florencio, L.L. Defining Post-COVID Symptoms (Post-Acute COVID, Long COVID, Persistent Post-COVID): An Integrative Classification. *Int. J. Environ. Res. Public Health* **2021**, *18*, 2621. [CrossRef]
7. World Health Organization. Post COVID-19 Condition (Long COVID). Available online: https://www.who.int/europe/news-room/fact-sheets/item/post-covid-19-condition (accessed on 8 August 2023).
8. Simani, L.; Ramezani, M.; Darazam, I.A.; Sagharichi, M.; Aalipour, M.A.; Ghorbani, F.; Pakdaman, H. Prevalence and correlates of chronic fatigue syndrome and post-traumatic stress disorder after the outbreak of the COVID-19. *J. Neurovirol.* **2021**, *27*, 154–159. [CrossRef] [PubMed]
9. Santos, S.; Flores, J.A. Musculoskeletal physiotherapy in physical sequelae of SARS-CoV-2 infection: A case report. *Physiother. Res. Int.* **2022**, *27*, e1938. [CrossRef]

10. Centre for Disease Control and Prevention. CDC Post-COVID Conditions: Information for Healthcare Providers. Available online: https://www.cdc.gov/coronavirus/2019-ncov/hcp/clinical-care/post-covid-conditions.html#print (accessed on 8 August 2023).
11. Donnachie, E.; Hapfelmeier, A.; Linde, K.; Tauscher, M.; Gerlach, R.; Greissel, A.; Schneider, A. Incidence of post-COVID syndrome and associated symptoms in outpatient care in Bavaria, Germany: A retrospective cohort study using routinely collected claims data. *BMJ Open* **2022**, *12*, e064979. [CrossRef]
12. Welch, V.; Petticrew, M.; Tugwell, P.; Moher, D.; O'Neill, J.; Waters, E.; White, H. Extensión PRISMA-Equidad 2012: Guías para la escritura y la publicación de revisiones sistemáticas enfocadas en la equidad en salud. *Rev. Panam. Salud Publica/Pan Am. J. Public Health* **2013**, *34*, 60–67. [CrossRef]
13. Psycbite SCED Scale. Available online: http://www.psycbite.com/docs/The_SCED_Scale.pdf (accessed on 27 March 2021).
14. Wagner, B.; Steiner, M.; Markovic, L.; Crevenna, R. Successful application of pulsed electromagnetic fields in a patient with post-COVID-19 fatigue: A case report. *Wien. Med. Wochenschr.* **2022**, *172*, 227–232. [CrossRef]
15. Rausch, L.; Puchner, B.; Fuchshuber, J.; Seebacher, B.; Löffler-Ragg, J.; Pramsohler, S.; Netzer, N.; Faulhaber, M. The Effects of Exercise Therapy Moderated by Sex in Rehabilitation of COVID-19. *Int. J. Sports Med.* **2022**, *43*, 1043–1051. [CrossRef]
16. Daynes, E.; Gerlis, C.; Chaplin, E.; Gardiner, N.; Singh, S.J. Early experiences of rehabilitation for individuals post-COVID to improve fatigue, breathlessness exercise capacity and cognition—A cohort study. *Chron. Respir. Dis.* **2021**, *18*, 147997312110156. [CrossRef] [PubMed]
17. Zha, M.; Chaffee, K.; Alsarraj, J. Trigger point injections and dry needling can be effective in treating long COVID syndrome-related myalgia: A case report. *J. Med. Case Rep.* **2022**, *16*, 31. [CrossRef] [PubMed]
18. Gómez Conesa, A. Escala PEDro. Available online: https://pedro.org.au/spanish/resources/pedro-scale/ (accessed on 27 March 2021).
19. Estebanez-Pérez, M.-J.; Pastora-Bernal, J.-M.; Martín-Valero, R. The Effectiveness of a Four-Week Digital Physiotherapy Intervention to Improve Functional Capacity and Adherence to Intervention in Patients with Long COVID-19. *Int. J. Environ. Res. Public Health* **2022**, *19*, 9566. [CrossRef] [PubMed]
20. Sharma, P.; Goswami, S.K. Pulmonary Tele-Rehabilitation in Patients (Post Covid-19) With Respiratory Complications: A Randomized Controlled Trial. *Indian J. Physiother. Occup. Ther.* **2022**, *16*, 182–189. [CrossRef]
21. Jimeno-Almazán, A.; Franco-López, F.; Buendía-Romero, Á.; Martínez-Cava, A.; Sánchez-Agar, J.A.; Sánchez-Alcaraz Martínez, B.J.; Courel-Ibáñez, J.; Pallarés, J.G. Rehabilitation for post-COVID-19 condition through a supervised exercise intervention: A randomized controlled trial. *Scand. J. Med. Sci. Sports* **2022**, *32*, 1791–1801. [CrossRef] [PubMed]
22. Sari, F.; Bayram, S.; Pala, G.G.; Çömçe, F.; Küçük, H.; Oskay, D. Effects of Inspiratory Muscle Training in Patients with post-COVID-19. *Harran Üniversitesi. Tıp Fakültesi. Derg.* **2022**, *19*, 581–588. [CrossRef]
23. Okan, F.; Okan, S.; Duran Yücesoy, F. Evaluating the Efficiency of Breathing Exercises via Telemedicine in Post-Covid-19 Patients: Randomized Controlled Study. *Clin. Nurs. Res.* **2022**, *31*, 771–781. [CrossRef]
24. McNarry, M.A.; Berg, R.M.G.; Shelley, J.; Hudson, J.; Saynor, Z.L.; Duckers, J.; Lewis, K.; Davies, G.A.; Mackintosh, K.A. Inspiratory muscle training enhances recovery post-COVID-19: A randomised controlled trial. *Eur. Respir. J.* **2022**, *60*, 2103101. [CrossRef]
25. Palau, P.; Domínguez, E.; Gonzalez, C.; Bondía, E.; Albiach, C.; Sastre, C.; Martínez, M.L.; Núñez, J.; López, L. Effect of a home-based inspiratory muscle training programme on functional capacity in postdischarged patients with long COVID: The InsCOVID trial. *BMJ Open Respir. Res.* **2022**, *9*, e001439. [CrossRef] [PubMed]
26. Pini, L.; Montori, R.; Giordani, J.; Guerini, M.; Orzes, N.; Ciarfaglia, M.; Arici, M.; Cappelli, C.; Piva, S.; Latronico, N.; et al. Assessment of respiratory function and exercise tolerance at 4–6 months after COVID-19 infection in patients with pneumonia of different severity. *Intern. Med. J.* **2023**, *53*, 202–208. [CrossRef] [PubMed]
27. Sociedad Española de Médicos Generales y de Familia. *Guía Clínica Para la Atención al Paciente Long COVID/COVID Persistente*; ERGON: Madrid, Spain, 2021.

biomedicines

MDPI

Article

Whole-Body Cryostimulation in Post-COVID Rehabilitation for Patients with Obesity: A Multidisciplinary Feasibility Study

Jacopo Maria Fontana [1,*], Angelo Alito [2], Paolo Piterà [1], Federica Verme [1], Stefania Cattaldo [3], Mauro Cornacchia [4], Stefania Mai [5], Amelia Brunani [1] and Paolo Capodaglio [1,6]

[1] Research Laboratory in Biomechanics, Rehabilitation and Ergonomics, IRCCS, Istituto Auxologico Italiano, San Giuseppe Hospital, 28824 Piancavallo, Italy; p.pitera@auxologico.it (P.P.); f.verme@auxologico.it (F.V.); brunani@auxologico.it (A.B.); p.capodaglio@auxologico.it (P.C.)
[2] Department of Biomedical, Dental Sciences and Morphological and Functional Images, University of Messina, 98125 Messina, Italy; alitoa@unime.it
[3] Laboratory of Clinical Neurobiology, IRCCS, Istituto Auxologico Italiano, San Giuseppe Hospital, 28824 Piancavallo, Italy; s.cattaldo@auxologico.it
[4] Pulmonary Rehabilitation Department, IRCCS, Istituto Auxologico Italiano, San Giuseppe Hospital, 28824 Piancavallo, Italy; m.cornacchia@auxologico.it
[5] Laboratory of Metabolic Research, IRCCS, Istituto Auxologico Italiano, San Giuseppe Hospital, 28824 Piancavallo, Italy; s.mai@auxologico.it
[6] Department of Surgical Sciences, Physical and Rehabilitation Medicine, University of Torino, 10121 Torino, Italy
* Correspondence: j.fontana@auxologico.it

Abstract: Background: A post-COVID condition can reduce activity and quality of life, resulting in a significant socioeconomic and health burden. Understanding its impact on patients' health is important for the development of personalized rehabilitation interventions. An independent association between obesity and post-COVID condition was found because of complications and comorbidities. Methods: Sixteen patients with obesity and post-COVID symptoms (i.e., dyspnea, pain, poor sleep quality, muscle fatigue), admitted to the Istituto Auxologico Italiano, Piancavallo (VB), Italy, were recruited for a four-week rehabilitation program including conventional exercise therapy, nutritional intervention, psychological support and whole-body cryostimulation (WBC). Results: All participants attended all sessions of the program. Anthropometric data showed statistically significant changes in weight, waist circumference and body mass index. Biochemical analyses showed significant reductions in lipid and inflammatory profiles. There was a significant improvement in physical performance, reduction in pain and improvement in psychological well-being. Conclusion: A multidisciplinary rehabilitation protocol including WBC, designed for patients with obesity and a post-COVID condition, is safe and feasible. The overall improvements demonstrate that multidisciplinary rehabilitation was effective on post COVID patients and suggest that the use of WBC is safe and could play a role as a booster in rehabilitation programs.

Keywords: multidisciplinary rehabilitation; obesity; personalized rehabilitation; post-COVID-19 condition; rehabilitation; whole-body cryostimulation

Citation: Fontana, J.M.; Alito, A.; Piterà, P.; Verme, F.; Cattaldo, S.; Cornacchia, M.; Mai, S.; Brunani, A.; Capodaglio, P. Whole-Body Cryostimulation in Post-COVID Rehabilitation for Patients with Obesity: A Multidisciplinary Feasibility Study. *Biomedicines* **2023**, *11*, 3092. https://doi.org/10.3390/biomedicines11113092

Academic Editor: César Fernández De Las Peñas

Received: 23 October 2023
Revised: 14 November 2023
Accepted: 15 November 2023
Published: 18 November 2023

1. Introduction

In December 2019, the world saw the emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2), which can range from an asymptomatic infection to acute respiratory distress syndrome (ARDS) [1,2]. The new coronavirus disease 2019 (COVID-19) put health systems in serious difficulties, infecting over 635 million people with more than 6.6 million deaths [3,4]. Nowadays, as the pandemic persists, more than 30% of people infected with COVID-19, including asymptomatic cases, and approximately 80% of patients hospitalized for COVID-19 have reported long COVID, or post COVID-19 condition (PCC) after infection [5]. PCC is defined as sequelae of symptoms that persist for

at least 4 weeks after acute infection or 3 months after infection, for at least 2 months and that cannot be explained by other causes [6]. This condition is characterized by dyspnea, coughing, fever, and persistent loss of smell or taste which eventually combines with musculoskeletal (e.g., fatigue and myalgias) and psychological (e.g., concentration and memory disorders, depression, and anxiety) problems [5,7,8]. PCC could reduce activity ability and health-related quality of life (HR-QoL), imposing significant socioeconomic and health burdens, and has attracted widespread attention [9]. Understanding PCC impact on patients' health and well-being is of great importance in developing effective and personalized rehabilitation and pharmacological interventions [10,11].

Obesity is linked to the progression of COVID-19 through a number of molecular pathways that increase SARS-CoV-2 infection vulnerability [12]. Adipose tissues in obese patients have a greater number of proteases and receptors for SARS-CoV-2 entry, suggesting that they could act as an accelerator and reservoir for this virus, enhancing the immune response and systemic inflammation [12].

Several studies have documented an independent association between obesity and PCC because of the complications and comorbidities, reporting a 25% higher risk of PCC with an additional burden on the immune system and involvement of physical and physiological processes [13–15].

Other studies have reported that patients with prolonged COVID symptoms are more likely to have obesity and any obesity degree (BMI $\geq 30\ kg/m^2$) was associated with a worse PCC prognosis [16]. Thus, an intensive multidisciplinary, tailored rehabilitation approach is required to maximize the patient's functional recovery and facilitate the returning to premorbid life, especially when PCC symptoms appear to persist or even worsen in susceptible individuals [17].

Conventional rehabilitative approaches for PCC include physiotherapy [18,19], breathing [20] and resistance and/or aerobic exercises [21], psychological counseling [22], and home-based programs [23].

Physical therapy in PCC focuses on improving physical strength, endurance, balance, and mobility, while respiratory therapy aims to improve lung function and breathing patterns [18]. Psychological counseling is essential to address the mental and emotional impact of PCC, including anxiety, depression, and post-traumatic stress disorder [22]. However, it is important to explore alternative interventions that can complement and enhance the conventional rehabilitation process.

Previous studies investigated Whole-Body Cryostimulation (WBC) as a treatment able to reduce pain and inflammatory status in several conditions [24] and to improve depression, anxiety [25], functional status and fatigue [26], and sleep quality [27]. WBC consists of exposure to cryogenic temperatures ($-110\ ^\circ C$ to $-140\ ^\circ C$) for a short period of time (2–3 min) and is a therapy with widely reported anti-inflammatory and less studied metabolic effects [28]. It is used as a post-exercise recovery technique and as an adjuvant therapy in conditions of rehabilitation interest [29–31] such as rheumatoid arthritis, fibromyalgia, multiple sclerosis, sleep disturbances, obesity. Moreover, the positive effects of ten serial sessions of WBC have been previously reported in patients with PCC [32].

The effect of adding WBC to a multidisciplinary rehabilitation program has not been studied extensively, particularly in the context of post-COVID care, although it is a unique approach [32]. The purpose of this study was to investigate the safety, acceptability and feasibility of a multidisciplinary personalized rehabilitation program including WBC in patients with obesity and PCC, admitted to a rehabilitation unit, and to provide additional data on cryostimulation as an adjuvant treatment for functional recovery.

2. Materials and Methods

2.1. Study Design

A single arm longitudinal study was performed. According to the literature [33], feasibility studies are an attempt to answer questions about whether some aspect of a future trial is feasible, in this case the authors seek to determine the acceptability of an

intervention and the perceived importance of types of outcomes. Acceptability can be interpreted as the participants' positive or negative opinion of a particular innovation. Patients were recruited from the Rehabilitation and Pneumology Unit of the San Giuseppe Hospital, Istituto Auxologico Italiano, Piancavallo (VB), Italy. The participants engaged in a 4-week multidisciplinary rehabilitation intervention. This study was conducted as part of a line of research aimed at defining better personalized rehabilitation programs for patients with obesity.

2.2. Participant Eligibility

Inclusion criteria were age 18–75 years, BMI $\geq$ 30 kg/m^2, and PCC. Exclusion criteria were severe psychiatric illness, acute respiratory disease, acute cardiovascular disease, unstable hypertension, cold intolerance, claustrophobia, pregnancy, recent change in usual medication, previous treatment with WBC, weight loss in the previous 3 months, and body temperature > 37.5 °C.

2.3. Participants

Between July 2021 and September 2022, 16 patients admitted to the Rehabilitation and Pneumology Unit of the San Giuseppe Hospital, Istituto Auxologico Italiano, Piancavallo (VB), Italy, agreed to participate in the study.

2.4. Study Variables

Anthropometric data, cardiovascular parameters, blood tests and functional test scores were collected at baseline (T0) and within 4 weeks at the end of the rehabilitation protocol (T10).

A schematic diagram of the protocol can be found in Figure 1. There were no follow-up measurements after discharge. Anthropometric measurements including weight, height, body mass index (BMI) and waist circumference (WC) were taken using a scale and tape measure. Resting cardiovascular parameters, including heart rate (HR) and systolic and diastolic blood pressure (SBP/DBP), were measured by a trained health professional using standard procedures. Hematological biomarkers analyzed from morning fasting blood samples included glucose, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG) and C-reactive protein (CRP) measured by standard laboratory techniques, specifically enzymatic methods for glucose, cholesterol, and triglycerides, immunoturbidimetric assay for CRP and spectrophotometric methods for HDL and LDL.

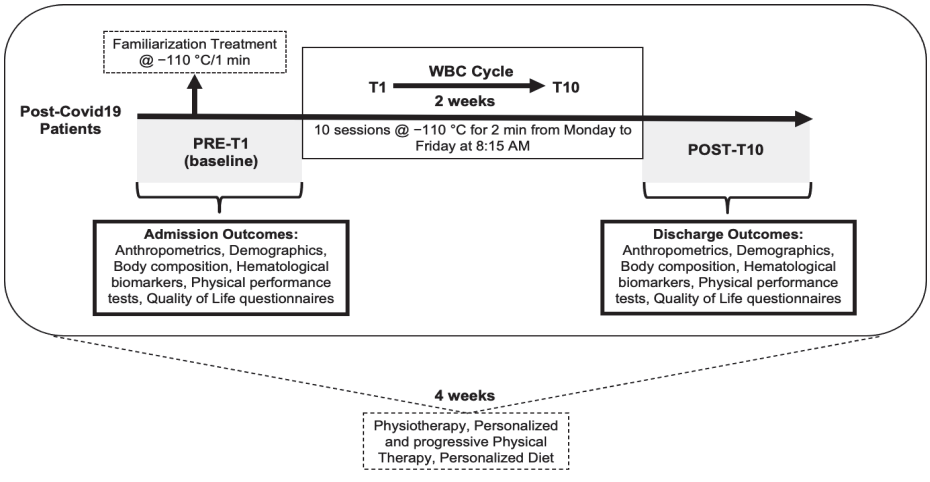


Figure 1. Schematic of the study design showing the timeline of the study protocol and research outcomes.

Physical performance tests included: a 6-min walk test (6MWT) [34] and the Timed Up and Go test (TUG) [35]. General pain was assessed using the Visual Analogue Scale (VAS) [36].

The Psychological General Well-Being Index (PGWBI) was used to measure subjective psychological well-being [37].

Patients admitted to the Pneumology Department had their basal SpO2 parameters measured by polysomnography on admission and discharge.

2.5. Intervention

The multidisciplinary rehabilitation program included individualized nutritional intervention, psychological support and supervised physical activity throughout the hospital stay. All patients received a balanced, hypocaloric Mediterranean diet consisting of three meals a day with 18–20% protein, 27–30% fat (of which <8% saturated fat) and 50–55% carbohydrates (<15% simple sugars), and 30 g of fiber from fresh vegetables. Under the supervision of a physiotherapist, two 60-min physiotherapy sessions were performed daily, consisting of personalized progressive aerobic training, postural control exercises and progressive strengthening exercises. The aerobic sessions were monitored with subjective perception of fatigue (Borg's CR10 scale) and oxygen saturation (SpO2). Exercise was stopped when a score of 5 was reached on the Borg scale. The first aerobic session, performed in the morning after WBC, consisted of walking at a predetermined cadence. The second session, performed in the afternoon, consisted of arm cranking at an intensity of 65% of HRmax according to Karvonen's equation $((220 - \text{age}) \times 0.65)$. This approach was individualized according to the patient's fitness, clinical status, and subjective perception of fatigue. All patients underwent ten sessions of WBC over a 2-week period (1 treatment per day, Monday to Friday, at 8:15 am, before exercise classes and physiotherapy).

2.6. Description of the WBC Session

Subjects were exposed to extremely cold, dry air at $-110\,^{\circ}\text{C}$ for 2 min in a cryochamber (Arctic, CryoScience, Rome, Italy). On the day before the first WBC session, a 1-min test session was performed. Sessions were conducted under the supervision of appropriately trained operators. On entering the cryochamber, the patients were asked to remove their glasses, contact lenses and jewelry, and to dry their bodies thoroughly to reduce the sensation of cold and avoid skin burns. Men were allowed to wear shorts or tracksuit bottoms (due to the severe cold sensation in some cases), a light t-shirt (or no shirt), mid-calf socks, clogs, gloves, headgear, and ear protection. Women also wore a sports bra or a light t-shirt. A surgical mask covered the nose and mouth. Subjects were encouraged to shift their weight, move their fingers, and breathe normally in the cryochamber. Visual and vocal contact with the volunteers was constant. For safety reasons, SBP and DBP blood pressure were measured before and after each treatment.

2.7. Feasibility

Adherence to the 4-week protocol treatment was monitored, and completion rates of tests and questionnaires before and after the intervention were assessed. Adverse events were monitored throughout the study. At the end of the intervention, an exit interview was conducted to collect qualitative information about the participants' experience of the feasibility and effects of the intervention.

2.8. Statistical Analysis

Statistical analysis was performed using Jamovi statistical software version 2.4.8. Data were expressed as mean (±standard deviation). The Shapiro–Wilk test was used to evaluate the normality of the distribution of the data. Student's paired t test for normal data and Wilcoxon's nonparametric paired test for nonnormal data were used to compare admission and discharge data. The level of significance was set at $p < 0.05$.

2.9. Ethical Considerations

Patients were fully informed of the scope and methodology of the study, which was conducted in accordance with the World Medical Association's Declaration of Helsinki and approved by the Ethics Committee of Istituto Auxologico Italiano (reference: 2021_05_18_14). Written and verbal informed consent was obtained from all experimental patients.

3. Results

3.1. Participant Flow

From July 2021 to September 2022, a total of 16 patients (three males, mean age 55.9 ± 7.51 years) met the eligibility criteria, were enrolled in this study, and started treatment. Eleven patients were recruited from the rehabilitation unit and five from the Pneumology unit. All patients completed the study protocol. Anthropometric, hematological, and functional data at baseline (T0) are shown in Table 1.

Table 1. Baseline clinical and demographic characteristics (N = 16).

	Mean	**SD**		**Mean**	**SD**
Age (y)	55.94	±7.50	CRP (mg/L)	0.57	±0.54
Weight (kg)	99.83	±29.19	HDL (mg/dL)	49.37	±11.41
BMI (kg/m^2)	37.99	±8.44	LDL (mg/dL)	130.50	±40.94
WC (cm)	114.40	±13.99	Tot. Col. (mg/dL)	196.96	±49.06
LOS (d)	30.6	±7.69	TG (mg/dL)	156.62	±51.38
6MWT (m)	381.80	±145.05	Glu (mg/dL)	112.25	±40.70
TUG (s)	12.65	±11.65	SBP (mmHg)	135.0	±19.0
VAS pain	53.06	±26.64	DBP (mmHg)	81.3	±11.9
PGWBI	63.50	±16.92	HR (bpm)	80.0	±10.4

6MWT, 6-Minute Walk Test; BMI, Body Mass Index; bpm, beat per minute; CRP, C-Reactive Protein; DBP, Diastolic Blood Pressure; HDL, high-density lipoprotein; HR, Heart Rate; LDL, Low-Density Lipoprotein; LOS, Length of Stay; PGWBI, Psychological General Well-Being Index; SBP, Systolic Blood Pressure; SpO2, Saturation of Peripheral Oxygen; TG, triglycerides; Tot Col., Total Cholesterol; TUG, Timed Up and Go test; VAS, Visual Analogue Scale; WC, Waist Circumference.

3.1.1. Admission and Discharge Data Comparison

After the four-week rehabilitation treatment, which included nutritional intervention, psychological support, supervised physical activity and WBC, the authors found the following changes. Anthropometric data showed statistically significant changes in weight, WC, and BMI, which were lower at discharge. Biochemical analyses showed a significant decrease in CRP, HDL, LDL, Tot Col, GLU at T10. Among the other parameters analyzed, no significant difference was found for TG at discharge. In terms of cardiovascular parameters, there was a significant reduction in SBP and HR but not in DBP. There was a significant improvement in performance capacity as measured by TUG and distance walked on the 6MWT, a significant reduction in pain as shown by VAS pain, and an improvement in psychological well-being as measured by the PGWBI scale.

The patients hospitalized at the Pneumology Department showed significant improvements of Sp02 basal evaluation. A comprehensive overview of the results is provided in Table 2.

Table 2. Comparison between the parameters at T0 and at T10 (N = 16).

	T0		T10		*p*-Value
Weight (kg)	99.83	±29.19	95.819	±27.10	**<0.001** [a]
BMI (kg/m^2)	37.99	±8.44	36.491	±7.81	**<0.001** [b]
WC (cm)	114.40	±13.99	108.800	±13.73	**0.002** [a]
6MWT (m)	381.80	±145.05	446.200	±84.56	**0.093** [a]
TUG (s)	12.65	±11.65	9.373	±4.67	**0.009** [a]
VAS pain	53.06	±26.64	33.938	±18.71	**<0.001** [b]
PGWBI	63.50	±16.92	76.188	±16.59	**0.002** [a]
CRP (mg/L)	0.57	±0.54	0.459	±0.41	**0.013** [a]
HDL (mg/dL)	49.37	±11.41	43.688	±9.90	**<0.001** [b]
LDL (mg/dL)	130.50	±40.94	107.563	±47.00	**0.005** [b]
Tot. Col. (mg/dL)	196.96	±49.06	176.838	±56.51	**0.013** [b]
TG (mg/dL)	156.62	±51.38	147.438	±49.77	0.473 [b]
Glu (mg/dL)	112.25	±40.70	97.563	±17.94	**0.003** [a]
SBP (mmHg)	135.0	±19.0	125.625	±14.59	**0.046** [a]
DBP (mmHg)	81.3	±11.9	76.250	±5.00	0.084 [b]
HR (bpm)	80.0	±10.4	73.188	±5.50	**0.014** [b]
SpO_2 *	91.5	±1.47	94.0	±2.05	**0.047** [b]

[a], Wilcoxon test; [b], t-Student test. * Only patients admitted to Pneumology Unit. 6MWT, 6-Minute Walk Test; BMI, Body Mass Index; bpm, beat per minute; CRP, C-Reactive Protein; DBP, Diastolic Blood Pressure; HDL, high-density lipoprotein; HR, Heart Rate; LDL, Low-Density Lipoprotein; PGWBI, Psychological General Well-Being Index; SBP, Systolic Blood Pressure; SpO2, Saturation of Peripheral Oxygen; TG, triglycerides; Tot. Col., Total Cholesterol; TUG, Timed Up and Go test; VAS, Visual Analogue Scale; WC, Waist Circumference.

3.1.2. Feasibility

All 16 participants attended all four weekly sessions of the program, indicating excellent compliance. The data collection completion rate was good overall. No adverse events occurred during the intervention period. Participants reported positive physical and mental changes and were generally satisfied with the program setting. Motivation was very high due to the innovative nature of the approach included in the protocol.

4. Discussion

The present study demonstrates the safety and the feasibility of a multidisciplinary rehabilitation program combined with WBC and provides some preliminary evidence in patients with obesity and PCC.

In general, cold exposure has been shown to have systemic effects on the neuromuscular, autonomic, endocrine, cardiovascular and immune systems [24]. As obesity is characterized by chronic low-grade inflammation, the effect of WBC may be due to its anti-inflammatory properties, such as the reduction of pro-inflammatory responses such as reduced levels of inflammatory markers (e.g., CRP), modulation of the pro-oxidant/antioxidant balance, and the cytokine levels observed (i.e., TNF-α, IL-6, and IL-1) may help counteract the inflammatory processes associated with the PCC [38]. In addition, the biological effects of WBC are thought to be enhanced recovery through increased activation of the parasympathetic system and improved oxygenation of muscle tissue [39].

The correct diagnosis and management of PCC is challenging for healthcare providers due to the heterogeneity and complexity of clinical manifestations and the likely need for multidisciplinary management approaches [40–42].

The importance of identifying outcome measures in PCC rehabilitation, such as physical function, quality of life, general symptoms, disability, activities of daily living and return to work, is crucial [43]. Global assessment before and after a rehabilitation program should be undertaken to provide more evidence for the development of effective management plans for Long COVID patients [44], particularly in people at higher risk of developing more severe PCC, such as those with obesity [45].

In primary care, general practitioner prescription of physical activity in a safe environment with a trained facilitator, could be an extremely effective way to manage the symptoms of long COVID from the outset [46,47]. This prescription should be preceded by an initial consultation to identify comorbidities and risks such as post-exertional symptom exacerbation, which is characterized by a worsening of symptoms after physical or mental exertion, usually 12–48 h after the activity and lasting for several days or (rarely) weeks [48].

Exercise-based rehabilitation is a therapeutic approach that may play an important role in improving sympathovagal balance and normalizing sympathetic index levels [49].

Several studies have focused on developing the most appropriate exercise protocol for patients with PCC, ranging from strength and endurance exercises to combined aerobic and resistance training [22,50,51], which can be varied in intensity and duration, and all show improvements in functional capacity [17,52].

According to our results, the effects of exercise therapy on SpO2 levels and cardiovascular fitness in patients with obesity and PCC have been widely documented [53].

Research has demonstrated the beneficial effects of exercise in reducing inflammation, improving immune function, and promoting overall physical and mental well-being in people with a post-COVID condition [54–56].

Given the existing evidence of clinical and functional benefits following WBC in musculoskeletal, neurological, and psychological conditions, the addition of such treatment aims to improve the patient's overall physical performance and perceived quality of life [57].

Indeed, WBC is a safe and innovative method capable of applying precise and homogeneous "doses" of cold and inducing a rapid systematic reduction of inflammation and oxidative stress [58] with therapeutic effects on fatigue, pain, thymic tone, depression and sleep [24,25], as well as metabolic effects [29] such as increased thermogenesis and improved lipid profile, insulin sensitivity and glucose utilization, and could thus enhance the beneficial effects of an exercise program, especially one of short duration [59–61].

Thus, in line with our previous studies, the benefits of WBC appear to be rapid, from the very first sessions, probably due to its rapid anti-inflammatory effect [62]. So, implementing the WBC seems feasible and the pre/post results are encouraging as well.

The results showed that a rehabilitation program including WBC had an impact on cardiometabolic profile, physical performance, sleep quality and overall well-being, suggesting that it may be an effective adjunct therapy in the rehabilitation of post-COVID obese patients.

Interestingly, an increase in parasympathetic tone is suggested by the significant reduction in HR observed. Autonomic dysfunction is a major hypothesis for symptom persistence in long COVID, and WBC may have a role to play as an adjuvant therapy as it can act as a 'training method' for the autonomic nervous system [63,64]. Indeed, it is well established that WBC is effective in increasing post-exercise and resting heart rate variability (HRV), an indicator of increased parasympathetic tone activation [65–67].

To the best of our knowledge, this study includes the largest sample of PCC in patients with obesity undergoing multidisciplinary rehabilitation combined with WBC.

Given its known rapid effects, WBC sessions started as early as a few days after admission and were performed early in the day with the aim of improving patients' overall physical performance and increasing adherence and motivation to rehabilitation.

In addition, no adverse events were observed in the sample patients, indicating that this type of treatment can be performed safely.

These findings may be of particular interest in cases in which rehabilitation programs may be hindered by pain, inflammation, or fatigue, and highlight the importance of early rehabilitation support. It is important to note that our results could lead to the possible application of the WBC in the rehabilitation of other respiratory diseases with similar symptoms. In fact, in obstructive lung disease, the sympathetic nervous system may be affected by recurrent episodes of hypoxemia, hypercapnia, elevated intrathoracic airway pressures, increased ventilatory effort, systemic inflammation and beta-sympathomimetics [68]. Rehabilitation of these patients should consider treatments that aim to re-establish the sym-

pathovagal balance to reduce resting sympathetic activity, such as WBC, exercise training, muscle stretching and breathing relaxation techniques [68,69].

Our data have some limitations, the main one being the lack of a control group of long-term COVID patients, which did not allow us to analyze both the evolution of the treated patients with the natural evolution of the symptoms, which is still unknown, and the effect of medical follow-up "alone", in a dedicated facility and group. In the absence of a control group, our results do not fully clarify the extent to which WBC, the multidisciplinary rehabilitation program, or a combination of the two may account for the observed improvements. It is not always clear whether improvements in anthropometrics, blood tests and general well-being are due to exercise, diet, and psychological intervention alone, or to the addition of WBC.

Due to the wide range of PCC symptoms, the sample was heterogeneous and consisted of different degrees of obesity, associated physical abilities and comorbidities.

Another limitation was the small number of participants involved in the study, therefore, larger studies with diverse populations should be conducted to determine the generalizability of these findings to a wider range of patients with PCC.

The reported results may have been influenced by motivational factors related to participation in a novel, well-tolerated treatment. Despite these limitations, the results of this feasibility study provide valuable insights into the potential efficacy and impact of a multidisciplinary rehabilitation program incorporating WBC in PCC patients with obesity. Future research should aim to further investigate the long-term effects and benefits of WBC in the rehabilitation of PCC. Also, it would be valuable to investigate the most appropriate frequency, time, and temperature protocols.

5. Conclusions

This study shows that a comprehensive multidisciplinary rehabilitation protocol that includes WBC developed for patients with PCC and obesity is safe and feasible. The overall improvement in physical performance, hematological and metabolic parameters, psychological and general well-being, and pain demonstrates that exercise rehabilitation was possibly an effective tool for long-term COVID patients. The clinical implications of this study are that WBC can be considered as an adjuvant and booster therapy in post-COVID rehabilitation of patients with obesity and PCC.

In addition, it is important to emphasize that the introduction of WBC was a turning point for all participants in terms of subjective and objective improvements in health and function, and that the overall improvement in clinical, physical, and biochemical parameters at discharge supports the use of WBC as an additional option in the multidisciplinary management of PCC.

In conclusion, considering that the severity and prevalence of PCC in the general population is still high, the identification of rehabilitation programs and adjuvants that can act as a booster for rehabilitation programs appears to be of paramount importance. However, due to the heterogeneity of this condition, rehabilitation protocols should be tailored to each patient's needs.

The small sample size makes it difficult to draw conclusions but underlines the importance of establishing a rehabilitation pathway for the care of patients with long COVID that can be adapted and tailored to the individual symptoms. Larger randomized trials with diverse populations should be conducted to determine the generalizability of these findings to a wider range of post-COVID patients.

Author Contributions: Conceptualization, P.P., P.C. and J.M.F.; methodology, A.B., M.C., S.M. and S.C.; validation, A.B., J.M.F. and P.C.; investigation, A.A., F.V. and P.P.; data curation, A.A., J.M.F. and P.P.; writing—original draft preparation, A.A., F.V., J.M.F. and P.P.; writing—review and editing, A.A., A.B., J.M.F. and P.C.; visualization, A.B., M.C. and P.C.; supervision, M.C., P.C., S.C., A.B. and S.M.; funding acquisition, P.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Italian Ministry of Health—Ricerca Corrente.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Istituto Auxologico Italiano (reference: 2021_05_18_14).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Brugliera, L.; Spina, A.; Castellazzi, P.; Cimino, P.; Tettamanti, A.; Houdayer, E.; Arcuri, P.; Alemanno, F.; Mortini, P.; Iannaccone, S. Rehabilitation of COVID-19 patients. *J. Rehabil. Med.* **2020**, *52*, jrm00046. [CrossRef] [PubMed]
2. Curci, C.; Pisano, F.; Bonacci, E.; Camozzi, D.M.; Ceravolo, C.; Bergonzi, R.; De Franceschi, S.; Moro, P.; Guarnieri, R.; Ferrillo, M.; et al. Early rehabilitation in post-acute COVID-19 patients: Data from an Italian COVID-19 Rehabilitation Unit and proposal of a treatment protocol. *Eur. J. Phys. Rehabil. Med.* **2020**, *56*, 633–641. [CrossRef]
3. Aidalina, M.; Khalsom, S. COVID-19 vaccination: A systematic review of vaccination strategies based on economic evaluation studies. *Med. J. Malaysia* **2023**, *78*, 411–420.
4. Beccia, F.; Di Pilla, A.; Causio, F.A.; Federico, B.; Specchia, M.L.; Favaretti, C.; Boccia, S.; Damiani, G. Narrative Review of the COVID-19 Pandemic's First Two Years in Italy. *Int. J. Environ. Res. Public. Health* **2022**, *19*, 15443. [CrossRef] [PubMed]
5. Ceban, F.; Ling, S.; Lui, L.M.W.; Lee, Y.; Gill, H.; Teopiz, K.M.; Rodrigues, N.B.; Subramaniapillai, M.; Di Vincenzo, J.D.; Cao, B.; et al. Fatigue and cognitive impairment in Post-COVID-19 Syndrome: A systematic review and meta-analysis. *Brain Behav. Immun.* **2022**, *101*, 93–135. [CrossRef] [PubMed]
6. Hope, A.A.; Evering, T.H. Postacute Sequelae of Severe Acute Respiratory Syndrome Coronavirus 2 Infection. *Infect. Dis. Clin. North. Am.* **2022**, *36*, 379–395. [CrossRef] [PubMed]
7. Ferioli, M.; Prediletto, I.; Bensai, S.; Betti, S.; Daniele, F.; Di Scioscio, V.; Modolon, C.; Rimondi, M.R.; De Molo, C.; Serra, C.; et al. Spontaneous Evolution of COVID-19 Lung Sequelae: Results from a Double-Step Follow-Up. *Respiration* **2022**, *101*, 381–393. [CrossRef]
8. Gupta, A.; Madhavan, M.V.; Sehgal, K.; Nair, N.; Mahajan, S.; Sehrawat, T.S.; Bikdeli, B.; Ahluwalia, N.; Ausiello, J.C.; Wan, E.Y.; et al. Extrapulmonary manifestations of COVID-19. *Nat. Med.* **2020**, *26*, 1017–1032. [CrossRef]
9. Lau, B.; Wentz, E.; Ni, Z.; Yenokyan, K.; Coggiano, C.; Mehta, S.H.; Duggal, P. Physical and mental health disability associated with long-COVID: Baseline results from a US nationwide cohort. *medRxiv* **2022**. [CrossRef]
10. Pouliopoulou, D.V.; Macdermid, J.C.; Saunders, E.; Peters, S.; Brunton, L.; Miller, E.; Quinn, K.L.; Pereira, T.V.; Bobos, P. Rehabilitation Interventions for Physical Capacity and Quality of Life in Adults with Post-COVID-19 Condition: A Systematic Review and Meta-Analysis. *JAMA Netw. Open* **2023**, *6*, e2333838. [CrossRef]
11. Alito, A.; Rodolico, C.; Palco, M.; Portaro, S. Steroid Management in a Patient with Myasthenia Gravis Undergoing COVID-19 Vaccination. *Innov. Clin. Neurosci.* **2022**, *19*, 8.
12. Behl, T.; Kumar, S.; Singh, S.; Bhatia, S.; Albarrati, A.; Albratty, M.; Meraya, A.M.; Najmi, A.; Bungau, S. Reviving the mutual impact of SARS-CoV-2 and obesity on patients: From morbidity to mortality. *Biomed. Pharmacother.* **2022**, *151*, 113178. [CrossRef]
13. Fernandez-de-Las-Penas, C.; Torres-Macho, J.; Elvira-Martinez, C.M.; Molina-Trigueros, L.J.; Sebastian-Viana, T.; Hernandez-Barrera, V. Obesity is associated with a greater number of long-term post-COVID symptoms and poor sleep quality: A multicentre case-control study. *Int. J. Clin. Pract.* **2021**, *75*, e14917. [CrossRef] [PubMed]
14. Shyam, S.; Gomez-Martinez, C.; Paz-Graniel, I.; Gaforio, J.J.; Martinez-Gonzalez, M.A.; Corella, D.; Fito, M.; Martinez, J.A.; Alonso-Gomez, A.M.; Warnberg, J.; et al. Coronavirus disease 2019 is associated with long-term depressive symptoms in Spanish older adults with overweight/obesity and metabolic syndrome. *Psychol. Med.* **2023**, 1–11. [CrossRef]
15. Varallo, G.; Suso-Ribera, C.; Ghiggia, A.; Veneruso, M.; Cattivelli, R.; Guerrini Usubini, A.; Franceschini, C.; Musetti, A.; Plazzi, G.; Fontana, J.M.; et al. Catastrophizing, Kinesiophobia, and Acceptance as Mediators of the Relationship Between Perceived Pain Severity, Self-Reported and Performance-Based Physical Function in Women with Fibromyalgia and Obesity. *J. Pain. Res.* **2022**, *15*, 3017–3029. [CrossRef] [PubMed]
16. Raman, B.; Bluemke, D.A.; Luscher, T.F.; Neubauer, S. Long COVID: Post-acute sequelae of COVID-19 with a cardiovascular focus. *Eur. Heart J.* **2022**, *43*, 1157–1172. [CrossRef]
17. Colas, C.; Le Berre, Y.; Fanget, M.; Savall, A.; Killian, M.; Goujon, I.; Labeix, P.; Bayle, M.; Feasson, L.; Roche, F.; et al. Physical Activity in Long COVID: A Comparative Study of Exercise Rehabilitation Benefits in Patients with Long COVID, Coronary Artery Disease and Fibromyalgia. *Int. J. Environ. Res. Public. Health* **2023**, *20*, 6513. [CrossRef]
18. Jimeno-Almazan, A.; Franco-Lopez, F.; Buendia-Romero, A.; Martinez-Cava, A.; Sanchez-Agar, J.A.; Sanchez-Alcaraz Martinez, B.J.; Courel-Ibanez, J.; Pallares, J.G. Rehabilitation for post-COVID-19 condition through a supervised exercise intervention: A randomized controlled trial. *Scand. J. Med. Sci. Sports* **2022**, *32*, 1791–1801. [CrossRef] [PubMed]
19. Zheng, C.; Chen, X.K.; Sit, C.H.; Liang, X.; Ming-Hui, L.I.; Ma, A.C.; Wong, S.H. Effect of Physical Exercise-Based Rehabilitation on Long COVID: A Systematic Review and Meta-analysis. *Med. Sci. Sports Exerc.* **2023**, *10*, 1249. [CrossRef] [PubMed]

20. McNarry, M.A.; Berg, R.M.G.; Shelley, J.; Hudson, J.; Saynor, Z.L.; Duckers, J.; Lewis, K.; Davies, G.A.; Mackintosh, K.A. Inspiratory muscle training enhances recovery post-COVID-19: A randomised controlled trial. *Eur. Respir. J.* **2022**, *60*. [CrossRef]
21. Saha, S.; Sharma, K. Modification of Lifestyle to Recover from Post-COVID Symptoms: A Short Review. *J. Lifestyle Med.* **2022**, *12*, 113–118. [CrossRef] [PubMed]
22. Araujo, B.T.S.; Barros, A.; Nunes, D.T.X.; Remigio de Aguiar, M.I.; Mastroianni, V.W.; de Souza, J.A.F.; Fernades, J.; Campos, S.L.; Brandao, D.C.; Dornelas de Andrade, A. Effects of continuous aerobic training associated with resistance training on maximal and submaximal exercise tolerance, fatigue, and quality of life of patients post-COVID-19. *Physiother. Res. Int.* **2023**, *28*, e1972. [CrossRef] [PubMed]
23. Cerfoglio, S.; Capodaglio, P.; Rossi, P.; Verme, F.; Boldini, G.; Cvetkova, V.; Ruggeri, G.; Galli, M.; Cimolin, V. Tele-Rehabilitation Interventions for Motor Symptoms in COVID-19 Patients: A Narrative Review. *Bioengineering* **2023**, *10*, 650. [CrossRef]
24. Fontana, J.M.; Gobbi, M.; Piterà, P.; Giusti, E.M.; Capodaglio, P. Whole-Body Cryostimulation in Fibromyalgia: A Scoping Review. *Appl. Sci.* **2022**, *12*, 4794. [CrossRef]
25. Rymaszewska, J.; Ramsey, D.; Chladzinska-Kiejna, S. Whole-body cryotherapy as adjunct treatment of depressive and anxiety disorders. *Arch. Immunol. Ther. Exp.* **2008**, *56*, 63–68. [CrossRef] [PubMed]
26. Miller, E.; Kostka, J.; Wlodarczyk, T.; Dugue, B. Whole-body cryostimulation (cryotherapy) provides benefits for fatigue and functional status in multiple sclerosis patients. A case-control study. *Acta Neurol. Scand.* **2016**, *134*, 420–426. [CrossRef] [PubMed]
27. Douzi, W.; Dupuy, O.; Tanneau, M.; Boucard, G.; Bouzigon, R.; Dugue, B. 3-min whole body cryotherapy/cryostimulation after training in the evening improves sleep quality in physically active men. *Eur. J. Sport. Sci.* **2019**, *19*, 860–867. [CrossRef] [PubMed]
28. Rose, C.; Edwards, K.M.; Siegler, J.; Graham, K.; Caillaud, C. Whole-body Cryotherapy as a Recovery Technique after Exercise: A Review of the Literature. *Int. J. Sports Med.* **2017**, *38*, 1049–1060. [CrossRef]
29. White, G.E.; Wells, G.D. Cold-water immersion and other forms of cryotherapy: Physiological changes potentially affecting recovery from high-intensity exercise. *Extrem. Physiol. Med.* **2013**, *2*, 26. [CrossRef]
30. Kujawski, S.; Zalewski, P.; Godlewska, B.R.; Cudnoch-Jedrzejewska, A.; Murovska, M.; Newton, J.L.; Sokolowski, L.; Slomko, J. Effects of whole-body cryotherapy and static stretching are maintained 4 weeks after treatment in most patients with chronic fatigue syndrome. *Cryobiology* **2023**, *112*, 104546. [CrossRef]
31. Poignard, M.; Guilhem, G.; Jubeau, M.; Martin, E.; Giol, T.; Montalvan, B.; Bieuzen, F. Cold-water immersion and whole-body cryotherapy attenuate muscle soreness during 3 days of match-like tennis protocol. *Eur. J. Appl. Physiol.* **2023**, *123*, 1895–1909. [CrossRef] [PubMed]
32. Piterà, P.; Gobbi, M.; Fontana, J.M.; Cattaldo, S.; Massucci, M.; Capodaglio, P. Whole-Body Cryostimulation: A Rehabilitation Booster in Post-COVID Patients? A Case Series. *Appl. Sci.* **2022**, *12*, 4830. [CrossRef]
33. Eldridge, S.M.; Lancaster, G.A.; Campbell, M.J.; Thabane, L.; Hopewell, S.; Coleman, C.L.; Bond, C.M. Defining Feasibility and Pilot Studies in Preparation for Randomised Controlled Trials: Development of a Conceptual Framework. *PLoS ONE* **2016**, *11*, e0150205. [CrossRef]
34. Enright, P.L. The six-minute walk test. *Respir. Care* **2003**, *48*, 783–785. [PubMed]
35. Bohannon, R.W. Reference values for the timed up and go test: A descriptive meta-analysis. *J. Geriatr. Phys. Ther.* **2006**, *29*, 64–68. [CrossRef] [PubMed]
36. Gift, A.G. Visual analogue scales: Measurement of subjective phenomena. *Nurs. Res.* **1989**, *38*, 286–288. [CrossRef]
37. Lundgren-Nilsson, A.; Jonsdottir, I.H.; Ahlborg, G., Jr.; Tennant, A. Construct validity of the Psychological General Well Being Index (PGWBI) in a sample of patients undergoing treatment for stress-related exhaustion: A Rasch analysis. *Health Qual. Life Outcomes* **2013**, *11*, 2. [CrossRef]
38. Ziemann, E.; Olek, R.A.; Grzywacz, T.; Kaczor, J.J.; Antosiewicz, J.; Skrobot, W.; Kujach, S.; Laskowski, R. Whole-body cryostimulation as an effective way of reducing exercise-induced inflammation and blood cholesterol in young men. *Eur. Cytokine Netw.* **2014**, *25*, 14–23. [CrossRef]
39. Bleakley, C.M.; Bieuzen, F.; Davison, G.W.; Costello, J.T. Whole-body cryotherapy: Empirical evidence and theoretical perspectives. *Open Access J. Sports Med.* **2014**, *5*, 25–36. [CrossRef]
40. Hoeg, T.B.; Ladhani, S.; Prasad, V. How methodological pitfalls have created widespread misunderstanding about long COVID. *BMJ Evid. Based Med.* **2023**. [CrossRef]
41. Parker, A.M.; Brigham, E.; Connolly, B.; McPeake, J.; Agranovich, A.V.; Kenes, M.T.; Casey, K.; Reynolds, C.; Schmidt, K.F.R.; Kim, S.Y.; et al. Addressing the post-acute sequelae of SARS-CoV-2 infection: A multidisciplinary model of care. *Lancet Respir. Med.* **2021**, *9*, 1328–1341. [CrossRef] [PubMed]
42. Sardella, A.; Chiara, E.; Alibrandi, A.; Bellone, F.; Catalano, A.; Lenzo, V.; Quattropani, M.C.; Basile, G. Changes in Cognitive and Functional Status and in Quality of Life of Older Outpatients during the COVID-19 Pandemic. *Gerontology* **2022**, *68*, 1285–1290. [CrossRef]
43. Munblit, D.; Nicholson, T.; Akrami, A.; Apfelbacher, C.; Chen, J.; De Groote, W.; Diaz, J.V.; Gorst, S.L.; Harman, N.; Kokorina, A.; et al. A core outcome set for post-COVID-19 condition in adults for use in clinical practice and research: An international Delphi consensus study. *Lancet Respir. Med.* **2022**, *10*, 715–724. [CrossRef] [PubMed]
44. Leisman, D.E.; Ronner, L.; Pinotti, R.; Taylor, M.D.; Sinha, P.; Calfee, C.S.; Hirayama, A.V.; Mastroiani, F.; Turtle, C.J.; Harhay, M.O.; et al. Cytokine elevation in severe and critical COVID-19: A rapid systematic review, meta-analysis, and comparison with other inflammatory syndromes. *Lancet Respir. Med.* **2020**, *8*, 1233–1244. [CrossRef] [PubMed]

45. Vimercati, L.; De Maria, L.; Quarato, M.; Caputi, A.; Gesualdo, L.; Migliore, G.; Cavone, D.; Sponselli, S.; Pipoli, A.; Inchingolo, F.; et al. Association between Long COVID and Overweight/Obesity. *J. Clin. Med.* **2021**, *10*, 4143. [CrossRef] [PubMed]
46. Greenhalgh, T.; Sivan, M.; Delaney, B.; Evans, R.; Milne, R. Long COVID-an update for primary care. *BMJ* **2022**, *378*, e072117. [CrossRef] [PubMed]
47. Wahlgren, C.; Divanoglou, A.; Larsson, M.; Nilsson, E.; Ostholm Balkhed, A.; Niward, K.; Birberg Thornberg, U.; Lilliecreutz Gudmundsson, E.; Levi, R. Rehabilitation needs following COVID-19: Five-month post-discharge clinical follow-up of individuals with concerning self-reported symptoms. *EClinicalMedicine* **2022**, *43*, 101219. [CrossRef]
48. Decary, S.; Gaboury, I.; Poirier, S.; Garcia, C.; Simpson, S.; Bull, M.; Brown, D.; Daigle, F. Humility and Acceptance: Working Within Our Limits with Long COVID and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. *J. Orthop. Sports Phys. Ther.* **2021**, *51*, 197–200. [CrossRef]
49. De Maria, B.; Lucini, D.; Gois, M.O.; Catai, A.M.; Perego, F.; Malacarne, M.; Pagani, M.; Porta, A.; Dalla Vecchia, L.A. Improvement of Sympathovagal Balance by Regular Exercise May Counteract the Ageing Process. A Study by the Analysis of QT Variability. *Front. Physiol.* **2022**, *13*, 880250. [CrossRef] [PubMed]
50. de Sire, A.; Andrenelli, E.; Negrini, F.; Lazzarini, S.G.; Cordani, C.; Ceravolo, M.G.; International Multiprofessional Steering Committee of Cochrane Rehabilitation, R.E.H.C.A. Rehabilitation and COVID-19: Update of the rapid living systematic review by Cochrane Rehabilitation Field as of February 28th, 2022. *Eur. J. Phys. Rehabil. Med.* **2022**, *58*, 498–501. [CrossRef] [PubMed]
51. Udina, C.; Ars, J.; Morandi, A.; Vilaro, J.; Caceres, C.; Inzitari, M. Rehabilitation in adult post-COVID-19 patients in post-acute care with Therapeutic Exercise. *J. Frailty Aging* **2021**, *10*, 297–300. [CrossRef] [PubMed]
52. Cattadori, G.; Di Marco, S.; Baravelli, M.; Picozzi, A.; Ambrosio, G. Exercise Training in Post-COVID-19 Patients: The Need for a Multifactorial Protocol for a Multifactorial Pathophysiology. *J. Clin. Med.* **2022**, *11*, 2228. [CrossRef] [PubMed]
53. Lemos, M.M.; Cavalini, G.R.; Pugliese Henrique, C.R.; Perli, V.A.S.; de Moraes Marchiori, G.; Marchiori, L.L.M.; Sordi, A.F.; Franzoi de Moraes, S.M.; de Paula Ramos, S.; Valdes-Badilla, P.; et al. Body composition and cardiorespiratory fitness in overweight or obese people post COVID-19: A comparative study. *Front. Physiol.* **2022**, *13*, 949351. [CrossRef] [PubMed]
54. Xiang, M.; Wu, X.; Jing, H.; Novakovic, V.A.; Shi, J. The intersection of obesity and (long) COVID-19: Hypoxia, thrombotic inflammation, and vascular endothelial injury. *Front. Cardiovasc. Med.* **2023**, *10*, 1062491. [CrossRef]
55. Zakynthinos, G.E.; Stavrou, V.T.; Malli, F.; Papathanasiou, I.V.; Zakynthinos, E.; Gourgoulianis, K.I.; Kalabakas, K.; Karagiannis, D.; Basdekis, G. The Effect of Whole-Body Cryostimulation in Healthy Adults: Whole-Body Cryostimulation According to Gender and Smoking Status. *Adv. Exp. Med. Biol.* **2021**, *1337*, 315–321. [CrossRef]
56. Cerfoglio, S.; Capodaglio, P.; Rossi, P.; Verme, F.; Boldini, G.; Cvetkova, V.; Galli, M.; Cimolin, V. Inertial-based Six-Minute Walking Test Assessment in Post-COVID Patients: Preliminary Results. In Proceedings of the EUROCON 2023—20th International Conference on Smart Technologies, Proceedings, Torino, Italy, 6–8 July 2023; pp. 520–524.
57. Tabisz, H.; Modlinska, A.; Kujawski, S.; Slomko, J.; Zalewski, P. Whole-body cryotherapy as a treatment for chronic medical conditions? *Br. Med. Bull.* **2023**, *146*, 43–72. [CrossRef]
58. Pilch, W.; Piotrowska, A.; Wyrostek, J.; Czerwinska-Ledwig, O.; Ziemann, E.; Antosiewicz, J.; Zasada, M.; Kulesa-Mrowiecka, M.; Zychowska, M. Different Changes in Adipokines, Lipid Profile, and TNF-Alpha Levels between 10 and 20 Whole Body Cryostimulation Sessions in Individuals with I and II Degrees of Obesity. *Biomedicines* **2022**, *10*, 269. [CrossRef] [PubMed]
59. Alito, A.; Quartarone, A.; Leonardi, G.; Tisano, A.; Bruschetta, A.; Cucinotta, F.; Milardi, D.; Portaro, S. Brown adipose tissue human biomarkers: Which one fits best? A narrative review. *Medicine* **2022**, *101*, e32181. [CrossRef]
60. Hanssen, M.J.; van der Lans, A.A.; Brans, B.; Hoeks, J.; Jardon, K.M.; Schaart, G.; Mottaghy, F.M.; Schrauwen, P.; van Marken Lichtenbelt, W.D. Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans. *Diabetes* **2016**, *65*, 1179–1189. [CrossRef]
61. Wiecek, M.; Szymura, J.; Sproull, J.; Szygula, Z. Whole-Body Cryotherapy Is an Effective Method of Reducing Abdominal Obesity in Menopausal Women with Metabolic Syndrome. *J. Clin. Med.* **2020**, *9*, 269. [CrossRef]
62. Verme, F.; Scarpa, A.; Varallo, G.; Pitera, P.; Capodaglio, P.; Fontana, J.M. Effects of Whole-Body Cryostimulation on Pain Management and Disease Activity in Active Rheumatic Polymyalgia: A Case-Report. *Biomedicines* **2023**, *11*, 1594. [CrossRef] [PubMed]
63. Allendes, F.J.; Diaz, H.S.; Ortiz, F.C.; Marcus, N.J.; Quintanilla, R.; Inestrosa, N.C.; Del Rio, R. Cardiovascular and autonomic dysfunction in long-COVID syndrome and the potential role of non-invasive therapeutic strategies on cardiovascular outcomes. *Front. Med.* **2022**, *9*, 1095249. [CrossRef] [PubMed]
64. Dani, M.; Dirksen, A.; Taraborrelli, P.; Torocastro, M.; Panagopoulos, D.; Sutton, R.; Lim, P.B. Autonomic dysfunction in 'long COVID': Rationale, physiology and management strategies. *Clin. Med.* **2021**, *21*, e63–e67. [CrossRef] [PubMed]
65. Hausswirth, C.; Schaal, K.; Le Meur, Y.; Bieuzen, F.; Filliard, J.R.; Volondat, M.; Louis, J. Parasympathetic activity and blood catecholamine responses following a single partial-body cryostimulation and a whole-body cryostimulation. *PLoS ONE* **2013**, *8*, e72658. [CrossRef] [PubMed]
66. Louis, J.; Theurot, D.; Filliard, J.R.; Volondat, M.; Dugue, B.; Dupuy, O. The use of whole-body cryotherapy: Time- and dose-response investigation on circulating blood catecholamines and heart rate variability. *Eur. J. Appl. Physiol.* **2020**, *120*, 1733–1743. [CrossRef] [PubMed]
67. Zalewski, P.; Bitner, A.; Slomko, J.; Szrajda, J.; Klawe, J.J.; Tafil-Klawe, M.; Newton, J.L. Whole-body cryostimulation increases parasympathetic outflow and decreases core body temperature. *J. Therm. Biol.* **2014**, *45*, 75–80. [CrossRef] [PubMed]

68. van Gestel, A.J.; Steier, J. Autonomic dysfunction in patients with chronic obstructive pulmonary disease (COPD). *J. Thorac. Dis.* **2010**, *2*, 215–222. [CrossRef]
69. McCarthy, B.; Casey, D.; Devane, D.; Murphy, K.; Murphy, E.; Lacasse, Y. Pulmonary rehabilitation for chronic obstructive pulmonary disease. *Cochrane Database Syst. Rev.* **2015**, *2015*, CD003793. [CrossRef]

biomedicines

MDPI

Article

Does COVID-19 Infection during Pregnancy Increase the Appearance of Congenital Gastrointestinal Malformations in Neonates?

Timea Elisabeta Brandibur [1,2], Nilima Rajpal Kundnani [3], Marioara Boia [1,2], Daciana Nistor [4,5], Daniel Milan Velimirovici [6], Leonard Mada [6,7], Aniko Maria Manea [1,2], Eugen Radu Boia [8,*], Marioara Nicula Neagu [9,*] and Calin Marius Popoiu [10]

1 Department of Neonatology and Puericulture, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania
2 Department of Neonatology and Puericulture, "Louis Ţurcanu" Children Emergency Hospital, 300011 Timisoara, Romania
3 Discipline of Internal Medicine and Ambulatory Care, Prevention and Cardiovascular Recovery, Department of Cardiology, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania; knilima@umft.ro
4 Discipline of Physiology, Department of Functional Sciences, Center of Immuno-Physiology and Biotechnologies (CIFBIOTEH), "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania; daciana_nistor@umft.ro
5 Centre for Gene and Cellular Therapies in Cancer, 300723 Timisoara, Romania
6 Doctoral School, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania; leo.mada@syonic.eu (L.M.)
7 Syonic SRL, 300254 Timisoara, Romania
8 Department of Oto-Rhino-Laryngology, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania
9 Discipline of Physiology, Faculty of Bioengineering of Animal Resources, University of Life Sciences "King Mihai I", 300645 Timisoara, Romania
10 Department XI of Pediatric Surgery, "Victor Babes" University of Medicine and Pharmacy, 300041 Timisoara, Romania; mcpopoiu@yahoo.com
* Correspondence: eugen.boia@umft.ro (E.R.B.); mnicula@animalsci-tm.ro (M.N.N.)

Citation: Brandibur, T.E.; Kundnani, N.R.; Boia, M.; Nistor, D.; Velimirovici, D.M.; Mada, L.; Manea, A.M.; Boia, E.R.; Neagu, M.N.; Popoiu, C.M. Does COVID-19 Infection during Pregnancy Increase the Appearance of Congenital Gastrointestinal Malformations in Neonates? *Biomedicines* **2023**, *11*, 3105. https://doi.org/10.3390/biomedicines11123105

Academic Editor: César Fernández De Las Peñas

Received: 21 October 2023
Revised: 14 November 2023
Accepted: 16 November 2023
Published: 21 November 2023

Abstract: Background: COVID-19 was an infection that was capable of bringing the entire world to a standstill position within a period of days to months. Despite the advancements in the medical sector, the contagion was difficult to control and costed the lives of millions of people worldwide. Many short- and long-term effects are witnessed even to date in people that contracted the disease. Pregnant females had to suffer not only the devastating effects of the virus, but also the psycho-social impact of the lockdown. The impact of COVID-19 infection during pregnancy causing decreased antenatal care or hypoxemic episodes due to severe respiratory distress and whether it could lead to the appearance of congenital gastrointestinal malformation in neonates is still unclear. The aim of our study was to analyze if COVID-19 infection during pregnancy could increase the incidence of gastric malformations in neonates born from these women. Materials and Methods: We sifted the files of all neonates admitted into our hospital between January 2022 and December 2022, and based on inclusion and exclusion criteria, we included the cases having gastrointestinal congenital malformations during the COVID-19 pandemic. We performed a single-center, retrospective, observational descriptive study. We further divided the patients based on the anatomical location of the malformation. We also took down details of the evolution of pregnancy and whether the mother had contracted a SARS-CoV-2 infection during the pregnancy. Details regarding the Apgar score, days of intensive care admission, sex, and nutrition were the key findings studied. Results: A total of 47 neonates were found to have digestive anomalies, among which, based on the anatomical locations, the number of malformation cases found at the level of the esophagus were 15, while 16 occurred at the level of the pylorus; we found 12 cases of malformation of the duodenum, and four cases had malformation of the rectum. Out of these 47 neonates, 38.3% were females and 61.7% were males. A total of 58% were preemies, among which 9% had intra-uterine growth retardation (IUGR), and 42% were full-term

newborns, among which 4% had intra-uterine growth retardation (IUGR). A total of 45% of the births were primiparous pregnancies and 55% were from multiparous females. A total of 14 mothers were found to have tested positive for COVID-19 during the course of pregnancy (p-value = 0.23); many had mild symptoms but were not tested. Conclusions: COVID-19 can affect the wellbeing of the pregnant female and their fetus. Larger studies can help gain extensive knowledge as to whether COVID-19 also has the potential to result in congenital gastrointestinal anomalies in children born from COVID-19 positive mothers. In our study, only a few infants born with this pathology were found to be born from COVID-19 positive mothers. Hence, it is difficult to conclude or exclude a direct correlation between the infection and the congenital malformations.

Keywords: COVID-19; neonates; gastrointestinal malformations; congenital anomalies

1. Introduction

COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spread quickly worldwide; this resulted in devastating effects on public health, affecting almost all organs and systems of human body, both during the acute phase of COVID-19 infection and during the immediate post COVID-19 period [1–3].

The risk of perinatal transmission, especially when breastfeeding, as well as the neonate's risk of developing COVID-19 during the perinatal period are still unknown [4,5]. However, members of the coronavirus family are known to be responsible for severe complications during pregnancy, such as miscarriage, fetal growth restriction, and congenital anomalies [6]. Whether or not COVID-19 affects fetuses in the same way requires further in-depth studies.

There is no reliable evidence for transplacental transmission of COVID-19 during the first or early second trimester of pregnancy; however, the current limited data does not indicate maternal-to-fetal transmission in the third trimester as well [7]. Meanwhile, a systematic review suggested that during delivery or while breastfeeding, the virus can enter the neonate and cause infection, but the chances of transplacental transmission are not yet documented [8]. During pregnancy, the maternal immune system and inflammatory responses are widely suppressed, and the fetus in the mother's womb remains safe without the mother's immune system attacking it, considering it a foreign entity [9]. Pregnant women were one of the most vulnerable groups during the COVID-19 pandemic, as pregnancy was found to be a strong risk factor for severity of COVID-19 infection [10].

Studies have shown that pregnant women may have an increased risk of maternal and neonatal complications due to COVID-19 infection [11]. Several clinical symptoms such as fever, disseminated intravascular coagulation, feeding intolerance, bleeding, cyanosis, complicated deliveries, rash, edema, dyspnea, and pneumonia have been reported in neonates born from mothers infected with COVID-19 [5,12,13]. Congenital anomalies include a wide range of anatomical or physiological abnormalities that can be present at birth or are diagnosed during the antenatal period. Primary prevention of congenital anomalies in the population, especially from the rural group, is of crucial priority, including pre-conceptional care and approaches involving the entire population in which education plays a pivotal role [14]. The urban population having easy access to healthcare units, awareness plans, and education helps immensely in maintaining the proper healthy state of pregnant women [15,16]. The global birth prevalence of congenital anomalies is approximately 2–3%. The pattern and prevalence of congenital anomalies may vary over time or with geographical location. Apart from the environmental factors, another key element is maternal age; the higher the age of the mother at the time of conceiving, the greater the chances of having an unhealthy child. Similarly, mothers suffering from chronic health issues often present stillbirths, low-birth weight infants, or miscarriages [17]. Proper antenatal care and deliveries at specialized units having neonatal intensive care units can further reduce the mortality.

Early diagnosis of congenital malformations during the regular follow-up visits of the pregnant women can provide both the parents and doctors enough time to intervene and take proper decisions for the child and mother [18]. Ultrasound examinations are safe and non-invasive procedures that can diagnose many malformations in the fetus and the newborn [19,20]. The ability to diagnose these malformations prenatally is influenced by the site of obstruction, the presence of associated anomalies, and the gestational age at the time of imaging [21]. Newborns should be rapidly transferred to a tertiary medical care center that ensures adequate medical and surgical treatment if they were born in small medical units [22].

The published literature indicates that viral illnesses during early pregnancy and several antiviral drugs are associated with an increased risk of cardiac and neurodevelopmental congenital anomalies in newborns [23–26]. Similarly, over-the-counter medications like paracetamol together with other NSAIDs can have harmful effects during pregnancy [27]. However, there is very limited evidence for an association between SARS-CoV-2 infection in early pregnancy or COVID-19 vaccination and the risk of congenital malformations [24,28,29].

We have described 51 cases of newborns with GI pathology during a previous study [30]. The former study included 39 cases of GI malformations and spanned a period of 3 years (1 January 2017 up to 31 December 2019). During the year 2022, we noticed a spike in GI malformations, which prompted us to initiate the present study.

The aim of the present study was to detect possible complications arising from COVID-19 infection. The focus was on gastrointestinal malformations and their relationship to a SARS-CoV-2 infection during pregnancy. The study was considered a research priority, motivated by the observed spike in GI congenital malformations.

2. Materials and Methods

Newborns. This single-center retrospective descriptive analysis of birth prevalence for digestive malformations was performed during the year 2022 and included newborns admitted to the regional level III Neonatal Intensive Care Unit (NICU) of 'Louis Turcanu' Emergency Clinical Hospital for Children in Timisoara, Romania. The population-based data were collected from the "Atlas-Med" S.C. GAMA IT S.R.L, address is Str. Zidului nr. 7, Sibiu, 550189 (RO).

Inclusion criteria: gestational age (GA) $\geq$ 28 weeks, birth weight of at least 1000 g, surgery for malformations of the digestive tract, not more than 7 days old at admission, complete medical history from maternity and pediatric surgery department.

Exclusion criteria: GA < 28 weeks, birth weight under 1000 g, newborns without digestive malformations, incomplete observation sheets, severe infections (sepsis or pneumonia), severe genetic malformations, and postoperative deaths.

A full feeding was defined as the completion of target calorie counts for premature neonates (150 kcal/kg/day) [31]. We followed the national neonatal enteral and parenteral nutrition guidelines in our country. Although, there was a slow rate of nutritional recovery found in all newborns [32].

Ethical approval and patient consent: The study was approved by the Ethics Committee for Scientific Research of the Emergency Hospital for Children 'Louis Turcanu' (approval no. 82/05.10.2023). The authors ensure that this study was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients/parents/legal guardian as a part of routine admission to our tertiary university hospital for future research and study purposes.

Demographic variables and clinical data were collected (sex, GA, Apgar scores, antenatal clinic visit details, presence of COVID-19 disease during pregnancy, COVID-19 vaccination details during pregnancy, environment factors, birth weight, maternal medication, weight at admission and discharge, other associated diseases, number of hospitalizations in the pediatric surgery department and our department, postoperative nutrition, and details regarding the digestive malformation and its time of diagnosis).

Statistical analysis: Descriptive statistics are presented as frequencies or as the median and interquartile range (IQR). Groups were compared using the *t*-test or the Kruskal–Wallis rank sum test and using Fisher's exact test or Pearson's chi-square test. Pathologies were grouped into 4 categories based on the anatomical location: esophagus, pylorus, duodenum (including the small intestine), and rectum. The number of rectal malformations was small (4 cases), and this group was excluded from some of the analysis. Inter-group comparisons between the various pathologies were performed using analysis of variance (ANOVA), and the differences were highlighted using boxplots. Specific predictors were also analyzed in bivariate models to test if they remained statistically significant when confounded with the type of malformation. A p-value of <0.05 was considered to indicate a statistically significant difference. The statistical analysis was performed using the R statistical framework and plotted using the ggplot2 package [33–35].

3. Results

During the study period, 477 newborn babies were admitted into our department, out of which 57 had congenital malformations, corresponding to a prevalence of 12%. Out of the total number of malformations, seven were cardiac malformations (12.5%), three were renal system malformations (5.35%), and the remaining 82.25% of cases (n = 47) presented gastrointestinal (GI) malformations (upper and lower gut abnormalities). It is important to mention that our department is not part of a maternity hospital; we accept transfers from four counties in Romania with a wide variety of neonatal pathologies, and we work in close collaboration with the pediatric surgery department as being part of a tertiary pediatric multispecialty hospital.

The patients (n = 47) with GI malformations were further divided into four categories, based on the anatomical location: malformations at the level of the esophagus (n = 15), pylorus (n = 16), duodenum (n = 12), and rectum (n = 4). A total of 58% were preemies, among which 9% had intra-uterine growth retardation (IUGR), and 42% were full-term newborns, among which 4% had intra-uterine growth retardation (IUGR).

The number of GI malformations was markedly higher during 2022 than during the 3 years prior to the COVID-19 epidemics (2017–2019, 39 cases, Fisher's exact test p-value = 2.2×10^{-6}). The total number of patients on the NICU was relatively stable during this time period (559, 626, and 678 cases vs. 679 cases during 2022). The increase in proportions was highly statistically significant (chi-square test trend in proportions: p-value = 1.2×10^{-7}).

The most common pathology during the previous 3 years corresponded to malformations of the duodenum (six, four, and seven cases; Figure 1), although the difference in the relative proportion did not reach statistical significance (chi-square test for trend in proportions: p = 0.06). Malformations of the rectum and colon occurred only infrequently during this time period (n = 3), a result which was also observed during 2022.

The second half of the study focused on the analysis of the 47 cases diagnosed during 2022. Almost half of the women were primiparous (45%), while the remaining 55% were multiparous.

The mothers were divided into three subgroups: those who had the COVID-19 disease (RT-PCR tested), those who did not have the disease, and the group of mothers who were not tested for IgG during pregnancy. Only a small number of mothers (approximately 13%) were found to be vaccinated against SARS-CoV-2. This could be due to limited knowledge regarding the disease and its potential side effects, as 51% of the mothers did not perform a COVID-19 test despite having mild symptoms indicative of a possible infection during the pregnancy. A history of COVID-19 disease could not be excluded in these women due to a lack of appropriate testing.

A positive history of COVID-19 (confirmed by RT-PCR) was present in 14 mothers, while another 8 did not experience an infection (negative RT-PCR). However, the status remained unknown in the remaining 25 mothers. Among the 14 COVID-19 positive

females, only 1 had contracted the infection in the last trimester, while the remaining 13 had COVID-19 in their first trimester.

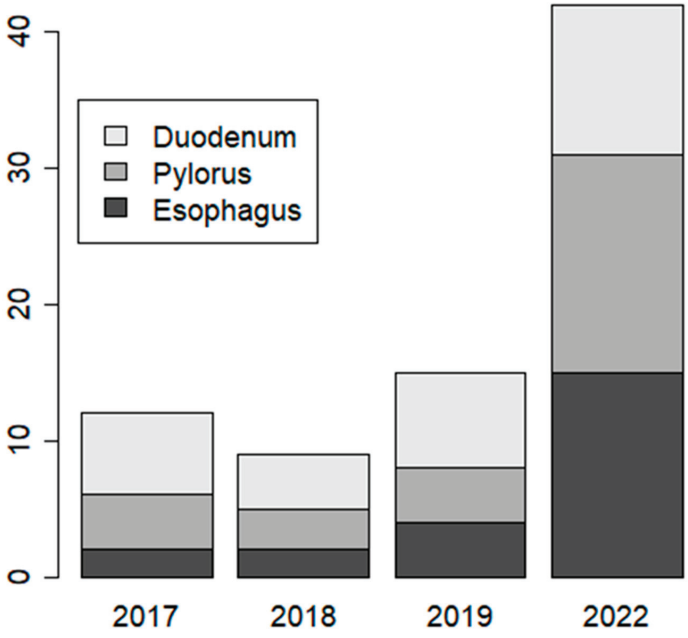


Figure 1. Bar plot with the number of GI malformations during the years 2017–2019 and 2022. There was a sharp increase during 2022. The number of patients was relatively stable during this time period. The increase in proportions was highly statistically significant. (p-value = 1.2×10^{-7}).

During the study period, we assessed the antenatal medication and found out that all pregnant women who came from dispensary pregnancies supplemented their diet with nutrients and antioxidants to cover the increased needs during pregnancy; acetaminophen was the most used analgesic and antipyretic drug [27] (Table 1).

Table 1. Demographic details of mothers and patients enrolled in the study.

	% or N (%)
Rural/Urban	50%/50%
Primipara (P/MP)	46%/54%
COVID-19 Status:	
Yes	14 (29.8%)
No	8 (17%)
Unknown	25 (53.2%)
COVID-19 Vaccine (Yes/No)	13%/87%
Vegan (Yes/No)	9%/91%
Sex (M/F)	61%/39%
Apgar Score at One Minute: <9 vs. ≥9	63%/37%
Nutrition (Diverse/Formula)	87%/13%

Out of the 47 analyzed cases, 61% were males (28 cases) and 39% were females. Abnormalities of the esophagus predominated in females (11 vs. 4), while those of the pylorus

were more common in males (13 vs. 3). There were also seven males and four females with malformations of the duodenum, while all four cases with rectal malformations were males (Fisher test: $p = 0.005$).

The Apgar score at one minute was similar in the groups of patients with malformations of the esophagus, pylorus, or duodenum. It was slightly lower in the fourth group (rectal malformations), although the difference did not reach statistical significance (p-value = 0.10) (Figure 2). The mean scores (esophagus: 7.8; pylorus: 8.4; duodenum: 8.0; and rectum: 6.8) closely followed the medians (8, 8.5, 8, and 6.5 respectively). The Apgar score was lower in patients from a rural setting (mean = 7.52 vs. 8.39; p-value = 0.01).

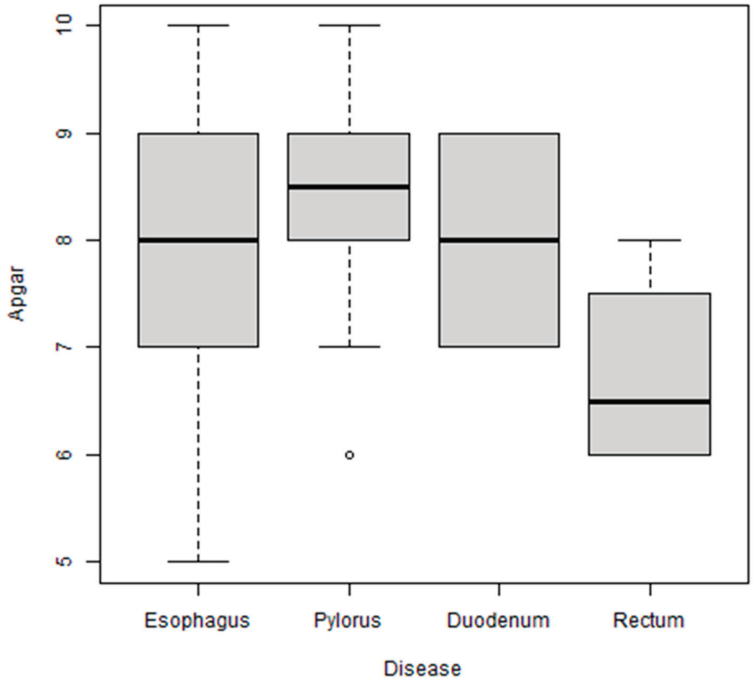


Figure 2. Boxplot with Apgar score grouped by pathology. The Apgar score was similar in the groups of patients with malformations of the esophagus, pylorus, or duodenum. There were only 4 patients with malformations of the rectum, with scores of 6, 6, 7, and 8. The difference did not reach statistical significance ($p = 0.10$), there was just one case having Apgar of 6 in the pylorus lot which is represented by a circle in the above figure.

The Apgar score did not vary significantly with the COVID status (Kruskal–Wallis $p = 0.70$; Figure 3) even after merging the group with unknown status with the negative group ($p = 0.41$). The proportion of duodenal malformations was higher in pregnancies with a positive history for COVID-19 virus as well as in the untested group. However, these results did not reach statistical significance ($p = 0.23$; $p = 0.76$ in the merged groups).

Infection with COVID-19 during pregnancy had no impact on the following outcomes: gestational age (p-value = 0.57), weight at admission (p-value = 0.88), weight at discharge (p-value = 0.74), or Apgar score below 9 (p-value = 0.71).

Apgar score was found to be much lower in the patients with a rural background (p-value = 0.01). COVID-19 infection in mothers was found to have no influence on the gestational age (p-value = 0.57), weight at admission (p-value = 0.88), weight at discharge (p-value = 0.74), or Apgar score (below 9) (p-value = 0.71).

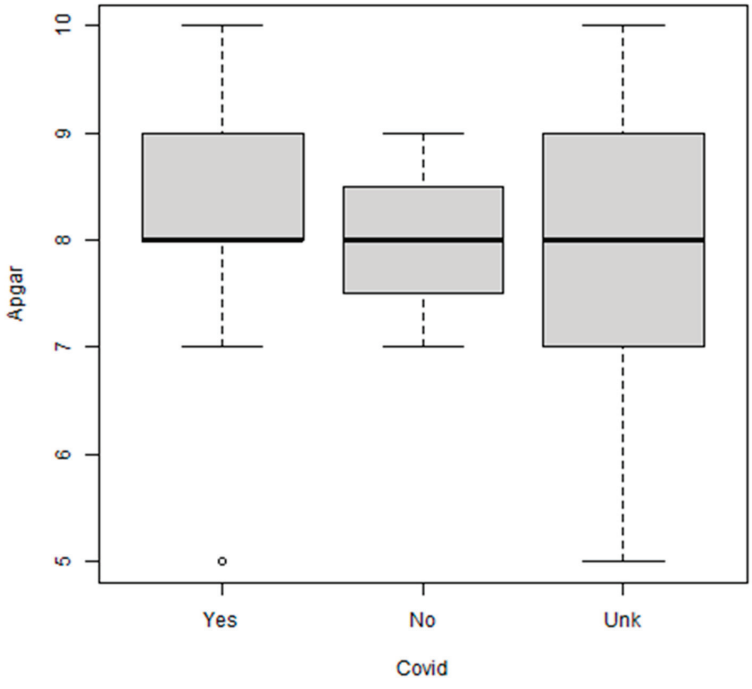


Figure 3. Apgar score and presence of COVID-19 infection. The Apgar score did not differ significantly with the COVID status of the mother ($p = 0.70$). However, there was a large number of mothers with unknown status ($n = 24$). Yes = infection; No = no infection; Unk = a previous infection could not be excluded. There was just one case having Apgar of 5 in the yes lot which is represented by a circle.

The birth weight, weight at admission to the NICU, and discharge weight varied significantly with the underlying pathology ($p = 0.01$, $p = 0.0005$, and $p = 0.005$). Newborns with an esophageal malformation or duodenal malformation had lower birthweights compared with the other groups (medians of 2520 g and 2400 g compared with >3000 g for the pylorus and rectum). These results remained statistically significant in a multivariate analysis (Figure 3) and after removing the small group of rectal malformations. Sex did not reach statistical significance in the bivariate model for birthweight ($p = 0.40$) but did have an impact on the discharge weight, even when confounding for the underlying pathology ($p = 0.033$ for sex and $p = 0.003$ for pathology) (Table 2).

Table 2. Median (IQR) for the gestational age, weight at birth, admission and discharge, Apgar scores, LOS during ICU and post ICU, and total LOS of the patients included in the study.

Median (IQR)	
37 (36.0–38.0) weeks	Gestational Age
2750 (2190–3200) g	Birth Weight
2760 (2390–3282.5) g	Admission Weight
3135 (2600–3500) g	Discharge Weight
8 (7–9)	Apgar score
8 (5–13) days	LOS (ICU)
11.5 (5–21.75) days	LOS (post- ICU)
21.5 (11.25–35.75) days	LOS (Total)

We observed that the proportion of duodenal malformations was higher in pregnancies with the COVID-19 virus and in the untested group. However, when evaluating the weight at admission and weight at discharge, it was found that all newborns born with digestive malformations were underweight (p = 0.01) (Figure 4).

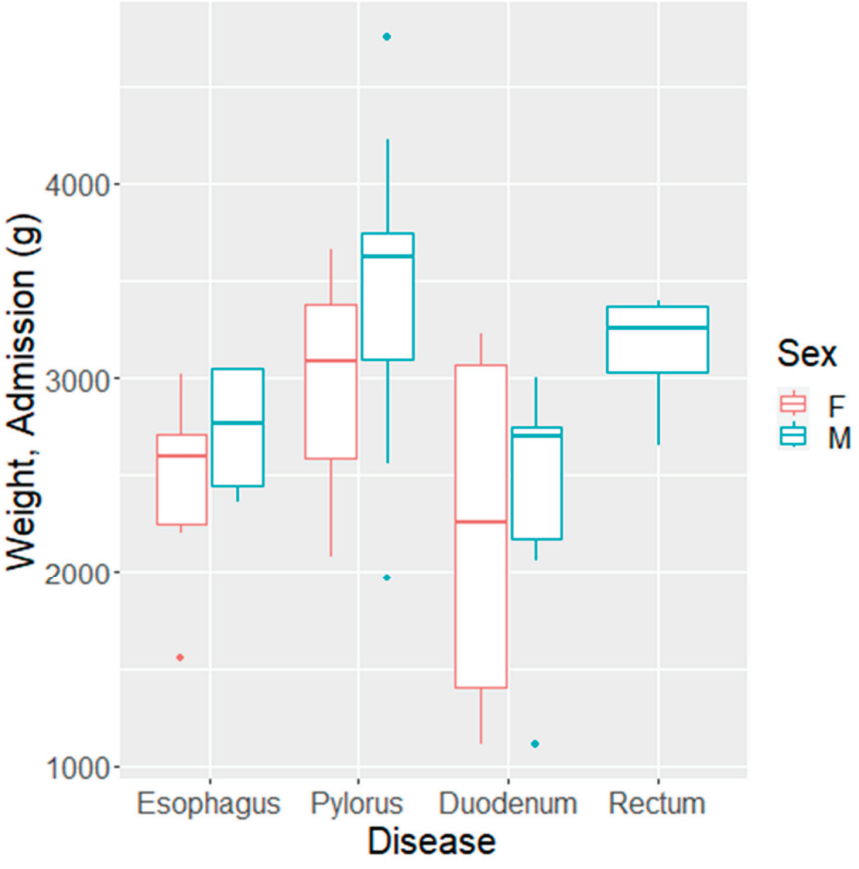


Figure 4. Boxplot of admission weight of newborns and their gastrointestinal malformations (F: females; M: males). The admission weight to the NICU varies with the type of gastrointestinal malformation and with sex. Females predominantly had abnormalities of the esophagus (11 vs. 4), while the pylorus was more commonly affected in males (13 vs. 3 cases). Males had also slightly more common malformations of the duodenum (7 vs. 4). All four infants with rectal malformations were males.

The number of days of hospitalization varied both with the type of GI malformation and the weight at admission, as visualized in Figure 5. Patients with pyloric malformations had a much shorter duration of hospitalization compared with those with esophageal or duodenal malformations (median LOS of 9 days vs. 35 and 29 days, p = 0.0007). They also required fewer days in the NICU (median 4.5 days vs. 13 and 10 days). All patients, except those with malformations of the pylorus, required prolonged care in the neonatal ward following the discharge from the NICU as well (median 4 days for pylorus vs. medians of 14–19 days for the remaining types). The dataset also contains two outliers. Notably, one patient with a malformation of the esophagus required 125 days of hospitalization; the median LOS was stable at 34.5 days after excluding this patient.

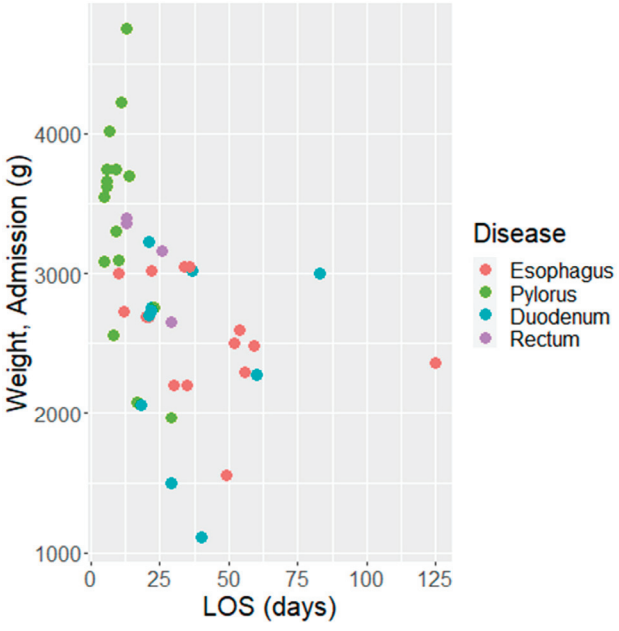


Figure 5. Total number of days of hospitalization depending on digestive malformations and weight at admission. The total number of days of hospitalization depends on the type of GI malformation and the weight at admission. Patients with malformations of the duodenum or esophagus had lower birth weights and were hospitalized longer ($p < 0.001$). The LOS in the NICU was lower for patients with malformations of the pylorus ($p < 0.001$, even in the bi-variate model) but did not differ with the COVID-19 status of the mothers ($p = 0.30$). The results did not change if the group with unknown COVID status was merged with the negative group and the group with malformations of the rectum was dropped ($p = 0.43$) (Figure 6).

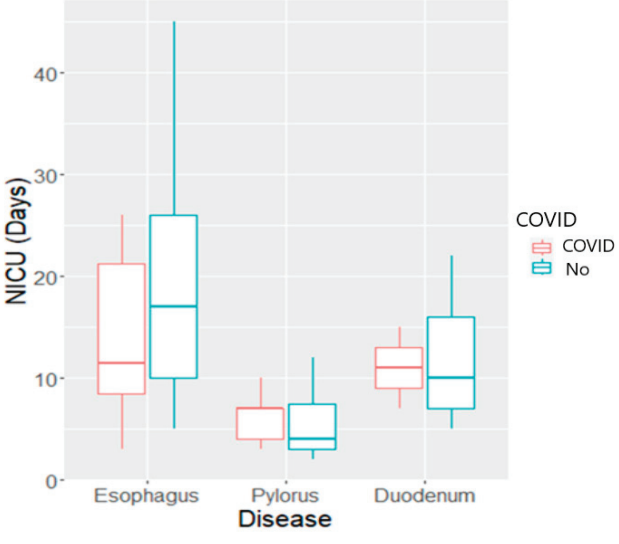


Figure 6. Boxplot of days of hospitalization in the intensive care unit grouped by COVID-19 and type of GI malformations. LOS in the NICU based on COVID-19 history of the mothers and type of GI malformation. The group with unknown COVID status was merged with the negative group.

Most of the patients were diagnosed antenatally (30 cases = 63.83%) during their dispensary/outpatient clinic visits, a fact that contributed to an early diagnosis and the approach of an effective therapeutic plan to favor the best possible evolution of the pregnancy. The children born from mothers who did not undergo follow-up during their entire pregnancy duration were diagnosed postnatally for their congenital digestive malformations (n = 15).

4. Discussion

It is documented that SARS-associated coronavirus infections result in a high incidence of premature birth, miscarriages, or maternal deaths [36]. Other viral infections in early pregnancy (e.g., rubella) are well-recognized causes of specific anomaly syndromes as well [37]. The most robust evidence published previously is a population-based cohort study from Israel conducted by Goldshtein et al. that highlighted the same fact [21]. They found no evidence that singleton live births to women who were vaccinated in the first trimester had a higher risk of congenital malformations compared with those not exposed to vaccination in pregnancy [36]. We believe that even if the virus does not directly affect the normal growth and development of the embryo, other variables related to a COVID-19 infection can indirectly cause harm to the fetus. The factors that could possibly have a negative impact on the fetus health are decreased quantity, quality, and routine visits to the antenatal care units; poverty due to the COVID-19 pandemic and lockdowns; and shortage of fetal screening and diagnosis possibilities, especially during pandemic situations. Furthermore, there are studies that highlight the fact that congenital COVID-19 infection can lead to neurodevelopmental disabilities, mainly resulting in epilepsy, cerebral palsy, and neurosensory disorders [37].

Furthermore, to see the prevalence of gastrointestinal congenital malformations, we sifted the files of neonates admitted in our hospital during a three-year duration in the immediate pre-COVID-19 era. We found that a total of 39 patients were born with gastrointestinal malformations during the three-year study period, whereas from the current study, we had 47 cases registered with the same pathology in a mere one-year time duration, a result that was highly statistically significant (Fisher exact test: p-value < 0.001). This difference points out to a possible correlation between COVID-19 infection and congenital digestive malformations. The proportions were stable during the prior 3 years (chi-square trend in proportions p-value = 0.88), excluding an underlying systematic long-range trend. Due to a lack of data from other maternity homes in our city, the results obtained are quiet limiting. Furthermore, large cohort studies can provide clarity on this hypothesis.

The mother's stress levels hinder the development of the fetus [38]. The state of anxiety and the factors that influence it may differ depending on the severity of the outbreak in each geographic region and the access to the healthcare units during the lockdown [38,39]. Uncertainty regarding the best treatment and clinical management of patients with COVID-19 can affect both the mind and psyche of the pregnant women [39–41].

Furthermore, due to the modern sedentary lifestyle, more and more younger people are diagnosed with chronic diseases like obesity, dyslipidemias, arterial hypertension, diabetes mellitus, metabolic syndrome, and rheumatic disease and their cardio-vascular complications (stroke, myocardial infraction, chronic coronary syndrome, atrial fibrillation) as compared with the former times, where these were once considered to be diseases of advanced age [42–44] and were rarely present in women of childbearing age [23–26,45–48]. González V.S.E. et al. reported that obesity, diabetes mellitus, and arterial hypertension were associated with a higher risk of developing severe forms of COVID-19 infection (if contracted), accounting for the odds ratio regarding mortality (1.413 (IC 95%, 1.11–1.78)), obesity (1.753 (IC 95%, 1.39–2.20)), and diabetes mellitus and hypertension, respectively (1.961 (IC 95%, 1.57–2.45)) [49].

Thrombo-embolic complications, arrhythmic complications, and even acute heart failure may occur during or post COVID-19 infection and may impose the use of very complex therapeutic algorithms that may involve the use of oral or parenteral anticoagulants, anti-

arrhythmic drugs (for the conversion of sinus rhythm for maintaining a proper heart rate), and renin–angiotensin–aldosterone system inhibitors [50,51]. Proper care should be taken while prescribing the treatment to pregnant females, keeping in mind not only the toxicity of the drugs but also that any sudden rhythm abnormality or hemodynamic changes can be fatal for the fetus. The anxiety and depression occurred in the acute phase or during the immediate post COVID-19 infection phase may necessitate the use of anxiolytics or antidepressive drugs, where again the safety of the fetus should be the prime priority [52]. Antibiotics were frequently used empirically and inadequately during the acute phase of COVID-19 infection, especially for severe cases with low oxygen saturation [53]. The use of such aggressive treatments in the periconceptual period or during the first weeks of pregnancy may result in multiple fetal malformations. This can frequently be seen in the case of unwanted or undiagnosed pregnancies in the initial phase of conception.

Due to both the placental and perinatal hypoxic-ischemic events, the fetus is exposed to higher risks of developing congenital malformations or growth retardations [54]. Furthermore, the ischemic placental events are assumed to be correlated with intestinal atresia (more frequently localized on the jejunal, ileal, and colonic segments), but no statistical significance was found when the incidences were compared with the pre-COVID-19 era and COVID-19 pandemic era in the pregnant females having hypoxemic episodes due to COVID-19 infection [55]. The diagnosis of esophageal atresia is difficult to establish based on ultrasound, especially by obstetricians with no experience or competence in maternal-fetal medicine. Hence, the majority of cases are diagnosed after birth [56].

In this study, all digestive malformations operated during the pandemic period for one year were evaluated. No direct correlation could be concluded between digestive anomalies and COVID-19 infection during pregnancy. However, cases of duodenal malformations were higher in pregnancies from COVID-19 positive mothers. The mothers did not take antivirals, and there was no previous history of births with congenital malformations.

Gastrointestinal malformations are often complicated by skeletal anomalies and intrauterine growth retardation [57]. We found statistically significant differences both in the case of admission weight and discharge weight in cases with digestive malformations, with the newborns being underweight. (p-value = 0.01) The average number of days of hospitalization in the intensive care unit was 16.2. Regarding premature babies or neonates suffering from certain conditions that occurred during the perinatal and neonatal period, newborns from the Neonatal Intensive Care Unit (NICU) have a high risk of developing neurological and developmental sequelae [58].

Furthermore, surgical infants can develop an aversion to oral feeding if oral feeding is delayed or if painful symptoms are associated with feeding, further hindering the growth and development of the child. Hence, clinical examination has a paramount role in the early detection of digestive malformations, in the effective management of specific necessary nutrition, and in the way of providing it [30]. Early trophic feeds may improve recovery time by increasing gut blood flow, improving motility and limiting the impact of starvation on the structure of the gut and its ability to absorb nutrients. Starting small-volume feeds of 10 mL/kg/day within 12–18 h of surgery may reduce the time needed until the full enteral nutrition is achieved [59]. Complicated cases (e.g., those with high stomas or extensive resection) may require either a hydrolyzed or lactose-free feed or a feed containing fats as medium-chain triglycerides (MCTs). The above-stated principles were followed for the best outcome in our hospital, keeping in mind the key elements of feeding in GI malformations [32,42].

The available data on vaccination against COVID-19 in pregnant women show that there is no specific cause for concern [32,59,60]. More data are clearly needed on the efficacy, safety, teratogenicity, and pharmacokinetics of drugs and biologics for pregnant and breastfeeding people with an active COVID-19 phase [61]. New agents are often licensed despite little information on key characteristics such as transplacental passage and drug labeling, which is unhelpful for informing clinical decisions for pregnant and breastfeeding people [62]. Clinical guidelines based on risk stratification for SARS-CoV-2

infection in children are needed to manage, monitor, and establish priority access for some groups to high medical care [63].

Limitations in Our Study

Although we found out with certainty that 14 mothers were COVID-19 positive during pregnancy, there remains a large group of patients, a total of 24, who were not tested for IgG SARS-CoV-2, which does not exclude the possibility of the presence of the disease during pregnancy and possible correlations between COVID-19 and digestive malformations. Lack of confirmatory tests in these 24 cases is one of the limitations of the study. On the other hand, the small number of cases and the inability to compare the data of the study group with a control group is another limitation of the study.

5. Conclusions

The results of the study indicate that SARS-CoV-2 infection during pregnancy is unlikely to cause congenital digestive malformations; however, due to the small cohort studied, it would be inappropriate to generalize the findings and reach a conclusion. Nevertheless, no significant differences were witnessed with regard to the Apgar score, days of admission, or severity in children born to COVID-19 positive mothers compared with those born from COVID-19 negative mothers. However, we plan to conduct further studies on larger cohorts on RT-PCR-tested COVID-19 positive mothers, which can provide a better understanding. Let us not forget that pregnancy is monitored by obstetricians and gynecologists, while COVID-19 is managed by infectious disease specialists. Therefore, a multidisciplinary approach is a key to success when the timely management of information on the diagnosis and treatment of COVID-19 are necessary to avoid complications in newborns. Precise antenatal care can further improve the outcomes and help earn time for a timely management, especially during pandemics like the one just faced.

Author Contributions: T.E.B., N.R.K. and C.M.P.: Conceptualization, validation, investigation, data curation, writing original draft, formal analysis; N.R.K. and M.B.: conceptualization, investigation, writing—review and editing; D.N. and E.R.B.: methodology, validation, writing—review and editing; D.M.V., L.M., E.R.B. and C.M.P.: data analysis, resources, project administration; L.M., A.M.M. and M.N.N.: data curation, software, formal analysis, visualization; T.E.B.: data primary analysis. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved by the Ethics Committee for Scientific Research of the Emergency Hospital for Children 'Louis Turcanu' (approval no. 82/5.10.2023). The authors ensure that this study was carried out in accordance with the Declaration of Helsinki.

Informed Consent Statement: Written informed consent was obtained from all patients/parents/legal guardian as a part of routine admission to our tertiary university hospital for future research and study purposes.

Data Availability Statement: Data will be made available on justified request.

Conflicts of Interest: Author Leonard Mada was employed by the company Syonic SRL. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Zhu, N.; Zhang, D.; Wang, W.; Li, X.; Yang, B.; Song, J.; Zhao, X.; Huang, B.; Shi, W.; Lu, R.; et al. Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **2020**, *382*, 727–733. [CrossRef]
2. Mocanu, V.; Bhagwani, D.; Sharma, A.; Borza, C.; Rosca, C.I.; Stelian, M.; Bhagwani, S.; Haidar, L.; Kshtriya, L.; Kundnani, N.R.; et al. COVID-19 and the Human Eye: Conjunctivitis, a Lone COVID-19 Finding—A Case-Control Study. *Med. Princ. Pract.* **2022**, *31*, 66–73. [CrossRef] [PubMed]
3. Rosca, C.I.; Branea, H.S.; Sharma, A.; Nicoras, V.A.; Borza, C.; Lighezan, D.F.; Morariu, S.I.; Kundnani, N.R. Rhythm Disturbances in Post-Acute COVID-19 Syndrome in Young Men without Pre-Existing Known Cardiovascular Disease—A Case Series. *Biomedicines* **2023**, *11*, 1146. [CrossRef] [PubMed]

4. Chen, H.; Guo, J.; Wang, C.; Luo, F.; Yu, X.; Zhang, W.; Li, J.; Zhao, D.; Xu, D.; Gong, Q.; et al. Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: A retrospective review of medical records. *Lancet* **2020**, *395*, 809–815. [CrossRef] [PubMed]
5. Zhu, H.; Wang, L.; Fang, C.; Peng, S.; Zhang, L.; Chang, G.; Xia, S.; Zhou, W. Clinical analysis of 10 neonates born to mothers with 2019-nCoV pneumonia. *Transl. Pediatr.* **2020**, *9*, 51–60. [CrossRef] [PubMed]
6. Di Mascio, D.; Khalil, A.; Saccone, G.; Rizzo, G.; Buca, D.; Liberati, M.; Vecchiet, J.; Nappi, L.; Scambia, G.; Berghella, V.; et al. Outcome of coronavirus spectrum infections (SARS, MERS, COVID-19) during pregnancy: A systematic review and meta-analysis. *Am. J. Obstet. Gynecol. MFM* **2020**, *2*, 100107. [CrossRef]
7. Yang, H.; Wang, C.; Poon, L.C. Novel coronavirus infection and pregnancy. *Ultrasound Obstet. Gynecol.* **2020**, *55*, 435–437. [CrossRef]
8. Sánchez-García, J.C.; Moreno, N.P.C.; Tovar-Gálvez, M.I.; Cortés-Martín, J.; Liñán-González, A.; Olmedo, L.A.; Rodríguez-Blanque, R. COVID-19 in Pregnant Women, Maternal—Fetal Involvement, and Vertical Mother-to-Child Transmission: A Systematic Review. *Biomedicines* **2022**, *10*, 2554. [CrossRef]
9. Prabhudas, M.; Bonney, E.; Caron, K.; Dey, S.; Erlebacher, A.; Fazleabas, A.; Fisher, S.; Golos, T.; Matzuk, M.; McCune, J.M.; et al. Immune mechanisms at the maternal-fetal interface: Perspectives and challenges. *Nat. Immunol.* **2015**, *16*, 328–334. [CrossRef]
10. Rasmussen, S.A.; Jamieson, D.J. COVID-19 and Pregnancy. *Infect. Dis. Clin. N. Am.* **2022**, *36*, 423–433. [CrossRef]
11. Yang, Z.; Wang, M.; Zhu, Z.; Liu, Y. Coronavirus disease 2019 (COVID-19) and pregnancy: A systematic review. *J. Matern. Neonatal Med.* **2020**, *35*, 1619–1622. [CrossRef] [PubMed]
12. Zamaniyan, M.; Ebadi, A.; Aghajanpoor, S.; Rahmani, Z.; Haghshenas, M.; Azizi, S. Preterm delivery, maternal death, and vertical transmission in a pregnant woman with COVID-19 infection. *Prenat. Diagn.* **2020**, *40*, 1759–1761. [CrossRef] [PubMed]
13. Khan, S.; Jun, L.; Nawsherwan; Siddique, R.; Li, Y.; Han, G.; Xue, M.; Nabi, G.; Liu, J. Association of COVID-19 with pregnancy outcomes in health-care workers and general women. *Clin. Microbiol. Infect.* **2020**, *26*, 788–790. [CrossRef] [PubMed]
14. Dolk, H.; Loane, M.; Garne, E. The prevalence of congenital anomalies in Europe. *Adv. Exp. Med. Biol.* **2010**, *686*, 349–364. [PubMed]
15. He, R.; Zhang, J.; Mao, Y.; Degomme, O.; Zhang, W.-H. Preparedness and Responses Faced during the COVID-19 Pandemic in Belgium: An Observational Study and Using the National Open Data. *Int. J. Environ. Res. Public Health* **2020**, *17*, 7985. [CrossRef] [PubMed]
16. Bin Nisar, Y.; Aurangzeb, B.; Dibley, M.J.; Alam, A. Qualitative exploration of facilitating factors and barriers to use of antenatal care services by pregnant women in urban and rural settings in Pakistan. *BMC Pregnancy Childbirth* **2016**, *16*, 42.
17. Patra, C.; Sarkar, S.; Dasgupta, M.; Nayek, K.; Karmakar, P. Prevalence of congenital anomalies in neonates and associated risk factors in a tertiary care hospital in eastern India. *J. Clin. Neonatol.* **2013**, *2*, 131–134. [CrossRef]
18. Boyle, M.I.; Jespersgaard, C.; Brøndum-Nielsen, K.; Bisgaard, A.M.; Tümer, Z. Cornelia de Lange syndrome. *Clin. Genet.* **2015**, *88*, 1–12. [CrossRef]
19. Liao, Y.; Wen, H.; Ouyang, S.; Yuan, Y.; Bi, J.; Guan, Y.; Fu, Q.; Yang, X.; Guo, W.; Huang, Y.; et al. Routine first-trimester ultrasound screening using a standardized anatomical protocol. *Am. J. Obstet. Gynecol.* **2020**, *224*, 396.e1–396.e15. [CrossRef]
20. Menon, P.; Binu, V.; Rao, K.L.N.; Suri, V. Trends in referral pattern of antenatally diagnosed surgical abnormalities in a tertiary care center in North India. *J. Indian Assoc. Pediatr. Surg.* **2018**, *23*, 198–202. [CrossRef]
21. Goldshtein, I.; Steinberg, D.M.; Kuint, J.; Chodick, G.; Segal, Y.; Ben David, S.S.; Ben-Tov, A. Association of BNT162b2 COVID-19 Vaccination During Pregnancy With Neonatal and Early Infant Outcomes. *JAMA Pediatr.* **2022**, *176*, 470–477. [CrossRef] [PubMed]
22. Fung, A.C.H.; Kan, A.S.Y.; Chung, P.H.; Shek, N.W.M.; Chan, I.H.Y.; Wong, K.K.Y. Antenatal counselling of congenital surgical anomalies: A decade of experience in a local tertiary centre. *J. Paediatr. Child Health* **2021**, *57*, 710–714. [CrossRef] [PubMed]
23. Leung, A.K.; Hon, K.; Leong, K. Rubella (German measles) revisited. *Hong Kong Med. J.* **2019**, *25*, 134–141. [CrossRef]
24. Hoffman, M.C.; Freedman, R.; Law, A.J.; Clark, A.M.; Hunter, S.K. Maternal nutrients and effects of gestational COVID-19 infection on fetal brain development. *Clin. Nutr. ESPEN* **2021**, *43*, 1–8. [CrossRef] [PubMed]
25. Faizan, I.; Abdullah, M.; Ali, S.; Naqvi, I.H.; Ahmed, A.; Parveen, S. Zika Virus-Induced Microcephaly and Its Possible Molecular Mechanism. *Intervirology* **2016**, *59*, 152–158. [CrossRef]
26. Luteijn, J.M.; Brown, M.J.; Dolk, H. Influenza and congenital anomalies: A systematic review and meta-analysis. *Hum. Reprod.* **2013**, *29*, 809–823. [CrossRef]
27. Zafeiri, A.; Raja, E.A.; Mitchell, R.T.; Hay, D.C.; Bhattacharya, S.; A Fowler, P. Maternal over-the-counter analgesics use during pregnancy and adverse perinatal outcomes: Cohort study of 151 141 singleton pregnancies. *BMJ Open* **2022**, *12*, e048092. [CrossRef]
28. Rasmussen, S.A.; Smulian, J.C.; Lednicky, J.A.; Wen, T.S.; Jamieson, D.J. Coronavirus Disease 2019 (COVID-19) and pregnancy: What obstetricians need to know. *Am. J. Obstet. Gynecol.* **2020**, *222*, 415–426. [CrossRef]
29. Heidarzadeh, M.; Taheri, M.; Mazaheripour, Z.; Abbasi-Khameneh, F. The incidence of congenital anomalies in newborns before and during the COVID-19 pandemic. *Ital. J. Pediatr.* **2022**, *48*, 174. [CrossRef]
30. Brandibur, T.E.; Manea, A.M.; Sharma, A.; Kundnani, N.R.; Popoiu, M.C.; Ahmad, B.; Dahdal, D.S.; Cioboata, D.; Lungu, N.; Doandes, F.M.; et al. Macronutrients Management for Growth in Neonates with Congenital Gastrointestinal Malformation. *Experiment* **2022**, *28*, e938106. [CrossRef]

31. Ben, X.-M. Nutritional management of newborn infants: Practical guidelines. *World J. Gastroenterol.* **2008**, *14*, 6133–6139. [CrossRef] [PubMed]
32. Available online: https://old.ms.ro/?pag=181 (accessed on 20 June 2023).
33. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2021. Available online: https://www.R-project.org/ (accessed on 20 June 2023).
34. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2009.
35. Bland, M. *An Introduction to Medical Statistics*, 3rd ed.; Oxford University Press: Oxford, UK, 2000.
36. Yu, W.; Hu, X.; Cao, B. Viral Infections During Pregnancy: The Big Challenge Threatening Maternal and Fetal Health. *Matern. Med.* **2021**, *4*, 72–86. [CrossRef] [PubMed]
37. Ravaldi, C.; Wilson, A.; Ricca, V.; Homer, C.; Vannacci, A. Pregnant women voice their concerns and birth expectations during the COVID-19 pandemic in Italy. *Women Birth* **2020**, *34*, 335–343. [CrossRef]
38. Ayaz, R.; Hocaoğlu, M.; Günay, T.; Yardımcı, O.D.; Turgut, A.; Karateke, A. Anxiety and depression symptoms in the same pregnant women before and during the COVID-19 pandemic. *JPME* **2020**, *48*, 965–970. [CrossRef]
39. Tomfohr-Madsen, L.M.; Racine, N.; Giesbrecht, G.F.; Lebel, C.; Madigan, S. Depression and anxiety in pregnancy during COVID-19: A rapid review and meta-analysis. *Psychiatry Res.* **2021**, *300*, 113912. [CrossRef] [PubMed]
40. Liu, C.H.; Erdei, C.; Mittal, L. Risk factors for depression, anxiety, and PTSD symptoms in perinatal women during the COVID-19 Pandemic. *Psychiatry Res.* **2021**, *295*, 113552. [CrossRef] [PubMed]
41. Andersson, C.; Vasan, R.S. Epidemiology of cardiovascular disease in young individuals. *Nat. Rev. Cardiol.* **2017**, *15*, 230–240. [CrossRef]
42. Wang, X.; Liu, J.; Cheng, Z.; Zhong, Y.; Chen, X.; Song, W. Triglyceride glucose-body mass index and the risk of diabetes: A general population-based cohort study. *Lipids Health Dis.* **2021**, *20*, 99. [CrossRef]
43. Conklin, A.I.; Guo, S.X.; Tam, A.C.; Richardson, C.G. Gender, stressful life events and interactions with sleep: A systematic review of determinants of adiposity in young people. *BMJ Open* **2018**, *8*, e019982. [CrossRef]
44. Fortin, O.; Mulkey, S.B. Neurodevelopmental outcomes in congenital and perinatal infections. *Curr. Opin. Infect. Dis.* **2023**, *36*, 405–413. [CrossRef]
45. Rosca, C.I.; Sharma, A.; Nisulescu, D.-D.; Otiman, G.; Duda-Seiman, D.-M.; Morariu, S.I.; Lighezan, D.F.; Kundnani, N.R. Prevalence of Cardio-Embolic Brain Complications in Permanent and Paroxysmal Atrial Fibrillation Patients. *Healthcare* **2023**, *11*, 175. [CrossRef] [PubMed]
46. MacDonell, N.; Hancox, R.J. Childhood and Adolescent Television Viewing and Metabolic Syndrome in Mid-Adulthood. *Pediatrics* **2023**, *152*, e2022060768. [CrossRef] [PubMed]
47. Sharma, A.; Christodorescu, R.; Agbariah, A.; Duda-Seiman, D.; Dahdal, D.; Man, D.; Kundnani, N.R.; Cretu, O.M.; Dragan, S. Cardiovascular Risk Prediction Parameters for Better Management in Rheumatic Diseases. *Healthcare* **2022**, *10*, 312. [CrossRef] [PubMed]
48. Rosca, C.I.; Lighezan, D.F.; Nisulescu, D.-D.; Sharma, A.; Neagu, M.N.; Nistor, D.; Georgescu, D.; Kundnani, N.R. Metabolic Syndrome: A Strange Companion of Atrial Fibrillation; A Blessing in Disguise from the Neuropsychiatric Point of View. *Biomedicines* **2023**, *11*, 2012. [CrossRef]
49. Villarreal, S.E.G.; Pacheco, S.M.S.; Alaniz, F.V.; Conde, M.I.B.; Pacheco, J.M.S.; Vazquez, K.C.C.; Leal, A.C.S.; Maldonado, O.A.T.; Contreras, J.A.R.; Cosain, E.I.H.; et al. Risk Factors Associated with COVID-19 Mortality in the State of Durango, Mexico. *Int. J. Med. Sci.* **2023**, *20*, 993–999. [CrossRef]
50. Rosca, C.I.; Kundnani, N.R.; Tudor, A.; Rosca, M.-S.; Nicoras, V.-A.; Otiman, G.; Ciurariu, E.; Ionescu, A.; Stelian, M.; Sharma, A.; et al. Benefits of prescribing low-dose digoxin in atrial fibrillation. *Int. J. Immunopathol. Pharmacol.* **2021**, *35*, 20587384211051955. [CrossRef]
51. Kundnani, N.R.; Rosca, C.I.; Sharma, A.; Tudor, A.; Rosca, M.S.; Nisulescu, D.D.; Branea, H.S.; Mocanu, V.; Crisan, D.C.; Buzas, D.R.; et al. Selecting the right anticoagulant for stroke prevention in atrial fibrillation. *Eur. Rev. Med. Pharmacol. Sci.* **2021**, *25*, 4499–4505.
52. Liu, E.N.; Yang, J.H.; Patel, L.; Arora, J.; Gooding, A.; Ellis, R.; Graves, J.S. Longitudinal analysis and treatment of neuropsychiatric symptoms in post-acute sequelae of COVID-19. *J. Neurol.* **2023**, *270*, 4661–4672. [CrossRef]
53. Bauer, K.A.; Puzniak, L.A.; Yu, K.C.; Klinker, K.P.; Watts, J.A.; Moise, P.A.; Finelli, L.; Gupta, V. Association of SARS-CoV-2 status and antibiotic-resistant bacteria with inadequate empiric therapy in hospitalized patients: A US multicenter cohort evaluation (July 2019–October 2021). *BMC Infect Dis.* **2023**, *23*, 490. [CrossRef]
54. Turdybekova, Y.G.; Kopobayeva, I.L.; Kamyshanskiy, Y.K.; Turmukhambetova, A.A. Comparative clinical and placental pathologic characteristics in pregnancies with and without SARS-CoV-2 infection. *J. Perinat. Med.* **2023**, *51*, 1179–1188. [CrossRef]
55. Garabedian, C.; Vaast, P.; Bigot, J.; Sfeir, R.; Michaud, L.; Gottrand, F.; Verpillat, P.; Coulon, C.; Subtil, D.; Houfflin Debarge, V. Esophageal atresia: Prevalence, prenatal diagnosis and prognosis. *J. Gynecol. Obstet. Biol. Reprod.* **2014**, *43*, 424–430. [CrossRef] [PubMed]
56. Reppucci, M.; Kaizer, A.; Prendergast, C.; Acker, S.; Mandell, E.; Euser, A.; Diaz-Miron, J. Incidence of congenital complications related to COVID-19 infection during pregnancy. *J. Neonatal-Perinatal Med.* **2023**, *16*, 227–234. [CrossRef] [PubMed]
57. Tárnok, A.; Méhes, K. Gastrointestinal Malformations, Associated Congenital Abnormalities, and Intrauterine Growth. *J. Pediatr. Gastroenterol. Nutr.* **2002**, *34*, 406–409. [CrossRef] [PubMed]

58. Doandes, F.M.; Manea, A.-M.; Lungu, N.; Cioboata, D.; Brandibur, T.; Costescu, O.; Hudisteanu, A.; Boia, E.R.; Boia, M. Clinical, biological and electroencephalographic monitoring of newborns with neurological risk in the Neonatal Intensive Care Unit. *Exp. Ther. Med.* **2021**, *22*, 760. [CrossRef] [PubMed]
59. Luxi, N.; Giovanazzi, A.; Capuano, A.; Crisafulli, S.; Cutroneo, P.M.; Fantini, M.P.; Ferrajolo, C.; Moretti, U.; Poluzzi, E.; Raschi, E.; et al. COVID-19 Vaccination in Pregnancy, Paediatrics, Immunocompromised Patients, and Persons with History of Allergy or Prior SARS-CoV-2 Infection: Overview of Current Recommendations and Pre- and Post-Marketing Evidence for Vaccine Efficacy and Safety. *Drug Saf.* **2021**, *44*, 1247–1269. [CrossRef] [PubMed]
60. Goldshtein, I.; Nevo, D.; Steinberg, D.M.; Rotem, R.S.; Gorfine, M.; Chodick, G.; Segal, Y. Association Between BNT162b2 Vaccination and Incidence of SARS-CoV-2 Infection in Pregnant Women. *JAMA* **2021**, *326*, 728–735. [CrossRef]
61. Ekingen, G.; Ceran, C.; Guvenc, B.H.; Tuzlaci, A.; Kahraman, H. Early enteral feeding in newborn surgical patients. *Nutrition* **2005**, *21*, 142–146. [CrossRef]
62. Jorgensen, S.C.; Tabbara, N.; Burry, L. A review of COVID-19 therapeutics in pregnancy and lactation. *Obstet. Med.* **2022**, *15*, 225–232. [CrossRef]
63. Domnicu, A.; Mogoi, M.; Manea, A.; Boia, E.R.; Boia, M. Clinical Factors Associated with COVID-19 Severity in Chronic Hospitalized Infants and Toddlers: Data from a Center in the West Part of Romania. *Healthcare* **2022**, *10*, 808. [CrossRef]

biomedicines

MDPI

Article

Improvement of Symptoms and Cardiac Magnetic Resonance Abnormalities in Patients with Post-Acute Sequelae of SARS-CoV-2 Cardiovascular Syndrome (PASC-CVS) after Guideline-Oriented Therapy

Mariann Gyöngyösi [1,*], Ena Hasimbegovic [1], Emilie Han [1], Katrin Zlabinger [1], Andreas Spannbauer [1], Martin Riesenhuber [1], Kevin Hamzaraj [1], Jutta Bergler-Klein [1], Christian Hengstenberg [1], Andreas Kammerlander [1], Stefan Kastl [1], Christian Loewe [2] and Dietrich Beitzke [2]

[1] Division of Cardiology, 2nd Department of Internal Medicine, Medical University of Vienna, 1090 Vienna, Austria; ena.hasimbegovic@meduinwien.ac.at (E.H.); emilie.han@meduniwien.ac.at (E.H.); katrin.zlabinger@meduniwien.ac.at (K.Z.); andreas.spannbauer@meduniwien.ac.at (A.S.); martin.riesenhuber@meduniwien.ac.at (M.R.); kevin.hamzaraj@meduniwien.ac.at (K.H.); jutta.bergler-klein@meduniwien.ac.at (J.B.-K.); christian.hengstenberg@meduniwien.ac.at (C.H.); andreas.kammerlander@meduniwien.ac.at (A.K.); stefan.kastl@meduniwien.ac.at (S.K.)

[2] Department of Biomedical Imaging and Image-Guided Therapy, Medical University of Vienna, 1090 Vienna, Austria; christian.loewe@meduniwien.ac.at (C.L.); dietrich.beitzke@meduniwien.ac.at (D.B.)

* Correspondence: mariann.gyongyosi@meduniwien.ac.at; Tel.: +43-1-40400-46140

Citation: Gyöngyösi, M.; Hasimbegovic, E.; Han, E.; Zlabinger, K.; Spannbauer, A.; Riesenhuber, M.; Hamzaraj, K.; Bergler-Klein, J.; Hengstenberg, C.; Kammerlander, A.; et al. Improvement of Symptoms and Cardiac Magnetic Resonance Abnormalities in Patients with Post-Acute Sequelae of SARS-CoV-2 Cardiovascular Syndrome (PASC-CVS) after Guideline-Oriented Therapy. *Biomedicines* **2023**, *11*, 3312. https://doi.org/10.3390/biomedicines11123312

Academic Editor: César Fernández De Las Peñas

Received: 12 November 2023
Revised: 11 December 2023
Accepted: 12 December 2023
Published: 14 December 2023

Abstract: Cardiac magnetic resonance (CMR) studies reported CMR abnormalities in patients with mild–moderate SARS-CoV-2 infection, suggesting ongoing myocardial inflammation. Patients (n = 278, 43 ± 13 years, 70.5% female) with post-acute sequelae of SARS-CoV-2 cardiovascular syndrome (PASC-CVS) were included prospectively into the Vienna POSTCOV Registry between March 2021 and March 2023 (clinicaltrials.gov NCT05398952). Clinical, laboratory, and CMR findings were recorded. Patients with abnormal CMR results were classified into isolated chronic pericardial (with/without pleural) effusion, isolated cardiac function impairment, or both (myopericarditis) groups. Medical treatment included a nonsteroidal anti-inflammatory agent (NSAID) for pericardial effusion and a condition-adapted maximal dose of heart failure (HF) treatment. Three months after medical therapy, clinical assessment and CMR were repeated in 82 patients. Laboratory analyses revealed normal hematological, inflammatory, coagulation, and cardiac biomarkers. CMR abnormalities were found in 155 patients (55.8%). Condition-adapted HF treatment led to a significant increase in the left ventricular ejection fraction (LVEF) in patients with initially reduced LVEF (from 49 ± 5% to 56 ± 4%, p = 0.009, n = 25). Low–moderate doses of NSAIDs for 3 months significantly reduced pericardial effusion (from 4/3;5.75/mm to 2/0;3/mm, median/interquartile ranges/p < 0.001, n = 51). Clinical symptoms improved markedly with a decrease in CMR abnormalities, which might be attributed to the maintenance of NSAID and HF medical treatment for PASC-CVS.

Keywords: long COVID; COVID-19; PASC-CVS; cardiac magnetic resonance imaging; CMR; myopericarditis; chronic pericardial effusion

1. Introduction

Cardiovascular symptoms, such as arrhythmias, exercise-induced dyspnea, chest pain, and cardiac fatigue syndrome, are common in patients with long COVID syndrome (post-acute sequelae of SARS-CoV-2 infection cardiovascular symptoms, PASC-CVS) [1–3]. Several studies have reported cardiac abnormalities detected by cardiac magnetic resonance imaging (CMR) in many non-hospitalized SARS-CoV-2-infected patients several months after the acute infection [4–7]. However, such findings (e.g., nonsignificant pericardial effusion or mildly enlarged left and/or right ventricle) were generally considered

clinically insignificant, not requiring further treatment [4–7]. Even if idiopathic chronic mild pericardial effusion has a good prognosis with rare complications [8], the risk of developing chronic pericarditis or a deterioration in heart function with consequences after SARS-CoV-2 infection is currently not calculable. Furthermore, 6 month mortality was significantly higher in SARS-CoV-2-infected patients if they had pericarditis compared with COVID-19-positive patients without pericarditis, even with a small amount of pericardial effusion [9,10]. Additionally, recent findings underlie the presence of ongoing myocardial tissue inflammation due to dysregulated immune system components [3,4], indicating low-dose anti-inflammatory maintenance therapy with steroids combined with angiotensin-receptor blocker (ARB) (MYOFLAME study, NCT05619653). In the absence of active inflammation with normal laboratory values for the inflammatory parameter, we treated symptomatic patients with PASC-CVS displaying abnormal CMR findings in accordance with the current guidelines for cardiac dysfunction and with the maintenance of low-dose nonsteroidal anti-inflammatory drugs (NSAID) in case of chronic pericardial effusion.

The aim of our study was to investigate the effects of our treatment regimen on cardiovascular symptoms and abnormalities found by CMR in symptomatic patients with PASC-CVS.

2. Materials and Methods

2.1. Study Design

Our POSTCOV study is an ongoing prospective registry study (ClinicalTrials.gov Identifier: NCT05398952). The presented methods and results conform with the STROBE guidelines [11].

Patients with PASC-CVS and CMR scans were included prospectively in the Vienna POSTCOV Registry between March 2021 and March 2023. Written informed consent was obtained from all patients before study entry. The study was approved by the local Ethical Committee of the Medical University of Vienna, Austria (EC: 1008/2021 and 1758/2022), and was performed in accordance with the Declaration of Helsinki. Clinical data, CMR findings, and blood sampling results were recorded. CMR was performed if clinically indicated by chest pain, persisting cough, ongoing subfebrility, palpitation, orthostatic intolerance, or ECG abnormalities with suspected chronic myopericarditis. CMR was repeated after medical therapy if clinically justified (Figure 1).

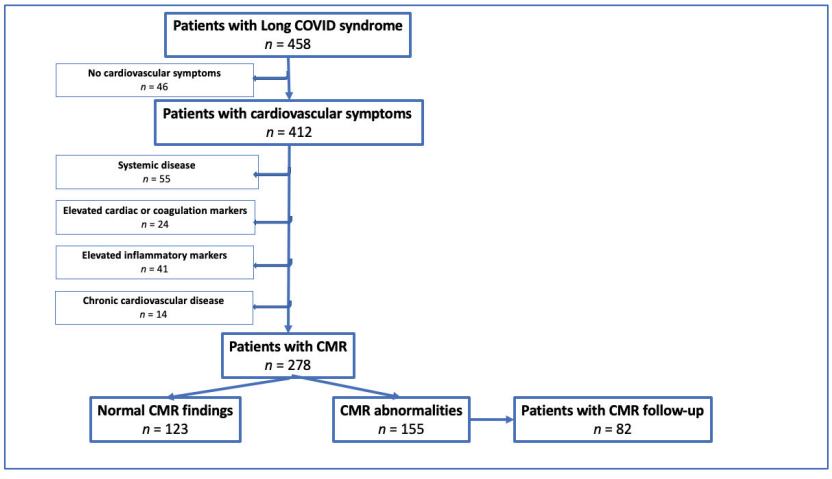


Figure 1. Flow chart of the study. CMR: cardiac magnetic resonance imaging.

2.2. Inclusion and Exclusion Criteria

Patients with PASC-CVS [12–14] were included if they had previous mild or moderate COVID-19 infection confirmed by quantitative real-time polymerase chain reaction (PCR) and were not hospitalized during the acute illness, had no actual or previous systemic diseases (e.g., systemic inflammatory, rheumatic, oncological, cardiovascular, or renal illnesses), and had at least three symptoms from three different organs [1,3]. The main exclusion criteria were signs of active infection with elevated inflammatory parameters (e.g., C-reactive protein, leukocytes, or fibrinogen), missing COVID-19 PCR test, and systemic disease, as well as reasons for secondary pericardial effusion (e.g., traumatic, drug-induced, pulmonary hypertension, metabolic, amyloidosis, rheumatic, or oncologic).

2.3. Clinical Data

Clinical data were collected during the outpatient visit and included detailed anamnesis, including age, sex, cardiovascular risk factors (hypertension, diabetes, hyperlipidemia, or smoking), previous or current systemic disease, current medical treatment, time of COVID positivity, time between SARS-CoV-2 infection and CMR (days), time between first COVID-19 vaccine and CMR (days), and number of patients with past SARS-CoV2 infection with probable lasting immunity and at least one COVID-19 vaccine.

2.4. Laboratory Data

Blood sampling was performed at the first outpatient visit and at the follow-up. The clinical laboratory data included hematological, inflammatory, coagulation, and cardiac markers. In addition, QuantiFERON-TB Gold and Borrelia tests were performed if clinically indicated. The laboratory examinations were performed at the Department of Laboratory Medicine, Medical University of Vienna, Wien, Austria [15]. The laboratory methods can be found at the institution's homepage (https://www.akhwien.at/default.aspx?pid=3985, accessed on 1 March 2021).

2.5. CMR Acquisition and Analysis

All CMR examinations were performed using either 1.5T or 3T MR systems (Philips Ingenia, Eindhoven, The Netherlands, and Siemen Avanto Fit, Siemens Vida, Munich, Germany), with dedicated protocols to screen for inflammation according to the SCMR guidelines [16,17]. All CMR protocols included short-axis cine images for the evaluation of cardiac function, edema-sensitive sequences for the detection of myocardial edema, and late gadolinium enhancement (0.15 mmol/kg gadobutrol/Gadovist; Bayer Vital GmbH, Leverkusen, Germany/if the estimated glomerular filtration rate was >30 mL/min/1.73 m^2) sequences for the detection of myocardial scarring. Image postprocessing and reporting was conducted by experienced cardiac imaging specialists according to recent guidelines [18]. Briefly, we used a stack of short-axis SSFP cine views to determine the end-diastolic volumes (EDVs, mL) and end-systolic volumes (ESVs, mL) of the left (LV) and right ventricle according to standard protocols [16]. The ejection fraction (EF, %) was calculated as the difference between EDV and ESV (stroke volume) divided by EDV and given as a percentage. T1 parametric mapping was performed using a modified Look–Locker inversion recovery (MOLLI) sequence as described previously [19]. The presence and quantification of pericardial and pleural effusion were determined in SSFP cine views, black-blood sequences, and parametric mapping slices as appropriate, and the amount was measured at the largest diameter and given as millimeters.

2.6. Definitions

The definition of chronic pericardial effusion was in accordance with the ESC and ACC/AHA guidelines [20,21]. Briefly, chronic hemodynamically nonsignificant pericardial effusion was diagnosed if the patient had mildly or moderately sized circumferential pericardial fluid longer than 1 months after the SARS-CoV-2 infection. Cardiac morphologic and/or functional impairment was defined based on guidelines [22,23] and involved mono-

lateral or bilateral enlargement of the ventricles with/without a decrease in monolateral or bilateral ventricular function or myocardial edema, T1 signal increase, or late gadolinium enhancement. Myopericarditis was diagnosed if the patient had both chronic pericardial effusion and cardiac morphological and/or functional impairment [20].

2.7. Treatments

Patients with CMR abnormalities were divided into three main groups: isolated chronic pericardial effusion with/without pleural effusion; signs of post-COVID myocarditis with/without ventricular systolic dysfunction or enlargement of the ventricles or the morphological abnormalities described above; and combined myopericarditis.

Patients with reduced left ventricular systolic function with/without monoventricular or biventricular enlargement or normal systolic function with enlarged ventricles were treated in accordance with the relevant heart failure (HF) guidelines [9,10], including with beta blockers, angiotensin-converting enzyme (ACE) inhibitors, or ARB, with/without diuretics (hydrochlorothiazide, HCT) and aldosterone antagonists (Figure 2). As our patients had no previous cardiac or other comorbidities, and the majority of the patients were middle-aged women, a blood-pressure- and condition-adapted maximal tolerated dose of HF therapy was applied.

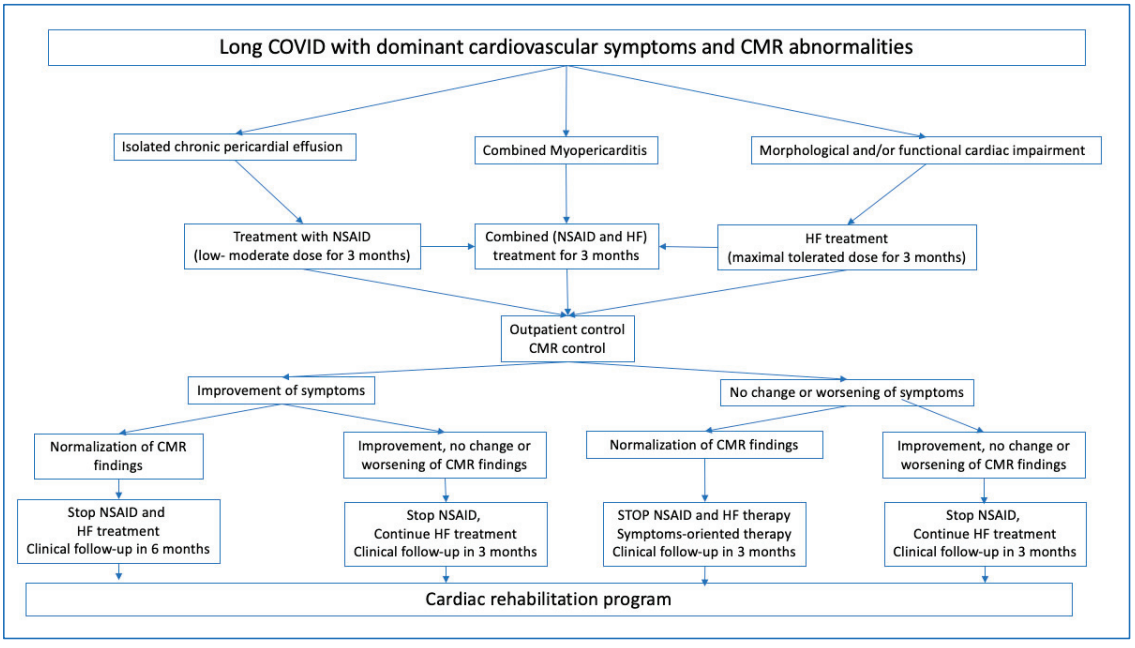


Figure 2. Therapy regimen for patients with long COVID-19 syndrome and cardiovascular symptoms and cardiac magnetic resonance imaging (CMR) abnormalities. NSAID: non-steroidal anti-inflammatory drug; HF: heart failure.

Patients with chronic pericardial effusion with/without pleural effusion received NSAIDs at low–moderate doses, in most cases ibuprofen at a 2 × 200 mg daily oral dose for 3 months, with H2-receptor antagonists at a daily dose of 20 or 40 mg if necessary (Figure 2).

This treatment was established based on the guidelines for chronic pericardiac effusion therapy [7,8] (Class I, Level C) and a routine clinical treatment regimen based on the literature [8,24] because there are no evidence-based therapy guidelines for idiopathic (probably post-viral) chronic hemodynamically nonsignificant pericardiac effusion without

signs of acute inflammation in symptomatic patients. Our combined guideline-oriented and literature-based therapy was established based on the following facts: (1) because all the inflammatory parameters were in the normal range for all patients, high-dose NSAID therapy, eventually combined with colchicine or corticosteroids, was not indicated; (2) three patients had received colchicine previously for 3 months, prescribed at the primary care level, without any efficacy; (3) short-term moderate or high-dose treatment (2 to 4 weeks) did not result in any changes in pericardial effusion controlled by CMR in some patients; (4) there are no evidence-based data on the optimal treatment duration for chronic, hemodynamically nonsignificant but symptomatic pericardial effusion [8,24]; (5) the ongoing MYOFLAME study suggests anti-inflammatory cardioprotective treatment for 4 months (NCT05619653).

Patients with both pericardial effusion and cardiac dysfunction were treated with a combination of the abovementioned therapies.

2.8. Statistics

Continuous variables were tested for normal or nonnormal distribution and expressed as mean ± standard deviation or median with interquartile range (IQRs), respectively. Nominal variables were categorized as frequencies. Baseline and follow-up data were compared using the two-sided Student's *t*-test with repeated measurements (normally distributed variables) or the nonparametric Wilcoxon test or the chi-square test for nominal variables. Statistical significance was defined as $p < 0.05$.

3. Results

We included a total of 278 patients (43 ± 13 years, 70.5% female). The main cardiac symptoms were reported in patients with indications for CMR imaging: chest pain: n = 187 (67.3%), dyspnea: n = 153 (55.0%), palpitation: n = 147 (52.9%), tachycardia: n = 92 (33.1%), thoracic discomfort with/without cough: n = 177 (63.9%), reduced physical activity with/without post-exertional malaise: n: 166 (59.7%), orthostatic incompetence: n = 33 (11.9%).

Laboratory analyses did not reveal elevated acute inflammation; hematological, inflammatory, or coagulation parameters; or cardiac biomarkers (Table 1).

Table 1. Clinical, electrocardiographic, and laboratory data in the long COVID-19 cohort with cardiac symptoms after COVID-19 infection.

Clinical Characteristics and Laboratory Findings	***n* = 278**
Time between COVID-19 infection and CMR (days)	328 ± 214
Number of patients with at least one COVID-19 vaccine (prior to or after COVID-19 infection)	227 (81.7%)
Anti-spike protein titer (AU/mL)	1546 ± 1093
Female sex *n* (%)	196 (70.5%)
Age (years)	43 ± 13
Body mass index (kg/m^2)	25.2 ± 5.2
Diabetes mellitus *n* (%)	7 (2.5%)
Hypertension *n* (%)	44 (15.8%)
Hyperlipidemia *n* (%)	39 (14.0%)
Smoking *n* (%)	9 (3.2%)
Systolic blood pressure (mmHg)	129 ± 17
Diastolic blood pressure (mmHg)	82 ± 11
Heart rate (bpm)	73 ± 12
Cumulative ECG abnormalities *n* (%)	66 (23.7%)

Table 1. *Cont.*

Clinical Characteristics and Laboratory Findings	***n* = 278**
Cardiac arrhythmias *n* (%)	13 (4.7%)
Conduction abnormalities *n* (%)	59 (21.2%)
QRS width (ms)	89.2 ± 12.7
Leukocyte (G/L)	7.0 ± 2.1
Fibrinogen (mg/dL)	313 ± 67
D-dimer (ug/mL)	0 (0;0.37)
Cardiolipin IgG (U/mL)	1.2 (0;1.7)
Cardiolipin IgM (U/mL)	1.4 (0;2.3)
Creatine kinase (U/L)	89 (62;122)
Creatine kinase myocardial subfraction (U/L)	14.1 (12;18.5)
Troponin T (ng/L)	0 (0;6)
NT-proBNP (pg/mL)	44.0 (23.7;82.0)
C-reactive protein (mg/dL)	0.08 (0.04;0.20)
Rheumafactor latex (IU/mL)	0.0 (0.0;0.0)
Alpha 1 antitrypsin (mg/dL)	138 ± 24.2
Interleukin-6 (pg/mL)	0 (0;2.14)
Procalcitonin (ng/mL)	0.03 (0;0.04)
Transferrin (mg/dL)	267.5 ± 45.6
Transferrin saturation (%)	24.3 ± 10.4

Values are given as mean ± SD, median with interquartile range, or *n* (%).

CMR abnormalities were found in 155 patients (55.8%) (Table 2). In total, 58 male (37.4%) and 97 female patients 62.6%) exhibited CMR abnormalities ($p = 0.001$). There were no differences between patients with/without pathological CMR findings regarding clinical (age, time to COVID infection, cardiovascular risk factors, blood pressure, heart rate), ECG, or laboratory parameters.

Table 2. Cardiac magnetic resonance (CMR) findings in the long COVID-19 cohort with cardiac symptoms after COVID-19 infection.

CMR Findings	***n* = 278**
No abnormalities	123 (44.2%)
Cumulative CMR abnormalities *n* (%)	155 (55.8%)
Isolated pericardial effusion (without functional impairment) *n* (%)	34/278 (12.2%)
Morphological and functional impairment *n* (%)	79/278 (28.4%)
Combined myopericarditis *n* (%)	42/278 (15.1%)
Pericardial effusion (w/wo functional impairment) *n* (%)	72 (25.9%)
Reduced LVF *n* (%)	39 (14.0%)
Reduced RVF *n* (%)	55 (19.8%)
Biventricular enlargement *n* (%)	21 (7.6%)
Isolated LV enlargement *n* (%)	56 (20.1%)
Isolated RV enlargement *n* (%)	47 (16.9%)
Myocardial edema *n* (%)	9 (3.2%)
T1 increase *n* (%)	5 (1.8%)

Table 2. *Cont.*

CMR Findings	*n* = 278
Nonischemic late gadolinium enhancement	35 (12.6%)
Pleural effusion *n* (%)	16 (5.8%)
CMR LV EF (%)	59 ± 7
CMR LV EDV (mL)	142 ± 36
CMR LV ESV (mL)	60 ± 20
CMR RV EF (%)	55 ± 6
CMR RV EDV (mL)	152 ± 39
CMR RV ESV (mL)	70 ± 25

Abbreviations: LV, left ventricular; RV, right ventricular; LVF, left ventricular function; RVF, right ventricular function; EF, ejection fraction; EDV, end-diastolic volume; ESV, end-systolic volume.

Supplementary Table S1 shows the detailed CMR data in the patients with/without CMR abnormalities and the data of the CMR phenotype groups.

The time analysis showed the highest incidence of CMR abnormalities 3–5 months after acute SARS-CoV-2 infection, with persistence of cardiac CMR abnormalities even over 24 months post COVID-19 infection (Figure 3).

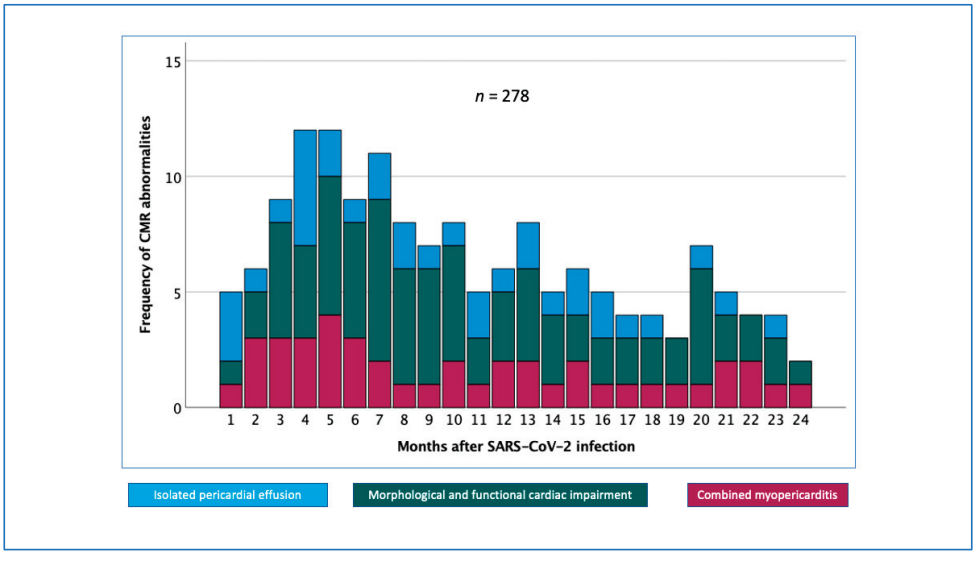


Figure 3. Time-dependent frequencies of cardiac magnetic resonance imaging (CMR) abnormalities.

Among the patients with CMR abnormalities (*n* = 155), 74 of them (47.7%) received NSAIDs (for chronic pericardial effusion), 81 (52.3%) ARB, 25 (16.1%) ARB/HCT, 6 (3.9%) ACE inhibitor, 68 (24.5%) beta blockers, 15 (5.4%) aldosterone antagonists (for cardiac impairment), and 71 (25.5%) H2-receptor blocker therapy.

All patients with abnormal CMR findings were controlled 3–4 months after the initial visit, and 82 patients underwent a follow-up CMR scan 131 ± 52 days after treatment start.

After medical therapy, the clinical symptoms improved markedly (Figure 4), with a decrease in CMR abnormalities (Table 3 and Figures 4 and 5) in terms of a decreasing amount of pericardial effusion (from 4/3;5.75/mm to 2/0;3/mm, median/IQRs/$p < 0.001$, *n* = 51) and a significant increase in the LVEF of patients with ventricular functional

impairment with/without pericardial fluid at first clinical presentation (from 49 ± 5% to 56 ± 4%, $p = 0.009$, $n = 25$).

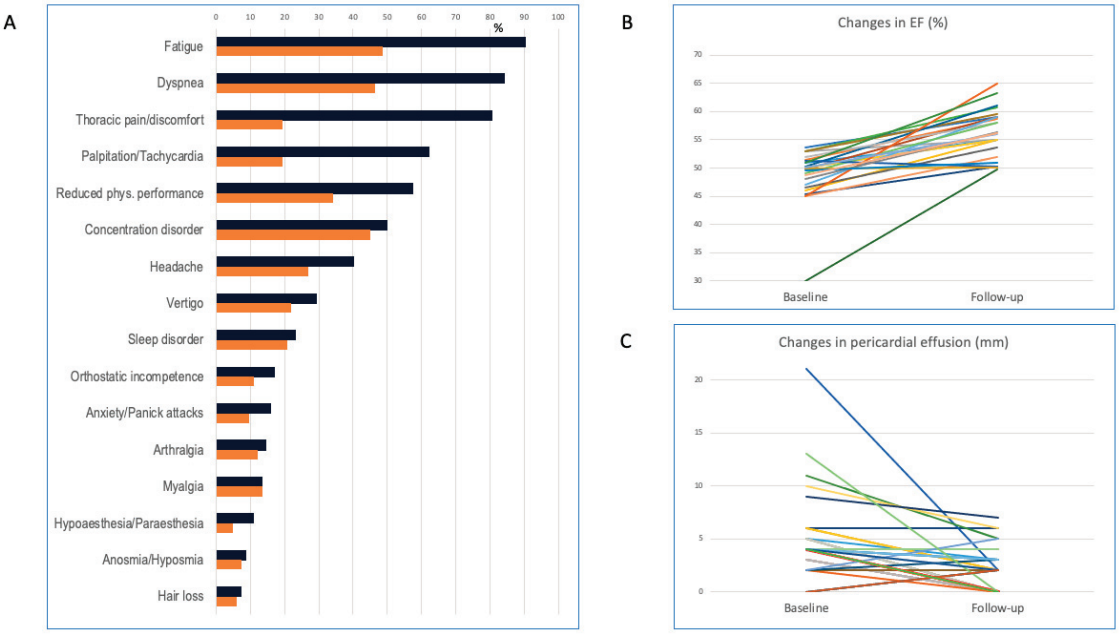


Figure 4. Improvement in clinical symptoms, increase in left ventricular ejection fraction, and decrease in pericardiac effusion after medical treatment in patients with long COVID-19 syndrome. (**A**) Frequency of symptoms at first (black columns) clinical presentation and after 3 months of medical treatment (orange columns); (**B**) individual changes in left ventricular ejection fraction in patients with reduced left ventricular function at first clinical presentation ($n = 25$); and (**C**) pericardial effusion ($n = 51$) between baseline and follow-up.

Table 3. Baseline and follow-up cardiac magnetic resonance (CMR) findings in patients with dominant cardiovascular syndromes and CMR abnormalities at baseline and after medical treatment.

CMR Findings Pre- and Post-Treatment	Baseline ($n = 82$)	Follow-up ($n = 82$)	p Value
Cumulative CMR abnormalities *n* (%)	82 (100%)	53 (64.6%)	<0.001
CMR phenotype *n* (%)			<0.001
No abnormalities *n* (%)		29 (35.4%)	
Isolated pericardial effusion (without functional impairment) *n* (%)	19 (23.2%)	22 (26.8%)	
Morphological and functional impairment *n* (%)	29 (35.4%)	20 (24.4%)	
Combined myopericarditis *n* (%)	34 (41.5%)	11 (13.4%)	
Pericardial effusion (w/wo functional impairment) *n* (%)	51 (62.2%)	32 (39.0%)	0.005
Reduced LVF *n* (%)	25 (30.5%)	4 (4.9%)	<0.001
Reduced RVF *n* (%)	27 (32.9%)	11 (13.4%)	0.039
Biventricular enlargement *n* (%)	14 (17.1%)	9 (11.0%)	
Isolated LV enlargement *n* (%)	29 (35.4%)	16 (19.5%)	0.035
Isolated RV enlargement *n* (%)	18 (22.0%)	16 (19.5%)	
Myocardial edema *n* (%)	8 (9.8%)	1 (1.2%)	0.034

Table 3. *Cont.*

CMR Findings Pre- and Post-Treatment	Baseline (n = 82)	Follow-up (n = 82)	p Value
T1 increase n (%)	1 (1.2%)	0 (0%)	
Nonischemic late gadolinium enhancement n (%)	20 (24.4%)	16 (19.5%)	
Pleural effusion n (%)	5 (6.2%)	8 (9.8%)	
Pericardial effusion (mm) *	4 (3;5.75)	2 (0;3)	<0.001
CMR LV EF (%)	57 ± 7	59 ± 5	0.034
CMR LV EDV (mL)	150 ± 39	151 ± 36	
CMR LV ESV (mL)	65 ± 21	64 ± 20	
CMR RV EF (%)	53 ± 7	557 ± 7	
CMR RV EDV (mL)	161 ± 44	167 ± 44	
CMR RV ESV (mL)	76 ± 25	76 ± 29	

* Median with interquartile range and Wilcoxon test. Abbreviations: LV, left ventricular; RV, right ventricular; LVF, left ventricular function; RVF, right ventricular function; EF, ejection fraction; EDV, end-diastolic volume; ESV, end-systolic volume.

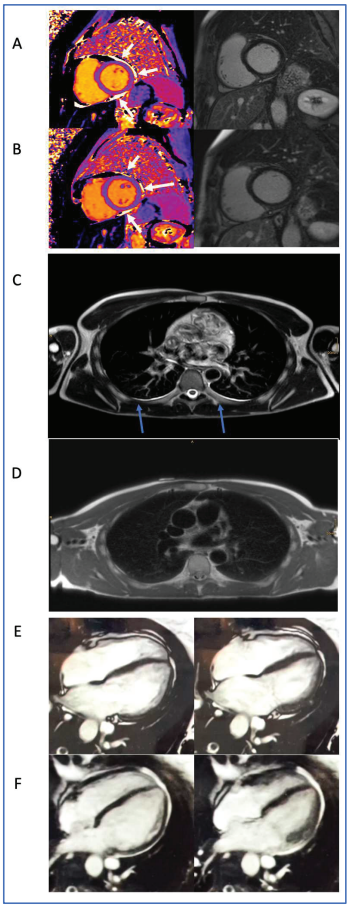


Figure 5. Cardiac magnetic resonance imaging (CMR) of patients with cardiovascular long COVID-19 syndrome at first clinical presentation and after treatment at control. (**A**) Non-physiological pericardial

effusion (white arrows) and (**B**) regression over a 3 month treatment period in a 53-year-old male patient. (**C**) Pleural effusion (blue arrows) at first clinical presentation in a 32-year-old male patient with long COVID-19 syndrome, with (**D**) complete regression after treatment 3 months later (bottom). (**E**) Baseline end-diastolic (left) and end-systolic (right) images of the left ventricle in a 42-year-old woman, with a left ventricular ejection fraction (LVEF) of 45%, and (**F**) 3 months later, after treatment with an LVEF of 52% calculated by CMR.

Table 4 summarizes the detailed changes in CMR abnormalities after 3 month therapy in the different CMR phenotype groups.

Table 4. Changes in CMR abnormalities 3 months after the recommended therapy.

Subgroups of CMR Phenotypes/Follow-up CMR Findings after Treatment	Normalized	Improved	Unchanged	Worsened	Total
Isolated pericardial effusion (without functional impairment) *n* (%)	6 (31.6%)	5 (26.3%)	5 (26.3%)	3 (15.8%)	19 (100%)
Morphological and functional impairment *n* (%)	12 (41.4%)	7 (24.1%)	8 (27.6%)	2 (6.9%)	29 (100%)
Combined myopericarditis *n* (%)	11 (32.4%)	22 (64.7%)	1 (2.9%)	0 (0%)	34 (100%)
Total	29 (35.4%)	34 (41.5%)	14 (17.1%)	5 (6.1%)	82 (100%)

4. Discussion

To the best of our knowledge, this is the first report on the treatment of patients with PASC-CVS based on CMR findings for chronic hemodynamically nonsignificant pericardial effusion with/without pleural effusion (polyserositis), isolated cardiac morphology and/or function impairment, or combined myopericarditis. As no causative therapy for long COVID-19 syndrome exists, we started the therapy by dividing the patients into three main groups and treated the disease entities with a guideline-oriented medical regimen, supplemented by literature-based and clinical routine therapy if the patients had chronic pericardial effusion.

CMR abnormalities were found in 55.8% of patients with cardiac/cardiovascular complaints. This number is in accordance with other reports stating that patients with COVID-19 exhibited abnormal CMR findings at rates of 18% to 78% [3–6], depending on the included patient cohort, the severity of the acute infection, hospitalization, and time after the viral infection. Our time analysis revealed a decrease in the prevalence of abnormal CMR findings after 3 to 5 months, probably due to repeated vaccination or to the less cardio-invasive SARS-CoV-2 variants (e.g., Omicron); these findings are in line with other reports [5,25]. However, the role of spontaneous improvement in cardiac abnormalities and the role of an arbitrary intake of diverse anti-inflammatory or antioxidant dietary supplements during the long COVID-19 period cannot be excluded. Puntmann et al. reported follow-up CMR findings at a median of 329 days after the first CMR and found no change in the LVEF (from 56.6 $\pm$ 4.6 to 56.9 $\pm$ 4.8%) and a significant increase in the RVEF (from 54.0 $\pm$ 5.6 to 55.4 $\pm$ 5.6%) without supplementary information on specific cardiac therapy [5].

The annual incidence of acute pericarditis before the outbreak of COVID-19 was 0.027% [8] and that of the common viral myocarditis was 0.001–0.01% of the general population [26]. Without evoking unnecessary anxiety, the relatively high incidence of chronic pericardial effusion and myocardial injury persisting for a long time in approximately 10% of the world population infected with SARS-CoV-2 virus requires attention [27,28].

At clinical presentation, acute viral myocarditis was excluded in all patients, based on normal cardiac enzymes, normal inflammatory parameters, and the lack of ECG signs or clinical symptoms of acute myocarditis. Few patients presented isolated myocardial edema, T1 increase, or nonischemic late gadolinium enhancement in CMR imaging, indicating chronic myocardial injury. However, through the lack of supportive acute clinical scenarios or laboratory signs, these CMR findings did not fulfill the modified Lake Louise criteria for

acute myocarditis [29,30]. In addition, no CMR was performed during the acute phase of the SARS-CoV-2 infection.

Polyserositis is characterized by inflammation and effusion of the serous membranes, e.g., the pleura, pericardium, and peritoneum. Combined pericarditis and pleuritis is the most common appearance of polyserositis, although diagnosis is difficult, with a lack of diagnostic and therapeutic guidelines [31,32]. Some case reports emphasize the diagnostic challenge of polyserositis in patients, especially in children with multiorgan inflammatory syndromes with acute SARS-CoV-2 infection [33]. We have detected simultaneous pericardial and pleural effusion in 13 patients. The long-time maintenance of polyserositis after the acute infection suggests either a chronic inflammation or autoimmune reaction; both processes require clinical controls.

In general, in the case of chronic pericardial effusion without definitive etiology (supposed SARS-CoV2-induced chronic pericardial effusion) and a lack of signs of systemic inflammation, diverse treatment regimens are recommended [20,21,24]. Less debatable is the treatment of morphological or functional cardiac injury. Patients with pericardial effusion received low or moderate doses of NSAIDs, combined with H2-receptor blocker antagonists if necessary. In accordance with the guideline definitions, patients had the diagnostic criteria for chronic pericardial effusion [20] without clinical (typical pericardial friction rubs) or imaging-proven (ECG or CMR) signs of acute pericarditis or acute viral infection. High doses of NSAIDs are recommended if inflammatory markers are elevated [9,10,20]. However, our patients had no elevated inflammatory or cardiac biomarkers at their first clinical presentation and had symptoms for a longer time, starting after the SARS-CoV-2 infection. In accordance with the ESC guidelines, a lower effective dose may eventually be applied for a shorter period [20]. However, longer NSAID therapy was associated with the decreased recurrence of idiopathic pericardial effusion [24]. Notably, as the figure shows, 3 months of a low–moderate dose of NSAID therapy decreased the amount of pericardial effusion, and it disappeared in several, even if not all, patients. Further steps of treatment with glucocorticoids were not justified in patients with no manifest acute inflammatory pericarditis. An explorative pericardial biopsy was not indicated due to its lack of consequences for decision making about the medical therapy [34].

Patients classified to the functional impairment group received the guideline-oriented HF treatment [22,23]. We observed an improvement in single or biventricular enlargement and/or single or biventricular function in almost all cases. Patients exhibiting myopericarditis were treated with both medical regimens. Their cumulative CMR abnormalities decreased significantly, in parallel with an improvement in symptoms, justifying their indication for treatment.

Most of the patients vaccinated against SARS-CoV-2 received their vaccine before the infection. Theoretically, the vaccine may also induce myocarditis, but its incidence is orders of magnitude lower than that of viral myocarditis [26].

Our study has several limitations. First, a routine CMR is not recommended in patients after COVID-19 disease without cardiac symptoms due to the occasional overinterpretation of nonsignificant CMR changes with questionable relation to previous SARS-CoV-2 infection, except in cases of suspected chronic pericardial effusion (Class IIa, Level C recommendation) [7]. In addition, previous data demonstrated the presence of non-physiological pericardial effusion in up to 30% of clinically asymptomatic patients [4,5]. In accordance with the consensus expert panel recommendation, a CMR should only be performed if it contributes to clinical decision making [32,35]. However, all of our patients presented PASC-CVS.

Several viruses, especially the Epstein–Barr virus, can be reactivated during coronavirus infection [36–38]. We cannot exclude the role of reactivated concomitant cardiotropic viruses (e.g., herpes virus, cytomegalovirus) causing chronic pericardial effusion and myopericarditis, even without signs of acute viral infections. Virus diagnostics from the pericardial punctatum may be informative, but with high risk and cost for low benefit and presumptively inadequate information. In addition, the decrease in or disappearance of

the pericardial fluid after NSAID treatment suggests a rather autoreactive immune process. In contrast with other CMR studies [39], all of our patients were home-quarantined with no medical record and a lack of information on inflammatory parameters, cardiac enzymes (e.g., troponin T or I), ECG, and echocardiography during the active phase of SARS-CoV-2 infection. Therefore, we cannot correlate real SARS-CoV-2-induced myopericarditis with the CMR findings. However, our patients reported typical PASC-CVS, which was not experienced before the COVID-19 disease. Eighty-two patients agreed with control CMR images after symptom- and CMR-abnormality-oriented treatments. The other patients, with pathological CMR findings, also underwent the medical therapy described above, which led to subjective wellbeing, making the control CMR clinically unnecessary.

Due to the lack of a control or placebo group, the efficacy of the suggested therapy might be overestimated. However, many patients had persistent CMR abnormalities for even longer than 1 year, which were resolved or improved after therapy, in parallel with the decrease in cardiovascular symptoms in our cohort, which suggests the beneficial effect of our therapy. Considering the psychological vulnerability of the patients with PASC-CVS, a blinded study with eventual randomization to a placebo arm was not accepted by our patients. Additionally, a CMR finding of morphological (e.g., enlarged ventricles) or functional post-viral cardiac injury represents an absolute indication for HF treatment.

5. Conclusions

Patients with PASC-CVS have a high incidence of CMR abnormalities. Improvement in cardiovascular symptoms and CMR findings might be attributed to NSAID maintenance and HF therapy. However, a randomized placebo-controlled study should be performed to confirm our findings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines11123312/s1, Table S1: Detailed CMR data in the patients with/without CMR abnormalities and the data of the CMR phenotype groups.

Author Contributions: M.G., E.H. (Ena Hasimbegovic), C.H. and D.B. conceptualized the trial. M.G., E.H. (Ena Hasimbegovic), E.H. (Emilie Han), D.B, A.S., M.R., J.B.-K., A.K. and S.K., coordinated the study. E.H. (Ena Hasimbegovic), E.H. (Emilie Han), A.S., M.R., S.K. and K.H. conducted the trial. A.K., C.L. and D.B. conducted the CMR analyses. E.H. (Ena Hasimbegovic), C.H. and A.S. obtained regulatory approval. M.G., E.H. (Ena Hasimbegovic), E.H. (Emilie Han), K.Z., J.B.-K., A.K., S.K., C.L. and D.B. analyzed and interpreted the data. M.G. and E.H. (Ena Hasimbegovic) drafted the manuscript. M.G., E.H. (Ena Hasimbegovic), A.K. and D.B. finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported by the Austrian Science Fund KLI 1064-B and Medical Scientific Fund of the Mayor of the City of Vienna 21176 to G.M. Open Access Funding by the Austrian Science Fund (FWF).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Medical University of Vienna (EC: 1008/2021 and 1758/2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data underlying this article cannot be shared publicly due to the small cohort and the possible risk of identification of individual patients. The data will be shared on reasonable request to the corresponding author.

Conflicts of Interest: M.G. received speaker fees from Medizinische Ausstellungs- u. Werbegesellschaft, General Reinsurance AG, Merck GmbH, FomF GmbH, Medacad, and Wiener Medizinische Akademie für Postgraduelle Ärztliche Fortbildung und Forschung (WMA), outside the submitted work. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Gyongyosi, M.; Alcaide, P.; Asselbergs, F.W.; Brundel, B.; Camici, G.G.; Martins, P.D.C.; Ferdinandy, P.; Fontana, M.; Girao, H.; Gnecchi, M.; et al. Long COVID and the cardiovascular system-elucidating causes and cellular mechanisms in order to develop targeted diagnostic and therapeutic strategies: A joint Scientific Statement of the ESC Working Groups on Cellular Biology of the Heart and Myocardial and Pericardial Diseases. *Cardiovasc. Res.* **2023**, *119*, 336–356. [CrossRef] [PubMed]
2. Xie, Y.; Xu, E.; Bowe, B.; Al-Aly, Z. Long-term cardiovascular outcomes of COVID-19. *Nat. Med.* **2022**, *28*, 583–590. [CrossRef] [PubMed]
3. Writing, C.; Gluckman, T.J.; Bhave, N.M.; Allen, L.A.; Chung, E.H.; Spatz, E.S.; Ammirati, E.; Baggish, A.L.; Bozkurt, B.; Cornwell, W.K., 3rd; et al. 2022 ACC Expert Consensus Decision Pathway on Cardiovascular Sequelae of COVID-19 in Adults: Myocarditis and Other Myocardial Involvement, Post-Acute Sequelae of SARS-CoV-2 Infection, and Return to Play: A Report of the American College of Cardiology Solution Set Oversight Committee. *J. Am. Coll. Cardiol.* **2022**, *79*, 1717–1756. [CrossRef]
4. Puntmann, V.O.; Carerj, M.L.; Wieters, I.; Fahim, M.; Arendt, C.; Hoffmann, J.; Shchendrygina, A.; Escher, F.; Vasa-Nicotera, M.; Zeiher, A.M.; et al. Outcomes of Cardiovascular Magnetic Resonance Imaging in Patients Recently Recovered From Coronavirus Disease 2019 (COVID-19). *JAMA Cardiol.* **2020**, *5*, 1265–1273. [CrossRef] [PubMed]
5. Puntmann, V.O.; Martin, S.; Shchendrygina, A.; Hoffmann, J.; Ka, M.M.; Giokoglu, E.; Vanchin, B.; Holm, N.; Karyou, A.; Laux, G.S.; et al. Long-term cardiac pathology in individuals with mild initial COVID-19 illness. *Nat. Med.* **2022**, *28*, 2117–2123. [CrossRef] [PubMed]
6. Vidula, M.K.; Rajewska-Tabor, J.; Cao, J.J.; Kang, Y.; Craft, J.; Mei, W.; Chandrasekaran, P.S.; Clark, D.E.; Poenar, A.M.; Gorecka, M.; et al. Myocardial Injury on CMR in Patients With COVID-19 and Suspected Cardiac Involvement. *JACC Cardiovasc. Imaging* **2023**, *16*, 609–624. [CrossRef] [PubMed]
7. Ferreira, V.M.; Plein, S.; Wong, T.C.; Tao, Q.; Raisi-Estabragh, Z.; Jain, S.S.; Han, Y.; Ojha, V.; Bluemke, D.A.; Hanneman, K.; et al. Cardiovascular magnetic resonance for evaluation of cardiac involvement in COVID-19: Recommendations by the Society for Cardiovascular Magnetic Resonance. *J. Cardiovasc. Magn. Reason.* **2023**, *25*, 21. [CrossRef]
8. Imazio, M.; Gaita, F.; LeWinter, M. Evaluation and Treatment of Pericarditis: A Systematic Review. *JAMA* **2015**, *314*, 1498–1506. [CrossRef]
9. Buckley, B.J.R.; Harrison, S.L.; Fazio-Eynullayeva, E.; Underhill, P.; Lane, D.A.; Lip, G.Y.H. Prevalence and clinical outcomes of myocarditis and pericarditis in 718,365 COVID-19 patients. *Eur. J. Clin. Investig.* **2021**, *51*, e13679. [CrossRef]
10. Mitiku, T.Y.; Heidenreich, P.A. A small pericardial effusion is a marker of increased mortality. *Am. Heart J.* **2011**, *161*, 152–157. [CrossRef]
11. Zavada, J.; Dixon, W.G.; Askling, J.; EULAR Study Group on Longitudinal Observational Registers and Drug Studies. Launch of a checklist for reporting longitudinal observational drug studies in rheumatology: A EULAR extension of STROBE guidelines based on experience from biologics registries. *Ann. Rheum. Dis.* **2014**, *73*, 628. [CrossRef] [PubMed]
12. Greenhalgh, T.; Knight, M.; A'Court, C.; Buxton, M.; Husain, L. Management of post-acute COVID-19 in primary care. *BMJ* **2020**, *370*, m3026. [CrossRef] [PubMed]
13. Subramanian, A.; Nirantharakumar, K.; Hughes, S.; Myles, P.; Williams, T.; Gokhale, K.M.; Taverner, T.; Chandan, J.S.; Brown, K.; Simms-Williams, N.; et al. Symptoms and risk factors for long COVID in non-hospitalized adults. *Nat. Med.* **2022**, *28*, 1706–1714. [CrossRef] [PubMed]
14. Herrera, J.E.; Niehaus, W.N.; Whiteson, J.; Azola, A.; Baratta, J.M.; Fleming, T.K.; Kim, S.Y.; Naqvi, H.; Sampsel, S.; Silver, J.K.; et al. Response to letter to the editor regarding "Multi-Disciplinary collaborative consensus guidance statement on the assessment and treatment of fatigue in patients with Post-Acute sequelae of SARS-CoV-2 infection (PASC)". *PM&R* **2021**, *13*, 1439–1440. [CrossRef]
15. Haslacher, H.; Gerner, M.; Hofer, P.; Jurkowitsch, A.; Hainfellner, J.; Kain, R.; Wagner, O.F.; Perkmann, T. Usage Data and Scientific Impact of the Prospectively Established Fluid Bioresources at the Hospital-Based MedUni Wien Biobank. *Biopreserv. Biobank.* **2018**, *16*, 477–482. [CrossRef] [PubMed]
16. Kramer, C.M.; Barkhausen, J.; Bucciarelli-Ducci, C.; Flamm, S.D.; Kim, R.J.; Nagel, E. Standardized cardiovascular magnetic resonance imaging (CMR) protocols: 2020 update. *J. Cardiovasc. Magn. Reason.* **2020**, *22*, 17. [CrossRef] [PubMed]
17. Kelle, S.; Bucciarelli-Ducci, C.; Judd, R.M.; Kwong, R.Y.; Simonetti, O.; Plein, S.; Raimondi, F.; Weinsaft, J.W.; Wong, T.C.; Carr, J. Society for Cardiovascular Magnetic Resonance (SCMR) recommended CMR protocols for scanning patients with active or convalescent phase COVID-19 infection. *J. Cardiovasc. Magn. Reason.* **2020**, *22*, 61. [CrossRef] [PubMed]
18. Schulz-Menger, J.; Bluemke, D.A.; Bremerich, J.; Flamm, S.D.; Fogel, M.A.; Friedrich, M.G.; Kim, R.J.; von Knobelsdorff-Brenkenhoff, F.; Kramer, C.M.; Pennell, D.J.; et al. Standardized image interpretation and post-processing in cardiovascular magnetic resonance—2020 update: Society for Cardiovascular Magnetic Resonance (SCMR): Board of Trustees Task Force on Standardized Post-Processing. *J. Cardiovasc. Magn. Reason.* **2020**, *22*, 19. [CrossRef]
19. Kammerlander, A.A.; Marzluf, B.A.; Zotter-Tufaro, C.; Aschauer, S.; Duca, F.; Bachmann, A.; Knechtelsdorfer, K.; Wiesinger, M.; Pfaffenberger, S.; Greiser, A.; et al. T1 Mapping by CMR Imaging: From Histological Validation to Clinical Implication. *JACC Cardiovasc. Imaging* **2016**, *9*, 14–23. [CrossRef]
20. Adler, Y.; Charron, P. The 2015 ESC Guidelines on the diagnosis and management of pericardial diseases. *Eur. Heart J.* **2015**, *36*, 2873–2874. [CrossRef]

21. Chiabrando, J.G.; Bonaventura, A.; Vecchie, A.; Wohlford, G.F.; Mauro, A.G.; Jordan, J.H.; Grizzard, J.D.; Montecucco, F.; Berrocal, D.H.; Brucato, A.; et al. Management of Acute and Recurrent Pericarditis: JACC State-of-the-Art Review. *J. Am. Coll. Cardiol.* **2020**, *75*, 76–92. [CrossRef] [PubMed]
22. McDonagh, T.A.; Metra, M.; Adamo, M.; Gardner, R.S.; Baumbach, A.; Bohm, M.; Burri, H.; Butler, J.; Celutkiene, J.; Chioncel, O.; et al. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. *Eur. Heart J.* **2021**, *42*, 3599–3726. [CrossRef] [PubMed]
23. Heidenreich, P.A.; Bozkurt, B.; Aguilar, D.; Allen, L.A.; Byun, J.J.; Colvin, M.M.; Deswal, A.; Drazner, M.H.; Dunlay, S.M.; Evers, L.R.; et al. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: Executive Summary: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *J. Am. Coll. Cardiol.* **2022**, *79*, 1757–1780. [CrossRef] [PubMed]
24. Massaro, M.G.; Rigante, D.; Sicignano, L.L.; Verrecchia, E.; De Vito, F.; Gasbarrini, A.; Manna, R. Therapeutic management of idiopathic recurrent serositis: A retrospective study. *Eur. Rev. Med. Pharmacol. Sci.* **2020**, *24*, 3352–3359. [CrossRef] [PubMed]
25. Nishijima, Y.; Hader, S.N.; Beyer, A.M. Differential impacts of COVID-19 variants on human microvascular function. *Cardiovasc. Res.* **2023**, *119*, e115–e117. [CrossRef] [PubMed]
26. Heymans, S.; Cooper, L.T. Myocarditis after COVID-19 mRNA vaccination: Clinical observations and potential mechanisms. *Nat. Rev. Cardiol.* **2022**, *19*, 75–77. [CrossRef] [PubMed]
27. Petersen, S.E.; Friedrich, M.G.; Leiner, T.; Elias, M.D.; Ferreira, V.M.; Fenski, M.; Flamm, S.D.; Fogel, M.; Garg, R.; Halushka, M.K.; et al. Cardiovascular Magnetic Resonance for Patients With COVID-19. *JACC Cardiovasc. Imaging* **2022**, *15*, 685–699. [CrossRef] [PubMed]
28. Yancy, C.W.; Fonarow, G.C. Coronavirus Disease 2019 (COVID-19) and the Heart—Is Heart Failure the Next Chapter? *JAMA Cardiol.* **2020**, *5*, 1216–1217. [CrossRef]
29. Ferreira, V.M.; Schulz-Menger, J.; Holmvang, G.; Kramer, C.M.; Carbone, I.; Sechtem, U.; Kindermann, I.; Gutberlet, M.; Cooper, L.T.; Liu, P.; et al. Cardiovascular Magnetic Resonance in Nonischemic Myocardial Inflammation: Expert Recommendations. *J. Am. Coll. Cardiol.* **2018**, *72*, 3158–3176. [CrossRef]
30. Sundaramoorthy, E.; Leonard, M.; Mak, R.; Liao, J.; Fulzele, A.; Bennett, E.J. ZNF598 and RACK1 Regulate Mammalian Ribosome-Associated Quality Control Function by Mediating Regulatory 40S Ribosomal Ubiquitylation. *Mol. Cell.* **2017**, *65*, 751–760.e4. [CrossRef]
31. Losada, I.; Gonzalez-Moreno, J.; Roda, N.; Ventayol, L.; Borjas, Y.; Dominguez, F.J.; Fernandez-Baca, V.; Garcia-Gasalla, M.; Payeras, A. Polyserositis: A diagnostic challenge. *Intern. Med. J.* **2018**, *48*, 982–987. [CrossRef] [PubMed]
32. Harris, E.; Shanghavi, S.; Viner, T. Polyserositis secondary to COVID-19: The diagnostic dilemma. *BMJ Case Rep.* **2021**, *14*, 1439–1440. [CrossRef] [PubMed]
33. Delmonaco, A.G.; Carpino, A.; Raffaldi, I.; Pruccoli, G.; Garrone, E.; Del Monte, F.; Riboldi, L.; Licciardi, F.; Urbino, A.F.; Parodi, E. First diagnosis of multisystem inflammatory syndrome in children (MIS-C): An analysis of PoCUS findings in the ED. *Ultrasound J.* **2021**, *13*, 41. [CrossRef] [PubMed]
34. Lazaros, G.; Imazio, M.; Tsioufis, P.; Lazarou, E.; Vlachopoulos, C.; Tsioufis, C. Chronic Pericardial Effusion: Causes and Management. *Can. J. Cardiol.* **2023**, *39*, 1121–1131. [CrossRef]
35. Lamontagne, F.; Stegemann, M.; Agarwal, A.; Agoritsas, T.; Siemieniuk, R.; Rochwerg, B.; Bartoszko, J.; Askie, L.; Macdonald, H.; Al-Maslamani, M.; et al. A living WHO guideline on drugs to prevent COVID-19. *BMJ* **2021**, *372*, n526. [CrossRef] [PubMed]
36. Hatayama, Y.; Hashimoto, Y.; Motokura, T. Frequent co-reactivation of Epstein-Barr virus in patients with cytomegalovirus viremia under immunosuppressive therapy and/or chemotherapy. *J. Int. Med. Res.* **2020**, *48*, 300060520972880. [CrossRef] [PubMed]
37. Chen, T.; Song, J.; Liu, H.; Zheng, H.; Chen, C. Positive Epstein-Barr virus detection in coronavirus disease 2019 (COVID-19) patients. *Sci. Rep.* **2021**, *11*, 10902. [CrossRef]
38. Gyongyosi, M.; Lukovic, D.; Mester-Tonczar, J.; Zlabinger, K.; Einzinger, P.; Spannbauer, A.; Schweiger, V.; Schefberger, K.; Samaha, E.; Bergler-Klein, J.; et al. Effect of monovalent COVID-19 vaccines on viral interference between SARS-CoV-2 and several DNA viruses in patients with long-COVID syndrome. *NPJ Vaccines* **2023**, *8*, 145. [CrossRef]
39. Yar, A.; Uusitalo, V.; Vaara, S.M.; Holmstrom, M.; Vuorinen, A.M.; Helio, T.; Paakkanen, R.; Kivisto, S.; Syvaranta, S.; Hastbacka, J. Cardiac magnetic resonance -detected myocardial injury is not associated with long-term symptoms in patients hospitalized due to COVID-19. *PLoS ONE* **2023**, *18*, e0282394. [CrossRef]

biomedicines

MDPI

Article

Red Blood Cell Adenylate Energetics Is Related to Endothelial and Microvascular Function in Long COVID

Marzena Romanowska-Kocejko [1], Agata Jędrzejewska [2], Alicja Braczko [2], Klaudia Stawarska [2], Oliwia Król [2], Marika Frańczak [2], Gabriela Harasim [2], Ryszard T. Smoleński [2], Marcin Hellmann [1,†] and Barbara Kutryb-Zając [2,*,†]

[1] Department of Cardiac Diagnostics, Medical University of Gdansk, 80-210 Gdansk, Poland; marzena.romanowska-kocejko@gumed.edu.pl (M.R.-K.); marcin.hellmann@gumed.edu.pl (M.H.)
[2] Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland; agata.jedrzejewska@gumed.edu.pl (A.J.); alicja.braczko@gumed.edu.pl (A.B.); klaudia.stawarska@gumed.edu.pl (K.S.); oliwia.krol@gumed.edu.pl (O.K.); marika.franczak@gumed.edu.pl (M.F.); gabriela.harasim@gumed.edu.pl (G.H.); rt.smolenski@gumed.edu.pl (R.T.S.)
* Correspondence: b.kutryb-zajac@gumed.edu.pl; Tel.: +48-58-349-14-64
† These authors contributed equally to this work.

Abstract: Adenine nucleotides play a critical role in maintaining essential functions of red blood cells (RBCs), including energy metabolism, redox status, shape fluctuations and RBC-dependent endothelial and microvascular functions. Recently, it has been shown that infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) might lead to morphological and metabolic alterations in erythrocytes in both mild and severe cases of coronavirus disease (COVID-19). However, little is known about the effects of COVID-19 on the nucleotide energetics of RBCs nor about the potential contribution of nucleotide metabolism to the long COVID syndrome. This study aimed to analyze the levels of adenine nucleotides in RBCs isolated from patients 12 weeks after mild SARS-CoV-2 infection who suffered from long COVID symptoms and to relate them with the endothelial and microvascular function parameters as well as the rate of peripheral tissue oxygen supply. Although the absolute quantities of adenine nucleotides in RBCs were rather slightly changed in long COVID individuals, many parameters related to the endothelial and microcirculatory function showed significant correlations with RBC adenosine triphosphate (ATP) and total adenine nucleotide (TAN) concentration. A particularly strong relationship was observed between ATP in RBCs and the serum ratio of arginine to asymmetric dimethylarginine—an indicator of endothelial function. Consistently, a positive correlation was also observed between the ATP/ADP ratio and diminished reactive hyperemic response in long COVID patients, assessed by the flow-mediated skin fluorescence (FMSF) technique, which reflected decreased vascular nitric oxide bioavailability. In addition, we have shown that patients after COVID-19 have significantly impaired ischemic response parameters (IR max and IR index), examined by FMSF, which revealed diminished residual bioavailability of oxygen in epidermal keratinocytes after brachial artery occlusion. These ischemic response parameters revealed a strong positive correlation with the RBC ATP/ADP ratio, confirming a key role of RBC bioenergetics in peripheral tissue oxygen supply. Taken together, the outcomes of this study indicate that dysregulation of metabolic processes in erythrocytes with the co-occurring endothelial and microvascular dysfunction is associated with diminished intracellular oxygen delivery, which may partly explain long COVID-specific symptoms such as physical impairment and fatigue.

Keywords: long COVID; red blood cells; nucleotides; microcirculation; endothelium

Citation: Romanowska-Kocejko, M.; Jędrzejewska, A.; Braczko, A.; Stawarska, K.; Król, O.; Frańczak, M.; Harasim, G.; Smoleński, R.T.; Hellmann, M.; Kutryb-Zając, B. Red Blood Cell Adenylate Energetics Is Related to Endothelial and Microvascular Function in Long COVID. *Biomedicines* **2024**, *12*, 554. https://doi.org/10.3390/biomedicines12030554

Academic Editor: César Fernández De Las Peñas

Received: 1 February 2024
Revised: 22 February 2024
Accepted: 24 February 2024
Published: 1 March 2024

1. Introduction

Infection with SARS-CoV-2 can lead to a range of temporary health problems, varying from mild to severe. According to NIH and WHO guidelines, mild COVID-19 is character-

ized in individuals who have any of the various signs and symptoms, e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, and loss of taste and smell [1]. It has been shown that many individuals after SARS-CoV-2 infection reported experiencing long-lasting COVID-19 sequelae and complications such as fatigue, dyspnea or chest pain. This condition, called long COVID, according to NICE (the National Institute for Clinical Excellence) guidelines, is commonly used to describe signs and symptoms that continue or develop after the acute phase of infection, including both ongoing symptomatic COVID-19 (from 4 to 12 weeks) and post-COVID-19 syndrome (PCS, 12 weeks or more) [2].

The mechanisms underlying PCS involve viral toxicity, immune dysregulation, hyperinflammation, hypercoagulability or endothelial damage. It has been also suggested that peripheral factors limiting O_2 supply may explain the reduced cardiovascular fitness and muscular weakness, as persistently impaired systemic tissue oxygenation beyond an acute COVID-19 infection has been demonstrated. One potential reason for tissue hypoxemia in PCS might be associated with dysfunction in the microcirculation. It was shown that SARS-CoV-2 affects the microcirculation, causing endothelial cell swelling and damage (endotheliitis), microscopic blood clots (microthrombosis), capillary congestion, and damage to pericytes that are integral to capillary integrity and barrier function, tissue repair (angiogenesis) and scar formation [3].

On the other hand, decreased tissue perfusion in PCS may be linked to changes in oxygen uptake into the red blood cells (RBCs), oxygen binding or oxygen release. These occurrences may be connected to harm to the beta-chain of hemoglobin or elevated production of methemoglobin, leading to increased oxygen affinity in the unaffected hemoglobin [4]. During the active phase of the infection, there may be changes in the hematological profile, such as a decrease in RBC count or a shift in RBC distribution width. Additionally, alterations in the morphology, structure and function of RBCs could take place, offering an additional explanation for the symptoms described [5,6]. COVID-19 is also reported to enhance RBC deformability and aggregation, which can affect blood flow and reduce tissue oxygen supply [7]. Recently, it has been shown that not only does severe COVID-19 induce prominent RBC structural and rheological changes, but these effects can occur also in patients after a mild course of the disease. Impairment of RBC deformability, aggregated strength and morphological changes were shown to affect blood flow dynamics and, together with the left shifting of the oxygen dissociation curve, possibly oxygen supply in the microcirculation [8].

RBCs, unlike other cell types, cannot generate purine nucleotides through the de novo pathway. Instead, RBCs must engage the salvage reactions, recycling purine bases and nucleosides [9]. The synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) in RBCs is exclusively dependent on the anaerobic conversion of glucose via the Embden–Meyerhof–Parnas pathway, as matured RBCs lack mitochondria [10]. The energy stored in ATP is crucial for various essential functions in erythrocytes, including oxygen delivery to the tissues, maintenance of the electrolyte gradient across erythrocyte membrane, synthesizing glutathione, preserving the asymmetry of the phospholipid membrane and keeping iron of hemoglobin (Hgb) in the ferrous state [10].

Multiple factors can impact the energy status of erythrocytes, leading to reductions in ATP concentration as well as in the adenosine triphosphate/adenosine diphosphate ratio (ATP/ADP) and adenylate energy charge (AEC). These factors include RBC enzymopathies [11], decreased erythrocyte deformability [12], a sedentary lifestyle [13], and neurodegenerative [14] and metabolic [15] disorders. However, very little is known about the metabolic alterations in erythrocytes after COVID-19. An individual study revealed significant changes in RBCs related to an increase in the glycolytic pathway to the detriment of the pentose phosphate pathway (PPP), highlighted by a characteristic increase in glucose consumption accompanied by an accumulation of intermediates of glycolysis and higher levels of phosphofructokinase (PFK), the rate-limiting enzyme of glycolysis [16]. Despite this knowledge, there is limited information available regarding the long-term influence of COVID-19 on the energy status of RBCs. This study aimed to analyze the levels

of adenine nucleotides in RBCs isolated from patients, on average 12 weeks after mild SARS-CoV-2 infection, suffering from long COVID symptoms. RBC nucleotide levels were then related to the parameters of peripheral tissue oxygen supply as well as endothelial and microvascular function.

2. Materials and Methods

2.1. Participants

All participants in the study gave written consent following the principles of the Declaration of Helsinki. The study received approval from the Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk, Poland (no. NKBBN/55/2021). The participants enrolled in the study consisted of cardiology outpatients exhibiting persistent symptoms associated with long COVID and healthy individuals with no previous diagnosis of SARS-CoV-2 infection (controls), as described in Tables 1 and S1.

Table 1. General characteristics of long COVID participants compared with the healthy control group. Results are shown as mean ± SEM. Na—not applicable.

Parameter	Control (n = 20)	Long COVID (n = 19)
Age (years)	40 ± 3	38 ± 2
Sex (F/M)	14/6	12/7
BMI (kg/m^2)	23.3 ± 0.9	23.5 ± 0.8
Long COVID symptoms		
Fatigue	Na	16 (84%)
Tachycardia	Na	3 (16%)
Chest pain	Na	6 (32%)
Dyspnea	Na	1 (5%)
Headache	Na	1 (5%)
Comorbidities		
Hypothyroidism	5 (25%)	5 (26%)
Asthma	0 (0%)	1 (5%)
Atopic dermatitis	0 (0%)	1 (5%)
Irritable bowel syndrome	1 (5%)	1 (5%)
Depression	0 (0%)	1 (5%)
Hyperlipidemia	0 (0%)	1 (5%)
Diabetes	0 (0%)	1 (5%)
Thrombosis	0 (0%)	1 5%)
Drugs taken		
Levothyroxine	5 (25%)	5 (26%)
Antidepressants	0 (0%)	2 (11%)
Metformin	0 (0%)	1 (5%)
Acetylsalicylic acid	0 (0%)	1 (5%)
Drugs prescribed		
Beta-adrenolytics	0 (0%)	6 (32%)
ACE-inhibitors	0 (0%)	3 (16%)
Statins	0 (0%)	1 (5%)

The diagnosis of COVID-19 was established through confirmation via polymerase chain reaction (PCR), serological testing or a rapid antigen test, meeting the sensitivity and specificity criteria recommended by the World Health Organization (WHO) for rapid antigen tests, within a maximum time frame of 4 months from the initial positive test result. The sample collection occurred before the vaccination period, specifically during February–March 2021. A specific time frame from the diagnosis to sample collection and flow-mediated skin fluorescence (FMSF) testing for each patient is provided in Table S1. None of the participants had received any vaccine doses during the study period. De-

tailed characteristics of the patients recruited for the study, including comorbidities and medications taken, are presented in Tables 1 and S1.

2.2. Peripheral Blood Sampling and Morphology

Blood samples were collected into three separate tubes with ethylenediaminetetraacetic acid (EDTA) (2.7 mL), lithium heparinate (4.9 mL) as an anticoagulant and without any anticoagulant (S-monovette, Sarstedt, Nümbrecht, Germany). The first tube was used for the determination of peripheral blood morphology parameters using standard methods. The whole blood in the second tube was centrifuged (1000× *g*, 5 min, rt). The plasma and buffy coat were removed and the erythrocytes were washed three times with the buffered 0.9% sodium chloride (NaCl) solution and centrifuged each time (1000× *g*, 5 min, 4 °C). After a final wash, the resulting erythrocyte pellet was resuspended with a small volume of phosphate-buffered saline (PBS). Next, the samples of washed erythrocytes were deproteinized with an equal volume of 1.3 mol/L $HClO_4$, mixed and then centrifuged at 16,000× *g* for 5 min at 4 °C. The supernatant (600 µL) was neutralized with 130–160 µL of 3 mol/L K_3PO_4 (to pH 5–7). The samples were centrifuged again under the same conditions as before, and the supernatant was immediately deep-frozen at −80 °C until the analysis of erythrocyte purine nucleotides (ATP, ADP and AMP). Whole blood in the third tube was centrifuged (5000 rpm, 5 min, rt) to obtain serum that was immediately frozen at −80 °C for later analyses.

2.3. Erythrocyte Nucleotide Measurements

The measurements were performed using ultra-high-performance liquid chromatography (UHPLC) with a UV–Vis detection system according to a previous method [17]. Briefly, 2 µL of supernatant was injected into a UHPLC system consisting of a Nexera LC40 set and an SPD-M30A diode array detector equipped with a high-sensitivity, 85 mm optical path cell (Shimadzu, Kyoto, Japan). Analytes were separated on a ReproSil-Pur 120 C18-AQ (150 × 2.0 mm ID, 4 µm) column using gradient elution at a flow rate of 500 µL/min. Peaks were detected by absorbance at 254 nm. After conversion to Hct, the intra-erythrocyte concentrations of purine nucleotides were expressed as µmol/L RBC. The values of ATP/ADP, ADP/AMP, total adenine nucleotide pool (TAN = ATP + ADP + AMP) and adenylate energy charge (AEC = [ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP]) were later calculated.

2.4. Serum Amino Acid Measurements

Serum amino acid concentrations, including glycine, arginine, citrulline, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), were determined using liquid chromatography/mass spectrometry (LC/MS) as previously described [18]. Briefly, an aliquot of serum (50 µL) was enriched with internal standards and extracted using 100 µL of acetonitrile for 15 min on ice. Subsequently, the samples were centrifuged at 4 °C, 20,800× *g* for 10 min. The supernatants were collected and freeze-dried. The obtained sediments were dissolved in 100 µL of distilled water and analyzed by using ion-pair high-performance liquid chromatography with mass detection in positive mode electrospray ionization. To identify individual amino acids, their molecular weight, chromatographic retention time and fragmentation pattern were used, as described previously [19].

2.5. Serum hs-CRP Measurement

The concentration of serum high-sensitive C-reactive protein (hs-CRP) was measured using an Automated Photometer (ERBA XL-180, Mannheim, Germany) and specific ERBA kits according to the manufacturer's instructions.

2.6. Microvascular Function Measurements

Microvascular function was evaluated in both female and male individuals who had recovered from COVID-19 (n = 19) and age/sex-matched controls (n = 5) without a history

of COVID-19. The assessment utilized flow-mediated skin fluorescence (FMSF), a non-invasive optical technique that examines microcirculation and metabolic regulation by measuring NADH fluorescence intensity in the epidermis. The quantification of FMSF was carried out using AngioExpert, developed by Angionica Ltd. (Lodz, Poland), as previously outlined [20]. Upon reaching the microcirculation laboratory, participants were positioned within a temperature-regulated environment (24 ± 1 °C). Following a 15-min adjustment period, the baseline intensity of a reduced form of nicotinamide adenine dinucleotide (NADH) fluorescence was measured for 3 min on the forearm. Subsequently, blood flow within the brachial artery was temporarily halted for 3 min by applying pressure to a cuff positioned on the left upper arm, inflated to 50 mmHg above systolic blood pressure. Throughout the occlusion phase, NADH fluorescence was continuously monitored within the same region of the forearm. Upon cuff release, the reduction in NADH fluorescence was observed and recorded for 3 min.

Various parameters were recorded during NADH fluorescence measurement, including ischemic response (IR max; IR index) and hyperemic response (HR max; HR index). Direct measurements of oscillations in the reperfusion stage allowed for the evaluation of hypoxia sensitivity (HS), representing the intensity of flow motion associated with myogenic oscillations. The reactive hyperemia response (RHR) parameter, derived from the sum of IR max and HR max, reflected vascular endothelial function in connection with nitric oxide production during occlusion-induced hyperemia in blood vessels.

2.7. Statistical Analysis

The statistical analysis of the collected data was conducted utilizing InStat software (GraphPad Prism 9.0, San Diego, CA, USA). To assess normality, the Kolmogorov–Smirnov test, Shapiro–Wilk test, or D'Agostino and Pearson Omnibus test were employed. Group mean values were compared through unpaired Student's *t*-test or Mann–Whitney test. Correlations were examined using Pearson correlation coefficient. The specific value of 'n' was provided for each experiment, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Long COVID Patients Demonstrate Decreased Peripheral Tissue Oxygenation with Changes in Endothelial and Microvascular Function Parameters

In our study, we demonstrated lower levels of skin tissue oxygenation together with endothelial and microvascular dysfunction estimated by the flow-mediated skin fluorescence (FMSF) technique (Table 2). FMSF is one of the available techniques for assessing the function of microcirculation, tissue oxygenation and nutrient supply. This technique is based on the registration of the cutaneous fluorescence intensity of NADH [21]. Excitation of the forearm with ultraviolet light at 340 nm results in the emission of a NADH fluorescence signal from human keratinocytes, which is detected by the receiver diode at 460 nm. The test involves inducing NADH fluorescence during 3 min of brachial artery occlusion [21]. In this way, the ischemic (IR) and hyperemic (HR) responses are recorded. IR reflects tissue sensitivity to hypoxia and includes parameters, such as IR max and IR index. The IR max indicates the ratio of the relative to maximal baseline increase in NADH fluorescence intensity observed over the occlusion period, while the IR index corresponds to the area under the curve. In our previous work [18], as well as in the group of patients recruited in this study, we showed lower IR max and IR index parameters in long COVID compared to healthy controls (Table 2). HR reflects microvascular reactivity and it is quantitatively described by two parameters: HR max and HR index. The first is expressed as the relative to maximal baseline decrease in NADH fluorescence intensity during the reperfusion phase, while the latter is defined as the area under the curve. Although long COVID patients revealed rather minor changes in HR parameters compared to the control group (Table 2), they had lower RHR (reactive hyperemia response), which characterizes endothelial function related predominantly to the production of nitric oxide (NO) in the vasculature due to reactive hyperemia [22]. In addition, long COVID patients revealed

a decrease in serum glycine concentration (Table 2), which has been demonstrated as a mitigator of cytokine storm with anti-inflammatory properties in COVID-19 [20]. There were no changes in red and white blood cell parameters in peripheral blood cell count. Meanwhile, differences were noticed in platelet distribution width (PDW) and the percentage of large platelets in favor of these higher parameters in patients after COVID-19 (Table 2).

Table 2. Microcirculation function parameters were assessed by flow-mediated skin fluorescence (FMSF) technique, serum circulating inflammatory and endothelial function parameters, and peripheral blood cell count in long COVID participants compared with healthy control group. Results are shown as mean ± SEM with corresponding *p*-value by unpaired Student's *t*-test or Mann–Whitney test as appropriate. IR—ischemic response, HR—hyperemic response, RHR—reactive hyperemic response, log(HS)—hypoxia sensitivity parameter, hs-CRP—high-sensitive C-reactive protein, RBCs—red blood cells, Hct—hematocrit, Hgb—hemoglobin, MCV—mean corpuscular volume, MCH—mean corpuscular hemoglobin, MCHC—mean corpuscular hemoglobin concentration, RDW—red blood cell distribution width, WBC—white blood cells, NEU—neutrophils, LYMPH—lymphocytes, MONO—monocytes, EOS—eosinophils, BAS—basophils, PLT—platelets, PDW—platelet distribution width, PCT—plateletcrit, NLR—neutrophil-to-lymphocyte ratio, LMR—lymphocyte-to-monocyte ratio, LCR—lymphocyte-to-C-reactive protein ratio and PLR—platelet-to-lymphocyte ratio.

Parameter	Control	Long COVID	*p* Value
Microcirculatory function parameters			
IR index [%]	14.5 ± 1.74	6.06 ± 1.09	<0.001
IR max [%]	19.5 ± 2.12	9.30 ± 1.32	<0.001
HR index [%]	15.1 ± 1.15	13.3 ± 0.73	0.20
HR max [%]	22.3 ± 2.29	20.3 ± 0.72	0.42
RHR [%]	38.7 ± 2.24	30.8 ± 1.32	<0.01
Log (HS)	114 ± 38.7	49.6 ± 9.85	<0.05
Serum inflammatory parameters			
hs-CRP [mg/L]	2.1 ± 0.30	2.4 ± 0.31	0.489
Serum amino acid compounds			
Arginine [μmol/L]	112 ± 6.92	126 ± 5.27	0.12
Citrulline [μmol/L]	15.5 ± 0.93	17.2 ± 0.76	0.17
SDMA [μmol/L]	0.83 ± 0.04	0.86 ± 0.05	0.64
Arginine/ADMA	162 ± 8.00	151 ± 8.08	0.34
Glycine [μmol/L]	314 ± 37.4	219 ± 12.9	<0.05
Peripheral blood cell count			
RBCs [T/L]	4.86 ± 0.10	4.70 ± 0.09	0.26
Hct [%]	42.4 ± 0.74	42.2 ± 0.75	0.25
Hgb [g/dL]	14.3 ± 0.29	14.2 ± 0.29	0.79
MCV [fL]	88.7 ± 0.91	87.7 ± 0.95	0.45
MCH [pg]	30.5 ± 0.32	30.2 ± 0.46	0.60
MCHC [g/dL]	34.3 ± 0.32	34.4 ± 0.31	0.81
RDW [%]	12.9 ± 0.18	12.8 ± 0.20	0.85
WBC [G/L]	6.22 ± 1.81	6.28 ± 0.47	0.96
NEU [G/L]	3.52 ± 1.22	3.65 ± 0.37	0.92
NEU [%]	56.6 ± 2.11	56.5 ± 2.21	0.98
LYMPH [G/L]	2.31 ± 0.23	1.94 ± 0.11	0.16
LYMPH [%]	37.1 ± 2.43	32.3 ± 2.03	0.14
MONO [G/L]	0.48 ± 0.05	0.51 ± 0.04	0.64
MONO [%]	7.72 ± 0.19	8.22 ± 0.48	0.33
EOS [G/L]	0.12 ± 0.03	0.15 ± 0.02	0.42
EOS [%]	1.93 ± 0.21	2.35 ± 0.26	0.21
BAS [G/L]	0.04 ± 0.01	0.04 ± 0.01	0.99
BAS [%]	0.64 ± 0.06	0.56 ± 0.05	0.31
PLT [G/L]	216 ± 12.0	222 ± 11.0	0.72

Table 2. *Cont.*

Parameter	Control	Long COVID	*p* Value
PDW [fL]	10.9 ± 0.51	13.2 ± 0.41	<0.01
PCT [%]	0.23 ± 0.01	0.24 ± 0.01	0.48
Large PLT [%]	26.7 ± 2.31	32.3 ± 1.44	<0.05
NLR	1.52 ± 0.12	1.93 ± 0.18	0.06
LMR	4.81 ± 0.32	4.11 ± 0.29	0.11
LCR	1.10 ± 0.09	0.94 ± 0.08	0.19
PLR	93.5 ± 10.4	120 ± 8.80	0.06

3.2. Adenine Nucleotides' Concentration in the Erythrocytes of Long COVID Patients Is at Similar Levels as in the Healthy Controls

As erythrocytes play a critical role in sufficient tissue oxygenation as well as possibly regulating endothelial and microvascular functions via adenine nucleotide metabolism and signaling, we analyzed the adenine nucleotides in RBCs. The concentrations of ATP, ADP and AMP in the erythrocytes of patients with long COVID did not differ from those in healthy controls (Figure 1). Similarly, no significant differences were observed in the ATP/ADP ratio and adenylate energy charge (AEC). However, the ADP/AMP ratio was higher in long COVID patients.

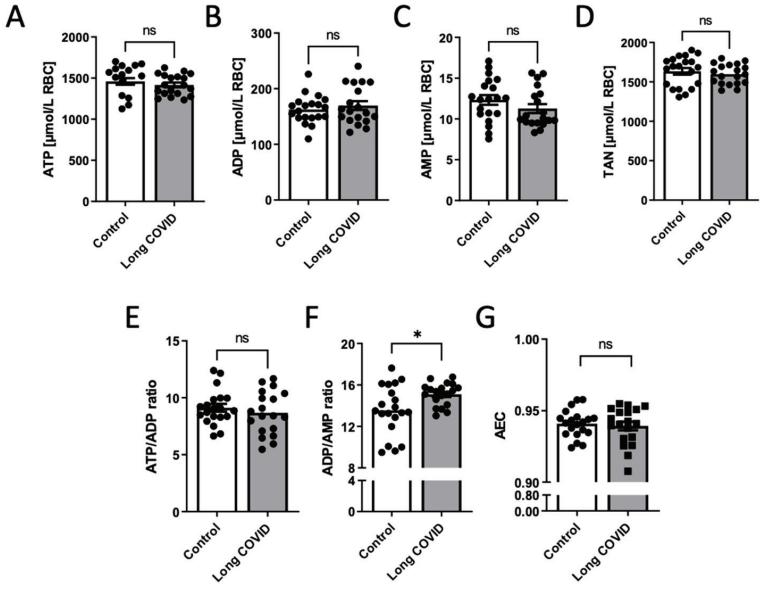


Figure 1. Adenine nucleotides' concentration in the erythrocytes of long COVID patients is not different from that in healthy controls. The concentration of (**A**) adenosine triphosphate (ATP), (**B**) adenosine diphosphate (ADP), (**C**) adenosine monophosphate (AMP), (**D**) total adenine nucleotide (TAN), (**E**,**F**) adenine nucleotide ratios (ATP/ADP; ADP/AMP) and (**G**) adenylate energy charge (AEC) in red blood cells of post-COVID-19 participants (n = 19) compared with healthy control group (n = 20). Results are shown as mean ± SEM; * $p < 0.05$ by unpaired Student's t-test (**A**,**B**,**D**–**G**) or Mann–Whitney test (**C**), ns—not significant.

3.3. Adenosine Triphosphate (ATP) Concentration in the Erythrocytes of Long COVID Patients Correlates with Markers of Endothelial and Microcirculatory Function

Despite the lack of differences in red blood cell ATP and ADP concentrations between post-COVID-19 patients and healthy controls, we demonstrated a positive correlation of IR max and IR index parameters with erythrocyte ATP concentration (Figure 2) and a negative

correlation with ADP (Table S2). This resulted in a strong positive correlation between the IR index, IR max and red blood cell ATP/ADP ratio and AEC (Table S3). In addition, the RHR parameter that reflects the endothelial ability to produce NO negatively correlated with RBC ADP concentration (Table S2) and positively correlated with the RBC ATP/ADP ratio and AEC (Table S3). We also found a negative correlation between log(HS), the other parameter of the microcirculatory response to hypoxia, and the RBC ADP/AMP ratio (Table S3). Log(HS) reflects myogenic microcirculatory oscillations, which are stimulated on the reperfusion line following transient hypoxia [21].

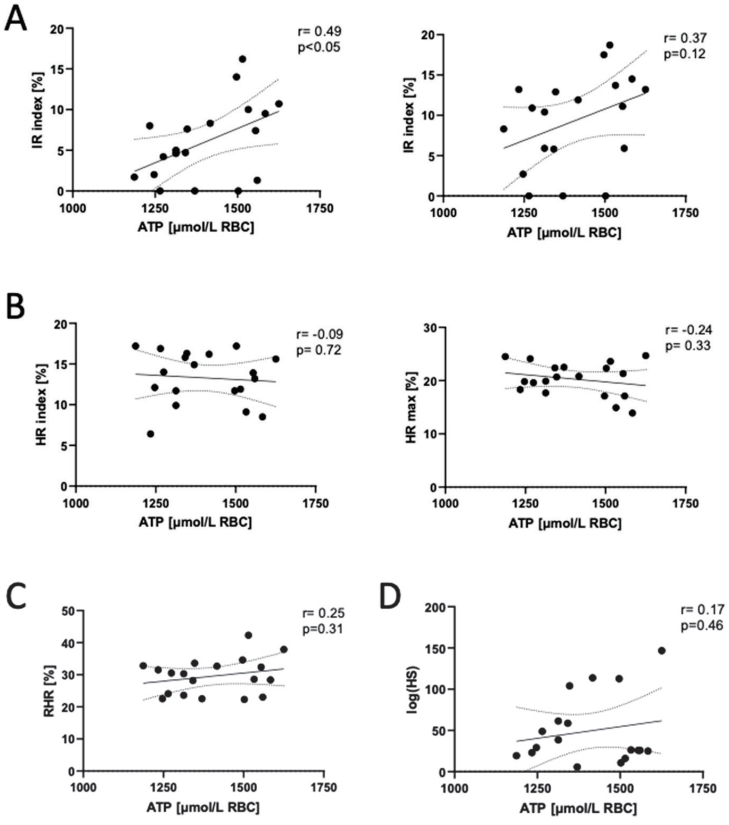


Figure 2. Adenosine triphosphate (ATP) concentration in the erythrocytes of long COVID patients correlates with ischemic response parameters measured by flow-mediated skin fluorescence (FMSF) technique. Correlations of red blood cell adenosine triphosphate (ATP) concentration with (**A**) ischemic response parameters (IR index; IR max), (**B**) hyperemic response parameters (HR index; HR max), (**C**) reactive hyperemic response (RHR) and (**D**) hypoxia sensitivity (logHS) in post-COVID-19 participants. Results are shown as correlation plots with corresponding Pearson (**A**–**C**) or Spearman (**D**) coefficient (r) and *p* value (*p*). Solid line—regression line, dotted line—error bars.

Additionally, we determined a significant positive correlation between ATP concentration in RBCs and serum arginine/ADMA (a ratio of nitric oxide substrate to NO synthase inhibitor). Then, we found positive relationships between RBC ATP and serum arginine and citrulline concentrations, which are a substrate and co-product in the reaction of NO synthesis, as well as a negative trend between RBC ATP and SDMA (arginine transport inhibitor) (Figure 3). In addition, ATP and TAN concentration in erythrocytes positively correlated with serum glycine concentration, an amino acid with anti-inflammatory properties (Figure 3, Table S2) [23].

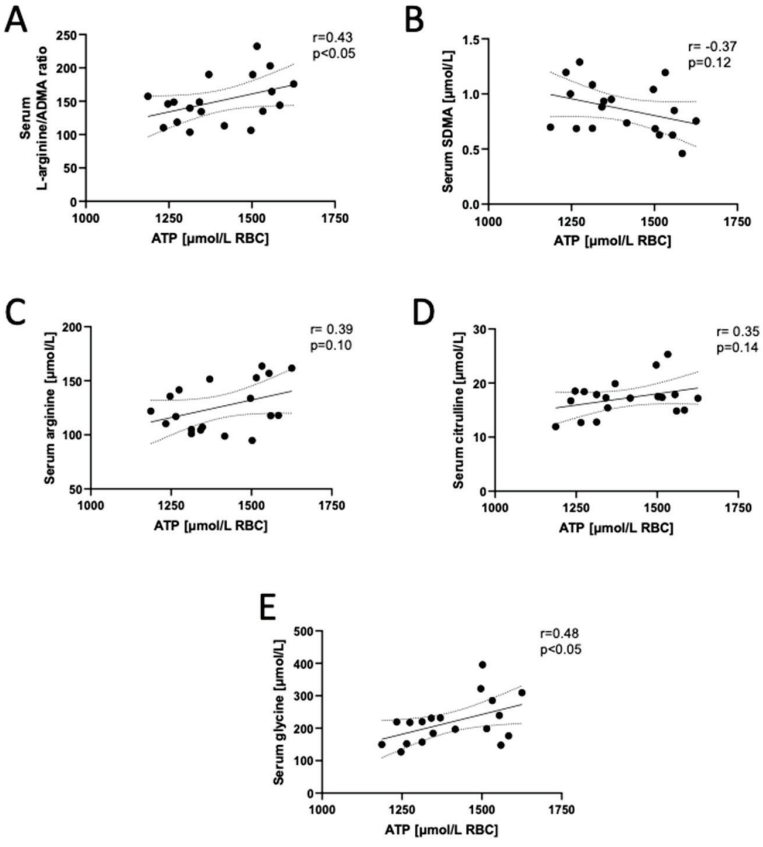


Figure 3. Adenosine triphosphate (ATP) concentration in the erythrocytes of long COVID patients correlates with circulating endothelial function parameters. Correlations of red blood cell adenosine triphosphate (ATP) concentration with (**A**) L-arginine/ADMA (asymmetric dimethyl-L-arginine) ratio, (**B**) symmetric dimethyl L-arginine (SDMA), (**C**) arginine, (**D**) citrulline and (**E**) glycine concentration in long COVID participants. Results are shown as correlation plots with corresponding Pearson coefficient (r) and p value (p). Solid line—regression line, dotted line—error bars.

3.4. Adenosine Triphosphate (ATP) Concentration in the Erythrocytes of Long COVID Patients Correlates with Markers of Systemic Inflammation Reactivation

Then, we determined no significant correlations of erythrocyte adenine nucleotide concentrations, ATP/ADP or ADP/AMP ratios and AEC with red blood cell parameters (Tables 3 and S4). However, there was a tendency toward negative relationships between the ATP concentration in erythrocytes and the number of red blood cells, the hematocrit, hemoglobin concentration or red blood cell distribution width (RDW). Interestingly, these trends became weaker or completely disappeared in the correlation analysis with ADP and AMP. Additionally, there were significant relationships between erythrocyte ATP, total adenine nucleotide (TAN) concentration and the percentage of neutrophils (NEU) and lymphocytes (LYMPH) (Table 3), which translated into a strong positive correlation with the neutrophil-to-lymphocyte ratio (NLR) in long COVID participants (Figure 4).

Table 3. Adenosine triphosphate (ATP) and total adenine nucleotide (TAN) concentration in the erythrocytes of long COVID patients correlates with peripheral blood neutrophil and lymphocyte percentage. Correlations of red blood cell adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and total adenine nucleotide (TAN) concentration with peripheral blood cell count in long COVID participants (n = 19). Results are shown as Pearson or Spearman (as appropriate) correlation coefficient (r) and p value (p).

Parameter	ATP [µmol/L RBC]		ADP [µmol/L RBC]		AMP [µmol/L RBC]		TAN [µmol/L RBC]	
	r	*p* Value	r	*p* Value	r	*p* Value	r	*p* Value
RBC [T/L]	−0.28	0.26	0.12	0.63	0.08	0.75	−0.23	0.36
Hct [%]	−0.27	0.28	0.14	0.57	0.04	0.87	−0.21	0.39
Hgb [g/dL]	−0.21	0.42	0.13	0.61	0.07	0.79	−0.16	0.53
MCV [fL]	0.08	0.74	0.04	0.87	−0.04	0.87	0.09	0.73
MCH [pg]	0.23	0.37	0.18	0.47	0.03	0.90	0.23	0.37
MCHC [g/dL]	0.14	0.59	−0.01	0.99	0.07	0.77	0.13	0.61
RDW [%]	−0.34	0.17	−0.17	0.50	−0.17	0.49	−0.37	0.13
WBC [G/L]	0.26	0.30	−0.10	0.70	−0.16	0.54	0.22	0.37
NEU [G/L]	0.39	0.11	−0.03	0.91	−0.12	0.64	0.37	0.13
NEU [%]	0.55	<0.05	0.07	0.78	−0.06	0.80	0.55	<0.05
LYMPH [G/L]	−0.24	0.34	−0.17	0.50	−0.11	0.66	−0.27	0.27
LYMPH [%]	−0.52	<0.05	0.01	0.97	−0.13	0.60	−0.50	<0.05
MONO [G/L]	0.01	0.98	−0.42	0.08	−0.40	0.10	−0.11	0.66
MONO [%]	−0.28	0.26	−0.40	0.10	−0.32	0.20	−0.38	0.12
EOS [G/L]	0.12	0.63	0.06	0.83	0.02	0.95	0.13	0.60
EOS [%]	−0.04	0.89	0.07	0.78	0.09	0.71	−0.02	0.95
BAS [G/L]	0.01	0.97	−0.15	0.54	−0.14	0.59	−0.04	0.89
BAS [%]	−0.27	0.28	−0.02	0.94	0.05	0.84	−0.27	0.28
PLT [G/L]	0.07	0.79	0.08	0.75	0.03	0.92	0.09	0.73
PDW [fL]	0.33	0.18	−0.21	0.40	−0.28	0.26	0.26	0.30
PCT [%]	0.18	0.48	0.01	0.97	−0.06	0.80	0.17	0.49
Large PLT [%]	0.32	0.20	−0.25	0.31	−0.32	0.20	0.24	0.35

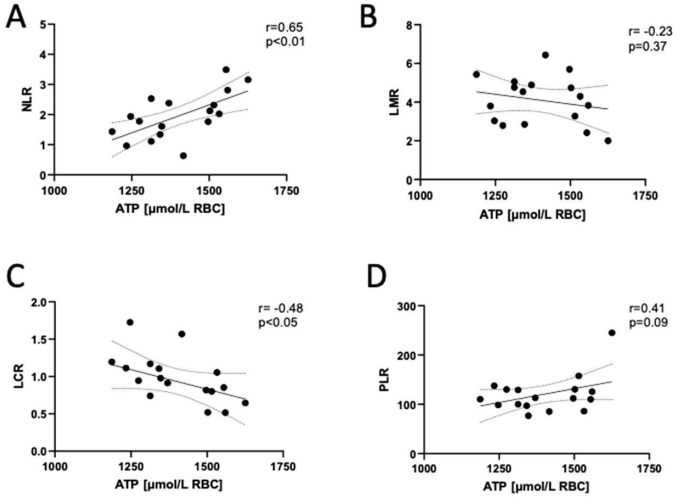


Figure 4. Adenosine triphosphate (ATP) concentration in the erythrocytes of long COVID patients correlates with inflammatory hematological ratios. Correlations of red blood cell adenosine triphosphate (ATP) concentration with (**A**) peripheral blood neutrophil-to-lymphocyte ratio (NLR), (**B**) lymphocyte-to-monocyte ratio (LMR), (**C**) lymphocyte-to-C-reactive protein ratio (LCR) and (**D**) platelet-to-lymphocyte ratio (PLR) in post-COVID-19 participants. Results are shown as correlation plots with corresponding Pearson (**A**–**C**) or Spearman (**D**) correlation coefficient (r) and p value (p). Solid line—regression line, dotted line—error bars.

It was observed very soon after the beginning of the COVID-19 pandemic that a high NLR can be used as a reliable indicator to determine disease severity, with a cut-off point above 3.0 [24]. In addition, the NLR has been proposed as a marker of systemic inflammation reactivation when monitoring long COVID patients [25]. It was shown that after normalization to approximately 2.5, the NLR gradually re-elevated to about 3.5 in patients with sustained long COVID symptoms. Our findings indicate that the NLR as well as the lymphocyte-to-C-reactive protein ratio (LCR) significantly correlated with the ATP and TAN concentration in erythrocytes (Figure 3, Table S4). Other indicators, such as the lymphocyte-to-monocyte ratio (LMR) and platelet-to-lymphocyte ratio (PLR), tended to correlate with ATP and TAN concentration in erythrocytes.

4. Discussion

This work highlights the potential role of red blood cell adenylate energetics in tissue oxygenation as well as endothelial and microvascular function in patients with long COVID. We demonstrated the correlation of many parameters related to the immune response and endothelial, and microcirculatory function with erythrocyte ATP and TAN concentrations in long COVID patients. A particularly strong positive relationship was observed between ATP concentration in RBCs and the serum ratio of arginine to asymmetric dimethylarginine, an indicator of vascular nitric oxide production capacity. Consistently, a positive correlation was observed between the ATP/ADP ratio in RBCs and diminished reactive hyperemic response in post-COVID-19 patients, assessed by flow-mediated skin fluorescence (FMSF), which reflected decreased vascular NO bioavailability. On the other hand, we have shown that patients with long COVID symptoms have significantly impaired ischemic response parameters (IR max and IR index), examined by FMSF, which revealed diminished residual bioavailability of oxygen in epidermal keratinocytes after brachial artery occlusion. IR max and IR index parameters revealed a strong correlation with the ATP/ADP ratio in RBCs. Taken together, this study indicates that a decrease in peripheral tissue oxygenation in long COVID patients may be associated with diminished intracellular oxygen delivery through the circulatory system due to dysregulation of metabolic processes in erythrocytes, with simultaneous endothelial and microvascular dysfunction. A lack of evident differences in the concentration of adenine nucleotides in the erythrocytes of all post-COVID-19 patients compared to healthy controls may indicate heterogeneity of post-COVID-19 patients, of which a large proportion had restored metabolic equilibrium.

The maintenance of metabolic balance relies significantly on the evolved mechanisms through which hemoglobin in RBCs senses the need for oxygen and responds suitably [26]. The coordinated regulation of ATP production and antioxidant systems within RBCs also takes advantage of Hgb-based oxygen sensitivity to address various physiological and pathological stresses [11]. For instance, during oxygen offloading, glycolysis is promoted to generate both 2,3-DPG (2,3-diphosphoglycerate, a negative allosteric effector of hemoglobin–oxygen binding) and ATP [27]. Conversely, under oxygen-rich conditions, the production via the PPP of nicotinamide adenine dinucleotide phosphate (NADPH), crucial for reducing systems, is favored [27]. The dynamic control of ATP not only ensures the maintenance of the ionic and structural balance in RBCs but also contributes to the availability of vasoregulatory ATP that can be released in hypoxia or during RBC deformation in microvessels [28]. The export of ATP from erythrocytes in response to hypoxia or deformation serves to dilate blood vessels, facilitating efficient oxygen delivery [29].

It has been demonstrated that the adaptability of RBCs to the metabolic environment through the control of the above mechanisms is compromised during COVID-19 infection [27]. It was revealed that RBCs from severe COVID-19 patients displayed signatures of oxidation and fragmentation of key structural and functional proteins including band 3 (AE1), spectrin beta and ankyrin, as well as revealing increased glycolytic intermediates, including 2,3-DPG, without significant changes in ATP levels [16]. Elevations in glycolytic metabolites within RBCs align with the potential enhancement of the capacity of Hgb to offload oxygen as a function of allosteric modulation by high-energy phosphate compounds

(a right shift of the oxygen dissociation curve) and may counteract COVID-19-induced hypoxia [30]. However, in spite of high 2,3-DPG levels in COVID-19 patients, no change in hemoglobin affinity was detected in three independent investigations, while in a large cohort study, even a left shift of the oxygen dissociation curve was calculated [4]. The most likely reason for this finding is the formation of methemoglobin, which enhances oxygen affinity and seems to thus counteract the impact of 2,3-DPG. A lack of this back shift would further impede oxygen loading in the damaged lung. The problem is thus partly transferred from oxygen uptake in the lung to oxygen transport from capillaries to the cells that consume it.

In our study, we have shown that patients with long COVID have significantly impaired ischemic response parameters (IR max and IR index), which were examined using the non-invasive FMSF technique. The IR parameters indicate the response to brachial artery occlusion, resulting in the complete blockage of oxygen delivery to the epidermis, and should be treated as a metabolic indicator of the changes in the $NADH/NAD^+$ equilibrium in keratinocytes due to transient ischemia [31]. Thus, the gradual shift of the $NADH/NAD^+$ equilibrium toward reduction, seen as an increase in NADH fluorescence (ischemic response), depends on the residual bioavailability of oxygen in epidermal keratinocytes after brachial artery occlusion. Therefore, the outcomes of our study confirmed that decreased oxygenation of peripheral tissues may be associated with diminished intracellular oxygen delivery through the circulatory system due to dysregulation of metabolic processes in erythrocytes. This may partly explain long COVID-specific symptoms such as physical impairment and fatigue. In line with that, we have found a decreased log(HS) parameter in post-COVID-19 patients, which mirrors a disturbed microcirculatory response to hypoxia. In another study, low HS values were related to a more severe course of COVID-19, suggesting that this parameter is a prognostic factor of the disease [32]. Interestingly, in this study, log(HS) negatively correlated with the RBC ADP/AMP ratio.

In addition, we revealed a decreased RHR parameter in long COVID patients, reflecting reduced nitric oxide production in the vasculature [22]. This observation is also in line with some other studies [18,33]. Disturbed tissue perfusion may result from both impaired oxygen transport by erythrocytes and dysfunction of the microvascular endothelium. Mounting evidence links SARS-CoV-2 infection with endothelial dysfunction, which has been recognized by reduced nitric oxide bioavailability, oxidative stress, leukocyte adhesion, hyperpermeability, glycocalyx disruption, endothelial-to-mesenchymal transition, hypercoagulability and thrombosis [34]. However, it has been described that COVID-19-induced endotheliitis is predominately a systemic microvessel vasculitis not involving the large arteries such as the main coronaries [35]. In our long COVID patients with typical chest pain and the evidence of ischemia in non-invasive tests, no significant changes were revealed by invasive coronary angiography or cardiac computed tomography angiography. We defined it as a microvascular angina-like phenomenon. In addition, cardiac troponin levels were within reference ranges, as we did not observe any patients during acute coronary syndrome. On the other hand, it should be noted that microvascular and endothelial dysfunction can manifest the autonomic dysfunction in long COVID syndrome, with local symptoms such as headache, brain fog, chest pain, the microvascular angina-like phenomenon, dyspnea and peripheral circulatory symptoms, including skin discoloration, oedema or Raynaud-like phenomena [36]. It is well documented that cardiovascular autonomic dysfunction occurs from a malfunction of the autonomic control of the circulation, and can involve failure or inadequate or excessive activation of the sympathetic and parasympathetic components of the autonomic nervous system [36].

It should be emphasized that beyond the fundamental role of erythrocytes in oxygen transport, RBCs are also critical modulators of endothelial and microvascular function via controlled ATP release [37]. When the mechanisms of ATP synthesis and release function properly, ATP exported from RBCs subserves efficient blood flow, including vasodilation in proportion to the degree of hypoxia, the inhibition of intercellular adhesion and the prevention of unwanted capillary permeability [38]. In our study, low RHR, reflecting

lower vascular NO production in long COVID patients, was associated with an increase in ADP concentration in RBCs and a decrease in the ATP/ADP ratio. This is consistent with reports on the stimulation of the first ATP-consuming glycolytic reactions in COVID-19 RBCs. However, it is not known whether this translates into the deregulated release of ATP from erythrocytes. It has been shown that RBC-induced NO-associated vasodilation under hypoxic conditions is the effect of ATP release from RBCs and its interaction via purinergic receptors to stimulate the synthesis of NO by endothelial NO synthase (eNOS) [39]. However, our study shows a positive relationship between the decrease in the serum arginine/ADMA ratio in long COVID patients and the ATP concentration in erythrocytes. This suggests disturbances in ATP release and/or its purinergic signaling cascade. Indeed, recent studies have revealed that long COVID RBCs demonstrated a damaged cell membrane, in particular through the oxidation of band 3 and binding with S1 spike proteins [16,40]. These alterations to band 3 can lead to significant disturbances in RBC functions, including the ATP release mechanism [41]. In such cases, hypoxia seen in COVID-19 patients is related to SARS-CoV-2-mediated band 3 alterations, which may decrease the ability of RBCs to release ATP, reducing vasodilation and oxygen delivery to tissues.

5. Conclusions

In this study, we revealed that many parameters related to the endothelial and microvascular function showed significant correlations with red blood cell adenine nucleotide concentration in patients with long COVID. A particularly strong relationship was observed between adenosine triphosphate concentration in erythrocytes and the serum ratio of arginine to asymmetric dimethylarginine—an indicator of endothelial function. In line with that, a positive correlation was observed between the ATP/ADP ratio and diminished reactive hyperemic response in long COVID patients, assessed by the flow-mediated skin fluorescence (FMSF) technique, which reflected decreased vascular nitric oxide bioavailability. In addition, we have shown that patients with long COVID have significantly impaired ischemic response parameters, examined by FMSF, which revealed diminished residual bioavailability of oxygen in epidermal keratinocytes after brachial artery occlusion. Taken together, this study indicates that the dysregulation of metabolic processes in erythrocytes that coexists with endothelial and microcirculatory dysfunction is associated with diminished intracellular oxygen delivery, which can explain long COVID cardiovascular complications, physical impairment and fatigue. In addition, functional assessment of the degree of skin tissue oxygenation using FMSF turned out to be a sensitive method to track the changes in hypoxia occurring after COVID-19. Further studies using this methodology, as well as large-scale analyses of erythrocyte energy metabolism in a larger group of patients, should be also performed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines12030554/s1, Table S1. Dates of COVID-19 diagnosis, peripheral blood sample collection and FMSF testing in recruited patients. F—female. M—Male. Table S2. Correlations of red blood cell adenosine diphosphate (ADP), adenosine monophosphate (AMP) and total adenine nucleotide (TAN) concentration with L-arginine/ADMA (asymmetric dimethyl-L-arginine) ratio and symmetric dimethyl L-arginine (SDMA) concentration in long COVID participants. Results are shown as Pearson correlation coefficient (r) and *p* value (*p*). Table S3. Correlations of red blood cell adenine nucleotide ratios and adenylate energy charge (AEC) with peripheral blood cell count in long COVID participants. Results are shown as Pearson correlation coefficient (r) and *p* value (*p*). Table S4. Correlations of red blood cell adenine nucleotide ratios and adenylate energy charge (AEC) with peripheral blood cell count in long COVID participants. Results are shown as Pearson correlation coefficient (r) and *p* value (*p*). Table S5. Correlations of red blood cell adenosine diphosphate (ADP), adenosine monophosphate (AMP) and total adenine nucleotide (TAN) concentration with peripheral blood cell count in long COVID participants. Results are shown as Pearson correlation coefficient (r) and *p* value (*p*).

Author Contributions: Conceptualization, M.H. and B.K.-Z.; methodology, M.R.-K., A.J., A.B., M.H. and B.K.-Z.; software, M.R.-K., K.S., A.J. and B.K.-Z.; validation, M.R.-K., A.J., A.B. and B.K.-Z.; formal analysis, M.R.-K., A.J., A.B. and B.K.-Z.; investigation, M.R.-K., A.J., K.S. and B.K.-Z.; resources, M.R.-K., M.F., O.K., G.H. and B.K.-Z.; data curation, M.R.-K., A.J. and A.B.; writing—original draft preparation, M.R.-K. and B.K.-Z.; writing—review and editing, R.T.S., M.H. and B.K.-Z.; visualization, M.R.-K., A.J., A.B. and B.K.-Z.; supervision, M.H. and B.K.-Z.; project administration, B.K.-Z.; funding acquisition, B.K.-Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Science Centre of Poland, grant number 2019/35/D/NZ3/03512.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk, Poland, under the license number NKBBN/55/2021 (approval date: 3 February 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Touma, M. COVID-19: Molecular Diagnostics Overview. *J. Mol. Med.* **2020**, *98*, 947. [CrossRef]
2. Staffolani, S.; Iencinella, V.; Cimatti, M.; Tavio, M. Long COVID-19 Syndrome as a Fourth Phase of SARS-CoV-2 Infection. *Infez. Med.* **2022**, *30*, 22–29. [CrossRef]
3. Østergaard, L. SARS CoV-2 Related Microvascular Damage and Symptoms during and after COVID-19: Consequences of Capillary Transit-Time Changes, Tissue Hypoxia and Inflammation. *Physiol. Rep.* **2021**, *9*, e14726. [CrossRef]
4. Böning, D.; Kuebler, W.M.; Bloch, W. The Oxygen Dissociation Curve of Blood in COVID-19. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2021**, *321*, L349–L357. [CrossRef] [PubMed]
5. Gérard, D.; Ben Brahim, S.; Lesesve, J.F.; Perrin, J. Are Mushroom-Shaped Erythrocytes an Indicator of COVID-19? *Br. J. Haematol.* **2021**, *192*, 230. [CrossRef] [PubMed]
6. Gagiannis, D.; Umathum, V.G.; Bloch, W.; Rother, C.; Stahl, M.; Witte, H.M.; Djudjaj, S.; Boor, P.; Steinestel, K. Antemortem vs Postmortem Histopathologic and Ultrastructural Findings in Paired Transbronchial Biopsy Specimens and Lung Autopsy Samples from Three Patients with Confirmed SARS-CoV-2. *Am. J. Clin. Pathol.* **2022**, *157*, 54–63. [CrossRef]
7. Renoux, C.; Fort, R.; Nader, E.; Boisson, C.; Joly, P.; Stauffer, E.; Robert, M.; Girard, S.; Cibiel, A.; Gauthier, A.; et al. Impact of COVID-19 on Red Blood Cell Rheology. *Br. J. Haematol.* **2021**, *192*, e108–e111. [CrossRef] [PubMed]
8. Grau, M.; Ibershoff, L.; Zacher, J.; Bros, J.; Tomschi, F.; Diebold, K.F.; Predel, H.G.; Bloch, W. Even Patients with Mild COVID-19 Symptoms after SARS-CoV-2 Infection Show Prolonged Altered Red Blood Cell Morphology and Rheological Parameters. *J. Cell. Mol. Med.* **2022**, *26*, 3022–3030. [CrossRef]
9. Dudzinska, W.; Hlynczak, A.J.; Skotnicka, E.; Suska, M. The Purine Metabolism of Human Erythrocytes. *Biochemistry* **2006**, *71*, 467–475. [CrossRef]
10. Van Wijk, R.; Van Solinge, W.W. The Energy-Less Red Blood Cell Is Lost: Erythrocyte Enzyme Abnormalities of Glycolysis. *Blood* **2005**, *106*, 4034–4042. [CrossRef]
11. McMahon, T.J.; Darrow, C.C.; Hoehn, B.A.; Zhu, H. Generation and Export of Red Blood Cell ATP in Health and Disease. *Front. Physiol.* **2021**, *12*, 754638. [CrossRef] [PubMed]
12. Racine, M.L.; Dinenno, F.A. Reduced Deformability Contributes to Impaired Deoxygenation-Induced ATP Release from Red Blood Cells of Older Adult Humans. *J. Physiol.* **2019**, *597*, 4503–4519. [CrossRef] [PubMed]
13. Dudzinska, W.; Suska, M.; Lubkowska, A.; Jakubowska, K.; Olszewska, M.; Safranow, K.; Chlubek, D. Comparison of Human Erythrocyte Purine Nucleotide Metabolism and Blood Purine and Pyrimidine Degradation Product Concentrations before and after Acute Exercise in Trained and Sedentary Subjects. *J. Physiol. Sci.* **2018**, *68*, 293–305. [CrossRef]
14. Kosenko, E.A.; Tikhonova, L.A.; Montoliu, C.; Barreto, G.E.; Aliev, G.; Kaminsky, Y.G. Metabolic Abnormalities of Erythrocytes as a Risk Factor for Alzheimer's Disease. *Front. Neurosci.* **2018**, *11*, 728. [CrossRef]
15. Papandreou, C.; Li, J.; Liang, L.; Bulló, M.; Zheng, Y.; Ruiz-Canela, M.; Yu, E.; Guasch-Ferré, M.; Razquin, C.; Clish, C.; et al. Metabolites Related to Purine Catabolism and Risk of Type 2 Diabetes Incidence; Modifying Effects of the TCF7L2-Rs7903146 Polymorphism. *Sci. Rep.* **2019**, *9*, 2892. [CrossRef] [PubMed]
16. Thomas, T.; Stefanoni, D.; Dzieciatkowska, M.; Issaian, A.; Nemkov, T.; Hill, R.C.; Francis, R.O.; Hudson, K.E.; Buehler, P.W.; Zimring, J.C.; et al. Evidence of Structural Protein Damage and Membrane Lipid Remodeling in Red Blood Cells from COVID-19 Patients. *J. Proteome Res.* **2020**, *19*, 4455–4469. [CrossRef]

17. Kutryb-Zajac, B.; Kawecka, A.; Caratis, F.; Urbanowicz, K.; Braczko, A.; Furihata, T.; Karaszewski, B.; Smolenski, R.T.; Rutkowska, A. The Impaired Distribution of Adenosine Deaminase Isoenzymes in Multiple Sclerosis Plasma and Cerebrospinal Fluid. *Front. Mol. Neurosci.* **2022**, *15*, 998023. [CrossRef] [PubMed]
18. Jedrzejewska, A.; Kawecka, A.; Braczko, A.; Romanowska-Kocejko, M.; Stawarska, K.; Deptuła, M.; Zawrzykraj, M.; Franczak, M.; Krol, O.; Harasim, G.; et al. Changes in Adenosine Deaminase Activity and Endothelial Dysfunction after Mild Coronavirus Disease-2019. *Int. J. Mol. Sci.* **2023**, *24*, 13140. [CrossRef]
19. Olkowicz, M.; Debski, J.; Jablonska, P.; Dadlez, M.; Smolenski, R.T. Application of a New Procedure for Liquid Chromatography/Mass Spectrometry Profiling of Plasma Amino Acid-Related Metabolites and Untargeted Shotgun Proteomics to Identify Mechanisms and Biomarkers of Calcific Aortic Stenosis. *J. Chromatogr. A* **2017**, *1517*, 66–78. [CrossRef]
20. Tarnawska, M.; Dorniak, K.; Kaszubowski, M.; Dudziak, M.; Hellmann, M. A Pilot Study with Flow Mediated Skin Fluorescence: A Novel Device to Assess Microvascular Endothelial Function in Coronary Artery Disease. *Cardiol. J.* **2018**, *25*, 120–127. [CrossRef]
21. Romanowska-Kocejko, M.; Dudziak, M.; Hellmann, M. Nicotinamide Adenine Dinucleotide Fluorescence to Assess Microvascular Disturbances in Post-COVID-19 Patients. *Cardiol. J.* **2022**, *29*, 154–156. [CrossRef]
22. Katarzynska, J.; Zielinski, J.; Marcinek, A.; Gebicki, J. New Approach to Non-Invasive Assessment of Vascular Circulation Based on the Response to Transient Ischemia. *Vasc. Health Risk Manag.* **2022**, *18*, 113–116. [CrossRef]
23. Li, C.Y. Can Glycine Mitigate COVID-19 Associated Tissue Damage and Cytokine Storm? *Radiat. Res.* **2020**, *194*, 199. [CrossRef]
24. Toori, K.U.; Qureshi, M.A.; Chaudhry, A.; Safdar, M.F. Neutrophil to Lymphocyte Ratio (NLR) in COVID-19: A Cheap Prognostic Marker in a Resource Constraint Setting. *Pak. J. Med. Sci.* **2021**, *37*, 1435. [CrossRef]
25. Fisher, L.; Goka, B.; Pater, J.; Chang, F.-L. Neutrophil-to-Lymphocyte Ratio (NLR) to Monitor Neuroinflammation Status During Long COVID. *Proc. IMPRS* **2023**, *6*, 1. [CrossRef]
26. Jensen, F.B. The Dual Roles of Red Blood Cells in Tissue Oxygen Delivery: Oxygen Carriers and Regulators of Local Blood Flow. *J. Exp. Biol.* **2009**, *212*, 3387–3393. [CrossRef]
27. Płoszczyca, K.; Czuba, M.; Chalimoniuk, M.; Gajda, R.; Baranowski, M. Red Blood Cell 2,3-Diphosphoglycerate Decreases in Response to a 30 Km Time Trial Under Hypoxia in Cyclists. *Front. Physiol.* **2021**, *12*, 670977. [CrossRef]
28. McMahon, T.J. Red Blood Cell Deformability, Vasoactive Mediators, and Adhesion. *Front. Physiol.* **2019**, *10*, 1417. [CrossRef] [PubMed]
29. Grygorczyk, R.; Orlov, S.N. Effects of Hypoxia on Erythrocyte Membrane Properties—Implications for Intravascular Hemolysis and Purinergic Control of Blood Flow. *Front. Physiol.* **2017**, *8*, 1110. [CrossRef] [PubMed]
30. Kosenko, E.; Tikhonova, L.; Alilova, G.; Montoliu, C. Erythrocytes Functionality in SARS-CoV-2 Infection: Potential Link with Alzheimer's Disease. *Int. J. Mol. Sci.* **2023**, *24*, 5739. [CrossRef] [PubMed]
31. Marcinek, A.; Katarzynska, J.; Sieron, L.; Skokowski, R.; Zielinski, J.; Gebicki, J. Non-Invasive Assessment of Vascular Circulation Based on Flow Mediated Skin Fluorescence (FMSF). *Biology* **2023**, *12*, 385. [CrossRef]
32. Gebicki, J.; Katarzynska, J.; Marcinek, A. Can Efficient Stimulation of Myogenic Microcirculatory Oscillations by Transient Ischemia Predict Low Incidence of COVID-19 Infection? *Respir. Physiol. Neurobiol.* **2021**, *286*, 103618. [CrossRef] [PubMed]
33. Chudzik, M.; Cender, A.; Mordaka, R.; Zielinski, J.; Katarzynska, J.; Marcinek, A.; Gebicki, J. Chronic Fatigue Associated with Post-COVID Syndrome versus Transient Fatigue Caused by High-Intensity Exercise: Are They Comparable in Terms of Vascular Effects? *Vasc. Health Risk Manag.* **2022**, *18*, 711–719. [CrossRef] [PubMed]
34. Xu, S.; Ilyas, I.; Weng, J. Endothelial Dysfunction in COVID-19: An Overview of Evidence, Biomarkers, Mechanisms and Potential Therapies. *Acta Pharmacol. Sin.* **2023**, *44*, 695–709. [CrossRef] [PubMed]
35. Maccio, U.; Zinkernagel, A.S.; Shambat, S.M.; Zeng, X.; Cathomas, G.; Ruschitzka, F.; Schuepbach, R.A.; Moch, H.; Varga, Z. SARS-CoV-2 Leads to a Small Vessel Endotheliitis in the Heart. *EBioMedicine* **2021**, *63*, 103182. [CrossRef] [PubMed]
36. Fedorowski, A.; Fanciulli, A.; Raj, S.R.; Sheldon, R.; Shibao, C.A.; Sutton, R. Cardiovascular Autonomic Dysfunction in Post-COVID-19 Syndrome: A Major Health-Care Burden. *Nat. Rev. Cardiol.* **2024**. [CrossRef] [PubMed]
37. Richardson, K.J.; Kuck, L.; Michael, J.S. Beyond Oxygen Transport: Active Role of Erythrocytes in the Regulation of Blood Flow. *Am. J. Physiol. Heart Circ. Physiol.* **2020**, *319*, H866–H872. [CrossRef]
38. Kirby, B.S.; Sparks, M.A.; Lazarowski, E.R.; Lopez Domowicz, D.A.; Zhu, H.; McMahon, T.J. Pannexin 1 Channels Control the Hemodynamic Response to Hypoxia by Regulating O2-Sensitive Extracellular ATP in Blood. *Am. J. Physiol. Heart Circ. Physiol.* **2021**, *320*, H1055–H1065. [CrossRef]
39. Da Silva, C.G.; Specht, A.; Wegiel, B.; Ferran, C.; Kaczmarek, E. Mechanism of Purinergic Activation of Endothelial Nitric Oxide Synthase in Endothelial Cells. *Circulation* **2009**, *119*, 871–879. [CrossRef]
40. Cosic, I.; Cosic, D.; Loncarevic, I. RRM Prediction of Erythrocyte Band3 Protein as Alternative Receptor for SARS-CoV-2 Virus. *Appl. Sci.* **2020**, *10*, 4053. [CrossRef]
41. Misiti, F. SARS-CoV-2 Infection and Red Blood Cells: Implications for Long Term Symptoms during Exercise. *Sports Med. Health Sci.* **2021**, *3*, 181–182. [CrossRef] [PubMed]

biomedicines

MDPI

Article

Pituitary–Adrenal Axis and Peripheral Immune Cell Profile in Long COVID

Jaume Alijotas-Reig [1,2,3,*], Ariadna Anunciacion-Llunell [1], Enrique Esteve-Valverde [3,4], Stephanie Morales-Pérez [5], Sergio Rivero-Santana [5], Jaume Trapé [6,7], Laura González-García [6,7], Domingo Ruiz [5], Joana Marques-Soares [1,2,3] and Francesc Miro-Mur [1,*]

1 Systemic Autoimmune Diseases Research Unit, Vall d'Hebron Institut de Recerca (VHIR), 08035 Barcelona, Spain; ariadna.anunciacion@vhir.org (A.A.-L.); joana.marquessoares@vallhebron.cat (J.M.-S.)
2 Systemic Autoimmune Diseases Unit, Department of Internal Medicine, Hospital Universitari Vall d'Hebron (HUVH), 08035 Barcelona, Spain
3 Department of Medicine, Faculty of Medicine, Universitat Autònoma de Barcelona (UAB), 08035 Barcelona, Spain; doctor.esteve@gmail.com
4 Systemic Autoimmune Diseases Unit, Department of Internal Medicine, Hospital Universitari Parc Taulí, 08208 Sabadell, Spain
5 Systemic Autoimmune Disease Unit, Internal Medicine Department, Althaia Healthcare University Network of Manresa, 08243 Manresa, Spain; emoralesp@althaia.cat (S.M.-P.); srivero@althaia.cat (S.R.-S.); druizh@altahaia.cat (D.R.)
6 Laboratory Medicine, Althaia Healthcare University Network of Manresa, 08243 Manresa, Spain; jtrape@althaia.cat (J.T.); lgonzalezg@althaia.cat (L.G.-G.)
7 Tissue Repair and Regeneration Laboratory (TR2Lab), Institut de Recerca i Innovació en Ciències de la Vida i de la Salut a la Catalunya Central (IrisCC), 08500 Barcelona, Spain
* Correspondence: jaume.alijotas@vallhebron.cat (J.A.-R.); francesc.miro@vhir.org (F.M.-M.); Tel.: +34-934894194 (J.A.-R.); +34-934894047 (F.M.-M.)

Citation: Alijotas-Reig, J.; Anunciacion-Llunell, A.; Esteve-Valverde, E.; Morales-Pérez, S.; Rivero-Santana, S.; Trapé, J.; González-García, L.; Ruiz, D.; Marques-Soares, J.; Miro-Mur, F. Pituitary–Adrenal Axis and Peripheral Immune Cell Profile in Long COVID. *Biomedicines* **2024**, *12*, 581. https://doi.org/10.3390/biomedicines12030581

Academic Editors: César Fernández De Las Peñas and Toshihiro Kita

Received: 30 January 2024
Revised: 29 February 2024
Accepted: 4 March 2024
Published: 5 March 2024

Abstract: In Long COVID, dysfunction in the pituitary–adrenal axis and alterations in immune cells and inflammatory status are warned against. We performed a prospective study in a cohort of 42 patients who suffered COVID-19 at least 6 months before attending the Long COVID unit at Althaia Hospital. Based on Post-COVID Functional Status, 29 patients were diagnosed with Long COVID, while 13 were deemed as recovered. The hormones of the pituitary–adrenal axis, adrenocorticotropin stimulation test, and immune cell profiles and inflammatory markers were examined. Patients with Long COVID had significantly lower EuroQol and higher mMRC scores compared to the recovered individuals. Their symptoms included fatigue, myalgia, arthralgia, persistent coughing, a persistent sore throat, dyspnoea, a lack of concentration, and anxiety. We observed the physiological levels of cortisol and adrenocorticotropin in individuals with or without Long COVID. The results of the adrenocorticotropin stimulation test were similar between both groups. The absolute number of neutrophils was lower in the Long COVID patients compared to recovered individuals ($p < 0.05$). The total count of B lymphocytes remained consistent, but Long COVID patients had a higher percentage of mature B cells compared to recovered participants ($p < 0.05$) and exhibited a higher percentage of circulating resident memory CD8+ T cells ($p < 0.05$) and Treg-expressing exonucleases ($p < 0.05$). Our findings did not identify adrenal dysfunction related to Long COVID, nor an association between adrenal function and clinical symptoms. The data indicated a dysregulation in certain immune cells, pointing to immune activation. No overt hyperinflammation was observed in the Long COVID group.

Keywords: Long COVID; fatigue; dyspnea; cortisol; adrenal insufficiency; immune cells; inflammation

1. Introduction

Individuals infected by SARS-CoV-2 may experience a constellation of long-lasting symptoms, including fatigue, myalgia, a sore throat, dyspnea, and coughing, with others

including nervous system and neurocognitive disorders [1–3]. Similar long-lasting features were also observed in the SARS epidemic in 2003; one study reported that 17% of individuals infected with SARS-CoV-1 experienced long-term health issues one year after the infection [4], while others identified that symptoms resembling those of fibromyalgia were observed three years post-infection [5]. Researchers describe that 38% of SARS survivors encountered reduced lung oxygen flow 15 years after the initial infection [6]. During the recent pandemic, a group of COVID-19 survivors has been observed to battle with such long-term consequences [7]. Initially, physicians did not readily associate them with post-COVID-19 effects [8]. Despite uncertainties regarding attributing some cases to SARS-CoV-2 infection, recent data highlight the secondary effects of coronavirus infection, referring to them as Long COVID, persistent COVID, or post-acute COVID. In both the peer-reviewed literature [9] and public discussion [10], persistent symptoms have been reported among COVID-19 survivors, including those who initially experienced a mild acute illness [11–14]. Indeed, a study of Israeli healthcare workers underscored the Long-COVID risk following a breakthrough infection, even in fully vaccinated people [15]. These studies were conducted before the emergence of the dominant Omicron variants, which have been observed to decrease post-acute COVID symptoms [16]. Subsequently, treatment with Paxlovid in the acute phase of the infection has also been shown to be effective in reducing the risk of suffering post-acute COVID, regardless of whether patients were previously vaccinated or not [17]. Although vaccination before infection confers partial protection against Long COVID [18], the understanding that COVID-19 may extend beyond a transient respiratory disease and manifest as neurological and physical symptoms months after the initial infection, thereby increasing the overall burden of the disease, is gaining recognition among physicians [19].

The need to assess the multidimensional impact of certain conditions, including COVID-19, on patient health and quality of life using standardized scales is frequently established. Tools such as the Modified Medical Research Council (mMRC) Dyspnea Scale and the EuroQol (EQ)-5 Dimension (5D) or visual analogue scale (VAS) have been applied in previous studies to quantify symptom severity and impact on daily living, capturing the subtleties of the disease's long-term effects, which may elude more immediate clinical assessments [20–22]. This nuanced understanding of post-infection symptoms is especially pertinent as we consider demographic vulnerabilities. While older patients may be at a higher risk for severe disease and death, younger survivors have also reported persistent symptoms weeks or months after acute illness [23].

Efforts to characterise the aetiology and pathophysiology of the late sequelae are ongoing and may reveal organ damage sustained during the acute infection phase [24]. This post-acute viral phase, during which individuals test negative for SARS-CoV-2, is sometimes persistent and is hypothesised to be associated with the residual or mild hypercytokinemia or dysfunction of the neuro-suprarenal axis, with accompanying subclinical adrenal hypofunction [25,26]. The impairment of the autonomic nervous system through disruption of tryptophan metabolism cannot be excluded [27,28]. This situation may or may not ease over time. However, in some patients, fatigue and other non-specific symptoms may last for more than six months, leading to the case being designated as viral chronic fatigue syndrome (ME/CFS). ME/CFS is a complex and poorly understood condition. Some studies evidenced a clear increased risk of developing ME/CFS in people who have had COVID-19 compared with those who have not [2,29]. Although many people first exhibit symptoms following a viral infection, the exact causes of this syndrome remain to be elucidated. One potential cause could be mitochondrial dysfunction [30]. Some immune cells in ME/CFS patients, particularly CD8+ T cells, show disruptions in energy production and use [31]. Additionally, both CD8+ and CD4+ T cells from ME/CFS demonstrate reduced glycolysis after activation. This diminished metabolism is inversely correlated with inflammatory cytokines in CD8+ T cells in patients affected by ME/CFS [31]. Severe COVID-19 may also induce long-term changes in the innate immune system through epigenetic modifications [32].

This study explored the premise that Long COVID symptoms are linked to pituitary–adrenal axis disruption, concomitant with a hyperinflammatory state and immune dysregulation. This hypothesis was assessed in a cohort of patients who had tested positive for SARS-CoV-2 at least six months prior to the enrolment. This work ultimately aims to contribute to the ongoing efforts to understand the underlying causes and mechanisms of Long COVID, potentially guiding more tailored management strategies for affected individuals.

2. Materials and Methods

2.1. Inclusion Criteria for Patients

Patients attending the Long COVID unit consultation at "Fundació Althaia Xarxa Assistencial" in Manresa (Barcelona, Spain) were invited to participate in this study, which was approved by the local ethics committee (CEI 21/82) and conducted according to the Declaration of Helsinki. All participants provided signed informed consent. Inclusion criteria included being between 18 and 70 years old and recording a positive PCR test for SARS-CoV-2 infection more than six months prior. Three expert clinicians (EEV, SMP, and SRS) from the Long COVID unit employed visual and verbal screening based on Post-COVID Functional Status (PCFS) [33] to determine if patients could be classified as having Long COVID. Enrolled patients were asked to answer PCFS questions regarding symptoms, pain, depression, anxiety, and their ability to perform household duties or activities independently. Those responding affirmatively were classified as having Long COVID. None of the enrolled patients indicated being unable to live alone without assistance. Blood samples collected for clinical management were analysed to assess leukocyte populations via flow cytometry, and basic blood tests and standard biochemistry parameters were also examined. None of the enrolled participants were treated with glucocorticoids during the Long COVID phase of the study. Demographic, clinical, and laboratory data were compiled in a database and stored in our institutional repository.

2.2. Health-Related Quality-of-Life Tests

The standardised health-related quality-of-life instrument EQ-5D assessed individuals' overall health status. This generic tool quantitatively measures a person's health and well-being, allowing for comparisons across different health conditions and populations. The EQ-5D comprises five health dimensions, namely, mobility, self-care, usual activities, pain/discomfort, and anxiety/depression, with each dimension having three levels (1 = no problem, 2 = moderate problems, and 3 = severe problems). In our Catalan cohort of patients, EQ-5D was assessed by using coefficients reported by [34], yielding an EQ-5D score ranging from 0 (worst health state) to 1 (best health state).

The mMRC dyspnoea scale was used to assess the severity of breathlessness, which is common in Long COVID patients. This scale uses a patient-reported measure of the impairment of daily life caused by breathlessness, which can range from minor discomfort to a factor that severely limits daily life. The scale ranges from grade 0, where patients experience no breathlessness, even with regular physical activity, to grade 4, where they report severe breathlessness that restricts them in their home or makes them short of breath when getting dressed or undressed. The intermediate stages are defined as follows: Grade 1, mild breathlessness during physical activity; Grade 2, shortness of breath when walking at an average pace; and Grade 3, having to pause for breath after walking for a few minutes.

As part of the assessment of the wide-ranging impact of the condition, the Long COVID Score (LCS) was formulated as LCS = mMRC/α + absolute value [log(EQ-5D + ε)], where the scaling factor $\alpha = 4$. This balances the quantitative assessment of breathlessness with the multi-faceted evaluation of overall health and well-being, thus taking into account a broader range of health-related quality-of-life factors than just the respiratory system. The smoothing coefficient $\varepsilon = 0.0758$ is employed to guarantee that the resulting EQ-5D value is greater than 0 and allows for logarithm calculation.

2.3. Adrenal Function

Serum cortisol and adrenocorticotropin (ACTH) levels were assessed at baseline from blood samples drawn at 8:00 in the morning. The ACTH stimulation test was performed immediately via intramuscular injection of 0.25 mg of synthetic ACTH (Cigna Healthcare, Nashville, TN, USA) mixed with 2 mL of 0.9% sodium chloride. Blood was taken 60 min after ACTH injection. Cortisol levels in the serum were promptly assessed using an electrochemiluminescence immunoassay (ECLIA) with the Elecsys Cortisol II (Roche Diagnostics, Risch-Rotkreuz, Switzerland) in the Cobas e801 system (Roche Diagnostics). Basal serum ACTH levels were also determined via ECLIA with Elecsys ACTH (Roche Diagnostics) on the Cobas e801 system (Roche Diagnostics). A Δcortisol value represents the net increase in cortisol levels at 60 min post-ACTH injection, expressed as ΔCortisol = Cortisol $60' -$ Basal cortisol.

2.4. Flow Cytometry

Blood samples drawn with EDTA for laboratory analysis were used to stain cells for flow cytometry. A 30-microliter aliquot of whole blood was stained with a 1:1 (v/v) mix of fluorescently conjugated antibodies to decipher leukocyte populations. This was performed in the presence of a human FcR blocking reagent (dilution 1:30; Milteny Biotech, Bergisch Gladbach, Germany) and Aqua Live/Dead cell fixable dye (1:1000; Molecular Probes, Eugene, OR, USA) to discard dead cells. The antibodies, used at dilution of 1:200, were anti-CD45-BV786 (HI30; BD Biosciences, Bergen, NJ, USA), anti-CD3-BB515 (UCHT1; BD Biosciences), anti-CD19-BV605 (HIB19; BioLegend, San Diego, CA, USA), anti-CD56-PE/Cy5 (B159; BD Biosciences), anti-γδTCR-PE (B1; BioLegend), anti-CD14-PE/CF594 (MfP9; BD Biosciences), anti-CD66b-APC/Cy7 (G10F5; BioLegend), anti-CD4-APC/R700 (RPA-T4; BD Biosciences), anti-CD8-BV650 (RPA-T8; BioLegend), anti-CD27-AF647 (M-T271; BioLegend), anti-CD45RA-PerCP/eF710 (GRT22; eBioscience, San Diego, CA, USA), anti-CD103-BV421 (Ber-ACT8; BioLegend), anti-αβTCR-BV650 (IP26; BD Biosciences), anti-CD73-BB515 (AD2; BD Biosciences), anti-CD38-PerCP/eF710 (HB7; eBioscience), anti-CD39-PE/CF594 (TU66; BD Biosciences), anti-HLA-DR-PE/Vio770 (REA805; Miltenyi Biotec), anti-CD25-AF647 (BC96; BioLegend), and anti-CD69-APC/Vio770 (REA824; Miltenyi Biotec). The antibody mixture was prepared in phosphate-buffered saline (PBS) containing 5 mM EDTA and 0.1% BSA (FACS buffer), along with a 1:10 (v/v) brilliant stain buffer (BD Biosciences). To determine the absolute cell number, 30 microliters of fluorescent beads (1000 beads/microliter; Beckman Coulter, Brea, CA, USA) were added to each tube. After a 30 min staining period, cells were fixed in 2% paraformaldehyde FACS lysing buffer (BD Biosciences) and washed with FACS buffer. Cellular data were acquired in a FACS LSRII (BD Biosciences) flow cytometer operated with FACS Diva software (BD Biosciences) and was analysed with FlowJo v10.6.0 (FlowJo-BD, Ashland, OR, USA).

2.5. Cytokine Assessment

Serum samples taken from participants were preserved at -80 °C and analysed collectively in a single batch. Concentrations of cytokines, including tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-12p40, IL-12p70, IL-1β, IL-2, and IL-10, were assessed using the ProQuantum high-sensitivity immunoassay (Thermo Fisher Scientific, Waltham, MA, USA). This analysis was conducted on the QuantStudio 5 qPCR instrument (Thermo Fisher) utilising the ProQuantumTM Protein Biology software (Thermo Fisher). IL-6 serum concentration was measured via ECLIA with the Elecsys IL-6 reagent (Roche Diagnostics) on the Cobas e801 system (Roche Diagnostics). GDF-8 or myostatin was determined using an enzyme-linked immunosorbent assay (ELISA, Helsinki, Finland) kit (EH215RB, Invitrogen, Waltham, MA, USA), with readings taken on the Quanta-Lyser-2 plate reader (Werfen, San Diego, CA, USA).

2.6. Statistical Analysis

The Mann–Whitney U test, chi-square test, and Fisher's exact were employed to compare differences between participants with or without Long COVID concerning quantitative and categorical variables, respectively. Continuous variables were expressed as median [interquartile range (IQR)], while categorical variables were presented as *n* (%). Two-way ANOVA was used to test for differences between groups, categorised by Long COVID status and sex. A two-sided α level of less than 0.05 was considered statistically significant. Statistical analysis was performed using the Rstats package in R software (v4.3.2).

3. Results

3.1. Long COVID Assessment

A total of 42 patients were enrolled for the study of Long COVID. The dates of their SARS-CoV-2 infection spanned from December 2020 to July 2021. These enrolled patients were part of the third wave of SARS-CoV-2 infections in Catalonia, Spain [35], which was attributed to an outbreak of the B.1.1.7 alpha variant [36] in this area.

The assessment of Long COVID took place, on average, 286 days after the primary infection, with a range of 217 to 346 days. Of the 42 enrolled individuals, 29 (69.05%) were diagnosed with Long COVID, while 13 (30.95%) were classified as fully recovered (Table 1). The time lapse between primary virus infection and the Long COVID assessment visit was comparable for individuals with Long COVID and those without Long COVID (268 [57] days vs. 303 [44.5] days, *p*-value = 0.0645).

Table 1. Comparison of demographic, preclinical, and acute phase data between Long COVID and recovered patients.

	Long COVID		
Variables	**No (13)**	**Yes (29)**	***p*-Value** [1]
Age (years)	50 [8]	53 [18]	0.64
Gender (female)	6 (46.15)	17 (58.62)	0.52
HTA	1 (7.69)	5 (17.24)	0.65
Dyslipidaemia	1 (7.69)	5 (17.24)	0.65
DM2	0 (0)	5 (17.24)	0.30
Previous smoker	3 (23.08)	5 (17.24)	0.69
BMI > 30	1 (7.69)	2 (6.9)	1.00
Lung disease	1 (7.69)	1 (3.45)	0.53
AIDs	0 (0)	2 (6.9)	1.00
Chronic fatigue	0 (0)	1 (3.45)	1.00
Fibromyalgia	0 (0)	1 (3.45)	1.00
Chemical sensitivity	0 (0)	1 (3.45)	1.00
Complications in acute phase			
Hospitalization	7 (53.85)	17 (58.62)	1.00
Bacterial lung coinfection	2 (15.38)	1 (3.45)	0.22
Respiratory bacterial sepsis	1 (7.69)	1 (3.45)	0.53
Other bacterial infection	0 (0)	1 (3.57)	1.00
Pulmonary thromboembolism	1 (7.69)	2 (6.9)	1.00
Treatment			
No treatment	0 (0)	2 (6.9)	1.00
Paracetamol	1 (7.69)	3 (10.34)	1.00
NSAIDs	1 (7.69)	7 (24.14)	0.40
Dexamethasone	7 (53.85)	16 (55.17)	1.00
Heparin	7 (53.85)	17 (58.62)	1.00
Tociluzumab	1 (7.69)	7 (24.14)	0.40
Remdesivir	0 (0)	2 (6.9)	1.00
Antibiotics	2 (15.38)	2 (6.9)	0.58

Data are *n* (%), but age is median [interquartile range (IQR)]. HTA, hypertension $\geq$ 160 mm Hg; dyslipidaemia, total cholesterol $\geq$ 200 mg/dL; DM2, diabetes mellitus type 2; BMI, body mass index; AIDs, autoimmune diseases; NSAIDs, non-steroidal anti-inflammatory drugs. [1] Fisher's exact test used for all data but Mann–Whitney test applied for age.

3.2. Preclinical and Acute COVID-19 Profiles

Upon defining our two groups of participants, we examined differences in their preclinical history and the acute phase of COVID-19 (Table 1). The age of the participants was similar between both groups, whether diagnosed with Long COVID or not (53 [18] years old in Long COVID vs. 50 [8] years old in recovered participants, p-value = 0.64). Variables such as smoking, peripheral artery disease, previous arterial or venous thrombosis, active neoplasia, immunosuppressant treatments, HIV seropositivity, or chronic renal impairment were discarded as none of the enrolled patients presented these issues. There were no differences in the number of non-hospitalised individuals or the duration of hospitalisation between the two groups. The severity of COVID-19 among hospitalised patients was consistent between those with and without Long COVID. The treatments administered during the acute phase of COVID-19 and the medical complications observed were comparable between the group that later developed Long COVID and the group that fully recovered.

3.3. Symptom Evaluation and Quality of Life

Participants were evaluated using the mMRC scale to test for dyspnea related to activity, which ranges from 0 (no breathlessness) to 4 (severe breathlessness), and the EQ-5D questionnaire. In our cohort, breathlessness, which was measured with the mMRC scale (Figure 1A), demonstrated a significant association with Long COVID (p-value = 0.000123); additionally, Long COVID patients registered a lower score on the EQ-5D (Figure 1B) compared to those who had fully recovered (0.58 [0.19] vs. 1.00 [0.00], respectively; p-value = 0.000003).

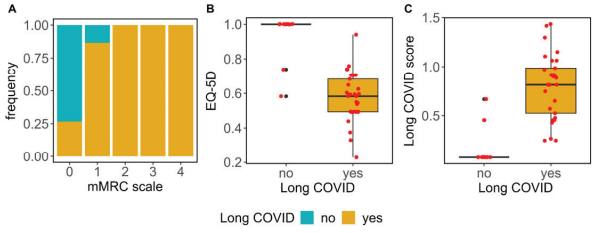


Figure 1. Quality of life and breathless affectation in Long COVID study participants. (**A**) Distribution of mMRC scores in patients with Long COVID and recovered participants. The figure classifies patients based on their mMRC scores. The recovered patients predominantly fall into the lower end of the mMRC scale, indicating minimal breathlessness. In contrast, Long COVID patients display higher mMRC scoring, suggesting more pronounced breathlessness during activity. Treating mMRC as an ordinal variable, the Fisher's statistics test indicated a significant difference between the Long COVID and recovered participants, with a p-value < 0.001. (**B**) EQ-5D scores in Long COVID. The box plot illustrated that Long COVID patients displayed lower EQ-5D scores, indicating poorer quality of life than recovered patients. The difference is statistically significant with a p-value of 2.913×10^{-6} (Mann–Whitney statistics test). (**C**) Long COVID score (LCS). The box plot illustrates that Long COVID patients displayed higher LCS, computed by combining mMRC and EQ-5D, indicating both more breathlessness and poorer quality of life than recovered patients. Red dots are patients and black dots refers to those patients that fall significantly outside the typical range of values The difference is statistically significant with a p-value of 4.0087×10^{-9} (Mann–Whitney statistics test).

Patients with Long COVID displayed a higher LCS than recovered individuals (0.84 [0.46] vs. 0.07 [0.00], respectively, p-value = 0.000000004) (Figure 1C). The above data suggested that Long COVID patients suffered more breathlessness and presented poorer quality of life than recovered individuals.

3.4. Vaccination and Seropositivity for SARS-CoV2, CMV and EBV Antibodies

Anti-SARS-CoV-2 vaccines were rolled out, starting in January 2021, with a prioritisation criterion in place. Our enrolled participants received their first shot, on average,

153 [31] days after their primary infection. At the time of their first Long COVID assessment, 88.24% of the participants had received at least one vaccine dose, and this distribution was similar among both Long COVID and fully recovered individuals (p-value = 0.55) (Supplementary Figure S1A).

No differences were observed in the number of individuals testing seropositive for IgG anti-SARS-CoV-2 Spike protein or IgG anti-CMV between Long COVID and recovered groups (Supplementary Figure S1B,C). Additionally, all participants were found to be IgG seropositive for anti-EBV. These data suggested that participants exhibited robust humoral immune responses.

3.5. Clinical Laboratory Parameters in Long COVID

We examined the biochemical and clinical laboratory parameters in patients with Long COVID to determine if there were any alterations compared to individuals who had fully recovered from COVID-19. Our analysis revealed that, on average, individuals with Long COVID had no dysfunctional laboratory parameters, similar to those who had recovered (Figure 2 and Supplementary Table S1).

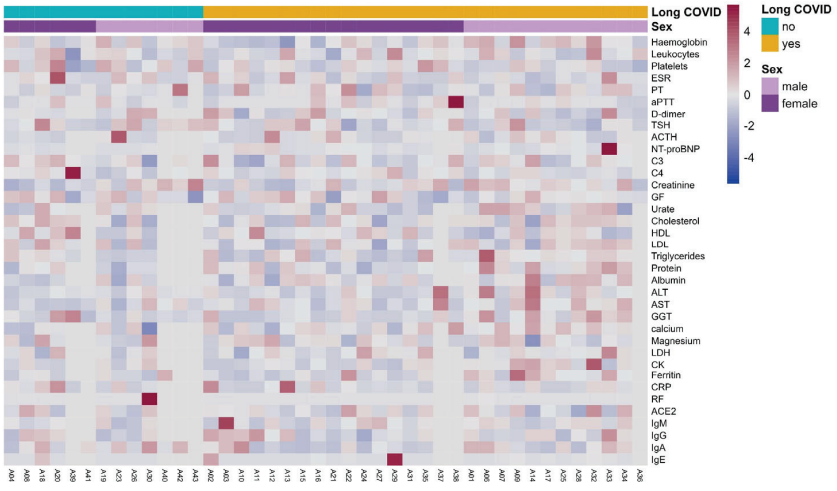


Figure 2. Clinical laboratory parameters in Long COVID cohort participants. Clinical parameters were measured during the medical visit to diagnose patient classification, either as having or not having Long COVID. A heatmap is plotted using the distribution of participants in columns depending on their diagnosis of Long COVID and sex. No clustering of clinical parameters was observed based on these two categories. ESR, erythrocyte sedimentation rate; PT, prothrombin time; aPTT, activated partial thromboplastin time; TSH, thyrotropin; NT-proBNP, N-terminal pro-B type natriuretic peptide; C3, complement component 3; C4, complement component 4; GF, glomerular filtrate; LDL, low-density lipoproteins; HDL, high-density lipoproteins; ALT, alanine amino transferase; AST, aspartate amino transferase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; CK creatine kinase; CRP, C-reactive protein; RF, rheumatoid factor; ACE2, angiotensin converting enzyme-2; Ig, immunoglobulin. Mann–Whitney U test showed no association of any clinical laboratory variable with Long COVID.

3.6. Long COVID Symptomatology

In our cohort, we assessed the presence of the clinical symptoms related to Long COVID (Figure 3 and Supplementary Table S2). Our study identified several significantly more prevalent symptoms in patients diagnosed with Long COVID. These included fatigue, myalgia, arthralgia, dyspnea, persistent coughing, a persistent sore throat, anxiety, and a lack of concentration.

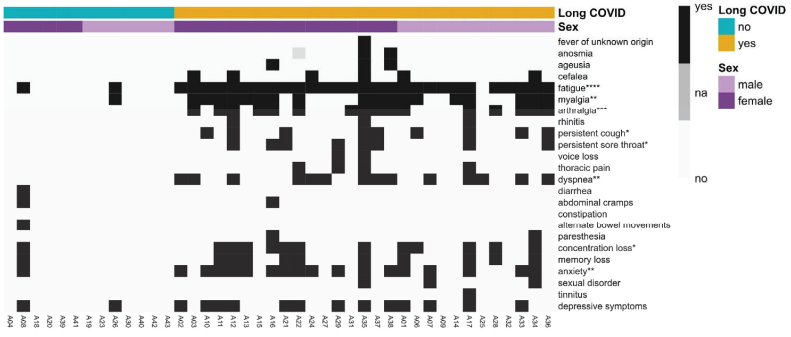


Figure 3. Presence of Long COVID symptoms in our Long COVID cohort. Long COVID symptoms were assessed as present (1, yes) or not (0, no) for all participants in our cohort and plotted in a heatmap. Participants were distributed along the columns based on their diagnosis of Long COVID and sex. Clinical symptoms showing a significant association with patients with Long COVID are indicated by asterisks, referring to their *p*-value significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Fisher's exact test was used.

3.7. Pituitary–Adrenal Axis Function in Long COVID

The aforementioned symptoms, which are more closely linked to Long COVID, indicated the possibility of altered pituitary–adrenal axis function in individuals with this disorder. Therefore, we examined the cortisol levels in both groups (Figure 4A, left). Although cortisol levels in patients with Long COVID (10.0 [5.3] µg/dL) were lower than those observed in individuals without Long COVID (11.1 [5.8] µg/dL), no significant statistical difference was observed between both groups (*p*-value = 0.52). The reference values for cortisol at 8 a.m. varied between 6 and 18 µg/dL. Our analysis only detected evidence of hypocortisolaemia in the Long COVID patients, where two individuals (6.9%) displayed lower cortisol levels. Notably, this proportion corresponded to the prevalence of hypocortisolaemia in cases of ME/CFS. We observed hypercortisolaemia across both groups in our cohort in equal measure. We also investigated the correlation between cortisol levels and symptom severity, as evaluated using the LCS (Supplementary Figure S2). The obtained results demonstrate no substantial correlation between cortisol levels and LCS (*p*-value = 0.98).

To determine whether Long COVID affected cortisol levels differently in women and men, we analysed cortisol variability, considering both Long COVID and sex (Figure 4A, right). The results of the two-way ANOVA statistical test indicate no significant differences (*p*-value = 0.26). However, it is noteworthy that the two cases of hypocortisolaemia were observed in female patients. In parallel with our observations on cortisol levels, we found that ACTH levels in patients with Long COVID (17.5 [11.7] µg/dL) were comparable to those without Long COVID (13.0 [4.7] µg/dL, *p*-value = 0.53) (Figure 4B).

Furthermore, we assessed the functional ability of the adrenal glands, focusing on the adrenal cortex, by conducting the ACTH stimulation test. As reflected in Figure 4C, the majority of the participants, comprising those with Long COVID, showed a typical response during the ACTH stimulation test. Following the intramuscular administration of ACTH (0.25 mg), cortisol levels exhibited significant escalation at 1 h. The rise in cortisol levels indicated that the adrenal glands were responding well to ACTH stimulation. The group with Long COVID experienced a comparable shift in cortisol levels (ΔCortisol = 15.0 [6.4] pg/dL) to that of the group without Long COVID (ΔCortisol = 16.1 [8.5] pg/dL, *p*-value = 0.58). The response pattern remained uniform when the data were examined by sex (Figure 4C, right). Nonetheless, a subset of individuals ($n = 12$, Supplementary Table S3) exhibited an inadequate cortisol response following ACTH administration. Adrenal insufficiency could be inferred when the cortisol levels increased by less than two-fold 60 min after ACTH stimulation (Supplementary Figure S3).

This inferior cortisol response was evenly distributed among individuals, regardless of their Long COVID status, and no statistically significant difference was observed (Fishers' Exact test *p*-value = 0.72).

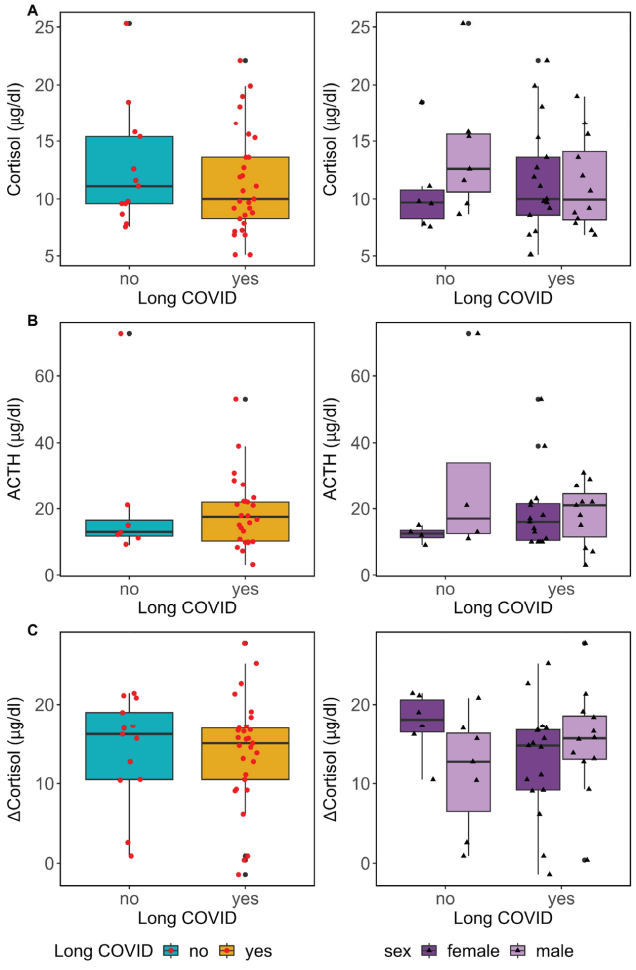


Figure 4. Pituitary–adrenal examination in our Long COVID cohort. Evaluation of basal cortisol and ACTH levels in the context of Long COVID. (**A**) Cortisol levels were measured at 8:00 a.m. for all participants and the results are subsequently presented in a box plot based on their Long COVID status. Left, the median levels of cortisol were equivalent in both groups (Mann–Whitney statistics test, *p*-value = 0.52). Right, participants with and without Long COVID were classified by sex, and cortisol levels were plotted for each group. The 2-way ANOVA statistical test showed no differences in any category (adjusted *p*-value: by Long COVID = 0.48; by sex = 0.54; interaction = 0.26). (**B**) ACTH levels were measured concomitantly to establish cortisol levels and the results are presented in a box plot according to Long COVID status. The median levels of ACTH were equivalent in both groups (*p*-value = 0.53) (Left), even when they were also analysed by sex (adjusted *p*-value: by Long COVID = 0.71; by sex = 0.45; interaction = 0.11). (**C**) Cortisol increases after 1 h of ACTH stimulation (ΔCortisol) were plotted based on the Long COVID status of participants (left) and also their sex (right). The 2-way ANOVA statistical test showed no differences in any category (adjusted *p*-value: by Long COVID = 0.29; by sex = 0.78; interaction = 0.67).

Regarding the symptoms described for Long COVID, those participants in our cohort exhibiting less than a two-fold cortisol induction in the ACTH stimulation test were found to be experiencing symptoms such as rhinitis, persistent coughing, and a persistent sore throat. However, there was no observed association between suboptimal cortisol response and depressive symptoms, anxiety, or fatigue (Table S3). The same results were obtained when the analysis was solely restricted to the group of patients with Long COVID.

3.8. Immune Profile in Long COVID Patients

The methodology used for examining the immune cell populations in the participants of this study is explained in Supplementary Figure S4, which outlines our gating strategy. We performed a comparative analysis to detect disparities in the number of assorted circulating immune cells between individuals who had been diagnosed with Long COVID and those who had completely recovered (Figure 5A and Supplementary Table S4).

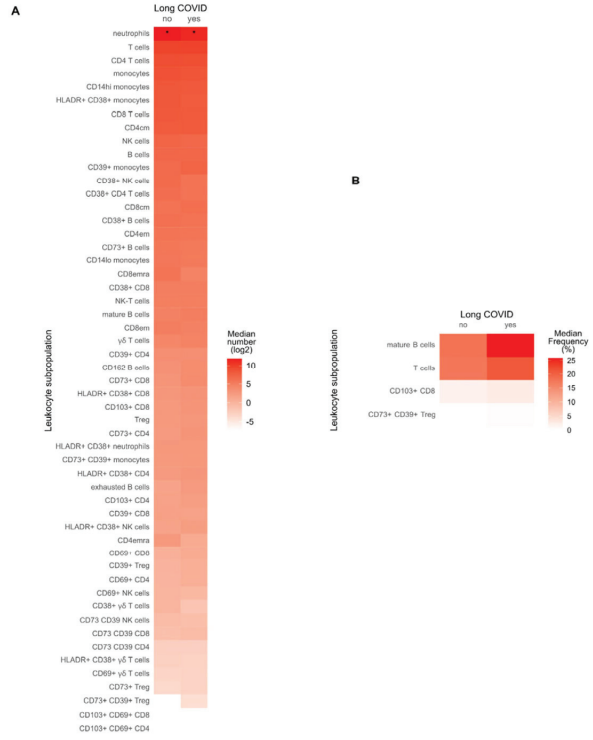


Figure 5. Blood immune cell predominance in the context of Long COVID. (**A**) Immune cell counts of different leukocyte subpopulations between patients with Long COVID (yes Long COVID) and recovered participants (no Long COVID) were plotted as the log2 of the median's group. Asterisks point out those leukocyte subpopulations with significant differences between both groups. Mann–Whitney U test, * $p < 0.05$. (**B**) Leukocytes subpopulations with significant ($p < 0,05$) differences in their percentages between participants with or without Long COVID. The frequency referred to their parental cells (see Supplementary Figure S4). cm, central memory; em, effector memory; emra, effector memory CD45RA-positive; NK, natural killer; Treg, regulatory T cells. The Mann–Whitney U test was performed.

The sole notable finding was that patients with Long COVID exhibited a lower number of circulating neutrophils in comparison with participants without Long COVID (2.6 [1.3] $\times$ 10^9 Cells/L vs. 3.8 [0.7] $\times$ 10^9 Cells/L, p-value = 0.0277, respectively). Nonetheless, their absolute neutrophil counts were still within the standard reference range

(2–7 × 10^9 Cells/L), precluding any clinical abnormality. No disparities were noted in the absolute tallies of overall T lymphocytes, T-helper lymphocytes, or cytotoxic lymphocytes. This uniformity was also evident in the tallies of memory and effector T cell populations within each lymphocyte subtype across both patient groups. Furthermore, the inquiry into the existence of HLA-DR+ CD38+ lymphocytes, which are indicative of viral infection, did not exhibit significant differences between the Long COVID and fully recovered cohorts. It is worth noting that the Long COVID group exhibited a higher proportion of Treg CD4+ HLA-DR+ CD38+ lymphocytes, although this disparity was not statistically significant (1.43% [0.55] vs. 1.11% [0.25], $p = 0.08$).

While the total B cell count was comparable between both groups, patients with Long COVID exhibited a higher proportion of mature B cells when compared to their recovered counterparts (Figure 5B and Supplementary Table S5). The median [IQR] was 25.8% [13.4] versus 17.9% [9.2], respectively (p-value = 0.0298). Long COVID patients exhibited a considerably greater proportion of CD8 T cells that expressed the CD103 integrin in circulation (2.8% [1.4] in Long COVID patients vs. 2.0% [0.5] in non-Long COVID patients, p-value = 0.033). This finding indicates more significant immune activation and an increase in the rate of resident memory CD8 cells in those experiencing Long COVID. The presence of regulatory T cells (Treg) was consistent in both groups. However, Long COVID patients had a greater proportion of Tregs expressing CD39 and CD73 markers (0.5% [1.1] vs. 0% [0], p= 0.027). No variations in other lymphocyte groups, including γδ-T cells, NK cells, and NK-T cells, were observed. In conclusion, our analysis suggests noticeable changes in particular lymphocyte subpopulations among patients with Long COVID, highlighting the intricate immune responses in these individuals.

3.9. Cytokine Evaluation

To investigate whether enduring immune cell activation is correlated to inflammation in Long COVID, we conducted tests on seven inflammatory markers in the sera of enrolled patients through ELISA (GDF-8), ECLIA (IL-6), or ProQuantum (TNF-α, IFN-γ, IL-1β, IL-2, IL-10, IL-12p40, and IL-12p70). Despite substantially higher levels of certain cytokines in some patients with Long COVID (Figure 6 and Supplementary Table S6), most of them did not show inflammation. Therefore, we did not discern a specific inflammatory cytokine with increased levels in patients with Long COVID relative to recovered individuals.

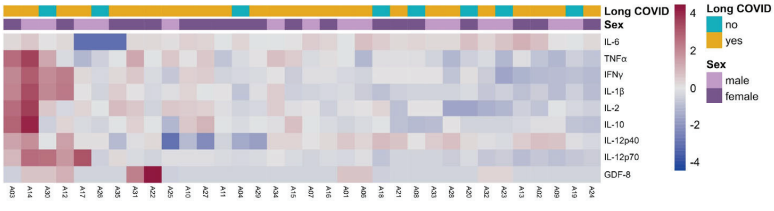


Figure 6. Inflammation in Long COVID. Heatmap plot of inflammatory molecules in participants with or without Long COVID, segregated by sex. Two-way ANOVA statistical test showed no differences in any category for each cytokine.

4. Discussion

In this preliminary investigation, we sought to analyse the functional physical status, adrenal function, and immune profiles of patients with Long COVID in comparison to individuals who had fully recovered from SARS-CoV-2 infection. Our study primarily ascertained that there was a noteworthy contrast in the EQ-5D score between Long COVID patients and those who had completely recovered. This disparity confirms that Long COVID patients experience a worsened quality of life, consistent with the persistent and debilitating nature of their symptoms. Furthermore, this study failed to identify any abnormalities in the pituitary–adrenal axis, particularly related to subclinical adrenal failure or insufficiency. One of the significant immunological findings in this study concerned

B lymphocytes. Although the absolute number of B cells remained steady between the two groups, Long COVID patients showcased a higher ratio of mature B cells. These observations warrant certain comments that will be explained below.

The finding of a higher ratio of fully developed B cells in patients with Long COVID hints at potential alterations in B cell maturation or function in these patients, which could affect antibody production and immune response to reinfection or vaccination. Further investigation is needed to understand the significance of this finding in the context of Long COVID pathophysiology. In contrast, our study did not reveal significant differences in several other immune cell populations, including total T lymphocytes, T-helper cells, cytotoxic lymphocytes, and central and effector memory T cell subsets, between Long COVID patients and those who had fully recovered. This suggests that Long COVID may not be primarily characterised by overt changes in these particular immune cell populations, at least in the peripheral circulation. An intriguing finding was the higher percentage of resident memory CD8+ T cells in Long COVID patients. Resident memory T cells play a crucial role in immune surveillance at mucosal surfaces and may contribute to ongoing immune responses. The significance of this finding warrants further exploration, as it could provide insights into the persistence of symptoms in Long COVID. Another notable result was the higher percentage of Treg cells expressing exonucleases in Long COVID patients. Treg cells are essential for immune regulation and tolerance, and higher exonuclease enzyme surface expression could be indicative of Treg functional activation. This finding suggests the potential dysregulation of immune tolerance mechanisms, which may contribute to the chronic inflammatory state seen in Long COVID [25], but we failed to observe it. The measurement of a panel of cytokines in the plasma of all participants indicated signs of inflammation in some participants, regardless of having Long COVID or not, but mostly results remained within normal ranges. This finding is in line with another study [37] in which, despite describing the persistence of circulating SARS-CoV-2 spike antigens 12 months after infection, no inflammation was found in the tested cohort of Long COVID patients.

Cortisol is a hormone secreted by the adrenal glands in response to stress. Chronic illnesses, like Long COVID, can lead to persistent stress, resulting in the prolonged over-stimulation of adrenal glands in order to maintain body balance. Over time, the adrenal cortex may fail to respond, despite the heightened activity of the hypothalamic–pituitary axis and the concurrent elevation of ACTH levels, leading to the development of subtle symptoms that may become apparent after a new stressor. Some clinical conditions align closely with these pathophysiological mechanisms. These include patients undergoing long-term corticosteroid treatment, cases of autoimmune adrenalitis, and those with post-viral chronic fatigue syndrome. Cortisol imbalance, i.e., slight adrenal hypofunction, can result in a range of symptoms. These may include fatigue, muscle and joint pain, feelings of weakness, mood disturbances, and cognitive deficits. Such symptoms are frequently cited by Long COVID patients, as previously reported [11–13] and our data agree. We assessed these distinct health conditions through various measures, including EQ-5D and mMRC. In a weighted LCS tool, we unified and equalised the scales. Our findings indicate that patients with Long COVID exhibited higher LCS than those who had recovered; however, these scores did not display any negative association with serum cortisol levels. Although we did not observe any dysfunction of the adrenal gland in our cohort of patients with Long COVID, two points should be highlighted. Firstly, we employed a standard and accepted method of stimulating the adrenal gland, which involved administering a 0.250 mg shot of ACTH and subsequently analysing the levels of blood cortisol over time. Studies argue against using this method to stimulate the pituitary–adrenal axis due to the supra-physiological doses of ACTH used [38,39]. There is a risk of misdiagnosing mild or early adrenal insufficiency [40]. Future studies using smaller ACTH doses should be conducted to analyse subclinical adrenal dysfunction. Secondly, 7% of patients in the Long COVID group exhibited slight hypocortisolemia. Nonetheless, these patients normalised their cortisol values after performing the ACTH stimulation test. For those patients suffering from

subclinical adrenal insufficiency, the administration of small doses prednisone or 5-alpha-fluorocortisone may assist in their improvement [41]. Other researchers have examined the correlation between the adrenal gland, cortisol production, and Long COVID [25,26]. In their study, [25] demonstrated that lower cortisol levels were effective predictors of Long COVID status. However, the study did not disclose the occurrence of hypocortisolaemia, despite reporting lower cortisol levels in patients with Long COVID. Our findings align with those of [26], who observed no variation in basal serum cortisol levels between healthy individuals and convalescent patients 3 months after experiencing the initial symptoms of COVID-19. Notably, [26] reported lower cortisol levels in those patients affected by respiratory symptoms 3 months after the onset of COVID-19. Again, these data have identified a specific clinical subset of patients who may benefit from cortisone therapeutic management. The physiological relevance of how cortisol levels could be affected without dysfunction in the supra-renal axis needs further examination.

Factors identified as predictive of persistent disease include Epstein–Barr virus viremia and type 2 diabetes [26]. Moreover, individuals with Long COVID may have reactivated immune responses against EBV [25]. In our cohort, all participants underwent a positive anti-EBV test, and although type 2 diabetes was more commonly diagnosed in patients with Long COVID, it was not statistically significant, nor was the association of Long COVID with acute disease severity [42,43].

Whether the breakthrough SARS-CoV-2 infection in vaccinated people results in post-acute sequelae is not clear. In our cohort, the majority of enrolled participants received one shot of anti-SARS-CoV-2 vaccination. Other strong predictors of Long COVID included elevated antibodies against EBV and reduced levels of certain immune cells, as indicated above [25]. While our data align with some immune cell population alterations, we did not identify a causal role for adrenal insufficiency in Long COVID. On the other hand, our study found that participants with Long COVID had comparable levels of antibody positivity to EBV or CMV, as recovered participants, suggesting that differences in immune cell populations could be specifically attributed to post-SARS-CoV-2 infection.

Limitations: our study provides a preliminary glimpse into the immunological and neuroendocrine landscape of Long COVID. However, it is essential to acknowledge the limitations of this pilot study, including its small sample size and the need for a more extensive, multi-centre study with a diverse patient population. The limited number of enrolled patients does not allow for in-depth analysis of the association of lower cortisol levels and clinical symptoms or whether vaccination has an effect on Long COVID prevention. Moreover, a longitudinal study would be needed in order to explore whether Long COVID symptoms are tied to adrenal insufficiency and how humans recover over time. Including patients from different waves will allow us to explore how different variants affect Long COVID. Finally, as discussed above, the ACTH stimulation test with lower doses of ACTH will disclose adrenal insufficiency more accurately.

5. Conclusions

Our findings suggest that Long COVID is associated with specific immunological alterations, such as changes in B cell maturity, the presence of resident memory CD8+ T cells, and Treg cell dysregulation. These findings lay the groundwork for future research aiming to unravel the mechanisms underlying Long COVID and develop targeted therapies, or for interventions aiming to alleviate its symptoms and improve the quality of life for affected individuals. Further investigations are required to validate and expand upon these initial observations and to explore potential therapeutic avenues.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines12030581/s1, Figure S1: anti-SARS-CoV-2 vaccination rates; Figure S2: Association of Long COVID Score (LCS) with cortisol levels; Figure S3: Fold cortisol induction in ACTH stimulation test; Figure S4: Strategy of gating in flow cytometry; Table S1: Comparison of clinical parameters between Long COVID and recovered patients; Table S2: Long COVID symptoms in participants with Long COVID or not; Table S3: Long COVID symptoms in

our cohort of participants having less than 2-fold cortisol induction in the ACTH stimulation test; Table S4: Number of leukocyte subpopulations in patients with and without Long COVID; Table S5: Frequency of leukocyte subpopulations in patients with and without Long COVID; Table S6: Serum cytokine levels in patients with Long COVID compared with those of recovered patients.

Author Contributions: Conceptualization, F.M.-M., E.E.-V. and J.A.-R.; methodology, F.M.-M., J.T., J.M.-S. and J.A.-R.; data curation, F.M.-M.; formal analysis, F.M.-M.; investigation, A.A.-L. and L.G.-G.; resources; F.M.-M., E.E.-V., S.M.-P., S.R.-S. and D.R.; supervision, J.A.-R. and F.M.-M.; visualization, F.M.-M., A.A.-L. and J.M.-S.; writing—original draft, F.M.-M.; writing—review and editing: F.M.-M., J.M.-S. and J.A.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundació Althaia and Fundació Aigües de Manresa (Barcelona, Spain; QIC-URil-01), Sociedad Española de Medicina y Cirugía Cosmética (SEMCC, Barcelona, Spain; SEMCC-JAR/2023), Gravida Fertilitat Avançada (Barcelona, Spain; Gravida-JAR/2023), and Synlab (Barcelona, Spain; Synlab-JAR/2023).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Althaia Xarxa Assistencial Foundation (CEI 21/82 on date 21 December 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Research data generated during this study are saved in our institutional repository of Vall d'Hebron Institut de Recerca and will be available upon reasonable request to the corresponding authors.

Acknowledgments: We acknowledge the High Technology Unit from Vall d'Hebron Research Institute (VHIR) for their help and technical assistance in flow cytometry. The authors thank the effort of nurses from Althaia Xarxa Assistencial for informing and collecting sera from Long COVID patients. The authors would like to thank the participants for their willingness to participate in this project.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Al-Aly, Z.; Xie, Y.; Bowe, B. High-dimensional characterization of post-acute sequelae of COVID-19. *Nature* **2021**, *594*, 259–264. [CrossRef]
2. Davis, H.E.; McCorkell, L.; Vogel, J.M.; Topol, E.J. Long COVID: Major findings, mechanisms and recommendations. *Nat. Rev. Microbiol.* **2023**, *21*, 133–146. [CrossRef]
3. Bowe, B.; Xie, Y.; Al-Aly, Z. Postacute sequelae of COVID-19 at 2 years. *Nat. Med.* **2023**, *29*, 2347–2357. [CrossRef]
4. Tansey, C.M.; Louie, M.; Loeb, M.; Gold, W.L.; Muller, M.P.; de Jager, J.; Cameron, J.I.; Tomlinson, G.; Mazzulli, T.; Walmsley, S.L.; et al. One-Year Outcomes and Health Care Utilization in Survivors of Severe Acute Respiratory Syndrome. *Arch. Intern. Med.* **2007**, *167*, 1312–1320. [CrossRef]
5. Moldofsky, H.; Patcai, J. Chronic widespread musculoskeletal pain, fatigue, depression and disordered sleep in chronic post-SARS syndrome; a case-controlled study. *BMC Neurol.* **2011**, *11*, 37. [CrossRef]
6. Cortinovis, M.; Perico, N.; Remuzzi, G. Long-term follow-up of recovered patients with COVID-19. *Lancet* **2021**, *397*, 173–175. [CrossRef] [PubMed]
7. Patient-Led Research Collaborative. Report: What Does COVID-19 Recovery Actually Look Like?—Patient Led Research Collaborative 2020. Available online: https://patientresearchcovid19.com/research/report-1/ (accessed on 30 November 2023).
8. Wang, Z.; Yang, L. Post-acute Sequelae of SARS-CoV-2 Infection: A Neglected Public Health Issue. *Front. Public Health* **2022**, *10*, 908757. [CrossRef] [PubMed]
9. Marshall, M. The lasting misery of coronavirus long-haulers. *Nature* **2020**, *585*, 339–341. [CrossRef] [PubMed]
10. Belluck, P. Covid Survivors with Long-Term Symptoms Need Urgent Attention, Experts Say. N Y Times 2021. Available online: https://www.nytimes.com/2021/01/08/health/Covid-survivors-longterm.html (accessed on 21 January 2021).
11. Groff, D.; Sun, A.; Ssentongo, A.E.; Ba, D.M.; Parsons, N.; Poudel, G.R.; Lekoubou, A.; Oh, J.S.; Ericson, J.E.; Ssentongo, P.; et al. Short-term and Long-term Rates of Postacute Sequelae of SARS-CoV-2 Infection: A Systematic Review. *JAMA Netw. Open* **2021**, *4*, e2128568. [CrossRef] [PubMed]
12. Jiang, D.H.; Roy, D.J.; Gu, B.J.; Hassett, L.C.; McCoy, R.G. Postacute Sequelae of Severe Acute Respiratory Syndrome Coronavirus 2 Infection: A State-of-the-Art Review. *JACC Basic Transl. Sci.* **2021**, *6*, 796–811. [CrossRef] [PubMed]
13. Parker, A.M.; Brigham, E.; Connolly, B.; McPeake, J.; Agranovich, A.V.; Kenes, M.T.; Casey, K.; Reynolds, C.; Schmidt, K.F.R.; Kim, S.Y.; et al. Addressing the post-acute sequelae of SARS-CoV-2 infection: A multidisciplinary model of care. *Lancet Respir. Med.* **2021**, *9*, 1328–1341. [CrossRef]

14. Tenforde, M.W.; Kim, S.S.; Lindsell, C.J.; Rose, E.B.; Shapiro, N.I.; Files, D.C.; Gibbs, K.W.; Erickson, H.L.; Steingrub, J.S.; Smithline, H.A.; et al. Symptom Duration and Risk Factors for Delayed Return to Usual Health Among Outpatients with COVID-19 in a Multistate Health Care Systems Network. *Morb. Mortal. Wkly. Rep.* **2020**, *69*, 993–998. [CrossRef]
15. Bergwerk, M.; Gonen, T.; Lustig, Y.; Amit, S.; Lipsitch, M.; Cohen, C.; Mandelboim, M.; Levin, E.G.; Rubin, C.; Indenbaum, V.; et al. Covid-19 Breakthrough Infections in Vaccinated Health Care Workers. *N. Engl. J. Med.* **2021**, *385*, 1474–1484. [CrossRef] [PubMed]
16. Antonelli, M.; Capdevila Pujol, J.; Spector, T.D.; Ourselin, S.; Steves, C.J. Risk of long COVID associated with delta versus omicron variants of SARS-CoV-2. *Lancet* **2022**, *399*, 2264. [CrossRef]
17. Xie, Y.; Choi, T.; Al-Aly, Z. Association of Treatment with Nirmatrelvir and the Risk of Post–COVID-19 Condition. *JAMA Intern. Med.* **2023**, *183*, 554–564. [CrossRef] [PubMed]
18. Al-Aly, Z.; Bowe, B.; Xie, Y. Long COVID after breakthrough SARS-CoV-2 infection. *Nat. Med.* **2022**, *28*, 1461–1467. [CrossRef]
19. Davis, H.E.; Assaf, G.S.; McCorkell, L.; Wei, H.; Low, R.J.; Re'Em, Y.; Redfield, S.; Austin, J.P.; Akrami, A. Characterizing long COVID in an international cohort: 7 months of symptoms and their impact. *EClinicalMedicine* **2021**, *38*, 101019. [CrossRef] [PubMed]
20. Agergaard, J.; Gunst, J.D.; Schiøttz-Christensen, B.; Østergaard, L.; Wejse, C. Long-term prognosis at 1.5 years after infection with wild-type strain of SARS-CoV-2 and Alpha, Delta, as well as Omicron variants. *Int. J. Infect. Dis.* **2023**, *137*, 126–133. [CrossRef]
21. Bildirici, Y.; Metintas, S.; Ucan, A.; Ak, G.; Guvenc, S.E.; Metintas, M. Post-COVID-19 syndrome in a cohort of hospitalized COVID-19 patients. *Eur. Rev. Med. Pharmacol. Sci.* **2023**, *27*, 8291–8300. [CrossRef] [PubMed]
22. Zhang, H.; Huang, C.; Gu, X.; Wang, Y.; Li, X.; Liu, M.; Wang, Q.; Xu, J.; Wang, Y.; Dai, H.; et al. 3-year outcomes of discharged survivors of COVID-19 following the SARS-CoV-2 omicron (B.1.1.529) wave in 2022 in China: A longitudinal cohort study. *Lancet Respir. Med.* **2023**, *12*, 55–66. [CrossRef]
23. Doykov, I.; Hällqvist, J.; Gilmour, K.C.; Grandjean, L.; Mills, K.; Heywood, W.E. 'The long tail of COVID-19'—The detection of a prolonged inflammatory response after a SARS-CoV-2 infection in asymptomatic and mildly affected patients. *F1000Research* **2020**, *9*, 1349. [CrossRef]
24. Nalbandian, A.; Sehgal, K.; Gupta, A.; Madhavan, M.V.; McGroder, C.; Stevens, J.S.; Cook, J.R.; Nordvig, A.S.; Shalev, D.; Sehrawat, T.S.; et al. Post-acute COVID-19 syndrome. *Nat. Med.* **2021**, *27*, 601–615. [CrossRef]
25. Klein, J.; Wood, J.; Jaycox, J.R.; Dhodapkar, R.M.; Lu, P.; Gehlhausen, J.R.; Tabachnikova, A.; Greene, K.; Tabacof, L.; Malik, A.A.; et al. Distinguishing features of long COVID identified through immune profiling. *Nature* **2023**, *623*, 139–148. [CrossRef] [PubMed]
26. Su, Y.; Yuan, D.; Chen, D.G.; Ng, R.H.; Wang, K.; Choi, J.; Li, S.; Hong, S.; Zhang, R.; Xie, J.; et al. Multiple early factors anticipate post-acute COVID-19 sequelae. *Cell* **2022**, *185*, 881–895.e20. [CrossRef] [PubMed]
27. Dani, M.; Dirksen, A.; Taraborrelli, P.; Torocastro, M.; Panagopoulos, D.; Sutton, R.; Lim, P.B. Autonomic dysfunction in 'long COVID': Rationale, physiology and management strategies. *Clin. Med.* **2021**, *21*, e63–e67. [CrossRef] [PubMed]
28. Wong, A.C.; Devason, A.S.; Umana, I.C.; Cox, T.O.; Dohnalová, L.; Litichevskiy, L.; Perla, J.; Lundgren, P.; Etwebi, Z.; Izzo, L.T.; et al. Serotonin reduction in post-acute sequelae of viral infection. *Cell* **2023**, *186*, 4851–4867.e20. [CrossRef] [PubMed]
29. Mancini, D.M.; Brunjes, D.L.; Lala, A.; Trivieri, M.G.; Contreras, J.P.; Natelson, B.H. Use of Cardiopulmonary Stress Testing for Patients with Unexplained Dyspnea Post– Coronavirus Disease. *JACC Heart Fail.* **2021**, *9*, 927–937. [CrossRef]
30. Guarnieri, J.W.; Dybas, J.M.; Fazelinia, H.; Kim, M.S.; Frere, J.; Zhang, Y.; Albrecht, Y.S.; Murdock, D.G.; Angelin, A.; Singh, L.N.; et al. Core mitochondrial genes are down-regulated during SARS-CoV-2 infection of rodent and human hosts. *Sci. Transl. Med.* **2023**, *15*, eabq1533. [CrossRef]
31. Mandarano, A.H.; Maya, J.; Giloteaux, L.; Peterson, D.L.; Maynard, M.; Gottschalk, C.G.; Hanson, M.R. Myalgic encephalomyelitis/chronic fatigue syndrome patients exhibit altered T cell metabolism and cytokine associations. *J. Clin. Investig.* **2020**, *130*, 1491–1505. [CrossRef]
32. Cheong, J.-G.; Ravishankar, A.; Sharma, S.; Parkhurst, C.N.; Grassmann, S.A.; Wingert, C.K.; Laurent, P.; Ma, S.; Paddock, L.; Miranda, I.C.; et al. Epigenetic memory of coronavirus infection in innate immune cells and their progenitors. *Cell* **2023**, *186*, 3882–3902.e24. [CrossRef]
33. Klok, F.A.; Boon, G.J.; Barco, S.; Endres, M.; Geelhoed, J.M.; Knauss, S.; Rezek, S.A.; Spruit, M.A.; Vehreschild, J.; Siegerink, B. The Post-COVID-19 Functional Status scale: A tool to measure functional status over time after COVID-19. *Eur. Respir. J.* **2020**, *56*, 2001494. [CrossRef] [PubMed]
34. Herdman, M.; Badia, X.; Berra, S. EuroQol-5D: A simple alternative for measuring health-related quality of life in primary care. *Aten Primaria* **2001**, *28*, 425–430. [CrossRef]
35. WHO COVID-19 Dashboard. Spain: WHO Coronavirus Disease (COVID-19) Dashboard with Vaccination Data 2020. Available online: https://covid19.who.int (accessed on 30 November 2023).
36. Troyano-Hernáez, P.; Reinosa, R.; Holguín, A. Evolution of SARS-CoV-2 in Spain during the First Two Years of the Pandemic: Circulating Variants, Amino Acid Conservation, and Genetic Variability in Structural, Non-Structural, and Accessory Proteins. *Int. J. Mol. Sci.* **2022**, *23*, 6394. [CrossRef] [PubMed]
37. Swank, Z.; Senussi, Y.; Manickas-Hill, Z.; Yu, X.G.; Li, J.Z.; Alter, G.; Walt, D.R. Persistent Circulating Severe Acute Respiratory Syndrome Coronavirus 2 Spike Is Associated with Post-acute Coronavirus Disease 2019 Sequelae. *Clin. Infect. Dis.* **2022**, *76*, e487–e490. [CrossRef] [PubMed]

38. Ceccato, F.; Selmin, E.; Antonelli, G.; Barbot, M.; Daniele, A.; Boscaro, M.; Plebani, M.; Scaroni, C. Low-dose short synacthen test with salivary cortisol in patients with suspected central adrenal insufficiency. *Endocr. Connect.* **2021**, *10*, 1189–1199. [CrossRef] [PubMed]
39. Thaler, L.M.; Blevins, L.S. The Low Dose (1-μg) Adrenocorticotropin Stimulation Test in the Evaluation of Patients with Suspected Central Adrenal Insufficiency. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 2726–2729. [CrossRef]
40. Gandhi, P.G.; Shah, N.S.; Khandelwal, A.G.; Chauhan, P.; Menon, P.S. Evaluation of low dose ACTH stimulation test in suspected secondary adrenocortical insufficiency. *J. Postgrad. Med.* **2002**, *48*, 280–282.
41. Sharma, A.; Lazarus, K.; Papadopoulou, D.; Prabhudev, H.; Tan, T.; Meeran, K.; Choudhury, S. Optimising prednisolone or prednisone replacement in adrenal insufficiency. *Endocr. Connect.* **2023**, *12*, e230097. [CrossRef]
42. Alijotas-Reig, J.; Anunciación-Llunell, A.; Morales-Pérez, S.; Trapé, J.; Esteve-Valverde, E.; Miro-Mur, F. Thrombosis and Hyperinflammation in COVID-19 Acute Phase Are Related to Anti-Phosphatidylserine and Anti-Phosphatidylinositol Antibody Positivity. *Biomedicines* **2023**, *11*, 2301. [CrossRef]
43. Blomberg, B.; Mohn, K.G.-I.; Brokstad, K.A.; Zhou, F.; Linchausen, D.W.; Hansen, B.-A.; Lartey, S.; Onyango, T.B.; Kuwelker, K.; Sævik, M.; et al. Long COVID in a prospective cohort of home-isolated patients. *Nat. Med.* **2021**, *27*, 1607–1613. [CrossRef]

biomedicines

MDPI

Article

Immunological Similarities and Differences between Post-COVID-19 Lung Sequelae and Idiopathic Pulmonary Fibrosis

Sara Gangi [1], Laura Bergantini [1], Paolo Cameli [1], Irene Paggi [1], Marco Spalletti [1], Fabrizio Mezzasalma [2], Elena Bargagli [1] and Miriana d'Alessandro [1,*]

[1] Respiratory Diseases and Lung Transplantation Unit, Department of Medical and Surgical Sciences & Neuro-Sciences, University of Siena, 53100 Siena, Italy; sara.gangi@student.unisi.it (S.G.); laurabergantini@gmail.com (L.B.); paolocameli88@gmail.com (P.C.); irene.paggi@student.unisi.it (I.P.); marcospalletti@live.it (M.S.); bargagli2@gmail.com (E.B.)

[2] Diagnostic and Interventional Bronchoscopy Unit, Cardio-Thoracic and Vascular Department, University Hospital of Siena, Azienda Ospedaliera Universitaria Senese—AOUS, 53100 Siena, Italy; fabriziomezzasalma@hotmail.it

* Correspondence: dalessandro.miriana@gmail.com; Tel.: +39-0577586713; Fax: +39-0577280744

Abstract: Introduction: Pulmonary fibrosis is an irreversible condition that may be caused by known (including viral triggers such as SARS-CoV-2) and unknown insults. The latter group includes idiopathic pulmonary fibrosis (IPF), which is a chronic, progressive fibrosing interstitial pneumonia of unknown cause. The longer the insult acts on lung tissue, the lower the probability of a complete resolution of the damage. An emerging clinical entity post-COVID-19 is pulmonary fibrosis (PCPF), which shares many pathological, clinical, and immunological features with IPF. The fibrotic response in both diseases—IPF and PCPF—is orchestrated in part by the immune system. An important role regarding the inhibitory or stimulatory effects on immune responses is exerted by the immune checkpoints (ICs). The aim of the present study was to analyse the similarities and differences between CD4+, CD8+, and NK cells in the peripheral blood of patients affected by fibrotic disease, IPF, and PCPF compared with sarcoidosis patients and healthy controls. The second aim was to evaluate the expression and co-expression of PD-1 and TIGIT on CD4, CD8, and NK cells from our patient cohort. Methods: One hundred and fifteen patients affected by IPF, PCPF, and sarcoidosis at the rare pulmonary disease centre of the University of Siena were enrolled. Forty-eight patients had an IPF diagnosis, 55 had PCPF, and 12 had sarcoidosis. Further, ten healthy controls were enrolled. PCPF patients were included between 6 and 9 months following hospital discharge for COVID-19. The peripheral blood samples were collected, and through flow cytometric analysis, we analysed the expression of CD4, CD8, NK cells, PD-1, and TIGIT. Results: The results show a greater depletion of CD4 and NK cells in IPF patients compared to other groups ($p = 0.003$), in contrast with CD8 cells ($p < 001$). Correlation analysis demonstrated an indirect correlation between CD4 and CD8 cells in IPF and sarcoidosis patients ($p < 0.001 = -0.87$ and $p = 0.042$; $r = -0.6$, respectively). Conversely, PCPF patients revealed a direct correlation between CD4 and CD8 cells ($p < 0.001$; $r = 0.90$) accentuating an immune response restoration. The expression of PD-1 and TIGIT was abundant on T and NK cell subsets of the two lung fibrotic groups, IPF and PCPF. Analogously, the co-expression of PD-1 and TIGIT on the surfaces of CD4 and CD8 were increased in such diseases. **Conclusions**: Our study shines a spotlight on the immune responses involved in the development of pulmonary fibrosis, idiopathic and secondary to SARS-CoV-2 infection. We observed a significant imbalance not only in CD4, CD8, and NK blood percentages in IPF and PCPF patients but also in their functional phenotypes evaluated through the expression of ICs.

Keywords: idiopathic pulmonary fibrosis; post-COVID pulmonary fibrosis; immune cells; immune checkpoint; CD4; CD8; NK; PD-1; TIGIT

Citation: Gangi, S.; Bergantini, L.; Cameli, P.; Paggi, I.; Spalletti, M.; Mezzasalma, F.; Bargagli, E.; d'Alessandro, M. Immunological Similarities and Differences between Post-COVID-19 Lung Sequelae and Idiopathic Pulmonary Fibrosis. *Biomedicines* **2024**, *12*, 630. https://doi.org/10.3390/biomedicines12030630

Academic Editor: César Fernández De Las Peñas

Received: 24 January 2024
Revised: 8 March 2024
Accepted: 9 March 2024
Published: 12 March 2024

1. Introduction

Pulmonary fibrosis is an irreversible condition characterised by scarring and thickening of the lung interstitium that leads to impaired gas transfer, loss of lung function, and in many cases, death. It may be caused by known (including viral triggers such as SARS-CoV-2) and unknown insults. The latter include idiopathic pulmonary fibrosis (IPF) and sarcoidosis. IPF is a chronic progressive idiopathic interstitial lung disease (ILD) [1] of unclear pathogenesis. Recurrent ongoing injury to alveolar epithelial cells triggers release of proinflammatory mediators (such as Transforming growth factor beta, TGF-β) and accumulation of immune and profibrotic cells in the lung, accompanied by deposition of a large amount of extracellular matrix (ECM). Moreover, pulmonary cellular damage induced by several factors (environmental, infections, mechanical damage) results in the disruption of lung parenchymal architecture. In this compromise microenvironment, resident and recruited immune cells such as macrophages and lymphocytes modulate existing responses through a variety of mechanisms [2]. These abnormalities contribute to the development and progression of IPF (ref [3]). Altered proportion or activation of T cells subsets, as well as specific receptors, were shown to negatively influence the progression of this disease [4].

T cells are diffusely present in the alveoli, lung tissues, and bloodstream of IPF patients, though their role is still controversial. CD4 and CD8 cells are both involved in the progression of the IPF. CD4 cell subsets play a profibrotic role in its pathogenesis, producing IL-4, IL-5 and IL-13 [5]. CD8 cells may impact the development of pulmonary fibrosis, infiltrating the lung parenchyma through the release of cytokines. Controversially, Koh et al. reported that CD8 cells may produce cytokines with pro- and anti-fibrotic properties [6]. Croft et al. demonstrated that an increased CD8 cell percentages was associated with severe lung injury [7]. An altered immune system has been reported in hospitalised COVID-19 patients, though few data are available about its impairment in the follow-up.

Post-COVID-19 pulmonary fibrosis (PCPF) is an emerging clinical entity following SARS-CoV-2 infection that shares many pathological, clinical, and immunological features with IPF [7,8]. The fibrotic response in IPF and PCPF is orchestrated in part by the immune system. The longer the insult acts on lung tissue, the lower the probability of complete resolution of lung damage. Among ILDs, sarcoidosis is a multisystemic inflammatory disorder that mainly affects the lungs, and its spontaneous resolution occurs in the early stages of disease, characterised by exaggerated immune cell activity.

Immune responses are regulated by immune checkpoints (ICs), which are crucial for maintaining self-tolerance. Programmed cell death protein-1 (PD-1), also known as CD279, and an emerging immune checkpoint T cell immunoglobulin and ITIM domain (TIGIT) are both expressed on T cells. PD-1 and TIGIT are two T-cell exhaustion markers involved in different fibrotic mechanisms. Under different conditions, PD-1 can regulate cell activation, phagocytosis, migration, invasion of immune and non-immune cells (such as fibroblasts and epithelial cells), and epithelial-to-mesenchymal transition [9]. TIGIT can directly induce T-cell suppression by blocking their activation, proliferation, and acquisition of effector functions [10].

The aim of the present study was to analyse the similarities and differences in $CD4^+$, $CD8^+$, and NK cells in the peripheral blood of patients with fibrotic disease (IPF and PCPF) compared with sarcoidosis and healthy controls (HCs). A second aim was to evaluate expression and co-expression of PD-1 and TIGIT on CD4, CD8, and natural killer (NK) cells from our patient cohorts.

2. Material and Methods

2.1. Study Population

We enrolled one hundred and fifteen patients with IPF, PCPF, and sarcoidosis monitored at the Rare Lung Disease Centre of Siena University Hospital. PCPF patients with comorbidities, including diabetes, heart failure, and hypertension, IPF patients with concomitant malignancies, and sarcoidosis patients with extrathoracic disease involvement and concomitant diseases were excluded. Patients who did not receive a diagnosis con-

firmation according to the international guidelines or PCPF patients without radiological confirmation of pulmonary fibrosis were excluded.

The IPF diagnosis was confirmed by a multidisciplinary group according to international American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines. Forty-eight IPF patients were enrolled before pharmacological treatment. Fifty-five PCPF patients were enrolled 6–9 months after discharge from hospital subsequent to admission with COVID-19 (hospitalised between March 2020 and May 2022) and returned to the regional program for disease monitoring. All PCPF patients underwent medical examination, including chest HRCT, blood tests, and lung function tests (LFTs). Twelve sarcoidosis patients were diagnosed according to international criteria based on clinical signs, chest radiography findings, and non-caseating granulomas in lymph nodes and/or endobronchial biopsy specimens. All patients with sarcoidosis enrolled in the study were in radiological stage II, characterised by mediastinal lymph node enlargement associated with micronodular lung parenchymal involvement. Ten healthy HCs without previous infectious or malignant diseases were enrolled as the HC group. Blood samples for immunological assay were drawn upon inclusion in the study.

All patients gave their written informed consent for participation in the study. The study was approved by the regional ethical review board of Siena, Italy (C.E.A.V.S.E. Markerlung 17431), and complied with the declaration of Helsinki.

2.2. Peripheral Blood Mononuclear Cells

PBMC collection and management of cells was performed at the laboratory of the Respiratory Disease Unit, Siena University Hospital (Italy). The blood samples were drawn into a tube containing EDTA anticoagulant (BD Vacutainer® EDTA tubes, BD Biosciences, CA, USA) and processed within 8 h. Subsequently, PBMCs were separated with gradient centrifugation (Ficoll Histopaque®-1077, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 1050 g without deceleration, then washed twice, resuspended in 80% RPMI 1640, 10% FBS, and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) at 2×10^6 cells per vial, and stored in liquid nitrogen until analysis.

2.3. Gating Strategy

Multicolour flow cytometric analysis was performed using mAb (Table 1).

Table 1. The features of monoclonal antibodies used for multicolour flow cytometric analysis, including clone, fluorochrome, and company.

Cluster of Differentiation (CD-)	Clone	Fluorochrome	Company
CD3	OKT3	APC	BioLegend (San Diego, CA, USA)
CD4	SK3	FITC	Becton Dickinson (Franklin Lakes, NJ, USA)
CD8	SK1	BV421	BioLegend
PD-1	PE	PD1.3.1.3	Miltenyi Biotec (Bergisch Gladbach, Germay)
TIGIT	APC-Cy7	A15153G	BioLegend
CD56	5.1H11	PerCP/Cy5.5	BioLegend

The gating strategy was performed with Kaluza Software 2.1 (Beckman Coulter, Brea, CA, USA). An assessment was conducted for T cell subsets and NK cells (Figure 1).

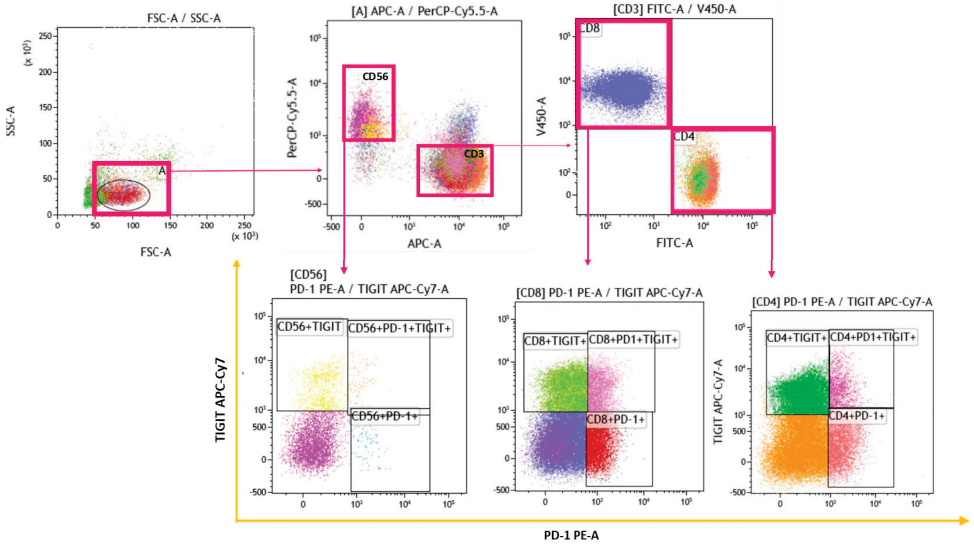


Figure 1. Lymphocytes were discriminated on the basis of forward (FSC) versus side (SSC) scatters. Then, a dot plot was performed to distinguished CD3- from CD56-expressing cells, and a second dot plot was performed to identify CD4 from CD8 cells. Using PD-1 and TIGIT markers, three dot plots were assessed on CD4-, CD8-, and CD56-positive cells to discriminate: $CD4^+PD\text{-}1^+$, $CD4^+TIGIT^+$, $CD4^+PD\text{-}1^+$ $TIGIT^+$; $CD8^+PD\text{-}1^+$, $CD8^+TIGIT^+$, $CD8^+PD\text{-}1^+TIGIT^+$; $CD56^+PD\text{-}1^+$, $CD56^+TIGIT^+$, $CD56^+PD\text{-}1^+TIGIT^+$.

Figure 2 reports the gating strategy performed with Kaluza Software 2.1 (Beckman Coulter, Brea, CA, USA) to identify $CD56^{dim}$, $CD56^{bright}$, and NKT-like cells.

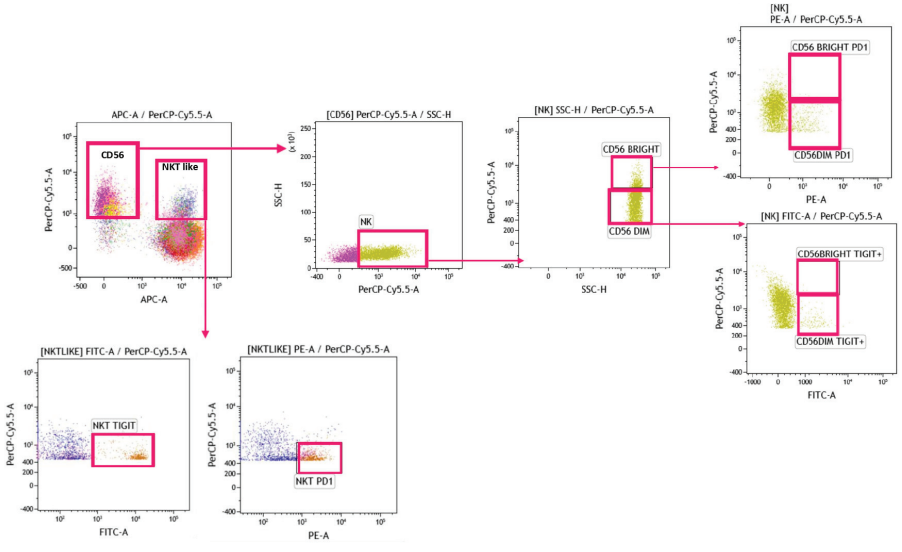


Figure 2. Two dot plots were performed to identify $CD56^{bright}$ and $CD56^{dim}$ expressing PD-1 or TIGIT. Further dot plots were performed to discriminate NKT-like cells expressing PD-1 and TIGIT.

2.4. Lung Function Tests

The following lung function parameters were recorded according to standard ATS/ERS criteria using a Jaeger Body Plethysmograph with correction for temperature and barometric: forced expiratory volume in the first second (FEV1), forced vital capacity (FVC), carbon monoxide diffusing capacity (DLCO). All parameters were expressed as percentages of predicted values.

2.5. Statistical Analysis

All values were expressed as medians and interquartile ranges (IQRs) or means ± standard deviations when appropriate. A non-parametric one-way ANOVA (Kruskal–Wallis test) and the Dunn test were performed for multiple comparisons. The Spearman test was used to correlate immunological and clinical findings. A *p*-value less than 0.05 was considered statistically significant. Statistical analysis was performed using the GraphPad 10.1.2 and Jamovi 2.3.21 softwares.

3. Results

3.1. Study Population

Demographic and clinical data of patients and HCs are reported in Table 2.

Table 2. Clinical and demographical data of enrolled patients. Data are reported as medians and ± standard deviations.

Clinical and Demographic Parameters	IPF (*n* = 48)	PCPF (*n* = 55)	Sarcoidosis (*n* = 12)	HC (*n* = 10)
Sex (F/M)	13/35	23/29	6/6	5/5
Age (median)	73 ± 8.11	75 ± 8.24	56 ± 6.76	69 ± 15.9
Smoking status (never/former)	41/7	52/3	5/7	10/0
Lung function test parameters (median and ±standard deviation):				
FEV1%	76.5 ± 20.42	94 ± 16.07	104 ± 10.09	
FVC%	73 ± 19.61	107 ± 10.98	110 ± 13.87	
DLCO%	45.5 ± 33.26	60 ± 12.35	76 ± 18.52	
Radiological findings	UIP (*n* = 48)	Fibrotic inter- or intralobular thickening (*n* = 55)/Air trapping (*n* = 43)/Groundglass (*n* = 40)	Scadding stage II (*n* = 12)	

We enrolled 48 patients with IPF (median age 73 (68–78) years; 73% males), 55 patients with PCPF (median age 75 (69–80) years; 53% males), 12 patients with sarcoidosis (median age 56 (54–58) years; 50% females), and 10 HCs (median age 69 (64–77) years; 50% males). IPF and PCPF patients were older than sarcoidosis patients ($p = 0.003$ and $p = 0.001$, respectively) and HCs ($p < 0.001$). A prevalence of smokers was found in the IPF cohort, whereas no-smokers were prevalent in PCPF patients ($p < 0.001$). No subject was on antifibrotic, steroid, or immunosuppressant treatment at the time of diagnosis.

3.2. Immunological Findings

Table 3 shows mean ± standard deviation and *p* values of cell subset percentages.

Table 3. T and NK cell subset percentages with and without PD-1 and TIGIT expression divided according to disease group: IPF, idiopathic pulmonary fibrosis; PCPF, post-COVID-19 pulmonary fibrosis; sarcoidosis; and HCs, healthy controls. All data were reported as means ± standard deviations. Abbreviations: CD-, cluster of differentiation; NK, natural killer; NKT-like cells, natural killer like T cells; PD-1, programmed death-1; TIGIT, T cell immunoglobulin and ITIM domain.

Cell Percentages	IPF (*n* = 48)	PCPF (*n* = 55)	Sarcoidosis (*n* = 12)	HCs (*n* = 10)	*p* Value
CD3	49.0 ± 26.0	51.1 ± 22.3	41.3 ± 22.8	55.8 ± 7.58	0.561
CD4	41.8 ± 17.8	55.2 ± 18.6	63.8 ± 14.4	46.9 ± 12.2	<0.001
CD8	49.3 ± 18.5	28.4 ± 17.5	22.5 ± 12.8	17.4 ± 5.14	<0.001
$CD4^+PD\text{-}1^+$	17.9 ± 11.7	24.7 ± 19.9	4.37 ± 5.52	14.1 ± 23.0	<0.001
$CD4^+TIGIT^+$	22.3 ± 21.3	15.6 ± 23.2	2.73 ± 2.02	9.09 ± 23.7	<0.001
$CD8^+PD\text{-}1^+$	26.5 ± 16.9	33.5 ± 19.6	4.04 ± 5.16	6.25 ± 5.05	<0.001
$CD8^+TIGIT^+$	23.4 ± 17.5	30.3 ± 29.2	1.61 ± 2.30	2.40 ± 2.17	<0.001
CD56	0.922 ± 2.70	1.27 ± 1.38	11.3 ± 5.88	7.23 ± 10.9	<0.001
$CD56^+PD\text{-}1^+$	14.1 ± 20.9	12.6 ± 20.4	0.394 ± 1.11	7.32 ± 10.0	<0.001
$CD56^+TIGIT^+$	28.6 ± 24.3	22.9 ± 27.7	5.82 ± 6.13	7.73 ± 9.25	0.001
NK	45.8 ± 23.9	37.3 ± 19.0	46.1 ± 19.2	21.6 ± 22.3	0.044
$CD56^{bright}$	3.33 ± 8.48	2.25 ± 3.49	4.70 ± 6.24	8.42 ± 3.35	0.029
$CD56^{dim}$	95.7 ± 9.30	96.7 ± 14.1	92.2 ± 5.34	81.7 ± 6.53	0.005
$CD56^{bright}PD\text{-}1^+$	0.965 ± 6.65	1.36 ± 13.7	1.34 ± 0.879	0.360 ± 1.51	0.328
$CD56^{bright}TIGIT^+$	0.665 ± 5.92	0.480 ± 2.79	0.395 ± 0.561	0.0600 ± 1.58	0.076
$CD56^{dim}PD\text{-}1^+$	10.9 ± 13.2	34.4 ± 18.1	6.93 ± 8.88	5.73 ± 7.18	<0.001
$CD56^{dim}TIGIT^+$	19.9 ± 16.8	2.92 ± 25.7	1.94 ± 2.36	0.240 ± 1.50	<0.001
NKT-like cells	6.33 ± 6.94	7.97 ± 6.33	10.00 ± 14.9	0.180 ± 2.65	0.030
$NKT\ PD\text{-}1^+$	28.6 ± 19.4	26.2 ± 14.9	6.93 ± 10.3	10.0 ± 5.43	<0.001
$NKT\ TIGIT^+$	31.5 ± 23.7	6.83 ± 26.5	8.22 ± 4.81	0.00 ± 1.50	<0.001

Matrix correlations, including all correlation data of immunological and lung function test parameters in HCs, sarcoidosis, IPF, and PCPF, are reported in Supplementary Materials (Figures S1–S4).

Statistically significant differences in immunological findings between IPF, PCPF, sarcoidosis, and HC are reported in Figure 3.

There was a direct correlation between $CD4^+$ and $CD8^+$ in PCPF patients ($p < 0.001$; r = 0.90), while in IPF and sarcoidosis patients, $CD4^+$ and $CD8^+$ were inversely correlated ($p < 0.001 = -0.87$ and $p = 0.042$; r = −0.6, respectively).

Figure 4 shows the statistically significant comparative analysis of CD4-, CD8-, and CD56-expressed PD-1 and TIGIT in the four groups, IPF, PCPF, sarcoidosis, and HC.

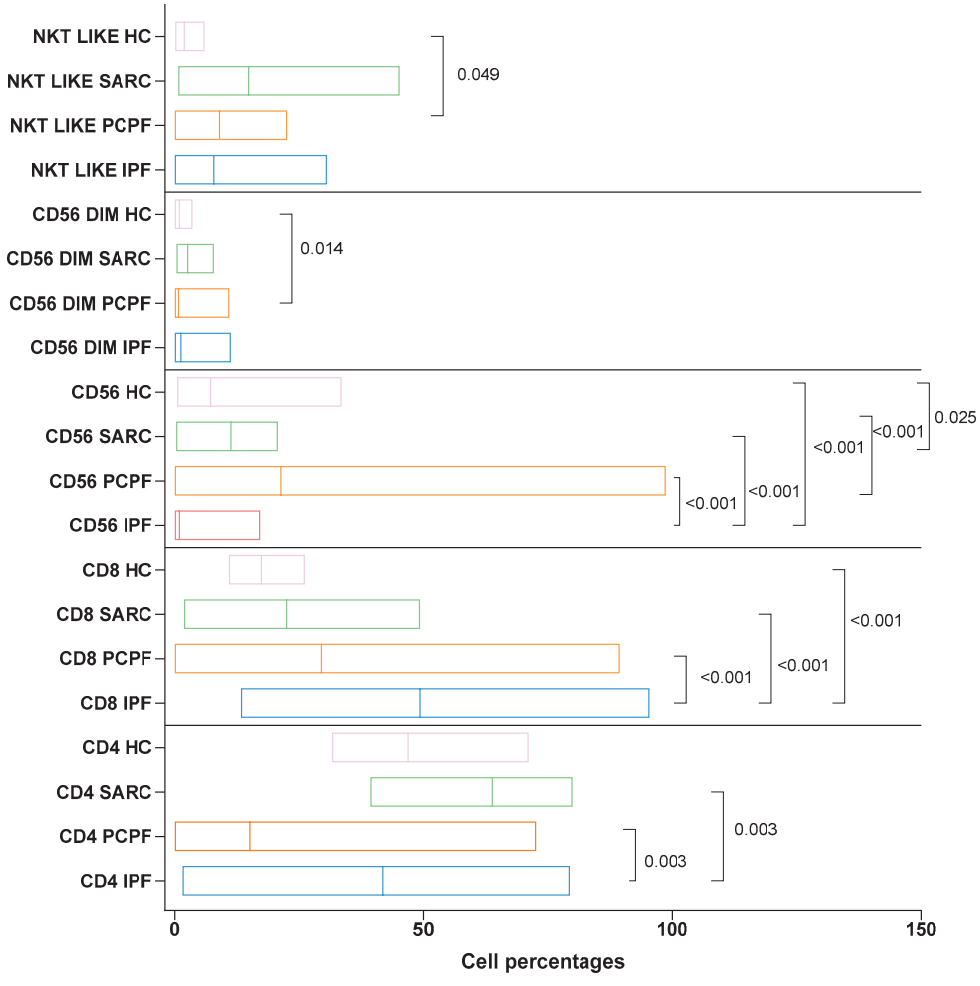


Figure 3. Comparison of CD4-, CD8-, and CD56-. CD56 ^{DIM} and NKT-like positive cell percentages in the three groups of diseases and healthy controls (HCs): IPF, idiopathic pulmonary fibrosis; PCPF, post-COVID-19 pulmonary fibrosis; SARC, sarcoidosis. Numerical values reported in the figure indicate the *p* values obtained via comparative analysis of groups: HCs, IPF, PCPF, and sarcoidosis.

A direct correlation was found in PCPF patients between $CD4^+$ cells and $CD8^+$, as well as $PD\text{-}1^+$ and $CD8^+$ $TIGIT^+$ ($r = 0.55$, $p < 0.001$ and $r = 0.55$, $p < 0.001$, respectively).

Table 4 shows the mean ± standard deviation of percentages of CD4, CD8, and CD56 cells co-expressing PD-1 and TIGIT.

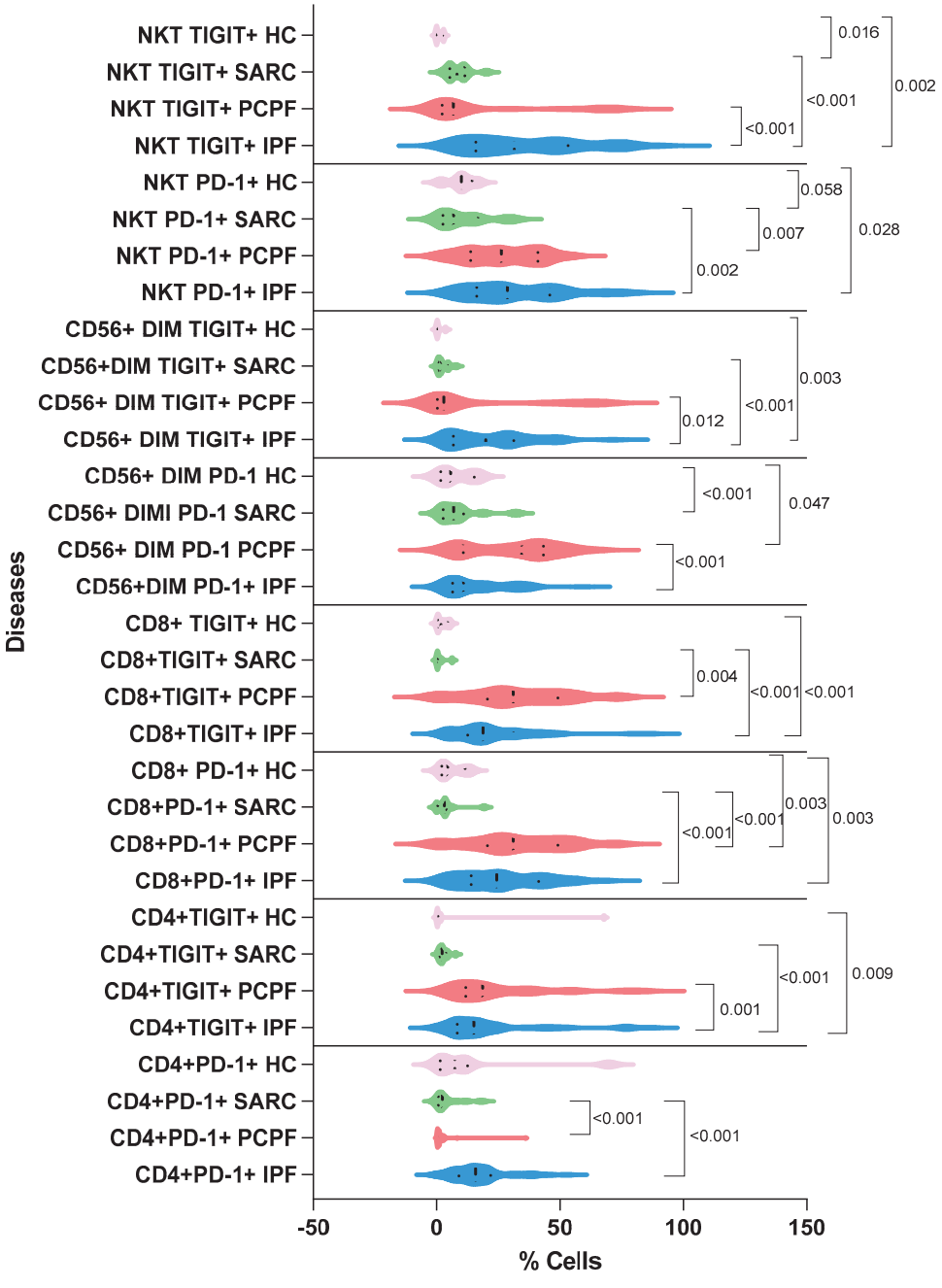


Figure 4. Comparison of CD4-, CD8-, and CD56-positive cell percentages expressing PD-1 and TIGIT in the three groups of diseases and healthy controls (HCs): IPF, idiopathic pulmonary fibrosis; SARC, sarcoidosis; PCPF, post-COVID-19 pulmonary fibrosis. Numerical values reported in the figure indicate the *p* values obtained via comparative analysis between HC, IPF, PCPF, and sarcoidosis groups.

Table 4. Co-expression of PD-1 and TIGIT on the surface of CD4, CD8, and CD56 cells. All data were expressed as means ± standard deviations. Lower $CD4^+PD\text{-}1^+TIGIT^+$, $CD8^+PD\text{-}1^+TIGIT^+$, and $CD56^+PD\text{-}1^+TIGIT^+$ cell percentages were in HCs compared to IPF, PCPF, and sarcoidosis ($p < 0.05$). Further statistically significant differences in $CD4^+PD\text{-}1^+TIGIT^+$ cell percentages were found between (a) IPF and sarcoidosis ($p = 0.013$). $CD8^+PD\text{-}1^+TIGIT^+$ was higher in (b) IPF than in sarcoidosis ($p < 0.001$), as well as in (c) PCPF compared to IPF (mettere p) and sarcoidosis ($p < 0.001$). Higher $CD56^+PD\text{-}1^+TIGIT^+$ cell percentages were in (d) IPF than in the PCPF and sarcoidosis groups ($p = 0.038$ and $p = 0.005$, respectively).

Double Positive Cell Percentages	IPF (*n* = 48)	PCPF (*n* = 55)	SARCOIDOSIS (*n* = 12)	HCs (*n* = 10)
$CD4^+PD\text{-}1^+TIGIT^+$	6.21 ± 5.97 [a]	8.67 ± 14.6	4.01 ± 9.83	0.0187 ± 0.0236
$CD8^+PD\text{-}1^+TIGIT^+$	7.12 ± 8.12 [b]	17.9 ± 20.3 [c]	0.0709 ± 0.235	0.374 ± 0.716
$CD56^+PD\text{-}1^+TIGIT^+$	15.7 ± 23.2 [d]	10.5 ± 21.9	3.39 ± 6.64	1.61 ± 2.80

3.3. Lung Function Tests

IPF patients showed significantly lower FEV1(%) and FVC (%) than the PCPF ($p = 0.016$ and $p < 0.001$, respectively) and sarcoidosis cohorts ($p = 0.041$ and $p = 0.001$, respectively). Accordingly, we observed a significant reduction in DLCO (%) between the IPF cohort and PCPF and sarcoidosis patients ($p < 0.001$; $p = 0.045$, respectively).

4. Discussion

Here, we compared T and NK cell percentages in peripheral blood from IPF and PCPF patients with those of sarcoidosis and HC groups. CD4 and NK cells were more depleted in the IPF than in the other groups, even in normal ranges, in contrast with CD8 cells. Correlation analysis demonstrated an indirect correlation between CD4 and CD8 cells in IPF and sarcoidosis patients. Conversely, PCPF patients showed a direct correlation between CD4 and CD8 cells, highlighting the restoration of an immune response.

Although data in the literature highlighted the similarities between post-COVID-19 syndrome and lung fibrosis, studies have reported the possible involvement of inflammatory cytokines, the renin–angiotensin system, the potential role of galectin-3, epithelial injuries in fibrosis, alveolar type 2 involvement, neutrophil extracellular traps, and other specific aspects (relationship with clinical and mechanical factors, epithelial transition mesenchymal, TGF-β signalling pathway, macrophages). Our study, for the first time, highlighted the similarities and differences between two fibrotic diseases, IPF and PCPF, in accordance with lymphocyte subsets and their functional phenotype.

Galati et al. showed a lack of significant differences in CD4 cell percentages between patients with IPF and healthy HCs [11], as confirmed by our results. Nevertheless, peripheral lymphopenia in IPF patients (with respect to those with other interstitial lung diseases (ILDs)) was confirmed to be a prognostic marker of disease progression [12]. This is the first time that researchers have compared two fibrotic diseases, IPF and PCPF, and demonstrated restoration of CD4 cells 6–9 months after SARS-CoV-2 infection. This finding suggests that lung fibrosis evolves differently after COVID-19 than in IPF, where it is chronic, progressive, and idiopathic.

The functional phenotype of T and NK cells in our patient cohort was demonstrated via expression of exhaustion T cell markers, PD-1, and TIGIT. Expression of PD-1 and TIGIT was abundant on T and NK cell subsets of the two lung fibrotic groups, IPF and PCPF. Likewise, co-expression of PD-1 and TIGIT on the surface of CD4 and CD8 cells increased in these diseases. IPF patients showed the highest co-expression of PD-1 and TIGIT on NK cells. Moreover, lung parameters showed more impaired respiratory function in IPF patients than in the PCPF and sarcoidosis groups. TIGIT expression was evaluated in our cohorts because it is a member of the second wave of IC receptors, which work in synergy with PD-1 [10]. Tumour-infiltrating T lymphocytes and NK cells express TIGIT in lung

tissues. Increased expression of TIGIT on CD4 and CD8 cells observed in our IPF cohort may suggest recruitment of T cells from peripheral blood (this explains the peripheral lymphopenia of IPF patients) to the lung interstitium, as described in the histopathological features of IPF.

Moreover, CD4 cells expressing PD-1 were higher in our fibrotic cohort (IPF and PCPF patients) than in the sarcoidosis group, suggesting involvement of PD-1 in fibrotic disorders but not granulomatous diseases. This is the first report of this finding, and it is in line with human models of lung fibrosis, where overexpression of $CD4^+PD1^+$ cells has been observed, suggesting dysregulation of immune checkpoint expression which influences the pathogenesis of IPF [13]. TIGIT is reported to be highly expressed on dysfunctional or exhausted T cells in chronic diseases such as chronic viral infection and cancer [14]. We observed lower percentages of $CD4^+$- and $CD8^+$-$TIGIT^+$ in sarcoidosis patients than in our fibrotic groups, IPF and PCPF, suggesting that the latter disorders may express a higher degree of exhaustion of T cells. In exhausted CD4, CD8, and NK cells, several immune checkpoints were co-expressed with PD-1 and provided a synergistic inhibitory effect. In addition, TIGIT indicated more severe exhaustion. In line with a more severely exhausted phenotype, our data show a more impaired immune system in IPF than in sarcoidosis patients, as demonstrated by the highest CD4-, CD8-, and CD56-$PD\text{-}1^+$ $TIGIT^+$ cell percentages.

Concerning CD8 cells, little data are available on their role in fibrotic diseases. Deng et al. suggested that they promote development of fibrosis in IPF through infiltration followed by differentiation into fibrotic tissues producing interleukins, such as Interferon-gamma (IFN-γ). Our results demonstrate higher CD8 cell percentages in IPF than in the other groups, inversely correlated with CD4 cell percentages, which are associated with severe lung injury demonstrated by low FEV1, FVC, and DLCO. Flow cytometry assessment of CD8 cell percentages could help physicians identify fibrotic patients in cases where ILD is suspected. Rha et al. found that a decrease in CD4 cells contributed to CD8 cell exhaustion in hospitalised COVID-19 patients [15]. Our study is the first to report a direct correlation between CD4 and the exhausted phenotype of CD8 cells expressing PD-1 and TIGIT in PCPF patients. This finding suggests that exhausted CD8+ cells may play a role in the active phase of COVID-19 and in long-term sequelae. In the stage II sarcoidosis patients (without pulmonary fibrosis) enrolled in the present study, lower CD8+PD-1+ cell percentages were found, in line with our previous original article [16].

Little is known about the biology of NK cells in the lungs, though NK cell percentages proved to be a potential marker of survival in IPF [17]. Our study is the first to compare peripheral $CD56^{dim}$ and $CD56^{bright}$ in two fibrotic lung diseases, IPF and PCPF. Lower percentages of $CD56^{bright}$ were found in PCPF than in the HC group, unlike the mature phenotype ($CD56^{dim}$), presumably due to the previous response to SARS-CoV-2 infection.

The role of PD-1 in healthy NK cells was investigated by Esen et al. [18], who demonstrated reduced expression of IFN-γ and Tumour necrosis factor-α (TNF-α), as well as reduced degranulation. Moreover, despite their lower expression in PBMC, the subgroup of NK cells expressing PD-1 was CD56dim. In line with this study, our data showed abundant expression of PD-1 on CD56dim, mainly in PCPF patients with respect to HC. Quatrini et al. [19] suggested that PD-1 expression is not associated with NK cell exhaustion but rather with acute activation. This may explain why more robust PD-1 expression can be observed in NK cells that are stimulated, for example, by COVID-19.

Studies on TIGIT expression in NK cells are limited. Faqrul Hasan et al. [20] suggested that expression of TIGIT is a marker of NK cell activation. However, chronic TIGIT engagement with its ligands in a tumour microenvironment leads to a functional decline in NK cells. In line with the literature, our results show that CD56dim TIGIT+ cells were more expressed in IPF patients, supporting the concept of immune system dysfunction [20]. In addition, NKT that expressed TIGIT were more numerous in IPF patients than in the other groups, which suggests a recruiter role of immune cells expressing TIGIT at the site of damage. Even though our study highlighted the similarities and differences between PCPF,

IPF, and sarcoidosis compared with HCs, our study did not include a larger multicenter cohort, and PCPF needs to be investigated in a longer follow-up. These findings are worth being analysed in other biological fluids.

5. Conclusions

Our study shines a light on the immune responses involved in the development of pulmonary fibrosis, both idiopathic and secondary to SARS-CoV-2 infection. We only observed a significant imbalance in CD4, CD8, and NK percentages in peripheral blood from IPF and PCPF patients, but also in their functional phenotypes, evaluated through immune checkpoint expression. Our study confirmed immunological similarities between IPF and PCPF. Further study of these immunological pathways with a longer follow-up for PCPF patients would be worthwhile.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/biomedicines12030630/s1. Figure S1. Matrix correlation of immunological and lung function test parameters in PCPF patients; Figure S2. Matrix correlation of immunological and lung function test parameters in IPF patients; Figure S3. Matrix correlation of immunological and lung function test parameters in sarcoidosis patients; Figure S4. Matrix correlation of immunological and lung function test parameters in healthy controls.

Author Contributions: S.G. and M.d., designing research studies; S.G., L.B., P.C. and I.P., conducting experiments; M.S., sample conservation; S.G., acquiring data; S.G., L.B. and M.d., analysing data; E.B., providing reagents; all authors, writing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee of Siena University Hospital (C.E.A.V.S.E.) (protocol code Markerlung 17431; date of approval 15 June 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors have declared that no conflict of interest exists.

Abbreviations

IPF, idiopathic pulmonary fibrosis; PCPF, post-COVID is pulmonary fibrosis; IC, immune checkpoint; HC, healthy control; TGF-β, Transforming growth factor beta; ECM, extracellular matrix; PD-1; Programmed cell death protein-1; TIGIT, T cell immunoglobulin and ITIM domain; NK, natural Killer; HRCT, highresolution computed tomography; IQR, interquartile range; p value, probability value; CD-, cluster of differentiation; ILD, interstitial lung disease; IFN-γ, Interferon-gamma; TNF-α, Tumor necrosis factor-α.

References

1. Mutsaers, S.E.; Miles, T.; Prêle, C.M.; Hoyne, G.F. Emerging role of immune cells as drivers of pulmonary fibrosis. *Pharmacol. Ther.* **2023**, *252*, 108562. [CrossRef] [PubMed]
2. Desai, O.; Winkler, J.; Minasyan, M.; Herzog, E.L. The Role of Immune and Inflammatory Cells in Idiopathic Pulmonary Fibrosis. *Front. Med.* **2018**, *5*, 43. [CrossRef] [PubMed]
3. Fernandez, I.E.; Greiffo, F.R.; Frankenberger, M.; Bandres, J.; Heinzelmann, K.; Neurohr, C.; Hatz, R.; Hartl, D.; Behr, J.; Eickelberg, O. Peripheral blood myeloid-derived suppressor cells reflect disease status in idiopathic pulmonary fibrosis. *Eur. Respir. J.* **2016**, *48*, 1171–1183. [CrossRef] [PubMed]
4. Patrucco, F.; Solidoro, P.; Gavelli, F.; Apostolo, D.; Bellan, M. Idiopathic Pulmonary Fibrosis and Post-COVID-19 Lung Fibrosis: Links and Risks. *Microorganisms* **2023**, *11*, 895. [CrossRef] [PubMed]

5. Deng, L.; Huang, T.; Zhang, L. T cells in idiopathic pulmonary fibrosis: Crucial but controversial. *Cell Death Discov.* **2023**, *9*, 62. [CrossRef] [PubMed]
6. Koh, C.-H.; Lee, S.; Kwak, M.; Kim, B.-S.; Chung, Y. CD8 T-cell subsets: Heterogeneity, functions, and therapeutic potential. *Exp. Mol. Med.* **2023**, *55*, 2287–2299. [CrossRef] [PubMed]
7. Croft, M.; Carter, L.; Swain, S.L.; Dutton, R.W. Generation of polarized antigen-specific CD8 effector populations: Reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J. Exp. Med.* **1994**, *180*, 1715–1728. [CrossRef] [PubMed]
8. Bergantini, L.; d'Alessandro, M.; Gangi, S.; Bianchi, F.; Cameli, P.; Perea, B.; Meocci, M.; Fabbri, G.; Marrucci, S.; Ederbali, M.; et al. Predictive Role of Cytokine and Adipokine Panel in Hospitalized COVID-19 Patients: Evaluation of Disease Severity, Survival and Lung Sequelae. *Int. J. Mol. Sci.* **2023**, *24*, 12994. [CrossRef] [PubMed]
9. Zhao, Y.; Qu, Y.; Hao, C.; Yao, W. PD-1/PD-L1 axis in organ fibrosis. *Front. Immunol.* **2023**, *14*, 1145682. [CrossRef] [PubMed]
10. Ge, Z.; Peppelenbosch, M.P.; Sprengers, D.; Kwekkeboom, J. TIGIT, the Next Step Towards Successful Combination Immune Checkpoint Therapy in Cancer. *Front. Immunol.* **2021**, *12*, 699895. [CrossRef] [PubMed]
11. Galati, D.; De Martino, M.; Trotta, A.; Rea, G.; Bruzzese, D.; Cicchitto, G.; Stanziola, A.A.; Napolitano, M.; Sanduzzi, A.; Bocchino, M. Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients. *Cytokine* **2014**, *66*, 119–126. [CrossRef] [PubMed]
12. Achaiah, A.; Rathnapala, A.; Pereira, A.; Bothwell, H.; Dwivedi, K.; Barker, R.; Iotchkova, V.; Benamore, R.; Hoyles, R.K.; Ho, L.-P. Neutrophil lymphocyte ratio as an indicator for disease progression in Idiopathic Pulmonary Fibrosis. *BMJ Open Respir. Res.* **2022**, *9*, e001202. [CrossRef] [PubMed]
13. Celada, L.J.; Kropski, J.A.; Herazo-Maya, J.D.; Luo, W.; Creecy, A.; Abad, A.T.; Chioma, O.S.; Lee, G.; Hassell, N.E.; Shaginurova, G.I.; et al. PD-1 up-regulation on $CD4^+$ T cells promotes pulmonary fibrosis through STAT3-mediated IL-17A and TGF-β1 production. *Sci. Transl. Med.* **2018**, *10*, eaar8356. [CrossRef] [PubMed]
14. Anderson, A.C.; Joller, N.; Kuchroo, V.K. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* **2016**, *44*, 989–1004. [CrossRef] [PubMed]
15. Rha, M.-S.; Shin, E.-C. Activation or exhaustion of CD8+ T cells in patients with COVID-19. *Cell. Mol. Immunol.* **2021**, *18*, 2325–2333. [CrossRef] [PubMed]
16. d'Alessandro, M.; Bergantini, L.; Mezzasalma, F.; Cavallaro, D.; Gangi, S.; Baglioni, S.; Armati, M.; Abbritti, M.; Cattelan, S.; Cameli, P.; et al. Immune-Checkpoint Expression on CD4, CD8 and NK Cells in Blood, Bronchoalveolar Lavage and Lymph Nodes of Sarcoidosis. *Mol. Diagn. Ther.* **2022**, *26*, 437–449. [CrossRef] [PubMed]
17. Bergantini, L.; d'Alessandro, M.; Cameli, P.; Otranto, A.; Finco, T.; Curatola, G.; Sestini, P.; Bargagli, E. Prognostic role of NK cell percentages in bronchoalveolar lavage from patients with different fibrotic interstitial lung diseases. *Clin. Immunol.* **2021**, *230*, 108827. [CrossRef] [PubMed]
18. Esen, F.; Deniz, G.; Aktas, E.C. PD-1, CTLA-4, LAG-3, and TIGIT: The roles of immune checkpoint receptors on the regulation of human NK cell phenotype and functions. *Immunol. Lett.* **2021**, *240*, 15–23. [CrossRef] [PubMed]
19. Quatrini, L.; Mariotti, F.R.; Munari, E.; Tumino, N.; Vacca, P.; Moretta, L. The Immune Checkpoint PD-1 in Natural Killer Cells: Expression, Function and Targeting in Tumour Immunotherapy. *Cancers* **2020**, *12*, 3285. [CrossRef] [PubMed]
20. Hasan, M.F.; Croom-Perez, T.J.; Oyer, J.L.; Dieffenthaller, T.A.; Robles-Carrillo, L.D.; Eloriaga, J.E.; Kumar, S.; Andersen, B.W.; Copik, A.J. TIGIT Expression on Activated NK Cells Correlates with Greater Anti-Tumor Activity but Promotes Functional Decline upon Lung Cancer Exposure: Implications for Adoptive Cell Therapy and TIGIT-Targeted Therapies. *Cancers* **2023**, *15*, 2712. [CrossRef] [PubMed]

MDPI AG
Grosspeteranlage 5
4052 Basel
Switzerland
Tel.: +41 61 683 77 34

Biomedicines Editorial Office
E-mail: biomedicines@mdpi.com
www.mdpi.com/journal/biomedicines

Academic Open
Access Publishing

mdpi.com

ISBN 978-3-7258-1506-7