nutrients

Biomarkers and Nutrients in Musculoskeletal Disorders

Edited by
César Calvo-Lobo, Ricardo Becerro de Bengoa Vallejo, Marta Elena Losa-Iglesias, Marta San-Antolín, David Rodríguez Sanz and Daniel López-López

Printed Edition of the Special Issue Published in *Nutrients*

www.mdpi.com/journal/nutrients

MDPI

Biomarkers and Nutrients in Musculoskeletal Disorders

Biomarkers and Nutrients in Musculoskeletal Disorders

Editors

César Calvo-Lobo
Ricardo Becerro de Bengoa Vallejo
Marta Elena Losa-Iglesias
Marta San-Antolín
David Rodríguez Sanz
Daniel López-López

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade • Manchester • Tokyo • Cluj • Tianjin

Editors

César Calvo-Lobo
Universidad Complutense de Madrid
Spain

Ricardo Becerro de Bengoa Vallejo
Universidad Complutense de Madrid
Spain

Marta Elena Losa-Iglesias
Universidad Rey Juan Carlos
Spain

Marta San-Antolín
Universidad Europea de Madrid
Spain

David Rodríguez Sanz
Universidad Complutense de Madrid
Spain

Daniel López-López
Universidade da Coruña
Spain

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Nutrients* (ISSN 2072-6643) (available at: https://www.mdpi.com/journal/nutrients/special_issues/biomarkers_musculoskeletal).

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

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

ISBN 978-3-0365-3351-3 (Hbk)
ISBN 978-3-0365-3352-0 (PDF)

Contents

nutrients

MDPI

Editorial

Biomarkers and Nutrients in Musculoskeletal Disorders

César Calvo-Lobo [1], Ricardo Becerro-de-Bengoa-Vallejo [1], Marta Elena Losa-Iglesias [2], David Rodríguez-Sanz [1,*], Daniel López-López [3] and Marta San-Antolín [4]

[1] Facultad de Enfermería, Fisioterapia y Podología, Universidad Complutense de Madrid, 28040 Madrid, Spain; cescalvo@ucm.es (C.C.-L.); ribebeva@ucm.es (R.B.-d.-B.-V.)
[2] Faculty of Health Sciences, Universidad Rey Juan Carlos, 28922 Alcorcón, Spain; marta.losa@urjc.es
[3] Research, Health and Podiatry Group, Department of Health Sciences, Faculty of Nursing and Podiatry, Universidade da Coruña, 150403 Ferrol, Spain; daniel.lopez.lopez@udc.es
[4] Department of Psychology, Universidad Europea de Madrid, 28670 Madrid, Spain; marta.sanantolin@universidadeuropea.es
* Correspondence: davidrodriguezsanz@ucm.es; Tel.: +34-91394-1535

Worldwide, the burden of musculoskeletal disorders is increasing with great variations between-countries, which makes it difficult for policymakers to provide resources and adequate interventions in order to provide for their appropriate management [1]. Thus, musculoskeletal disorders remain a public health problem and their incidence and trend is increasing for some specific conditions [2].

Indeed, nutrients and biological biomarkers play a key role in the prognosis, diagnosis and health status of patients suffering from musculoskeletal conditions, being the main indicators for understanding biological processes as well as tailoring therapeutic interventions and nutritional programs in patients with musculoskeletal disorders [3].

Thus, the purpose of this Special Issue was to provide an update on the state of the art, through current reviews as well as new insights and interventions, about the main role of nutrients and biomarkers in patients who suffer from musculoskeletal conditions from a multidisciplinary point of view. According to the aim of this Special Issue, a total of six papers summarized in Tables 1 and 2 were published between May and December, 2020, in Nutrients.

Among these publications, three papers were reviews providing an update of various topics including the current metabolic impact of COVID-19 pandemic confinement secondary to diet modifications and the reduction of physical activity [4]; the influence of nutrients for osteoporosis prevention in a specific population of patients suffering from inflammatory bowel disease [5]; and the relationship of nutrient and metabolic status with spinal muscular atrophy [6].

In addition, three papers were original research studies supporting key information from a multidisciplinary approach about the effects of curcumin and resveratrol on satellite cells as well as biomarkers for muscle regeneration process [7]; the fat rich diet effect on acetylcholine spontaneous release produced in mice neuromuscular junctions revealing key aspects for the relationship myofascial pain syndrome and nutrients or biomarkers [8]; and the effects of supplementation with vitamin D and L-cysteine on the biomarkers of musculoskeletal conditions in high-fat-diet–fed mice with vitamin D deficiency [9].

The main characteristics of the reviews provided in this Special Issue are presented in Table 1, updating the state of the art of the available scientific evidence on nutrients and biomarkers in musculoskeletal conditions about trending topics such as the effects of the COVID-19 pandemic, the prevention of osteoporosis related to inflammatory bowel disease, as well as the influence of nutrients and biomarkers in spinal muscular atrophy [4–6].

Firstly, Martínez-Ferran et al. [4] provided an interesting review which detailed the key metabolic consequences of the COVID-19 confinement, such as increased insulin resistance, body and abdominal fat, as well as inflammatory cytokines, which are linked to metabolic

Citation: Calvo-Lobo, C.; Becerro-de-Bengoa-Vallejo, R.; Losa-Iglesias, M.E.; Rodríguez-Sanz, D.; López-López, D.; San-Antolín, M. Biomarkers and Nutrients in Musculoskeletal Disorders. *Nutrients* **2021**, *13*, 283. https://doi.org/10.3390/nu13020283

Received: 14 January 2021
Accepted: 18 January 2021
Published: 20 January 2021

syndrome development and the appearance of musculoskeletal disorders, among others. The authors encouraged researchers to explore positive energy balance as a plausible mechanism for these nutritional and musculoskeletal disorders, and suggested that the restriction of calorie intake could prevent the COVID-19 confinement impact associated with physical inactivity.

Table 1. The characteristics of the review studies included in the Special Issue about nutrients and biomarkers in musculoskeletal diseases.

Month, Year and Authors	Study Design and Characteristics	Main Findings
May, 2020. Martínez-Ferrán et al. [4]	Narrative review including 66 references.	Metabolic impairment consequences of COVID-19 physical inactivity, overweight, sedentary lifestyle, overfeeding and dietary intake modifications may predispose to musculoskeletal disorders. The restriction of calorie intake was proposed to prevent the COVID-19 confinement impact on physical inactivity and musculoskeletal conditions.
June, 2020. Ratajczak et al. [5]	Narrative review including 134 references.	Protein, fat, carbohydrate, vitamin, mineral, microelement and polyphenol intakes may predispose to osteoporosis and reduced bone mineral density in patients suffering from inflammatory bowel diseases. The development of nutritional guidelines to prevent osteoporosis for patients suffering from inflammatory bowel diseases should especially take into account vitamin D and calcium for bones mineral density, as well as vitamins such as A, B_{12}, C and K, calcium, folic acid, magnesium, phosphorus, zinc, sodium, selenium and copper for bones mass formation.
December, 2020. Li et al. [6]	Narrative review including 85 references.	Lipid metabolic alterations, glucose metabolic modifications and vitamin levels alteration may be linked to neurodegenerative diseases such as spinal muscular atrophy. Dietary issues monitoring by biomarkers and nutrients may determine nutritional status and therapeutic target in patients who suffer from spinal muscular atrophy.

Secondly, Ratajczak et al. [5] reviewed the influence of nutrients in preventing osteoporosis and low bone mineral density in patients who suffered from inflammatory bowel disease, i.e. ulcerative colitis and Crohn's disease. Vitamin D and calcium were the most commonly studied nutrients for bone mineral density. In addition, vitamins such as A, B_{12}, C and K, calcium, folic acid, magnesium, phosphorus, zinc, sodium, selenium and copper were implicated in bone mass formation. These patients seemed to commonly consume inadequate amounts of these vitamins and minerals, impairing absorption and disturbing nutritional status with an increase in the risk of osteoporosis. Therefore, the authors also encouraged researchers to develop nutritional guidelines for patients suffering from inflammatory bowel diseases to prevent osteoporosis.

Lastly, the review carried out by Li et al. [6] highlighted the relationship between nutrient and metabolic status and neurodegenerative diseases. Concretely, various fatty acids' metabolic alterations and glucose tolerance impairment were linked to spinal muscular atrophy. Thus, nutritional support as well as monitoring of biomarkers and nutrients may play a key role in patients who suffer from spinal muscular atrophy. Furthermore, metabolomics may provide a promising support as therapeutic targets or specific biomarkers for metabolic alterations and for the quantification of specific metabolites in patients who suffer from this neurodegenerative disease.

The principal characteristics of the original research reports published in this Special Issue are presented in Table 2, providing new insights and interventions about the key role of nutrients and biomarkers in patients who suffer from musculoskeletal conditions, dealing with specific main topics such as the importance of satellite cells in the process of muscle regeneration, the release of acetylcholine in the neuromuscular junction for the myofascial pain syndrome development, as well as nutritional supplementation for musculoskeletal disorders [7–9].

Table 2. Characteristics of the original research reports included in the Special Issue about nutrients and biomarkers in musculoskeletal diseases.

Month, Year and Authors	Study Design and Characteristics	Main Findings
June, 2020. Mañas-García et al. [7]	Experimental laboratory study in female mice (10 weeks old, weight ~20 g).	Pharmacological agents increased sirtuin-1 activity related to curcumin and resveratrol enhanced muscle tissue regeneration. Authors claimed potential clinical effects of these phenolic compounds for muscle disuse and atrophy to improve muscle regeneration.
October, 2020. Gimenez-Donoso et al. [8]	Experimental laboratory study in young (45–50 days) adult Swiss male mice.	A fat rich diet produced acetylcholine spontaneous release in neuromuscular junction of mice. Authors claimed that hypercaloric diet supplementation increased spontaneous neurotransmission in the neuromuscular junction suggesting the consequent activation of myofascial trigger points to originate myofascial pain syndrome.
November, 2020. Parsanathan et al. [9]	Experimental laboratory study in male mice (5 weeks old, 20–24 g).	Vitamin D and L-cysteine cosupplementation produced an improvement of myogenic biomarkers of musculoskeletal conditions and gene expression. Authors revealed that this cosupplementation improved muscle biomarkers linked to musculoskeletal conditions more than monotherapy.

First, Mañas-García et al. [7] carried out an excellent experimental laboratory study detailing the curcumin and resveratrol effects on satellite cells by analyzing muscle regeneration biomarkers. Resveratrol and curcumin supplementation in the immobilized muscles of mice elicited an increase in muscle satellite cells. Curcumin treatment for reloaded muscles improved the cross-sectional area of hybrid muscle fibers and sirtuin-1 activity, while resveratrol treatment for reloaded muscles improved the cross-sectional area of fast-twitch muscle fibers, sirtuin-1 content and progenitor muscle cell count. In addition, curcumin and resveratrol treatment for unloaded muscles improved the satellite cell number. The authors encourage the use of curcumin and resveratrol due to their potential clinical effects against muscle disuse and atrophy to optimize the muscle regeneration process.

Second, Gimenez-Donoso et al. [8] performed an outstanding experimental laboratory study which supported novel findings in a prevalent musculoskeletal condition such as myofascial pain syndrome, showing the first relationship in the research literature between this syndrome and nutritional biomarker status due to a fat rich diet producing acetylcholine spontaneous release in neuromuscular junction of mice. Spontaneous acetylcholine release at the neuromuscular junction was previously proposed as the key mechanism activating a vicious circle which perpetuates myofascial pain syndrome as a group of motor, sensitive and autonomic signs and symptoms originating from myofascial trigger points. This experimental laboratory study was carried out in male Swiss mice evaluating intramuscular adipocytes with Sudan-III and the plaque noise of the neuromuscular junction suggesting spontaneous acetylcholine release by electromyography. An increased plaque noise was presented after the interruption of the proposed diets. Thus, supplementation by a hypercaloric diet increased spontaneous neurotransmission in the neuromuscular junction promoting the development of myofascial trigger points.

Finally, Parsanathan et al. [9] performed an experimental laboratory study in high-fat-diet–fed mice with vitamin D deficiency, showing that vitamin D and L-cysteine cosupplementation mitigated biomarkers of musculoskeletal conditions. Vitamin D and L-cysteine cosupplementation provided beneficial effects for gene expression regarding myogenic biomarkers. Therefore, cosupplementation of vitamin D and L-cysteine improved the skeletal muscle biomarkers of musculoskeletal conditions more than monotherapy in vitamin D deficient high-fat-diet mice.

Future studies should address the role of ultrasound imaging as a promising tool for nutrient and biomarker status in musculoskeletal disorders [10]. The analysis of muscle tissue by sonoelastography may provide an alternative tool to indirectly evaluate

sarcopenia and the lack of skeletal muscle mass from both qualitative and quantitative points of view, presenting a sensitivity of 77.3%, a specificity of 100% and a diagnostic accuracy of 87.5% [11]. The expression of mediators of neoangiogenesis and vascular density of the synovial characteristics in patients with rheumatoid arthritis provided a good correlation (r = 0.73) with the vascular area at histological level, linked to the cellular profile and pro-inflammatory cytokines, which provided considerable validity for using these measurements as objective assessments of synovial inflammation in clinical practice [12]. In addition, ultrasound analysis of the echo-texture, echo-intensity and echo-variation of the muscle tissue suggested promising results as biomarkers in neuromuscular pathologies such as amyotrophic lateral sclerosis [13]. Finally, the ultrasound software for the evaluation of muscle glycogen in the skeletal muscle may be decisive in detailing the musculoskeletal status and risk of injury in sport performance with promising results in this field, being a validated software with respect to muscle biopsy (r = 0.81) that allowed a non-invasive assessment of muscle glycogen [14,15].

In conclusion, the main findings of this Special Issue summarize the current scientific evidence available about nutrients and biomarkers in musculoskeletal diseases related to the metabolic impairment secondary to COVID-19 confinement, the prevention of osteoporosis for patients suffering from inflammatory bowel diseases, and nutritional issues in patients who suffer from spinal muscular atrophy, as well as new insights from experimental animal models on pharmacological agents to enhance muscle tissue regeneration, spontaneous acetylcholine release in the neuromuscular junction of mice by hypercaloric diet supplementation increasing spontaneous neurotransmission and the consequent activation of myofascial trigger points to originate myofascial pain syndrome, and the improvement of myogenic biomarkers of musculoskeletal conditions and gene expression due to vitamin D and L-cysteine cosupplementation. Future studies should investigate the role of ultrasound imaging as a promising tool for nutrient and biomarker status in musculoskeletal disorders.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

References

1. Safiri, S.; Kolahi, A.A.; Cross, M.; Carson-Chahhoud, K.; Almasi-Hashiani, A.; Kaufman, J.; Mansournia, M.A.; Sepidarkish, M.; Ashrafi-Asgarabad, A.; Hoy, D.; et al. Global, regional, and national burden of other musculoskeletal disorders 1990–2017: Results from the Global Burden of Disease Study 2017. *Rheumatology* **2020**, keaa315. [CrossRef] [PubMed]
2. Jin, Z.; Wang, D.; Zhang, H.; Liang, J.; Feng, X.; Zhao, J.; Sun, L. Incidence trend of five common musculoskeletal disorders from 1990 to 2017 at the global, regional and national level: Results from the global burden of disease study 2017. *Ann. Rheum. Dis.* **2020**, *79*, 1014–1022. [CrossRef] [PubMed]
3. Collino, S.; Martin, F.P.; Karagounis, L.G.; Horcajada, M.N.; Moco, S.; Franceschi, C.; Kussmann, M.; Offord, E. Musculoskeletal system in the old age and the demand for healthy ageing biomarkers. *Mech. Ageing Dev.* **2013**, *134*, 541–547. [CrossRef] [PubMed]
4. Martinez-Ferran, M.; de la Guía-Galipienso, F.; Sanchis-Gomar, F.; Pareja-Galeano, H. Metabolic Impacts of Confinement during the COVID-19 Pandemic Due to Modified Diet and Physical Activity Habits. *Nutrients* **2020**, *12*, 1549. [CrossRef] [PubMed]
5. Ratajczak, A.E.; Rychter, A.M.; Zawada, A.; Dobrowolska, A.; Krela-Kaźmierczak, I. Nutrients in the Prevention of Osteoporosis in Patients with Inflammatory Bowel Diseases. *Nutrients* **2020**, *12*, 1702. [CrossRef] [PubMed]
6. Li, Y.-J.; Chen, T.-H.; Wu, Y.-Z.; Tseng, Y.-H. Metabolic and Nutritional Issues Associated with Spinal Muscular Atrophy. *Nutrients* **2020**, *12*, 3842. [CrossRef] [PubMed]
7. Mañas-García, L.; Guitart, M.; Duran, X.; Barreiro, E. Satellite Cells and Markers of Muscle Regeneration during Unloading and Reloading: Effects of Treatment with Resveratrol and Curcumin. *Nutrients* **2020**, *12*, 1870. [CrossRef] [PubMed]
8. Gimenez-Donoso, C.; Bosque, M.; Vila, A.; Vilalta, G.; Santafe, M.M. Effects of a Fat-Rich Diet on the Spontaneous Release of Acetylcholine in the Neuromuscular Junction of Mice. *Nutrients* **2020**, *12*, 3216. [CrossRef] [PubMed]
9. Parsanathan, R.; Achari, A.E.; Manna, P.; Jain, S.K. l-Cysteine and Vitamin D Co-Supplementation Alleviates Markers of Musculoskeletal Disorders in Vitamin D-Deficient High-Fat Diet-Fed Mice. *Nutrients* **2020**, *12*, 3406. [CrossRef] [PubMed]

10. Romero-Morales, C.; Bravo-Aguilar, M.; Ruiz-Ruiz, B.; Almazán-Polo, J.; López-López, D.; Blanco-Morales, M.; Téllez-González, P.; Calvo-Lobo, C. Current advances and research in ultrasound imaging to the assessment and management of musculoskeletal disorders. *Dis. Mon.* **2020**, 101050. [CrossRef] [PubMed]
11. Kim, K.-C.; Park, J.-W. Assessing Low Skeletal Mass in Patients Undergoing Hip Surgery: The Role of Sonoelastography. *Hip Pelvis* **2020**, *32*, 132. [CrossRef] [PubMed]
12. Kelly, S.; Bombardieri, M.; Humby, F.; Ng, N.; Marrelli, A.; Riahi, S.; DiCicco, M.; Mahto, A.; Zou, L.; Pyne, D.; et al. Angiogenic gene expression and vascular density are reflected in ultrasonographic features of synovitis in early rheumatoid arthritis: An observational study. *Arthritis Res. Ther.* **2015**, *17*, 58. [CrossRef] [PubMed]
13. Martínez-Payá, J.J.; Ríos-Díaz, J.; Del Baño-Aledo, M.E.; Tembl-Ferrairó, J.I.; Vazquez-Costa, J.F.; Medina-Mirapeix, F. Quantitative Muscle Ultrasonography Using Textural Analysis in Amyotrophic Lateral Sclerosis. *Ultrason. Imaging* **2017**, *39*, 357–368. [CrossRef] [PubMed]
14. San-Millán, I.; Hill, J.C.; Calleja-González, J. Indirect assessment of skeletal muscle glycogen content in professional soccer players before and after a match through a non-invasive ultrasound technology. *Nutrients* **2020**, *12*, 971. [CrossRef]
15. Hill, J.C.; Millán, I.S. Validation of musculoskeletal ultrasound to assess and quantify muscle glycogen content. A novel approach. *Phys. Sportsmed.* **2014**, *42*, 45–52. [CrossRef] [PubMed]

nutrients

MDPI

Review

Metabolic Impacts of Confinement during the COVID-19 Pandemic Due to Modified Diet and Physical Activity Habits

María Martinez-Ferran [1], Fernando de la Guía-Galipienso [2,3,4], Fabián Sanchis-Gomar [5,6] and Helios Pareja-Galeano [1,*]

1 Faculty of Sports Sciences and Physiotherapy, Universidad Europea de Madrid, Villaviciosa de Odón, 28670 Madrid, Spain; maria.martinez.nutricion@gmail.com
2 Cardiology Service, Hospital Clínica Benidorm, Benidorm, 03501 Alicante, Spain; fdelaguia@gmail.com
3 Glorieta Policlinic, Dénia, 03700 Alicante, Spain
4 REMA Sports Cardiology Clinic, Denia, 03749 Alicante, Spain
5 Department of Physiology, Faculty of Medicine, INCLIVA Biomedical Research Institute, University of Valencia, 46010 Valencia, Spain; fabian.sanchis@uv.es
6 Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California, CA 94305, USA
* Correspondence: helios.pareja@universidadeuropea.es; Tel.: +34-912115200 (ext. 3010)

Received: 2 April 2020; Accepted: 20 May 2020; Published: 26 May 2020

Abstract: While the detrimental effects of a chronic positive energy balance due to a sedentary lifestyle have been well established, the impacts of a short period of abruptly reduced physical activity and overeating arising from strict confinement due to the COVID-19 pandemic will soon start to emerge. To reasonably anticipate major consequences according to the available evidence, we hereby review the literature for studies that have explored the health impacts of several weeks of a reduction in physical activity and daily step-count combined with modified eating habits. These studies identify as main metabolic consequences increases in insulin resistance, total body fat, abdominal fat and inflammatory cytokines. All these factors have been strongly associated with the development of metabolic syndrome, which in turn increases the risk of multiple chronic diseases. A plausible mechanism involved in these impacts could be a positive energy balance promoted by maintaining usual dietary intake while reducing energy expenditure. This means that just as calorie intake restriction could help mitigate the deleterious impacts of a bout of physical inactivity, overeating under conditions of home confinement is very likely to exacerbate these consequences. Moreover, hypertension, diabetes, and cardiovascular disease have been identified as potential risk factors for more severely ill patients with COVID-19. Thus, adequate control of metabolic disorders could be important to reduce the risk of severe COVID-19.

Keywords: COVID-19; acute sedentary lifestyle; step reduction; positive energy balance; metabolic consequences; insulin resistance; metabolic syndrome; sarcopenia

1. Introduction

The current COVID-19 pandemic has led governments of the mainly affected countries to impose strict confinement rules on their citizens. These include measures such as working from home and closing schools, shops, restaurants and any business or service considered non-essential in order to slow down the spread of the contagion and thereby prevent the collapse of health care systems. These measures have, however, had their impacts on the general health of the population because of both exercise restrictions and effects on diet. Exercise restrictions have been the consequence of closed gyms and sports centers, restrictions on walking distance, lack of space and infrastructure of homes for

physical exercise, and lack of technical knowledge of the population on appropriate training routines. Effects on nutrition include limited access to shops, a poorer quality of food products due to the already visible impacts on family income, and overeating. Before this pandemic, insufficient physical inactivity was already described as a global public health problem, with over a quarter of all adults not undertaking the levels of physical activity required for good health [1]. As a result of the current situation in which many people are confined to their homes, physical activity and exercise levels drastically decline while dietary habits remain unchanged or fail to offset this inactivity producing a positive energy balance. There is strong epidemiological evidence that a chronic sedentary lifestyle is detrimental for health [2,3]. Likewise, there is evidence that such negative effects persist even when performing physical exercise programs, revealing that it is just as important to reduce sitting times as it is to lengthen exercising periods [4].

Exercise plays a fundamental role in the prevention of most chronic diseases. Our body needs a relatively long period to benefit from the healthy adaptations that exercise generates, modulated by different molecular mechanisms such as epigenetics, metabolic modulation or reduced inflammation [5–13]. Unfortunately, it requires only a period of a few days to reverse these adaptations, and the body returns to a physiological situation similar to baseline or even worse [2]. This means that trying to maintain an active lifestyle during home quarantine is essential to avoid physical consequences and this approach may also help mitigate the psychological impacts of confinement, especially among the elderly [14–16].

To analyze the consequences of physical inactivity and an acute positive energy balance due to changes in eating habits, different models have been employed [17,18]. Nevertheless, these interventions do not reflect the current home confinement situation, in which daily physical activity is drastically reduced and there is a tendency to eat more and worse affecting the risk of metabolic-associated chronic diseases such as cardiovascular diseases (CVD) across a large part of the world population (Figure 1). This paper therefore reviews evidence of the metabolic-health impacts of a short period of a reduction in physical activity and a tendency to overeat.

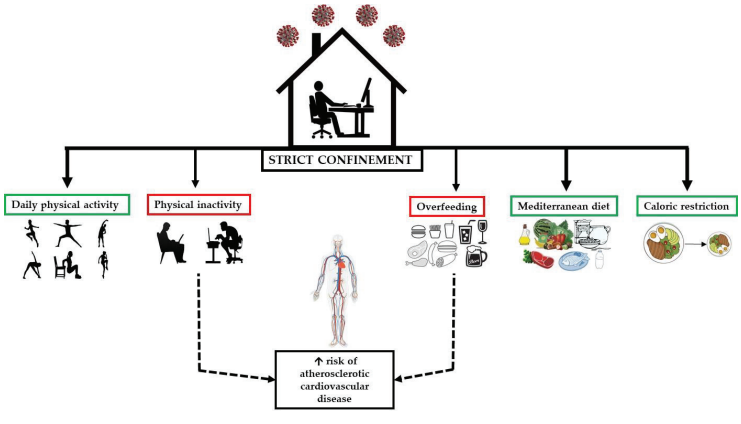


Figure 1. Consequences of overfeeding and reduced physical activity.

2. Methods

Electronic databases (Medline, EMBASE, and Web of Science) were searched without language restrictions to identify all reports on metabolic-related alterations, physical inactivity and overfeeding. Inclusion criteria were: (i) publication in a peer-reviewed journal, (ii) human study, (iii) studies examining the impacts of acute physical inactivity, and (iv) studies examining the impacts of acute changes in dietary habits. Reasons for exclusion were: (i) studies in non-adult subjects, (ii) no control group, (iii) data reported not usable.

3. Results and Discussion

3.1. Metabolic Consequences of Acute Physical Inactivity in Healthy Adults

In this section, we review the impact of acute physical inactivity on glycemic control, inflammatory markers, body composition and cardiorespiratory fitness (CRF) in healthy young adults (Table 1).

Pedersen et al. [19,20] employed a step reduction model to examine the consequences of current lifestyles involving prolonged periods of inactivity interspersed with short episodes of low to moderate physical activity. In their investigation, participants were instructed to reduce daily steps by taking lifts instead of stairs and using their cars instead of walking or cycling.

The two following studies were conducted on healthy young men who performed less than 2 h of regular exercise per week and walked more than 3500 steps per day. During the interventions, participants maintained their usual dietary habits. In the first study, participants were recruited for 2 sub-studies [19]. In the first sub-study [19], eight participants (27.1 (5.7) years; body mass index (BMI) 22.9 (4.0) kg/m^2) reduced their activity from 6203 steps/day (5135–7271) to 1394 steps/day (1261–1528) for three weeks. Results included a significant increase in the area under the curve (AUC) for plasma insulin during an oral glucose tolerance test (OGTT) from baseline to the third week. In the second sub-study of the first study [19], ten subjects (23.8 (4.6) years; BMI 22.1 (2.1) kg/m^2) reduced their mean activity level of 10,501 steps/day (8755–12,247) to 1344 steps/day (1272–1416) for 2 weeks. In this study, both plasma insulin AUC and plasma C-peptide levels increased significantly after the inactivity period. In the oral fat tolerance test, AUCs for plasma insulin, C-peptide and triglycerides (TG) also increased significantly. Further, while total fat mass (FM) remained unchanged, intra-abdominal FM increased (by 7%) after the two weeks of step reduction, and this was accompanied by a significant reduction in total free fat mass. In the second study [20], ten young healthy males (23.8 (1.5) years; BMI 22.1 (0.7) kg/m^2) reduced their daily steps from a baseline value of 10,501 (808) to 1344 (33) for two weeks. This step reduction led to a decreased glucose infusion rate due to a reduction in peripheral insulin sensitivity, and a concurrent decrease in insulin-provoked muscle Akt phosphorylation with no effect on endogenous glucose production in the liver. In contrast to the results of study 2 [19], there was no significant change in total FM after step reduction, but leg lean mass was lower. Finally, two weeks of physical inactivity did not produce changes in plasma levels of TG, free fatty acids (FFA), glucose, insulin or C-peptide.

Interestingly, the findings of a similar study indicated that just three days of step reduction from 12,956 (769) to 4319 (256) steps/day led to impaired glycemic control in twelve healthy active participants (8 men, 4 women; 29 (1) years; 23.6 (0.9) kg/m^2) [21]. In response to this short intervention, the authors also noted increased postprandial glucose levels, increased fasting plasma insulin and C-peptide responses to OGTT, along with increased insulin resistance and diminished insulin sensitivity.

Maximal aerobic capacity (VO_{2max}) is a major predictor of functional capacity and is the gold standard indicator of CRF. A person's VO_{2max} also reflects pulmonary and muscle function, nutritional status or the health state of other organ systems. While higher CRF is associated with better health, lower CRF is associated with increased mortality independently of other risk factors [2]. Several studies [20,22–24] have determined the effects of an acute period of inactivity on VO_{2max}. Results indicated that following two weeks of physical inactivity VO_{2max} was reduced when participants maintained their normal diet [20,24], when calorie intake was restricted [23] or when intake was increased by 50% kcal [22]. In two of these studies, nevertheless, it was found that VO_{2max} returned to baseline after normal physical activity was resumed [22,24].

Table 1. Summary of the studies reviewed examining the effects of acute physical inactivity and/or overfeeding.

Study	Subjects	Intervention	Blood Parameters	Inflammatory Parameters	Lipid Profile	Body Composition	CRF
Olsen et al. 2014 [19]	8 healthy men <2 h EX/week, >3500 steps/day 27.1 (5.7) years BMI 22.9 (4.0) kg/m^2	3-weeks SR 6203 (5135–7271) to 1394 (1261–1528) steps/day Maintained dietary habits	OGTT ↑ insulin AUC	Not reported	Not reported	Not reported	Not reported
	10 healthy men <2 h EX/week, >3500 steps/day 23.8 (4.6) years BMI 22.1 (2.1) kg/m^2	2-week SR 10,501 (8755–12,247) to 1344 (1272–1416) steps/day Maintained dietary habits	OGTT ↑ insulin AUC, C-peptide levels OFTT ↑ insulin, C-peptide, TG	Not reported	Not reported	↔ FM ↑ intra-abdominal fat ↓ fat free mass	Not reported
Krogh-Madsen et al. 2010 [20]	10 healthy men <2 h EX/week, >3500 steps/day 23.8 (1.5) years BMI 22.1 (0.7) kg/m^2	2-week SR 10,501 (808) to 1344 (33) Maintained dietary habits	↓ GIR during the clamp ↓ peripheral insulin sensitivity (H-E) ↓ insulin provoked muscle Akt phosphorylation ↔ hepatic glucose production, plasma glucose, insulin, C-peptide	↔ TNF, IL-6, IL-15, adiponectin, leptin	↔ TG, FFA	↔ FM ↓ BM, leg lean mass ↔ trunk and arm lean mass	↓ VO_{2max}
Mikus et al. 2012 [21]	12 healthy participants (4 F, 8 M) >10000 steps/day 29 (1) years BMI 23.6 (0.9) kg/m^2	3-day SR 12,956 (769) to 4319 (256) steps/day Maintained dietary habits	CGM ↑ post-prandial glucose, fasting insulin ↔ pre-meal blood glucose, 24 h average glucose OGTT ↑ insulin AUC, C-peptide, HOMA-IR ↔ glucose AUC ↓ Matsuda	Not reported	Not reported	Not reported	Not reported
Dixon et al. 2013 [25]	18 healthy men EX > 30 min/5 d/week 9 overweight: 49 (1.0) years BMI 29.3 (1.2) kg/m^2 9 lean men: 51.5 (1.4) years BMI 29.3 (1.2) kg/m^2	1-week SR <4000 steps/day Maintained dietary habits	OGTT ↑ insulin AUC, glucose AUC Overweight: >insulin AUC, glucose AUC	↔ CRP, IL-6, TNF-α, WBC, sICAM, ALT Overweight: >CRP, ALT	↑ TG ↔ FFA, TC, HDL-c, LDL-c Overweight: >FFA, TG	Not reported	Not reported
Bowden-Davies et al. 2018 [24]	45 healthy participants (28 F, 17 M) >10,000 steps/day, >2 h EX/week 16 FDR+ve: 40(14) yearsBMI 27 (5) kg/m^2 29 FDR-ve: 33 (13) years BMI 24 (3) kg/m^2	2-week SR <1500 steps/day (mean decreased: 10,285) Maintained dietary habits	OGTT ↑ insulin AUC, glucose AUC ↓ Matsuda, muscle insulin sensitivity ↔ hepatic insulin resistance, NEFA AUC FDR+ve: <insulin sensitivity (after SR and resuming of PA)	Not reported	↑ TC, LDL-c, TG	↑ FM. liver fat ↓ total lean mass, lower limb lean mass ↔ arm lean mass FDR+ve: >increase of android fat	↓ VO_{2max}
		2-week resumed usual PA					
Knudsen et al. 2012 [22]	9 healthy men >10,000 steps/day 24.3 (3.3) years BMI 21.6 (2.5) kg/m^2	2-week SR + overfeeding 10,278 (2399) to 1521 (488) steps/day 2762 (299) kcal to 4197 (290) kcal	OGTT ↑ insulin AUC (day 7, day 14), Matsuda (day 3, day 7) ↔ glucose AUC ↓ Peripheral insulin sensitivity (H-E) ↔ plasma glucose, C-peptide, hepatic glucose production	↔ TNF-α, IL-6 ↑ leptin, adiponectin	↔ FFA, TG	↑ BM, FM, android and visceral fat ↔ FFM	↓ VO_{2max}
		16-day resumed usual PA	Returned to baseline			Remained elevated	Returned to baseline
Winn et al. 2019 [23]	10 healthy participants (4 F, 6 M) >90 min PA 3 days/week and >10,000 steps/day 24 (1) years BMI < 28 kg/m^2	10-day SR (>10,000 to <50,000 step/day) + control diet (400 kcal/day deficit) 4-week washout 10-day SR + higher protein diet (400 kcal/day deficit)	OGTT ↔ Postprandial glucose, insulin, NEFA, 2-h glucose and 2-h insulin, C-peptide, hepatic insulin extraction, plasma glucose and insulin	Not reported	↔ TG, LDL-c, oxidized LDL ↓ HDL-C, TC	↓ BM abdominal FM ↔ FFM, FM	↓ VO_{2max}
		10-day SR + overfeeding (880 kcal/d)	OGTT ↑ HOMA-IR, 2h-glucose, 2h-insulin ↔ glucose AUC, NEFA ↑ plasma glucose, insulin, C-peptide ↔ hepatic insulin extraction	Not reported	↔ TG, TC, HDL-c, LDL-c, oxidized LDL	↑ BM, FM ↔ FFM	↓ VO_{2max}

Table 1. *Cont.*

Study	Subjects	Intervention	Blood Parameters	Inflammatory Parameters	Lipid Profile	Body Composition	CRF
Walhin et al. 2013 [26]	26 healthy men Vigorous-intensity EX > 30 min/3 day/week 25 (7) years BMI 23.8 (2.5)	1-week SR: 12,562 (3520) to 3762 (860) + overfeeding (+50% kcal) + 45 min/day treadmill running at 70% VO_{2max}	OGTT ↔Matsuda, B- cell function, insulin AUC	↔ ALT, CRP, IL-6, WBC	↔ HDL-C. LDL-C, NEFAs, TG, CT	↑ BM, waist/hip circumference, lean mass ↔ FM	Not reported
		1-week SR: 10,544 (2756) to 3690 (400) + overfeeding (+50% kcal) + not training	↓Matsuda, B- cell function ↑insulin AUC OGTT	↔ ALT, CRP, IL-6 ↑ adiponectin, WBC	↑ CT ↔ HDL-C. LDL-C, NEFAs, TG	↑ BM, waist/hip circumference, lean mass ↔ FM	Not reported
Breen et al. 2013 [27]	10 healthy older adults (5 F, 5 M) >3500 steps/day 72.3 (1.0) years; BMI 29.0 (1.8) kg/m^2	2-week SR: 5962 (695) to 1413 (110) steps/day Maintained dietary habits	OGTT ↑HOMA-IR, glucose AUC and AUC ↓Matsuda ↔ C-peptide AUC	↑ TNF-α, CRP ↔ IL-6	Not reported	↔ total FM, FFM ↓leg FFM ↑% FM ↓ MPS ↔ isometric MVC, SPPB	Not reported
McGlory et al. 2018 [28]	22 healthy older adults >3500 steps/day 12 M: 69 (3) years; BMI 27.3 (4.6) 10 F: 70 (5]) years; BMI 27.7 (5.1)	2-week SR: 7362 (3294) to 991 (97) steps/day Maintained dietary habits 2-week resumed usual PA	OGTT ↑glucose and insulin AUC, HOMA-IR ↓Matsuda	↑ TNF-α, CRP, IL-6	Not reported	↔BMI, %total FM, lean mass ↓ MPS ↔ isometric MVC	Not reported

Area under curve (AUC), alanine transaminase (ALT), body mass (BM), body mass index (BMI), cardiorespiratory fitness (CRF), continuous glucose monitoring (CGM), C-reactive protein (CRP), exercise (EX), fat mass (FM), females (F), first-degree relatives with type 2 diabetes (FDR+ve), first-degree relatives without type 2 diabetes (FDR−ve), free fatty acids (FFA), glucose infusion rate (GIR), high density lipoprotein cholesterol (HDL-c), homeostatic model assessment for insulin resistance (HOMA-IR), hyperinsulinemic-euglycemic during clamp (H-E), LDL-C (low-density lipoprotein cholesterol), males (M), maximal voluntary contraction (MVC), muscle protein synthesis (MPS), non-esterified fatty acids (NEFA), oral glucose tolerance test (OGTT), physical activity (PA), short physical performance battery (SPPB), soluble intercellular adhesion molecule (sICAM), step reduction (SR), triglycerides (TG), total cholesterol (TC), tumor necrosis factor alpha (TNF-α), white blood cells (WBC), ↔ no significant change, ↑ significant increase, ↓ significant decrease, > significantly higher, < significantly lower.

Collectively, the above data indicate that a reduction in daily physical activity of three days, two weeks or three weeks impairs glycemic control. In addition, two weeks of step reduction increased FM and reduced lean mass, and also had a negative impact on VO_{2max}.

3.2. Metabolic Consequences of Acute Physical Inactivity in Overweight Adults

In the previous articles, interventions were conducted on subjects within the normal weight range. Additionally, similar step reduction interventions have been tested on both overweight and normal weight populations to compare the consequences of acute physical inactivity (Table 1).

To examine whether reduced physical activity in adults with central overweight could lead to a rapid decline in metabolic and inflammatory homeostasis compared to what happens in lean counterparts, Dixon et al. [25] performed a study in which individuals were subjected to a week of step reduction to below 4000 steps/day. The participants of this study were nine active men with central overweight (49 (1.0) years; BMI 29.3 (1.2) kg/m^2) and nine active lean men (51.5 (1.4) years; BMI 29.3 (1.2) kg/m^2) who maintained their normal diet. In the former group, subjects had significantly higher total FM and abdominal FM levels before the intervention (these variables were not nevertheless determined after the intervention). Results indicated that, while insulin and glucose AUC responses to an OGTT and fasting TG concentrations increased in both groups, in the overweight group, glucose and insulin AUC, TG and C-reactive protein (CRP) and alanine transaminase were all higher before the intervention and remained so throughout. No changes were produced in total cholesterol, low-density lipoprotein (LDL) or high-density lipoprotein (HDL) cholesterol over the intervention period and neither did differences emerge between groups.

In another study, the metabolic consequences of a drop in physical activity from a daily step-count of <10,000 to 1500 for 14 days were examined in 45 active healthy participants who continued with their usual diet [24]. Of these 45 participants, 16 had first-degree relatives with type 2 diabetes (10 females, 6 males; 40 (14) years; BMI 27 (5) kg/m^2) and 29 did not (18 females, 11 males; 33 (13) years; BMI 24 (3)). Those in the former group had a significantly higher BMI classified as "overweight" and greater waist and hip circumferences, although there were no significant differences in FM. Both groups experienced a significant reduction in insulin sensitivity accompanied by a significant decrease in glucose and insulin AUC. Although both groups showed a reduction in muscle insulin sensitivity, the overweight group displayed a lower sensitivity. The period of reduced physical activity significantly lowered VO_{2max} across the study population without differences between groups. Total lean mass and lower limb lean mass decreased significantly and there was a significant increase in total FM and liver fat; those with overweight accumulated more android fat (1.5%) after step reduction. Lipid profiles were also modified in that higher total cholesterol, LDL-cholesterol and TG were recorded after step reduction, a greater TG increase being detected in the overweight subjects. All variables returned to baseline values 14 days after the subjects resumed their usual physical activity. After resuming normal activity, the overweight group engaged in lower amounts of vigorous activity and had lower insulin sensitivity.

According to the findings of a study by Bowden et al. [29], obese individuals with metabolic syndrome had lower CRF (measured as VO_{2max} peak) than both non-obese subjects without metabolic syndrome and non-obese individuals with metabolic syndrome, the latter being the most sedentary population. The authors also found that higher VO_{2max} peak, lesser sedentary time and average daily METS were correlated with lower liver fat. Results suggested that high levels of CRF in the overweight and obese population significantly reduced or eliminated the elevated risk of CVD and all-cause mortality. This indicates that CRF changes the relationship between body fat and its prognosis. We should underscore that many of the benefits of improved CRF are derived from an increase in physical activity [30].

In conclusion, in these studies a reduction in acute physical activity negatively influenced glycaemia control and lipid profile (TG, total cholesterol, LDL-cholesterol). Regarding body composition, step

reduction increased FM, liver fat mass and reduced lean mass in both overweight and normal weight subjects. However, in those overweight, consequences were usually somewhat greater.

3.3. Detrimental Health Effects of an Acute Sedentary Lifestyle in the Elderly

The prevalence of sarcopenia is high among the elderly. A loss of skeletal muscle mass and strength has several repercussions on health, and all conditions in which muscle activity is reduced can lead to sarcopenia [31]. Moreover, ageing is associated with abdominal obesity, an important contributor to insulin resistance and metabolic syndrome, along with a higher level of proinflammatory cytokines [32]. Accordingly, drastic decreases in physical activity could have worse consequences in elderly subjects by accelerating the ageing process and the appearance of age-related diseases. As an example, the two studies described below examine the impacts of a step reduction intervention in elderly subjects on glycemic control, body composition, inflammatory parameters and CRF (Table 1).

Breen et al. (2013) conducted a study in healthy older adults (5 males, 5 females; 72.3 (1.0) years; BMI 29.0 (1.8)) who were moderately active (>3500 steps/day). During the intervention, participants reduced their daily step-count by approximately 76% of habitual levels while maintaining their dietary habits. After this period, insulin resistance was increased and postprandial insulin sensitivity was reduced. Fasting insulin concentrations and its peak plasma concentrations at 30 min of OGTT were greater after step reduction and AUC for plasma glucose and insulin during OGTT increased. The body composition data revealed that after step reduction, body fat percentage increased and skeletal leg muscle mass was significantly reduced. Further findings were postprandial rates of myofibrillar protein synthesis reduced by approximately 26% after the intervention with no difference in postabsorptive rates.

A similar step-reduction intervention was conducted in 22 moderately active older adults (12 males: 69 (3) years, BMI 27.3 (4.6): 10 females; 70 (5) years, BMI 27.7 (5.1)) [28]. Participants reduced their daily step-count by 70% and maintained their usual dietary habits. Body composition variables remained unchanged. However, the authors reported that just a week of step reduction led to increased insulin resistance and reduced insulin sensitivity. Moreover, glucose and insulin AUC were elevated as were fasting plasma glucose and insulin concentration during OGTT. Also observed was a reduction in muscle protein synthesis. What it is interesting to point out is that after the step reduction protocol, participants were reassessed after 14 days of return to their habitual step-count. In this examination it was confirmed, however, that glycemic control and inflammatory markers had not recovered. In contrast, in a younger study population (36 (14) years) changes in metabolic variables produced were reversed when normal physical activity levels were recovered [28].

In both studies conducted in older subjects, plasma concentrations of inflammatory markers (TNF-α and CRP) were significantly increased after the step reduction intervention [27,28]. In the second study [28], IL-6 was also increased and after returning to normal activity, inflammatory markers were still elevated. In contrast, in the studies conducted in young individuals, no changes were produced in inflammatory parameters [20,22,25,26].

In summary, acute physical inactivity led to impaired glycemic control, increased inflammation and reduced muscle protein synthesis. Inactivity may also reduce fat free mass while increasing FM. In addition, recovering normal levels of activity in the elderly could be harder compared to younger subjects.

3.4. Metabolic Effects of Acute Physical Inactivity plus Overfeeding

Energy balance is the state in which energy intake equals energy expenditure. A positive energy balance, whereby energy intake exceeds expenditure, can lead to weight gain due to increased body fat [33]. In the studies described in the previous section, participants kept up their usual dietary intake while reducing energy expenditure, resulting in a positive energy balance that may have contributed to the observed metabolic changes [19,20,24,25]. In this section, we review the impact of physical inactivity when added to a diet intervention (Table 1).

In a study of the effects of two weeks of step reduction combined with overfeeding, nine healthy young men (24.3 (3.3) years; BMI 21.6 (2.5) kg/m^2) undertook 14 days of step reduction from 10,278 (2399) to 1521 (488) steps/day and increased their daily total energy intake by 50% kcal [22]. This study showed that insulin sensitivity reduction occurs after three days of inactivity and overfeeding. Clamp-derived insulin sensitivity was reduced after 14 days of inactivity and oral glucose tolerance remained unaffected. The insulin response to OGTT increased after the first and the second week. Body composition was affected by the intervention, in that body weight was higher due to an increase in total FM (1.5 (0.5) kg; $p < 0.05$), and in android, gynoid and visceral fat. Finally, plasma levels of leptin and adiponectin increased after the intervention and no changes in TG or FFA were observed. The authors of this study also reported that after 16 days of returning to an uncontrolled free-living environment, body weight and adiposity were still elevated while remaining variables returned to baseline values.

Interestingly, one study explored whether metabolic dysfunction caused by inactivity might be blunted by energy restriction [23]. Ten physically active men and women (24 (1) years) reduced their daily steps from 10,000 to 5000 for 10 days. Participants completed two periods of physical inactivity while consuming either a control diet (16% kcal from protein, 64% kcal from carbohydrate, 20% kcal from fat) or a higher-protein diet (30% kcal from protein, 50% kcal from carbohydrate, and 20% kcal from fat) in a randomized crossover design. In both diets, energy intake was decreased by 15–20% of total energy expenditure to offset the reduction in energy expenditure (400 kcal/day). As a positive control condition, a group of subjects from the initial sample (n = 5) repeated the same protocol of inactivity in association with overfeeding (35% kcal). The results of this study revealed that when diet was controlled, body fat was not altered by physical inactivity and body weight was significantly reduced; abdominal FM was also lowered. In contrast, when overfeeding was accompanied by inactivity, both body weight and body fat went up. Further, when overfeeding was combined with inactivity, the authors observed increases in fasting blood glucose, plasma insulin, plasma c-peptide, insulin resistance, and 2 h postprandial glucose and insulin concentrations. However, when the diet was controlled none of these changes were produced.

Other authors have looked at what happens when a reduction in physical activity and overfeeding are accompanied by an exercise intervention [26]. Over one week, 26 physically active men (25 (7) years; BMI 23.8 (2.5)) were randomly assigned to two groups. In both groups, physical activity was restricted to under 4000 steps/day and energy intake increased (50% kcal) while individuals in one of the groups undertook 45 min of daily treadmill running at 70% of VO_{2max}. In both groups, increases were recorded in body weight, waist/hip circumference and lean mass. In the group of subjects who did not train, insulin sensitivity and B-cell function were reduced and the insulin response to OGTT was increased. This group also showed an increase in total cholesterol and adiponectin. However, the addition of physical exercise was able to abolish these changes.

Taken together, these findings indicate that energy balance plays a key role in the metabolic consequences of acute physical inactivity. Accordingly, while overeating could worsen its repercussions, energy restriction could help avoid its impacts. Although physical exercise seems to improve glycemic control, a positive energy balance still affects body composition. In the studies reported, however, calorie intake was controlled, so it is unknown whether reduced physical activity may have led to reduced energy intake.

Physical activity plays an important role in energy balance, and subjects engaging in higher levels of physical activity may have improved sensitivity of the appetite control system [34]. Evidence shows that there is weak coupling between energy intake and expenditure in individuals displaying low levels of daily physical activity, but strong coupling with high levels of physical activity [35].

In 1956, Mayer [36] described that calorie intake increases with activity only within a certain zone of "normal activity". This author also confirmed that below a level of physical activity or so-called "sedentary zone", a further decrease in activity is not followed by a decrease in food intake. Recent investigations have also shown that a reduction in physical activity is not usually offset by a reduction in energy intake, resulting in a positive energy balance [37,38]. Further, it has been proposed that

sedentary activities not only reduce energy expenditure, but they also promote increased food intake. Hence activities such as watching television or cognitive tasks stimulate food intake such that the sensations of satiety and fullness are ignored leading to overconsumption [39]. This evidence suggests it is highly likely that a significant reduction in physical activity will not be accompanied by a reduction in energy intake and this will result in a positive energy balance likely worsening the metabolic effects of sedentary behavior.

3.5. Manipulating Dietary Intake to Offset the Metabolic Impacts of Confinement

While it is important to remain active to avoid the problems an acute sedentary lifestyle brings, what about our attitude to food during this period of confinement? On the one hand, there may be overfeeding, while on the other, calorie intake may be restricted due to reduced activity or physical inactivity. Restricted calorie intake could be an optimal option for our current situation. This means a diet lower in a given percentage of calories than our regular diet, but that, nevertheless, is balanced to include all the necessary nutrients. Research in some animals has shown that the intake of up to 40% fewer calories has an impressive positive effect on markers of disease and ageing [18]. In humans, a few randomized controlled clinical trials have examined the effects of calorie restriction on health. The findings of a study performed in healthy subjects who underwent a three-month period of calorie restriction, i.e., five consecutive days per week of a fasting-mimicking diet low in calories, proteins, and sugars but high in unsaturated fatty acids, were reduced BMI, trunk, and total body fat [40]. Likewise, reductions were recorded in blood pressure, triglycerides, total and low-density lipoprotein cholesterol, C-reactive protein, and insulin-like growth factor 1 (IGF-1). The authors concluded that cycles of a five-day fasting-mimicking diet were safe, feasible, and effective in reducing risk factors associated with metabolic-related diseases.

In a two-year follow-up clinical trial, the effect of two years of 15% calorie restriction was assessed in healthy individuals. Results indicated an average weight loss of 8.7 kg (70% was body fat) in the calorie restriction group versus an average gain of 1.8 kg in the control group. Further, subjects in the restricted calorie intake group showed a 10% reduction in the metabolic rate of sleep, associated with reduced levels of reactive oxygen species and thyroid activity (reduced T3 and T4), which are biomarkers of aging [41]. In another study carried out in eighteen healthy and physically active subjects, the effects of caloric restriction close to 40% of the standard calorie intake for six weeks were assessed [42]. Diet was based on three days of severe restriction (600–800 Kcal) per week and normal intake for the rest of the week. Results indicated considerable weight loss including reduced fat mass (mostly android) and a less appreciable effect on fat-free mass. Hence, the option of calorie restriction should be considered with caution due to the lack of evidence in humans.

The key during this period of confinement would be a balanced diet comprising all the necessary nutrients, including healthy fats with balanced levels of sugar and cholesterol. During confinement, low-calorie diets should not be recommended, as they are not effective in the long term, and do not provide sufficient energy for a person in this situation of staying at home. Carbohydrates are an appropriate source of energy and are needed daily, mainly if associated with aerobic exercise. Foods rich in carbohydrates with a low glycemic index (whole grains, brown rice, vegetables, legumes, fruits, etc.) and proteins are an essential part of the diet, especially during this period of greater inactivity and we should avoid carbohydrates with a high glycemic index such as sugars, sweets, or bread. Foods rich in proteins with a lower percentage of fat such as chicken and turkey meat, fish, cooked eggs, fresh cheeses, legumes (soy), as well as dairy products such as yogurt and cottage cheese, are recommended because proteins have a stimulating effect on metabolism and are involved in the elimination of fats. Therefore, the combination of an adequately balanced diet and regular physical exercise, should serve to maintain a stable metabolic balance.

3.6. Physical Activity to Mitigate the Metabolic Impacts of Confinement

In many countries, an undesirable effect of the COVID-19 pandemic is restricted outdoor physical exercise. Recommendations include indoor walking every 2 h in order to stimulate both cardiovascular and musculoskeletal systems. Aerobic exercise, such as jogging at home if there is enough space, is highly recommended, as well as performing flexo-joint extension (shoulders, elbows, wrists, back, hip, knees, and ankles) and strength, flexibility and stretching exercises of the main muscle groups. A central-question is: what type of training is appropriate for an individual with metabolic syndrome? In a meta-analysis examining the effects of aerobic exercise training, strength training, or both combined, on cardiovascular risk factors in patients with metabolic syndrome, it was observed that aerobic training improved waist circumference, fasting blood glucose, HDL-cholesterol, triglycerides, diastolic blood pressure, and VO_{2peak} [43]. No changes were related to strength training alone, probably due to the limited data available. Accordingly, high-intensity aerobic training performed over more than 12 weeks (3 days/week), shows the most marked effects on cardiovascular risk factors [43]. Inactivity slows the metabolic benefits of exercise, while exercise improves postprandial lipemia levels, glucose tolerance, and insulin sensitivity, all of which are risk factors for CVD. Another study highlights that physical inactivity (e.g., sitting 13.5 h/day and walking fewer than 4000 steps a day) provokes resistance to metabolic improvements that usually result from an acute episode of aerobic exercise, emphasizing that exercise, a heart-healthy diet, and an active lifestyle should be combined to achieve a healthy cardiometabolic profile [44].

Another added problem during this period of home confinement at is that the only form of exercise for many people is walking up and down the corridor of their house. Thus, the question arising is how many steps per day are recommended? Taking more steps per day (8000 vs. 4000 steps per day) is associated with lower all-cause mortality but a significant association has not been found between step intensity and mortality after adjusting for the total number of steps per day [45]. It is essential to walk as much as possible regardless of intensity, since muscle is an endocrine organ that modulates the production of substances according to their activity, requiring minimal muscle activation to obtain benefits [13]. During confinement, it is very likely that physical activity drops drastically possibly resulting in more hours of bed rest, with the consequent loss of muscle mass and its impaired function, and increases in glucose intolerance. Nutrition will play an even more significant role at this challenging moment and must include a consumption in the range of 1.4–2.0 g/kg per day protein to protect from the consequences of muscle inactivity [46,47]. Protein intake should be customized according to different factors, such as the type of population (young or older) [48], energy status, the quality of protein intake or the mode and intensity of exercise [46].

An exercise program for the confinement period has been proposed [49]. Recommendations include increasing the frequency of exercise to 5–7 days per week, 200–400 min of aerobic training and 2–3 days of resistance training. Mobility should be included every day as well as balance and coordination distributed through different training. This should be done at least two times per week. For older people, moderate intensity exercise is recommended during quarantine. Exercise may be performed without any specific training materials. Resistance training can be done through body weight exercises, such as squats, push-ups or sit-ups. Household items such as water bottles or packets of food can be used as weights. Different examples of aerobic exercise are dancing, stair climbs and walking or running on the spot. Balance exercises could be performed via stepping over obstacles or walking along a straight line marked out on the floor [49,50]. Additionally, yoga or traditional Tai Ji Quan can be considered as they do not require any equipment or large space [50].

Resistance training has been demonstrated to reduce the loss of muscle mass and muscle strength [51–53] and improve bone density [54], metabolic health and insulin resistance [53]. Resistance training should be properly designed for older adults following principles of individualization, periodization, and progression [53]. However, minimally supervised home-based training has also been shown to be a safe and effective method of increasing body muscle strength [51]. Resistance training can be useful to fight against the metabolic and physical consequences of COVID-19. However,

exercise programs should consider other important components: aerobic, balance, coordination and mobility [49].

4. Limitations

As the main limitation of the studies reviewed, with the exception of three studies [24,26,28] (n = 45, n = 26, n = 22, respectively), most had <20 participants, so statistical power was low. Participants were mainly males (n = 119) and there was a lower number of females (n = 51). Additionally, none of the studies reported on sex differences, an aspect that would be interesting to explore. Nevertheless, as results exist for both sexes, we believe that recommendations can be followed by both.

Another aspect we should highlight is the fact that not all the articles measured the same variables. Although all examined glycemic control, not all analyzed CRF, body composition, lipid profile or inflammatory factors. These variables could have provided much evidence of the deleterious impact of step reduction. We found no study including a short term step reduction intervention in obese persons or people with metabolic syndrome. According to the high prevalence of both syndromes and their pathogens, it is probably that in these populations, the health impacts of short term physical inactivity will be worse.

In most of the studies reviewed, while physical activity was reduced, participants maintained their dietary habits and, as a result, their energy balance was positive [19,20,24,25,27,28]. Another three articles added overfeeding to the intervention [22,23,26], and just one examined calorie restriction [23]. In these studies, most deleterious consequences were avoided. So it is unknown if the negative repercussions of acute physical inactivity are derived from the inactivity itself, the positive energy balance or from both aspects. According to the current evidence, home exercise as well as a healthy balanced diet avoiding overeating could be a good strategy to mitigate the impacts of acute physical inactivity. Further research on this topic is needed.

5. Conclusions

Metabolic syndrome, also known as "insulin resistance syndrome", is defined as "a constellation of interconnected physiological, biochemical, clinical, and metabolic factors that directly increases the risk of atherosclerotic CVD, type 2 diabetes mellitus, and all-cause mortality" [55]. Metabolic syndrome is strongly linked to insulin resistance, oxidative stress, inflammation, obesity, endothelial dysfunction and CVD [56]. In turn, physical inactivity has been related to every described risk factor for metabolic syndrome: dyslipidemia, hypertension, hyperglycemia, visceral obesity, and prothrombotic and proinflammatory events (Figure 2).

Insulin resistance is a central component of metabolic syndrome [56] and while high levels of daily physical activity can prevent insulin resistance, physical inactivity is a primary cause of insulin resistance and a loss of insulin sensitivity in skeletal muscle [2]. In the studies reviewed, it was observed that just two weeks of physical inactivity and a positive energy balance can increase insulin resistance and modify glycemic control [19–28]. Obesity and visceral obesity are also central components of metabolic syndrome [55,56] with an important role in CVD [57] through different mechanisms such as insulin resistance and the induction of a proinflammatory state [58]. The studies reviewed detected increases in body fat [22–24,27] and abdominal fat mass [19,22,24] after just one or two weeks of step reduction associated with a positive energy balance.

The role of inflammation in the pathogenesis of metabolic syndrome and CVD has been well documented [55,56,59]. In the studies conducted in elderly subjects reviewed here, it was confirmed that two weeks of physical inactivity led to increases in TNF-α, IL6 and CRP [27,28]. TNF-α and IL-6 are cytokines with endocrine, autocrine and paracrine functions, and their gene expression is increased in the adipocytes, macrophages and lymphocytes of obese individuals [56]. TNF-α acts locally on adipocytes and reduces insulin sensitivity via different mechanisms, increases FFA levels through the induction of lipolysis, and inhibits adiponectin release [60]. This cytokine also attenuates nitric oxide-mediated vasodilation and is involved in the vascular pathology of metabolic syndrome,

atherosclerosis and coronary disease [56]. IL-6 creates insulin resistance in the liver and enhances the hepatic synthesis of acute phase proteins such as CRP and fibrinogen. CRP shows high correlation with metabolic syndrome, diabetes and CDV, and fibrinogen leads to a prothrombotic state [56,60]. IL-6 also promotes the expression of adhesion molecules by endothelial cells and activates the local renin-angiotensin system, whose activation contributes to metabolic syndrome development [56,60].

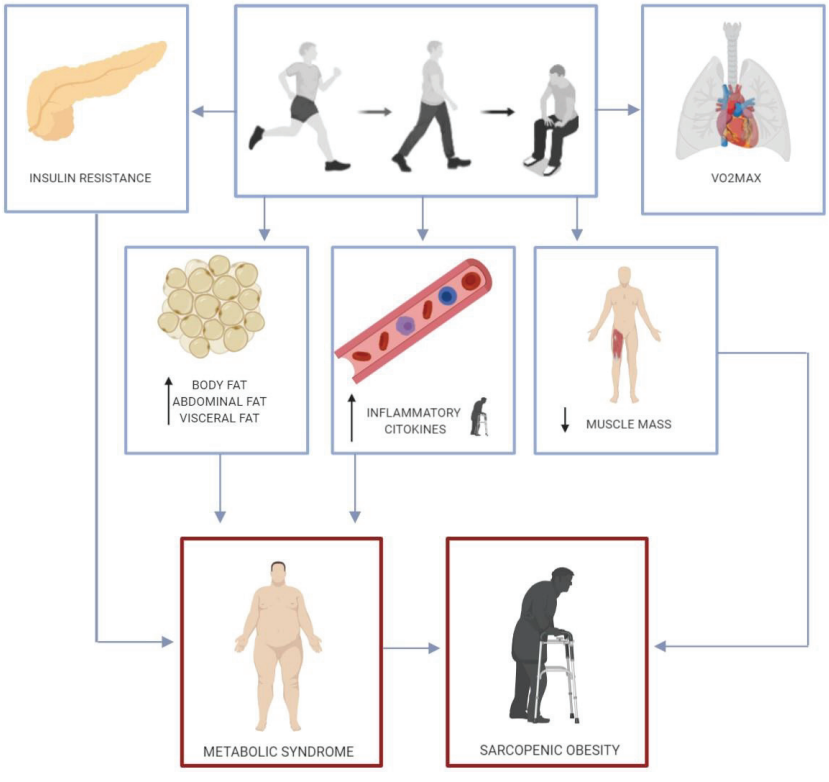


Figure 2. Consequences of a short-term reduction in physical activity.

Acute sedentarism can be deleterious for health though other mechanisms. It has been reported that short term physical inactivity lowers VO_{2max} [20,22–24] and also reduces lean mass and fat free mass, with greater impacts on the lower body [19,20,24,27]. Both low skeletal muscle mass and maximal aerobic capacity (VO_{2max}) are biomarkers associated with a shorter life expectancy [2]. The impact of this reduction in muscle mass could be especially important in the elderly, due to a higher prevalence of sarcopenia and its health impacts in these subjects. Moreover, sarcopenia combined with obesity (sarcopenic obesity) has been linked to a worse metabolic impact and increased risk of mortality [61].

More research is needed to examine whether energy restriction could avoid the consequences of acute physical inactivity as suggested in one of the articles reviewed [23]. Nevertheless, there is evidence to support that at lower levels of physical activity, energy intake is dysregulated leading to a positive energy balance [35–38]. Maintaining food glycemic control is a specific measure in patients with diabetes to help reduce infection risk and severity. In effect, it has been also recommended that attention be paid to nutrition and adequate protein intake, along with exercise to improve immunity [62]. Short-term physical inactivity and a positive energy balance can have several consequences for health related to reduced insulin sensitivity, higher total body and central fat, and a proinflammatory state,

which are all central risk factors for metabolic syndrome. For the elderly, consequences could be worse, increasing the risk of developing sarcopenic obesity.

According to recent evidence, adequate control of metabolic disorders is important to reduce the risk of severe COVID-19. We should try to avoid the deleterious consequences of physical inactivity and positive energy balance by maintaining physical activity and exercise levels in a safe home environment and adhering to a healthy diet. Of course, this is also important for people without metabolic disorders to avoid the reported deleterious effect of physical inactivity and positive energy balance, which may prompt the development of metabolic syndrome and its comorbidities. COVID-19 varies from a mild self-limiting flu-like illness to full-blown pneumonia, respiratory failure and death [62]. Hypertension, diabetes, and CVD have been identified as potential risk factors for the more severely ill patients. In addition, COVID-19 could also enhance damage to the heart in patients with CVD [63,64]. As obesity increases, so does risk of chronic disease related to metabolic syndrome and based on recent data, obese individuals are also being considered at high risk for severe complications of COVID-19 [65,66].

Accordingly, our recommendations during this period of confinement are to avoid overeating by following a healthy balanced diet. This diet should be based on carbohydrates with a low glycemic index, such as vegetables, legumes or fruits, healthy fats and food rich in proteins with a lower percentage of fat. Moreover, calorie intake may be restricted due to reduced activity or physical inactivity. This nutrition recommendation should be combined with an adequate daily physical activity program designed by sports science specialists to prevent metabolic-related health problems. This program should consider different components: resistance, aerobic, mobility, coordination and balance. The recommended frequency of training is 5–7 days per week, including at least 2–3 days of resistance training.

Once COVID-19 transmission is controlled, different countries are setting new regulations in which exercising in the street is allowed. Each set of regulation may have different rules, in terms of time, or number of people training together. Depending on the country, personal training or in small groups might be permitted. However, reopening of gym or sport centers to the population at large could take longer. For this reason, resistance training should continue at home as recommended here, as well as exercise for mobility, coordination and balance. As indicated by the studies reviewed in this article, short term physical inactivity can lead to a reduction in CFR and also in muscle mass. In some countries this inactivity has been much longer. Accordingly, people must consider that their fitness level is lower than before confinement if they begin to exercise in the street, such as running. Counselling by a sports science specialists could be useful to avoid injuries. Likewise, it is imperative that all the actions we carry out comply with the social distancing recommended by the health authorities.

Author Contributions: M.M.-F. and H.P.-G. designed the study; M.M.-F. and F.S.-G. analyzed the data; M.M.-F., F.d.l.G.-G. and F.S.-G. interpreted the results of experiments; M.M.-F. and F.d.l.G.-G. prepared the figures and tables; M.M.-F., F.S.-G. and H.P.-G. drafted the manuscript; H.P.-G., F.S.-G. and F.d.l.G.-G. edited and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Helios Pareja-Galeano is supported by a grant from Universidad Europea de Madrid (#2019/UEM01).

Acknowledgments: Fabian Sanchis-Gomar is supported by a postdoctoral contract granted by "Subprograma Atracció de Talent - Contractes Postdoctorals de la Universitat de València."

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

References

1. Guthold, R.; Stevens, G.A.; Riley, L.M.; Bull, F.C. Worldwide trends in insufficient physical activity from 2001 to 2016: A pooled analysis of 358 population-based surveys with 1·9 million participants. *Lancet Glob. Health* **2018**, *6*, e1077–e1086. [CrossRef]
2. Booth, F.W.; Roberts, C.K.; Laye, M.J. Lack of exercise is a major cause of chronic diseases. *Compr. Physiol.* **2012**, *2*, 1143–1211. [PubMed]

3. Sanchis-Gomar, F.; Lucia, A.; Yvert, T.; Ruiz-Casado, A.; Pareja-Galeano, H.; Santos-Lozano, A.; Fiuza-Luces, C.; Garatachea, N.; Lippi, G.; Bouchard, C.; et al. Physical inactivity and low fitness deserve more attention to alter cancer risk and prognosis. *Cancer Prev. Res.* **2015**, *8*, 105–110. [CrossRef] [PubMed]
4. Biswas, A.; Oh, P.I.; Faulkner, G.E.; Bajaj, R.R.; Silver, M.A.; Mitchell, M.S.; Alter, D.A. Sedentary time and its association with risk for disease incidence, mortality, and hospitalization in adults a systematic review and meta-analysis. *Ann. Intern. Med.* **2015**, *162*, 123–132. [CrossRef] [PubMed]
5. Pareja-Galeano, H.; Sanchis-Gomar, F.; Santos-Lozano, A.; Fiuza-Luces, C.; Garatachea, N.; Ruiz-Casado, A.; Lucia, A. Regular physical activity: A little is good, but is it good enough? *Am. J. Clin. Nutr.* **2015**, *101*, 1099–1101. [CrossRef] [PubMed]
6. Pareja-Galeano, H.; Sanchis-Gomar, F.; García-Giménez, J.L. Physical exercise and epigenetic modulation: Elucidating intricate mechanisms. *Sports Med.* **2014**, *44*, 429–436. [CrossRef] [PubMed]
7. Valenzuela, P.L.; Morales, J.S.; Pareja-Galeano, H.; Izquierdo, M.; Emanuele, E.; de la Villa, P.; Lucia, A. Physical strategies to prevent disuse-induced functional decline in the elderly. *Ageing Res. Rev.* **2018**, *47*, 80–88. [CrossRef] [PubMed]
8. Martinez-Gomez, D.; Lavie, C.J.; Hamer, M.; Cabanas-Sanchez, V.; Garcia-Esquinas, E.; Pareja-Galeano, H.; Struijk, E.; Sadarangani, K.P.; Ortega, F.B.; Rodríguez-Artalejo, F. Physical activity without weight loss reduces the development of cardiovascular disease risk factors—A prospective cohort study of more than one hundred thousand adults. *Prog. Cardiovasc. Dis.* **2019**, *62*, 522–530. [CrossRef]
9. Romagnoli, M.; Alis, R.; Aloe, R.; Salvagno, G.L.; Basterra, J.; Pareja-Galeano, H.; Sanchis-Gomar, F.; Lippi, G. Influence of training and a maximal exercise test in analytical variability of muscular, hepatic, and cardiovascular biochemical variables. *Scand. J. Clin. Lab. Investig.* **2014**, *74*, 192–198. [CrossRef]
10. Garatachea, N.; Pareja-Galeano, H.; Sanchis-Gomar, F.; Santos-Lozano, A.; Fiuza-Luces, C.; Morán, M.; Emanuele, E.; Joyner, M.J.; Lucia, A. Exercise attenuates the major hallmarks of aging. *Rejuvenation Res.* **2015**, *18*, 57–89. [CrossRef]
11. Inglés, M.; Serra-Añó, P.; Gambini, J.; Abu-Sharif, F.; Dromant, M.; Garcia-Valles, R.; Pareja-Galeano, H.; Garcia-Lucerga, C.; Gomez-Cabrera, M.C. Active paraplegics are protected against exercise-induced oxidative damage through the induction of antioxidant enzymes. *Spinal Cord* **2016**, *54*, 830–837. [CrossRef] [PubMed]
12. Pareja-Galeano, H.; Brioche, T.; Sanchis-Gomar, F.; Montal, A.; Jovaní, C.; Martínez-Costa, C.; Gomez-Cabrera, M.C.; Viña, J. Impact of exercise training on neuroplasticity-related growth factors in adolescents. *J. Musculoskelet. Neuronal Interact.* **2013**, *13*, 368–371. [PubMed]
13. Pareja-Galeano, H.; Garatachea, N.; Lucia, A. *Exercise as a Polypill for Chronic Diseases*, 1st ed.; Elsevier Inc.: Philadelphia, PA, USA, 2015; Volume 135.
14. Pareja-Galeano, H.; Sanchis-Gomar, F.; Lucia, A. Physical activity and depression: Type of exercise matters. *JAMA Pediatr.* **2015**, *169*, 288–289. [CrossRef] [PubMed]
15. Pareja-Galeano, H.; Mayero, S.; Perales, M.; Garatachea, N.; Santos-Lozano, A.; Fiuza-Luces, C.; Emanuele, E.; Gálvez, B.G.; Sanchis-Gomar, F.; Lucia, A. Biological Rationale for Regular Physical Exercise as an Effective Intervention for the Prevention and Treatment of Depressive Disorders. *Curr. Pharm. Des.* **2016**, *22*, 3764–3775. [CrossRef] [PubMed]
16. Santos-Lozano, A.; Pareja-Galeano, H.; Sanchis-Gomar, F.; Quindós-Rubial, M.; Fiuza-Luces, C.; Cristi-Montero, C.; Emanuele, E.; Garatachea, N.; Lucia, A. Physical Activity and Alzheimer Disease: A Protective Association. *Mayo Clin. Proc.* **2016**, *91*, 999–1020. [CrossRef]
17. Thyfault, J.P.; Krogh-Madsen, R. Metabolic disruptions induced by reduced ambulatory activity in free-living humans. *J. Appl. Physiol.* **2011**, *111*, 1218–1224. [CrossRef]
18. López-Otín, C.; Galluzzi, L.; Freije, J.M.P.; Madeo, F.; Kroemer, G. Metabolic Control of Longevity. *Cell* **2016**, *166*, 802–821. [CrossRef]
19. Olsen, R.H.; Krogh-Madsen, R.; Thomsen, C.; Booth, F.; Pedersen, B. Metabolic Responses to Reduced Daily Steps in Healthy Nonexercising Men. *JAMA J. Am. Med. Assoc.* **2008**, *299*, 1261–1263. [CrossRef]
20. Krogh-Madsen, R.; Thyfault, J.P.; Broholm, C.; Mortensen, O.H.; Olsen, R.H.; Mounier, R.; Plomgaard, P.; Van Hall, G.; Booth, F.W.; Pedersen, B.K.; et al. A 2-wk reduction of ambulatory activity attenuates peripheral insulin sensitivity. *J. Appl. Physiol.* **2010**, *108*, 1034–1040. [CrossRef]

21. Mikus, C.R.; Oberlin, D.J.; Libla, J.L.; Taylor, A.M.; Booth, F.W.; Thyfault, J.P. Lowering Physical Activity Impairs Glycemic Control in Healthy Volunteers. *Med. Sci. Sports Exerc.* **2017**, *25*, 1032–1057. [CrossRef]
22. Knudsen, S.H.; Hansen, L.S.; Pedersen, M.; Dejgaard, T.; Hansen, J.; Van Hall, G.; Thomsen, C.; Solomon, T.P.J.; Pedersen, B.K.; Krogh-Madsen, R. Changes in insulin sensitivity precede changes in body composition during 14 days of step reduction combined with overfeeding in healthy young men. *J. Appl. Physiol.* **2012**, *113*, 7–15. [CrossRef] [PubMed]
23. Winn, N.C.; Pettit-Mee, R.; Walsh, L.K.; Restaino, R.M.; Ready, S.T.; Padilla, J.; Kanaley, J.A. Metabolic Implications of Diet and Energy Intake during Physical Inactivity. *Med. Sci. Sports Exerc.* **2019**, *51*, 995–1005. [CrossRef] [PubMed]
24. Bowden Davies, K.A.; Sprung, V.S.; Norman, J.A.; Thompson, A.; Mitchell, K.L.; Halford, J.C.G.; Harrold, J.A.; Wilding, J.P.H.; Kemp, G.J.; Cuthbertson, D.J. Short-term decreased physical activity with increased sedentary behaviour causes metabolic derangements and altered body composition: Effects in individuals with and without a first-degree relative with type 2 diabetes. *Diabetologia* **2018**, *61*, 1282–1294. [CrossRef] [PubMed]
25. Dixon, N.C.; Hurst, T.L.; Talbot, D.C.S.; Tyrrell, R.M.; Thompson, D. Effect of short-term reduced physical activity on cardiovascular risk factors in active lean and overweight middle-aged men. *Metabolism* **2013**, *62*, 361–368. [CrossRef] [PubMed]
26. Bowden Davies, K.A.; Sprung, V.S.; Norman, J.A.; Thompson, A.; Mitchell, K.L.; Harrold, J.A.; Finlayson, G.; Gibbons, C.; Wilding, J.P.H.; Kemp, G.J.; et al. Physical Activity and Sedentary Time: Association with Metabolic Health and Liver Fat. *Med. Sci. Sports Exerc.* **2019**, *51*, 1169–1177. [CrossRef] [PubMed]
27. Lavie, C.J.; Ozemek, C.; Carbone, S.; Katzmarzyk, P.T.; Blair, S.N. Sedentary Behavior, Exercise, and Cardiovascular Health. *Circ. Res.* **2019**, *124*, 799–815. [CrossRef]
28. Marzetti, E.; Calvani, R.; Tosato, M.; Cesari, M.; Di Bari, M.; Cherubini, A.; Collamati, A.; D'Angelo, E.; Pahor, M.; Bernabei, R.; et al. Sarcopenia: An overview. *Aging Clin. Exp. Res.* **2017**, *29*, 11–17. [CrossRef]
29. Donato, K.A. Executive summary of the clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults. *Arch. Intern. Med.* **1998**, *158*, 1855–1867.
30. Breen, L.; Stokes, K.A.; Churchward-Venne, T.A.; Moore, D.R.; Baker, S.K.; Smith, K.; Atherton, P.J.; Phillips, S.M. Two weeks of reduced activity decreases leg lean mass and induces "anabolic resistance" of myofibrillar protein synthesis in healthy elderly. *J. Clin. Endocrinol. Metab.* **2013**, *98*, 2604–2612. [CrossRef]
31. McGlory, C.; Von Allmen, M.T.; Stokes, T.; Morton, R.W.; Hector, A.J.; Lago, B.A.; Raphenya, A.R.; Smith, B.K.; McArthur, A.G.; Steinberg, G.R.; et al. Failed recovery of glycemic control and myofibrillar protein synthesis with 2 wk of physical inactivity in overweight, prediabetic older adults. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* **2018**, *73*, 1070–1077. [CrossRef]
32. Walhin, J.P.; Richardson, J.D.; Betts, J.A.; Thompson, D. Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men. *J. Physiol.* **2013**, *591*, 6231–6243. [CrossRef] [PubMed]
33. Hill, J.O.; Wyatt, H.R.; Peters, J.C. The importance of energy balance. *Eur. Endocrinol.* **2013**, *9*, 111–115. [CrossRef] [PubMed]
34. Dorling, J.; Broom, D.R.; Burns, S.F.; Clayton, D.J.; Deighton, K.; James, L.J.; King, J.A.; Miyashita, M.; Thackray, A.E.; Batterham, R.L.; et al. Acute and chronic effects of exercise on appetite, energy intake, and appetite-related hormones: The modulating effect of adiposity, sex, and habitual physical activity. *Nutrients* **2018**, *10*, 1140. [CrossRef] [PubMed]
35. Hopkins, M.; Blundell, J.E. Energy balance, body composition, sedentariness and appetite regulation: Pathways to obesity. *Clin. Sci.* **2016**, *130*, 1615–1628. [CrossRef] [PubMed]
36. Mayer, J.; Roy, P.; Mitra, K.P. Relation between caloric intake, body weight, and physical work: Studies in an industrial male population in West Bengal. *Am. J. Clin. Nutr.* **1956**, *4*, 169–175. [CrossRef]
37. Stubbs, R.J.; Hughes, D.A.; Johnstone, A.M.; Horgan, G.W.; King, N.; Blundell, J.E. A decrease in physical activity affects appetite, energy, and nutrient balance in lean men feeding ad libitum. *Am. J. Clin. Nutr.* **2004**, *79*, 62–69. [CrossRef]
38. Shook, R.P.; Hand, G.A.; Drenowatz, C.; Hebert, J.R.; Paluch, A.E.; Blundell, J.E.; Hill, J.O.; Katzmarzyk, P.T.; Church, T.S.; Blair, S.N. Low levels of physical activity are associated with dysregulation of energy intake and fat mass gain over 1 year. *Am. J. Clin. Nutr.* **2015**, *102*, 1332–1338. [CrossRef]
39. Chaput, J.P.; Klingenberg, L.; Astrup, A.; Sjödin, A.M. Modern sedentary activities promote overconsumption of food in our current obesogenic environment. *Obes. Rev.* **2011**, *12*, 12–20. [CrossRef]

40. Wei, M.; Brandhorst, S.; Shelehchi, M.; Mirzaei, H.; Cheng, C.W.; Budniak, J.; Groshen, S.; Mack, W.J.; Guen, E.; Di Biase, S.; et al. Fasting-mimicking diet and markers/risk factors for aging, diabetes, cancer, and cardiovascular disease. *Sci. Transl. Med.* **2017**, *9*, eaai8700. [CrossRef]
41. Redman, L.M.; Smith, S.R.; Burton, J.H.; Martin, C.K.; Il'yasova, D.; Ravussin, E. Metabolic Slowing and Reduced Oxidative Damage with Sustained Caloric Restriction Support the Rate of Living and Oxidative Damage Theories of Aging. *Cell Metab.* **2018**, *27*, 805–815.e4. [CrossRef]
42. Sala, V.P.; Martínez, F.D.; Biescas, A.P. Restricción calórica, un método eficaz, sencillo y saludable para perder peso. *Nutr. Clin. Diet. Hosp.* **2017**, *37*, 77–86.
43. Wewege, M.A.; Thom, J.M.; Rye, K.A.; Parmenter, B.J. Aerobic, resistance or combined training: A systematic review and meta-analysis of exercise to reduce cardiovascular risk in adults with metabolic syndrome. *Atherosclerosis* **2018**, *274*, 162–171. [CrossRef] [PubMed]
44. Akins, J.D.; Crawford, C.K.; Burton, H.M.; Wolfe, A.S.; Vardarli, E.; Coyle, E.F. Inactivity induces resistance to the metabolic benefits following acute exercise. *J. Appl. Physiol.* **2019**, *126*, 1088–1094. [CrossRef] [PubMed]
45. Saint-Maurice, P.F.; Troiano, R.P.; Bassett, D.R.; Graubard, B.I.; Carlson, S.A.; Shiroma, E.J.; Fulton, J.E.; Matthews, C.E. Association of Daily Step Count and Step Intensity With Mortality Among US Adults. *JAMA* **2020**, *323*, 1151–1160. [CrossRef] [PubMed]
46. Campbell, B.; Kreider, R.B.; Ziegenfuss, T.; La Bounty, P.; Roberts, M.; Burke, D.; Landis, J.; Lopez, H.; Antonio, J. International Society of Sports Nutrition position stand: Protein and exercise. *J. Int. Soc. Sports Nutr.* **2007**, *4*, 8. [CrossRef] [PubMed]
47. Jäger, R.; Kerksick, C.M.; Campbell, B.I.; Cribb, P.J.; Wells, S.D.; Skwiat, T.M.; Purpura, M.; Ziegenfuss, T.N.; Ferrando, A.A.; Arent, S.M.; et al. International Society of Sports Nutrition Position Stand: Protein and exercise. *J. Int. Soc. Sports Nutr.* **2017**, *14*, 1–25. [CrossRef] [PubMed]
48. Witard, O.C.; Wardle, S.L.; Macnaughton, L.S.; Hodgson, A.B.; Tipton, K.D. Protein considerations for optimising skeletal muscle mass in healthy young and older adults. *Nutrients* **2016**, *8*, 181. [CrossRef]
49. Jiménez-Pavón, D.; Carbonell-Baeza, A.; Lavie, C.J. Physical exercise as therapy to fight against the mental and physical consequences of COVID-19 quarantine: Special focus in older people. Progress in Cardiovascular Diseases. *Prog. Cardiovasc. Dis.* **2020**, in press.
50. Fallon, K. Exercise in the time of COVID-19. *Aust. J. Gen. Pract.* **2020**, *49*. [CrossRef]
51. Kis, O.; Buch, A.; Stern, N.; Moran, D.S. Minimally supervised home-based resistance training and muscle function in older adults: A meta-analysis. *Arch. Gerontol. Geriatr.* **2019**, *84*, 103909. [CrossRef]
52. Peterson, M.D.; Rhea, M.R.; Sen, A.; Gordon, P.M. Resistance exercise for muscular strength in older adults: A meta-analysis. *Ageing Res. Rev.* **2010**, *9*, 226–237. [CrossRef] [PubMed]
53. Fragala, M.S.; Cadore, E.L.; Dorgo, S.; Izquierdo, M.; Kraemer, W.J.; Peterson, M.D.; Ryan, E.D. Resistance Training for Older Adults: Position Statement From the National Strength and Conditioning Association. *J. Strength Cond. Res.* **2019**, *33*, 2019–2052. [CrossRef] [PubMed]
54. Marques, E.A.; Mota, J.; Carvalho, J. Exercise effects on bone mineral density in older adults: A meta-analysis of randomized controlled trials. *Age (Omaha)* **2012**, *34*, 1493–1515. [CrossRef] [PubMed]
55. Kaur, J. A comprehensive review on metabolic syndrome. *Cardiol. Res. Pract.* **2014**, *2014*. [CrossRef]
56. Zafar, U.; Khaliq, S.; Ahmad, H.U.; Manzoor, S.; Lone, K.P. Metabolic syndrome: An update on diagnostic criteria, pathogenesis, and genetic links. *Hormones* **2018**, *17*, 299–313. [CrossRef]
57. Oikonomou, E.K.; Antoniades, C. The role of adipose tissue in cardiovascular health and disease. *Nat. Rev. Cardiol.* **2019**, *16*, 83–99. [CrossRef]
58. Gustafson, B.; Hammarstedt, A.; Andersson, C.X.; Smith, U. Inflamed adipose tissue: A culprit underlying the metabolic syndrome and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **2007**, *27*, 2276–2283. [CrossRef]
59. Eckel, R.H.; Grundy, S.M.; Zimmet, P.Z. The metabolic syndrome. *Lancet* **2005**, *366*, 1415–1428. [CrossRef]
60. Rochlani, Y.; Naga, V.P.; Swathi, K.; Jawahar, L.M. Metabolic syndrome: Pathophysiology, management, and modulation by natural compounds. *Ther. Adv. Cardiovasc. Dis.* **2017**, *11*, 215–225. [CrossRef]
61. Wannamethee, S.G.; Atkins, J.L. Muscle loss and obesity: The health implications of sarcopenia and sarcopenic obesity. *Proc. Nutr. Soc.* **2015**, *74*, 405–412. [CrossRef]
62. Gupta, R.; Ghosh, A.; Singh, A.K.; Misra, A. Clinical considerations for patients with diabetes in times of COVID-19 epidemic. *Diabetes Metab. Syndr. Clin. Res. Rev.* **2020**, *14*, 211–212. [CrossRef] [PubMed]
63. Bornstein, S.R.; Dalan, R.; Hopkins, D.; Mingrone, G.; Boehm, B.O. Endocrine and metabolic link to coronavirus infection. *Nat. Rev. Endocrinol.* **2020**, *16*, 297–298. [CrossRef] [PubMed]

64. Li, B.; Yang, J.; Zhao, F.; Zhi, L.; Wang, X.; Liu, L.; Bi, Z.; Zhao, Y. Prevalence and impact of cardiovascular metabolic diseases on COVID-19 in China. *Clin. Res. Cardiol.* **2020**, *109*, 531–538. [CrossRef] [PubMed]
65. Dietz, W.; Santos-Burgoa, C. Obesity and its Implications for COVID-19 Mortality. *Obesity* 2020. [CrossRef]
66. Ryan, D.H.; Ravussin, E.; Heymsfield, S. COVID 19 and the Patient with Obesity—The Editors Speak Out. *Obesity* 2020. [CrossRef]

nutrients

MDPI

Review

Nutrients in the Prevention of Osteoporosis in Patients with Inflammatory Bowel Diseases

Alicja Ewa Ratajczak *, Anna Maria Rychter, Agnieszka Zawada, Agnieszka Dobrowolska and Iwona Krela-Kaźmierczak *

Department of Gastroenterology, Dietetics and Internal Diseases, Poznan University of Medical Sciences, 49 Przybyszewskiego Street, 60-355 Poznan, Poland; a.m.rychter@gmail.com (A.M.R.); aga.zawada@gmail.com (A.Z.); agdob@ump.edu.pl (A.D.)

* Correspondence: alicjaewaratajczak@gmail.com (A.E.R.); krela@op.pl (I.K.-K.); Tel.: +48-667-385-996 (A.E.R.); +48-8691-343 (I.K.-K.); Fax: +48-8691-686 (A.E.R.)

Received: 12 May 2020; Accepted: 3 June 2020; Published: 6 June 2020

Abstract: The chronic character of inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, results in various complications. One of them is osteoporosis, manifested by low bone mineral density, which leads to an increased risk of fractures. The aetiology of low bone mineral density is multifactorial and includes both diet and nutritional status. Calcium and vitamin D are the most often discussed nutrients with regard to bone mineral density. Moreover, vitamins A, K, C, B12; folic acid; calcium; phosphorus; magnesium; sodium; zinc; copper; and selenium are also involved in the formation of bone mass. Patients suffering from inflammatory bowel diseases frequently consume inadequate amounts of the aforementioned minerals and vitamins or their absorption is disturbed, resulting innutritional deficiency and an increased risk of osteoporosis. Thus, nutritional guidelines for inflammatory bowel disease patients should comprise information concerning the prevention of osteoporosis.

Keywords: bowel diseases; diet; osteoporosis; bone density; nutrients

1. Introduction

Inflammatory bowel diseases (IBD), including Crohn's Disease (CD) and Ulcerative Colitis (UC), are chronic conditions, the aetiology of which is not entirely known. Possible risk factors comprise genetic predisposition, immunological disorders, and environmental conditions [1]. Furthermore, the chronic character of these diseases causes extra-intestinal complications such as osteoporosis, which is manifested by low bone mineral density, resulting in an increased risk of fractures [2].

The World Health Organisation (WHO) classifies Bone Mineral Density (BMD) on the basis of Dual-Energy X-ray Absorptiometry (DEXA)—i.e., the gold standard in the diagnosis of osteoporosis:

1. Normal: T-score ≥ −1 SD;
2. Osteopenia (low bone mass): T-score < −1 SD and >−2.5 SD;
3. Osteoporosis: T-score ≤ −2.5 SD;
4. Severe osteoporosis: T-score ≤ −2.5 SD with fragility fractures [3].

Risk factors of osteoporosis in IBD include early age onset; steroid therapy; malnutrition; low body mass; hormonal disorders, including a decreased oestrogen level; and malabsorption causing a nutritional deficiency, particularly in terms of vitamin D and calcium. However, osteoporosis may also stem from genetic factors. In the course of IBD, pro-inflammatory molecules TNF-α, IL-1β, IL-6, and IL-17 levels are elevated and lead to increased bone resorption, causing a decrease in bone mineral density [4]. In fact, low bone mineral density, osteopenia and osteoporosis were found in

22–77%, 32–36%, and 7–15% of IBD patients, respectively [1]. Additionally, low BMD is observed more frequently in CD than UC patients [5].

An important risk factor for BMD loss in IBD patients is the use of certain medications. Corticosteroids (CS) are a group of medications whose prolonged use is associated with side effects involving bone tissue [5]. CS increase apoptosis and decrease the formation of osteoblasts and promote osteoclastogenesis. Moreover, CS influence calcium balance (decreasing calcium absorption in the intestine and renal resorption) and the neuroendocrine system. The data indicate that following the first year of steroid therapy, bone mass may decrease by about 12%, and 2–3% per year in the following year. In addition, CS use decreases muscle mass, which leads to an elevated risk of fractures [6,7]. Corticosteroids also affect the receptor activator of nuclear factor κβ/receptor activator of nuclear factor κβ ligand/osteoprotegerin (RANK/RANKL/OPG) pathway. In fact, CS increase the expression of RANKL, which binds with the RANK receptor in osteoclasts, which in turn elevates their differentiation and activation. Moreover, corticosteroids decrease the level of OPG responsible for the inhibition of RANKL activity [8]. According to research studies, therapy with steroids was associated with a lower BMD in IBD patients [9]. Nevertheless, different results obtained in other studies may stem from differences in the methodology of the research. Wada et al. reported that steroid therapy was a risk factor for low BMD for UC patients but not for CD patients [10]. In our study, we also demonstrated a correlation between a cumulative prednisolone dose, administered in the course of the disease, and the lumbar spine (L2–L4) T-score, the femoral neck (FN) T-score, and the Z-score in UC patients. However, the above-mentioned association was not found in CD patients [5]. It is generally accepted that osteoporosis affects 30–50% of subjects treated with CS. Therefore, in order to prevent steroid-related osteoporosis, the BMD should be evaluated before and after every year of CS treatment. Additionally, the supplementation of calcium (1200 mg/day) and vitamin D (800 IU) should also be recommended [11].

As far as Infliximab (IFX) is concerned, research studies suggest that IFX treatment in CD patients increased the N-terminal telopeptide of type I collagen [12]. However, another study showed no difference in the level of N-terminal telopeptide in type I collagen, although it was pointed out that the bone-specific alkaline phosphatase and total osteocalcin were elevated in CD patients who had used IFX [13]. The BMD and bone mineral content of the lumbar spine increased in CD patients treated by IFX [14]. Lima et al. reported the association between azathioprine and infliximab use and low BMD in a patient with CD [15]. However, another study revealed that IFX did not affect BMD [16]. Krajcovicova et al. showed that a combined therapy with anti-TNFα and azathioprine improved the lumbar spine BMD by –3% per year, whereas corticosteroid therapy reduced the BMD by –3% per year [17]. Moreover, Hoffmann et al. reported that immunomodulatory treatment was associated with a BMD decrease [18], although another study revealed that biological and immunomodulatory treatment did not affect the risk of hip fracture [19].

The use of monoclonal antibodies in osteoporosis associated with IBD is the subject of several studies. Bone metabolism at the osteoclast/osteoblast level is regulated by the following factors belonging to the tumour necrosis factor superfamily: receptor activator of nuclear factor kappa B (κB; RANK), its ligand (receptor activator of nuclear factor κB ligand, RANKL), and osteoprotegerin (RANKL/RANK/OPG). Krela-Kaźmierczak I. et al. showed that low OPG levels may be associated with osteoporosis in UC. Nevertheless, in CD patients increased RANKL levels were observed [20,21]. Interleukin 6 can modulate BMD via the OPG/sRANKL system in the femoral neck, which can cause a loss of bone, particularly in the course of CD. Therefore, these studies may contribute to the development and implementation of new secondary osteoporosis therapy in the course of IBD [22]. It is worth emphasizing the effects of biological therapy employed in IBD on bones. Although studies focusing on the effects of anti-TNF-α therapy on bone metabolism in IBD are limited, it was observed that IFX treatment induced beneficial effects on bone metabolism in IBD patients [23,24], which suggests that monoclonal antibodies constitute an important factor in the treatment of osteoporosis. Understanding the role of RANKL and sclerostin in bone cell biology entirely changes the therapeutic perspective.

Sclerostin, an antagonist of the Wnt pathway, plays a key role in bone formation and is mainly secreted by osteocytes. In fact, high levels of RANKL and sclerostin were detected in osteoporosis, leading to the production of antibodies which are able to neutralize their activity. Denosumab (anti-RANKL antibody) is a fully human monoclonal antibody which inhibits osteoclastic-medicated bone resorption by binding to osteoblast-produced RANKL. Romosozumab, a monoclonal antibody binding sclerostin, increases bone formation and decreases bone resorption. Denosumab and romosozumab present promising results in the treatment of postmenopausal osteoporosis [25]. Therefore, the use of these antibodies in the therapy of secondary osteoporosis in IBD patients requires further research.

Diet and nutritional status constitute risk factors in the development of osteoporosis. Micro and macronutrients, vitamins, and mineral components intake is often below the recommended values, particularly when the disease is active. Hence, malnutrition and deficiency in nutrients essential to bone mineralization can affect patients with inflammatory bowel diseases [26].

2. Proteins, Fats, and Carbohydrates Intake in IBD and the Risk of Osteoporosis Development

The European Society for Clinical Nutrition and Metabolism (ESPEN) recommends supplying 1.2–1.5 g protein/kg body weight in active IBD. Patients in remission should consume 1 g protein/kg body weight. Improper diet and the loss of nutrients in the gastrointestinal tract leads to an increase in fat mass, as well as a decrease in free fat mass in patients suffering from IBD. On the basis of these guidelines, there is no special diet in the remission period [27]. The recommended macronutrient intake values for adults are listed below (Table 1) [28,29].

Table 1. References of macronutrient consumption.

Macronutrient	DRI * (% of Total Energy Intake)	RDA **	
		% of Total Energy Intake	Mass of Macronutrient
Protein	10–35	10–20	0.9 g/kg body weight/day
Carbohydrates	45–65	50–70	Minimum 130 g/day
Fat	20–35	20–35	53–158 g/day
n-6 fatty acids (linoleic acid)	5–10	4	-
n-3 fatty acids (alfalinolenic acid)	0.6–1.2	0.5	-

* DRI—Dietary Reference Intake [28]; ** RDA—Recommended Dietary Allowance [29].

2.1. Protein

Protein provides about 50% of bone volume, 1/3 of bone mass and forms the bone matrix, therefore its supply determines the maintenance of bone mass in adults [30]. On the other hand, an excessive intake of protein causes increased calcium excretion and negative calcium balance [31].

Moderate or severe hypoalbuminemia was diagnosed in 17% and 24% of IBD patients, respectively. In patients with albumin deficiency following surgical procedures, mortality within 30 days was higher than in patients with normal albumin levels [32]. Protein-energy wasting is observed more frequently in IBD patients than in healthy individuals, and the same observation can be made in Crohn's disease patients as compared to subjects suffering from ulcerative colitis [33]. There were no statistically significant differences between men with ulcerative colitis in remission and healthy subjects [34].

One of the research studies showed a statistically significant difference in protein intake between men over 63 years old with and without a femur fracture. Every 3% increase in protein daily intake caused a 20% decreased risk of femur fractures [35]. What is more, the percentage of protein in the daily caloric content of middle aged women (65–72 years old) correlated negatively with a femoral neck. Additionally, a protein intake higher than 1.2 g/kg body weight was associated with lower BMD in the femoral neck and lumbar spine [36].

2.2. Fats

There was no statistically significant differences in the intake of total fat, saturated fat, and mono–and poly–unsaturated fat between healthy individuals and CU patients [34]. The European

Society for Clinical Nutrition and Metabolism does not recommend n-3 fatty acid supplementation for maintaining remission in IBD [27].

The development of osteoporosis was correlated with the type of fatty acids. In fact, the risk of fractures was increased by n-3 and saturated fatty acid intake and it was decreased by monounsaturated and n-6 fatty acids. Dong et al. demonstrated that the supplementation of eicosapentaenoic and docosahexaenoic acid (1.2 g per day) did not result in statistically significant differences in osteocalcin and bone-specific alkaline phosphatase [37]. Furthermore, n-3 fatty acid supplementation did not significantly alter bone-specific alkaline phosphatase and type I collagen-telopeptide levels, although it decreased the osteocalcin level [38]. The twenty-four-week supplementation of n-3 fatty acid in combination with aerobic exercise reduced chronic inflammation and increased BMD in postmenopausal women [39].

2.3. Carbohydrates

Carbohydrates supply energy for host cells and the intestinal microbiome. Fermentable carbohydrates (especially mono–and di–saccharides) and fibre constitute important factors in inflammatory bowel diseases [40].

A meta-analysis demonstrated that a low-FODMAP diet (low-Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyol) decreased the incidence of abdominal pain, nausea, weakness, and flatulence, although no change in the rate of constipation was observed [41]. Patients with IBD in remission were supplied with insufficient amounts of carbohydrates relative to recommendations [42]. "National Health and Nutrition Examination Survey" research demonstrated a negative association between energy density (a diet rich in carbohydrates, sugar, fat, and saturated fatty acid) and BMD in the femur and femoral neck [43]. In animals, a high fructose diet decreased transepithelial active calcium ion transport and the 1.25(OH)2D3 level by about 30–40% [44].

On the other hand, the fructose content in breast milk triggered an increased BMC (Bone Mineral Content) in a 6-month-old infant compared to the first month of the baby's life. Nevertheless, lactose and glucose did not cause changes in BMC [45]. Another study pointed to the association between carbonated beverages consumption, which frequently are sources of sugar, and fractures [46]. In fact, a meta-analysis showed that the consumption of non-alcoholic beverages was negatively associated with calcium supply [47]. Additionally, fibre possibly prevents a decrease in BMD, as water-soluble fibre intake causes increased calcium retention in bones [48].

3. The Importance of Vitamin Intake in IBD and Bone Mineral Density

3.1. Vitamin D

Vitamin D comprises a group of chemical compounds such as ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). The active forms of vitamin D are 1.25(OH)2D and 24.25(OH)2D. Vitamin D acts directly and indirectly on the bone [49], regulates calcium absorption in the gastrointestinal tract, determines the proper serum calcium level, influences osteoblasts mineralization and differentiation, and decreases the synthesis of parathormone and the reabsorption of phosphates from bones. Vitamin D can be found in fatty sea fish, cod liver oil, and yolk [49,50].

IBD patients are a group presenting a higher risk of vitamin deficiency and, therefore, should control their 25(OH)D serum level. The recommended vitamin D supplementation for healthy adults is 800–2000 IU from September to April [51]. According to the available data, vitamin D serum level was lower than 30 ng/mL in IBD, but without statistically significant differences compared to the control group. It may stem from the low exposure to sunlight of the whole population and malabsorption caused by the inflammation in the gastrointestinal tract associated with IBD [52]. The deficiency was observed in 52% of children with IBD [53]. A sufficient vitamin D level was found more often in patients with a non-inflammatory disease activity; however, the serum concentration level was not

correlated with any inflammatory markers [54]. In terms of IBD patients, a vitamin D dose of 1820 IU was sufficient to maintain the normal 25(OH)D serum level [55].

Although the supplementation of vitamin D decreased the risk of fractures in general as well as the risk of femoral neck fractures, it did not alter the risk of osteoporosis [56]. In elderly patients, the 1-year supplementation of vitamin D (12,000, 24,000, or 48,000 IU per month) did not change the hip BMD but decreased the parathormone level [57]. A meta-analysis showed the supplementation of vitamin D in patients over 50 years of age was associated with an increased risk of fractures. The dose of 1200 mg calcium and minimum 800 IU of vitamin D was more effective than lower doses [58]. However, a high dose (4000 IU and more) of vitamin D decreased the volumetric BMD in healthy adults. The study did not reveal significant differences in bone strength [59].

3.2. Vitamin C (Ascorbic Acid)

Bone tissue is 90% made of collagen, and vitamin C is a necessary antioxidant for its synthesis [60]. Moreover, it is possible that ascorbic acid participates in the synthesis of osteoblasts and influences osteoclast differentiation [61]. In animals, vitamin C deficiency increases osteoclast and decreases bone formation [62].

Since patients with IBD fear the symptoms of the disease, they often eliminate vegetables and fruits from their diets. This, in turn, may lead to an insufficient vitamin C intake and to the deterioration of the patients' health. Children, the elderly, and patients with the malabsorption syndrome are particularly exposed to this deficiency. Ascorbic acid can be found in citrus fruits, potatoes, parsley, and berries without seeds; thus, their consumption should be encouraged in this patient group [63]. In fact, patients with inflammatory bowel diseases consumed significantly less fruits and fewer vegetables; hence, they presented lower serum vitamin C than healthy individuals [64]. Patients suffering from ulcerative colitis consumed a similar amount of vitamin C in comparison with the healthy subjects; however, the INQ (Index of Nutritional Quality) of UC patients was significantly lower than that of healthy adults [65]. What is more, children with IBD presented a significantly lower intake of vitamin C than healthy children [66]. Enteral nutrition decreased the serum vitamin C level in children with active Crohn's disease, whereas glutamine supplementation did not influence the ascorbic acid level [67].

Sahni et al. estimated vitamin C intake on the basis of the Food Frequency Questionnaire. Following a 17-year-long observation period, subjects who consumed the highest amount of vitamin C suffered femoral neck and non-spine fractures less frequently than individuals reporting the lowest intake. The authors suggested that vitamin C intake in the elderly can be beneficial to bone health [60]. Additionally, daily vitamin C intake was associated with BMD in postmenopausal women. Patients with osteoporosis consumed a smaller amount of ascorbic acid than women not suffering from osteoporosis [68]. On the other hand, an American study presented a lack of association between vitamin C intake (from food and supplements) and BMD in postmenopausal women. In fact, long-term vitamin C supplementation was connected with a higher BMD in 55 to 64-year-old women [69]. Furthermore, although it was just a pilot study comprising 34 subjects, it showed that anti-oxidative vitamins influenced BMD, vitamin E (600 mg), and vitamin C (1000 mg) with and without resistance training, as well as preventing a decrease in BMD [70]. Kim et al. reported that a higher vitamin C intake was associated with a lower risk of osteoporosis development in subjects over 50 years of age; however, this was only in patients reporting little physical activity [71].

3.3. Vitamin B12 and Folic Acid

Vitamins B9 (folic acid) and B12 (cobalamin) are water-soluble vitamins. Folic acid is necessary for DNA methylation, whereas cobalamin is a coenzyme found in many biochemical reactions in the human body, e.g., in the metabolization of folic acid, or DNA synthesis. Their deficiency causes megaloblastic anaemia as well as nervous system disorders [72]. Vitamin B12 and folic acid are involved in the remethylation of homocysteine to methionine; therefore, a deficiency in these vitamins leads to hyperhomocysteinemia. It is vital to bear in mind that the serum homocysteine level is correlated

to osteoporotic fractures [73]. A deficiency in folic acid and vitamin B12 is due to insufficient intake, abnormal absorption, or increasing demand. Furthermore, cobalamin connects with an intrinsic factor, allowing for the absorption of vitamins in the ileum, and folic acid is absorbed in the duodenum and jejunum. Both of these vitamins are not absorbed in the large intestine. The risk of B9 or B12 deficiency is lower in ulcerative colitis than in Crohn's disease, which may stem from the fact that CD can affect all parts of the gastrointestinal tract, including the small intestine, whereas inflammation in UC predominantly affects the large intestine [74].

A meta-analysis showed that the serum folic acid level was lower in IBD patients than in healthy individuals. This could be accounted for by an insufficient intake, higher utilization, or taking medications. The authors recommended the routine supplementation of folic acid for colorectal cancer prevention. However, the study did not demonstrate a significant difference in serum B12 levels between the patients and the control group [72].

Kim et al. pointed out a negative correlation between the serum homocysteine level and BMD in women under 50 years of age [75]. Women with a normal homocysteine concentration consumed a larger amount of vitamin B12 than women with a higher level. In the same group, subjects with a normal lumbar spine BMD presented a lower serum homocysteine level and higher folic acid concentration in erythrocyte than women with low BMD or osteoporosis [76]. There was no correlation between the serum vitamin B12 level and the risk of fractures in women and men. However, an association was found between homocysteine concentration and the risk of a femoral neck fracture, particularly in women [77]. Salari et al. investigated the influence of folic acid supplementation (1 mg/day) on the serum osteocalcin and homocysteine level in postmenopausal women. They observed a significant difference in osteocalcin concentration between the study and control group after 6 months. It was found that the serum homocysteine level decreased in both groups, although the changes were not significant within the groups or between them [78]. Vitamin B9 and B12 supplementation was demonstrated to decrease homocysteine concentration and increase serum folic acid and cobalamin levels. Serum alkaline phosphatase and C-terminal cross-linking collagen I telopeptide levels did not change significantly [79].

Hyperhomocysteinemia (diagnosed in 60% of patients) was associated with osteoporosis and low BMD in CD patients, whereas bone disorders affected 90% of the subjects [80].

3.4. Vitamin A

Vitamin A is vital for growth; vision; and osteoclastogenesis stimulation, which further influences BMD. A total of 16% of non-adult patients with IBD presented vitamin A deficiency [81].

There was no statistically significant difference in vitamin A intake between men suffering form UC and the control group [34]. In fact, vitamin A or beta-carotene supplementation within a 15-year period did not increase the risk of bone fractures [82]. The retinol serum level correlated negatively with the BMD of the lumbar spine and femoral neck in postmenopausal women [83]. A meta-analysis showed that a high vitamin A level but not beta-carotene intake increased the risk of a femoral neck fracture. Additionally, a high and a low serum vitamin A level increased the risk of a femoral neck fracture. The authors did not observe a significant difference in total fractures depending on the supply and vitamin A concentration [84].

3.5. Vitamin K

The enzyme participating in the synthesis of glutamic acid (Gla) found in osteocalcin created in osteoblast and in the matrix bone protein is vitamin K. It occurs in two forms: K1 (phylloquinone) and K2 (menaquinone, MK-n) [85].

The study, conducted among postmenopausal Japanese women with osteoporosis, indicated that the administration of risedronic acid with vitamin K (45 g/day) was not more beneficial than administration without vitamin K supplementation. In fact, there was no statistically significant difference in fractures within the studied group [86]. Another study demonstrated that supplementation

of menaquinone decreased carboxylated osteocalcin to a undercarboxylated osteocalcin ratio in young adults (47 ± 14 years old) and in postmenopausal women [87]. However, the supplementation of 360 ng of vitamin K (MK-7) for 12 months did not change bone mass as compared to the control group. The authors observed an increase in the serum carboxylated osteocalcin and a decrease in the undercarboxylated osteocalcin level in the study group in comparison with the placebo group [88].

4. Minerals and Bone Mineral Density in IBD

4.1. Calcium and Phosphate

Calcium (Ca) is responsible for proper BMD, blood coagulation, and the proper functioning of the cardiovascular system. In the human body, more than 99% of Ca is stored in bones. Therefore, a decreased serum calcium level leads to its release from bones and causes bone tissue resorption [50]. Furthermore, an insufficient calcium intake causes hormonal disorders, leading to a higher risk of fractures. Calcium can be found in such sources as milk, dairy products, and green leafy vegetables [89]. Additionally, the human body contains about 700 g of phosphorus (P), which is mainly stored in bones (80–90%). Hence, both its excessive and inadequate intake can develop osteoporosis. Phosphorus deficiency, or its insufficient supply to calcium supply ratio, causes bone resorption and inhibits bone mineralization and bone formation. On the other hand, an oversupply of P, particularly with insufficient Ca intake, results in excessive parathormone excretion and the loss of bone mass [90].

The insufficient intake of calcium was estimated in 80–86% of IBD patients, who avoid milk and dairy products due to lactose intolerance [91]. Patients with IBD have lower calcium and phosphate levels in comparison with healthy individuals [52].

Another cause of calcium malabsorption is the use of steroids as well as the occurrence of diarrhoea. The supplementation of calcium in a 1000–1500 mg/day dose is recommended for most patients with inflammatory bowel diseases. Furthermore, patients treated with steroids require calcium and vitamin D supplementation [91]. Calcium intake was correlated negatively with the femoral neck BMD but not with the lumbar spine BMD in IBD patients [92]. Premenopausal women suffering from IBD consumed insufficient amounts of calcium and vitamin D, and their intake of Ca and vitamin D was correlated [93]. Moreover, a low calcium serum level was observed in patients more frequently than in the control group, although it was insignificant. Additionally, the Ca serum level was negatively correlated with steroids [52]. A meta-analysis demonstrated that calcium supplementation without other substances (for example, vitamin D) did not alter the risk of femoral neck fractures in both sexes [94]. The study revealed that an increased intake of calcium by every 300 mg decreased the risk of fractures, although it was nonlinear. The highest risk was found in the intake below 751 mg of calcium. The fracture risk was unchanged in the intakes of more than 1137 mg and 882–996 mg of calcium [56]. Gutiérrez et al. demonstrated that a one-week diet rich in phosphorus (1677 ± 167 mg/day) increased Fibroblast Growth Factor 23 (FGF23), osteocalcin, and osteopontin levels. The aforementioned results suggest that a phosphorus-rich diet negatively affects health [95], and that women over 45 years of age, both with and without osteoporosis, consume similar amounts of calcium. Thus, Ca intake was not associated with the incidence of fractures [96].

4.2. Magnesium

Magnesium (Mg) is absorbed in the small intestine, and its absorption ranges from 30% to 80%. Bones store about 60% of the total body magnesium. The main sources of Mg are legumes, seeds, nuts, almonds, spinach, and buckwheat. Not only is this element responsible for the stability and permeability of cell membranes but it also maintains the DNA double helix integrity and regulates the activity of about 300 enzymes [97]. On the other hand, magnesium deficiency causes decreased osteoblast and osteoclast activity, resulting in bone metabolism disorders [98]. Chronic hypomagnesemia leads to the disturbance of parathyroid hormone production, leading to hypocalcaemia [99].

Patients with UC and CD consumed a lower amount of Mg than healthy adults. CD patients consumed 60–63% of the daily magnesium requirement [65,100]. Magnesium intake correlated with BMD, with a stronger correlation found in men than in women [98]. Postmenopausal women who consumed 422.5 mg and more of Mg per day presented a higher hip and total body BMD by 3% and 2%, respectively, than the individuals supplying <206.5 mg Mg/day. No association was observed between magnesium intake and the risk of fractures. On the other hand, a high magnesium dose was associated with a higher risk of forearm and wrist fractures in comparison with a low Mg intake. The authors paid attention to the subjects with a high supply of magnesium, since they reported much physical activity, which increases the frequency of falls [101]. The supplementation of magnesium (106 mg) and calcium (1200 mg) for 4 weeks in postmenopausal women did not change the serum parathyroid hormone level both in the study and the control group. However, the supplementation increased the serum CTX (C-terminal telopeptide) level—i.e., a bone resorption marker [102]. A conducted meta-analysis indicated that a high magnesium intake was not associated with a lower risk of hip fractures. On the other hand, magnesium dose was connected with the hip and femoral neck BMD, although no association was found with the lumbar spine BMD [103].

4.3. Sodium (Na)

The absorption of water and electrolytes, including sodium (Na), takes place in the colon. The lymphatic function of the large intestine can be impaired in the course of the mucosal inflammation [104].

In spite of the fact that the human body contains as much as 105 g of sodium, the intake of Na in the population is still too high, with some people consuming 9–12 g salt per day, which results in numerous disorders and also affects bones [105]. Na is known to improve taste and preserve products [106]; it is usually found in salt (40% of mass), meat and its preparations, grains, milk, and dairy products. This element constitutes the main extracellular cation excreted in urine and sweat. Moreover, sodium is responsible for the maintenance of the acid-base balance, cell work, and the transmission of nerve impulses. Although a normal sodium Na^+ level is 135–145 mmol, both too high and too low Na concentration levels constitute a threat to health and life. In fact, hypernatremia causes weakness, headache, vomiting, loss of appetite, weak nerve reflexes, and cardiac disorders. On the other hand, hyponatremia induces neuromuscular excitability, confusion, and cardiac arrest.

Patients with UC in remission consumed non-significantly lower sodium amounts than healthy individuals [34]. The sodium intake was lower in malnourished subjects than in properly nourished patients [107].

The Korea National Health and Nutrition Examination Survey indicated that osteoporosis was observed more frequently in postmenopausal women consuming ≥ 4001 mg of salt per day than in those consuming ≤ 2000 mg/day. A salt intake of ≥ 5001 mg was associated with a higher risk of osteoporosis in the femoral neck compared to the consumption of ≤ 2000 mg/day [108]. A sodium-rich diet (11.2 g of salt per day) increased calcium excretion in urine and changed the serum NTX (N-terminal telopeptide) level in comparison with a low salt intake (3.9 g). However, there was no significant change in the concentration of Pyr (pyridoxine) and Dpyr (deoxypyridoline) [109]. A meta-analysis demonstrated that a high intake of Na is a factor associated with a higher risk of osteoporosis. There was no significant correlation between the amount of calcium excretion in urine and bone mineral density [110].

A low sodium diet (2 g salt/day) for 6 months decreased calcium excretion with urine in patients who consumed 3.4 g or more salt per day and reduced the concentration of P1NP (propeptide of type 1 collagen). There was no significant change in the serum NTX level.

On the other hand, a low-sodium diet (2 g salt per day) of 6-month duration in persons consuming 3.4 g or more salt per day increased the amount of excreted calcium and the serum P1NP level. The authors did not observe any changes in the serum NTX level [111].

5. Microelements and Bone Mineral Density in IBD

5.1. Zinc

Zinc (Zn) catalyses about 100 enzymes, which makes it an indispensable element for the proper functioning of the immune system as well as for protein and DNA synthesis [112]. Zinc participates in the stabilization of the mucous membrane structure and inhibits mast cells degranulation. On the other hand, zinc deficiency can lead to the release of endogenous heparin, which contributes to the development of osteoporosis. Nevertheless, the exact mechanism of zinc influence on the prevention of osteoporosis is unknown [113].

About 15% of patients with UC or CD exhibited zinc deficiency. In diarrhoea, which often affects IBD patients, Zn demand in the body increases, and its supplementation is essential [112].

The serum zinc level of postmenopausal women with osteoporosis was lower than that of the control group, suggesting the influence of Zn on bone mineralization [113]. Zinc intake correlated negatively with the excretion of Pyr and Dpyr, which are specificity and sensitivity bone resorption markers. No association with serum osteocalcin and alkaline phosphatase was observed, and more patients presented a normal serum zinc level [114].

5.2. Copper

The human body contains about 80–150 mg copper (Cu), stored mainly in the muscles, bones, brain, and liver. Cu is absorbed in the small intestine and constitutes a cofactor for many enzymes, such as lysyl oxidase, participating also in collagen synthesis [115]. Thus, copper deficiency results in anaemia, the rupture of blood vessels, fractures, and depression [116].

Although women with IBD did not consume enough copper [42], Nangliyai et al. demonstrated that the copper serum level was statistically higher in IBD patients than in the control group. In fact, BMD correlated negatively with the serum Cu level [117].

The serum Cu level was statistically lower in postmenopausal women with osteoporosis than in the group without bone disorders. The administration of calcitonin to women with osteoporosis caused an increase in the serum Cu level. Hence, the results suggested that Cu impacts the development of osteoporosis [112]. In contrast, Cu supplementation (3 or 6 mg per day) for 4 weeks did not cause a change in the level of urine osteocalcin, creatinine, and the daily excretion of Pyr and DPyr [118].

5.3. Selenium

Selenium (Se) is an antioxidant and a cofactor for numerous enzymes in the human body. Additionally, it decreases inflammation and inactivates osteoclasts, thus influencing BMD [119]. Furthermore, since deiodinase contains Se, this element determines thyroid function. The main sources of selenium are eggs, meat, and milk, but only if it was added to fodder [120].

It was observed that the serum selenium level was lower in CD patients than in healthy adults. In men with UC, the selenium concentration depended on the location of the inflammatory lesions [121].

A study showed a negative correlation between the amount of selenium intake and osteoporosis [122]. On the other hand, the serum selenoprotein level correlated positively with total BMD, and the serum selenium level with the total and femoral neck BMD in aging men from Europe. It is vital to note that the observed relationships were independent of the thyroid function [123].

The demand for selected vitamins and minerals for adults is presented in Table 2.

Table 2. Requirements for the selected vitamins and minerals in adults [29].

Nutrient	Male	Female
Vitamin A * (μg equivalent of retinol)	900	700
Vitamin D ** (μg cholecalciferol)	15	15
Vitamin K ** (μg phylloquinone)	65	55
Vitamin C * (mg)	90	75
Folic acid * (μg equivalent of folate)	400	400
Vitamin B12 * (μg cobalamin)	2.4	2.4
Calcium * (mg)	1000–1200	1000–1200
Phosphorus * (mg)	700	700
Magnesium *(mg)	400–420	310–320
Sodium ** (mg)	1200–1500	1200–1500
Zinc * (mg)	11	8
Copper * (mg)	0.9	0.9
Selenium * (μg)	55	55

* RDA—Recommended Dietary Allowance; ** AI—Adequate Intake.

6. Polyphenols

Polyphenols are bioactivity chemical compounds which occur in plants, primarily in fruits, vegetables, coffee, tea, cocoa, herbs, and spices [124]. They are antioxidants and possess anti-inflammatory properties, which may influence the course of inflammatory diseases, such as IBD [125]. Polyphenols may influence the activity of nuclear factor erythroid 2–related factor 2 (Nrf2), which protects against oxidative stress and inflammation [126]. Moreover, polyphenols affect gut microbiota composition, stimulating bifidium, and lactobacilli [127]. There only a few studies regarding the influence of selected polyphenols on the IBD course. The available research is more frequently focused on animal models.

In addition, it is crucial to acknowledge the importance of green tea polyphenols. They decrease the TNF-α level and increase COX-2 (cyclooxygenase-2) synthesis. [128].

A study based on animal models showed that green tea polyphenols increase antioxidants levels, reduce serum TNF-α and Il-6, and decrease intestinal inflammation. [129]. Rezaei et al. reported that feeding rats with honey (which contains phenols and flavonoids) and Spirulina Platensis (containing antioxidants) may protect against UC development induced by acetic acid. The supplementation of polyphenols influences, for instance, the downregulation of various inflammatory pathways [130].

Another study presented no association between the total polyphenol intake and CD or UC, but polyphenols and resveratrol may protect against the development of Crohn's disease [124]. Moreover, the 6-week supplementation of resveratrol (500 mg/day) in UC patients decreased the TNF-α level and the clinical colitis activity index score [131].

What is more, polyphenols are vital elements in the prevention of osteoporosis. The consumption of products with high amounts of polyphenols (olive oil, soy, tea, fruits, vegetables) probably protects against osteoporosis development. However, the mechanism of action is not well understood [132]. A higher intake of flavonoids was associated with a higher BMD of the spine, but no association was found with the hip BMD [133]. Hardcastle et al. reported a negative correlation between the amount of flavonoids intake and bone resorption markers [134].

7. Conclusions

The diet of IBD patients often proves to be inadequate, which affects the peak bone mass, thus leading to osteopenia, osteoporosis, and fractures. Nutrition guidelines for IBD patients should include osteoporosis prevention.

In conclusion, it is worth emphasizing that:

1. The vitamin D concentration in patients with IBD should be examined routinely, since IBD constitutes a risk factor of vitamin D deficiency. Individual doses of vitamin D are recommended.

2. Most IBD patients require calcium supplementation (1000–1500 mg/day).
3. We recommend the assessment of the fruit and vegetable intake in IBD patients. In patients with low BMD and a simultaneous inadequate consumption of fruits and vegetables, vitamin C supplementation may be considered.
4. Vitamin B12 and folic acid supplementation may be introduced in patients with hyperhomocysteinemia, since a high level of homocysteine correlates with a low bone mineral density.
5. Vitamin A supplementation is not recommended. A high level of this vitamin may have a negative influence on bone health.
6. There are no sufficient data to recommend the supplementation of vitamin K, magnesium, zinc, copper, and selenium in IBD patients for the prevention of osteoporosis.
7. IBD patients consume less sodium than the rest of the population, in particular the malnourished patients. On the other hand, sodium intake in developed countries is higher than recommended, and a high dose of Na correlates with a low BMD. Therefore, it is not necessary to encourage patients to use more salt.
8. Polyphenols may be beneficial for patients' health; thus, they should be included in the diet in the form of herbs, fruits, or vegetables.

The importance of diet in the prevention of osteoporosis in the group of patients with IBD is indisputable. Hence, it is vital to adhere to the recommendations of a clinical dietitian, as an important member of the coordinated IBD patient care team.

Author Contributions: Conceptualization, A.E.R. and I.K.-K.; writing—original draft preparation, A.E.R.; critical revision of the manuscript, I.K.-K., A.D., A.Z., A.M.R.; supervision, I.K.-K.; acceptance of the final version: all authors. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We would like to thank Translation Lab, a biomedical translation company, for revising the language and style of the manuscript.

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

References

1. Sgambato, D.; Gimigliano, F.; De Musis, C.; Moretti, A.; Toro, G.; Ferrante, E.; Miranda, A.; De Mauro, D.; Romano, L.; Iolascon, G.; et al. Bone alterations in inflammatory bowel diseases. *World J. Clin. Cases* **2019**, *7*, 1908–1925. [CrossRef] [PubMed]
2. Miranda-Bautista, J.; Verdejo, C.; Díaz-Redondo, A.; Bretón, I.; Bellón, J.M.; Pérez-Valderas, M.D.; Caballero-Marcos, A.; de Dios-Lascuevas, M.; González-Río, E.; García-Sánchez, C.; et al. Metabolic bone disease in patients diagnosed with inflammatory bowel disease from Spain. *Ther. Adv. Gastroenterol.* **2019**, *12*. [CrossRef] [PubMed]
3. Prevention and management of osteoporosis. *World Health Organ. Tech. Rep. Ser.* **2003**, *921*, 1–164.
4. Mirza, F.; Canalis, E. Management of endocrine disease: Secondary osteoporosis: Pathophysiology and management. *Eur. J. Endocrinol.* **2015**, *173*, R131–R151. [CrossRef] [PubMed]
5. Krela-Kaźmierczak, I.; Michalak, M.; Szymczak-Tomczak, A.; Łykowska-Szuber, L.; Stawczyk-Eder, K.; Waszak, K.; Kucharski, M.A.; Dobrowolska, A.; Eder, P. Prevalence of osteoporosis and osteopenia in a population of patients with inflammatory bowel diseases from the Wielkopolska Region. *Pol. Arch. Intern. Med.* **2018**, *128*, 447–454. [CrossRef]
6. Whittier, X.; Saag, K.G. Glucocorticoid-induced Osteoporosis. *Rheum. Dis. Clin. North Am.* **2016**, *42*, 177–189. [CrossRef]
7. Hardy, R.S.; Zhou, H.; Seibel, M.J.; Cooper, M.S. Glucocorticoids and Bone: Consequences of Endogenous and Exogenous Excess and Replacement Therapy. *Endocr. Rev.* **2018**, *39*, 519–548. [CrossRef]
8. Chmielnicka, M.; Woźniacka, A.; Torzecka, J.D. The influence of corticosteroid treatment on the OPG/RANK/RANKL pathway and osteocalcin in patients with pemphigus. *Postepy Dermatol. Alergol.* **2014**, *31*, 281–288. [CrossRef]

9. Even Dar, R.; Mazor, Y.; Karban, A.; Ish-Shalom, S.; Segal, E. Risk Factors for Low Bone Density in Inflammatory Bowel Disease: Use of Glucocorticoids, Low Body Mass Index, and Smoking. *Dig. Dis. Basel Switz.* **2019**, *37*, 284–290. [CrossRef]
10. Wada, Y.; Hisamatsu, T.; Naganuma, M.; Matsuoka, K.; Okamoto, S.; Inoue, N.; Yajima, T.; Kouyama, K.; Iwao, Y.; Ogata, H.; et al. Risk factors for decreased bone mineral density in inflammatory bowel disease: A cross-sectional study. *Clin. Nutr. Edinb. Scotl.* **2015**, *34*, 1202–1209. [CrossRef]
11. Caplan, A.; Fett, N.; Rosenbach, M.; Werth, V.P.; Micheletti, R.G. Prevention and management of glucocorticoid-induced side effects: A comprehensive review: A review of glucocorticoid pharmacology and bone health. *J. Am. Acad. Dermatol.* **2017**, *76*, 1–9. [CrossRef] [PubMed]
12. Sugimoto, K.; Ikeya, K.; Iida, T.; Kawasaki, S.; Arai, O.; Umehara, K.; Watanabe, F.; Tani, S.; Oishi, S.; Osawa, S.; et al. An Increased Serum N-Terminal Telopeptide of Type I Collagen, a Biochemical Marker of Increased Bone Resorption, Is Associated with Infliximab Therapy in Patients with Crohn's Disease. *Dig. Dis. Sci.* **2016**, *61*, 99–106. [CrossRef] [PubMed]
13. Ryan, B.M.; Russel, M.G.V.M.; Schurgers, L.; Wichers, M.; Sijbrandij, J.; Stockbrugger, R.W.; Schoon, E. Effect of antitumour necrosis factor-alpha therapy on bone turnover in patients with active Crohn's disease: A prospective study. *Aliment. Pharmacol. Ther.* **2004**, *20*, 851–857. [CrossRef] [PubMed]
14. Mauro, M.; Radovic, V.; Armstrong, D. Improvement of lumbar bone mass after infliximab therapy in Crohn's disease patients. *Can. J. Gastroenterol.* **2007**, *21*, 637–642. [CrossRef]
15. Lima, C.A.; Lyra, A.C.; Mendes, C.M.C.; Lopes, M.B.; Coqueiro, F.G.; Rocha, R.; Santana, G.O. Bone mineral density and inflammatory bowel disease severity. *Braz. J. Med. Biol. Res.* **2017**, *50*. [CrossRef]
16. Pazianas, M.; Rhim, A.D.; Weinberg, A.M.; Su, C.; Lichtenstein, G.R. The effect of anti-TNF-alpha therapy on spinal bone mineral density in patients with Crohn's disease. *Ann. N. Y. Acad. Sci.* **2006**, *1068*, 543–556. [CrossRef]
17. Krajcovicova, A.; Hlavaty, T.; Killinger, Z.; Miznerova, E.; Toth, J.; Letkovsky, J.; Nevidanska, M.; Cierny, D.; Koller, T.; Zelinkova, Z.; et al. Combination therapy with an immunomodulator and anti-TNFα agent improves bone mineral density in IBD patients. *J. Crohns Colitis* **2014**, *8*, 1693–1701. [CrossRef]
18. Hoffmann, P.; Krisam, J.; Kasperk, C.; Gauss, A. Prevalence, Risk Factors and Course of Osteoporosis in Patients with Crohn's Disease at a Tertiary Referral Center. *J. Clin. Med.* **2019**, *8*. [CrossRef]
19. Ludvigsson, J.F.; Mahl, M.; Sachs, M.C.; Björk, J.; Michaelsson, K.; Ekbom, A.; Askling, J.; Backman, A.-S.; Olén, O. Fracture Risk in Patients with Inflammatory Bowel Disease: A Nationwide Population-Based Cohort Study from 1964 to 2014. *Am. J. Gastroenterol.* **2019**, *114*, 291–304. [CrossRef]
20. Krela-Kaźmierczak, I.; Kaczmarek-Ryś, M.; Szymczak, A.; Michalak, M.; Skrzypczak-Zielińska, M.; Drwęska-Matelska, N.; Marcinkowska, M.; Eder, P.; Łykowska-Szuber, L.; Wysocka, E.; et al. Bone Metabolism and the c.-223C > T Polymorphism in the 5'UTR Region of the Osteoprotegerin Gene in Patients with Inflammatory Bowel Disease. *Calcif. Tissue Int.* **2016**, *99*, 616–624. [CrossRef]
21. Krela-Kaźmierczak, I.; Wysocka, E.; Szymczak, A.; Eder, P.; Michalak, M.; Łykowska-Szuber, L.; Stawczyk-Eder, K.; Klimczak, K.; Linke, K.; Horst-Sikorska, W. Osteoprotegerin, s-RANKL, and selected interleukins in the pathology of bone metabolism in patients with Crohn's disease. *Przeglad Gastroenterol.* **2016**, *11*, 30–34. [CrossRef] [PubMed]
22. Krela-Kaźmierczak, I.; Szymczak-Tomczak, A.; Łykowska-Szuber, L.; Wysocka, E.; Michalak, M.; Stawczyk-Eder, K.; Waszak, K.; Linke, K.; Eder, P. Interleukin 6, osteoprotegerin, sRANKL and bone metabolism in inflammatory bowel diseases. *Adv. Clin. Exp. Med. Off. Organ Wroclaw Med. Univ.* **2018**, *27*, 449–453. [CrossRef] [PubMed]
23. Veerappan, S.G.; Healy, M.; Walsh, B.; O'Morain, C.A.; Daly, J.S.; Ryan, B.M. A 1-year prospective study of the effect of infliximab on bone metabolism in inflammatory bowel disease patients. *Eur. J. Gastroenterol. Hepatol.* **2016**, *28*, 1335–1344. [CrossRef] [PubMed]
24. Veerappan, S.G.; O'Morain, C.A.; Daly, J.S.; Ryan, B.M. Review article: The effects of antitumour necrosis factor-α on bone metabolism in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* **2011**, *33*, 1261–1272. [CrossRef]
25. Faienza, M.F.; Chiarito, M.; D'amato, G.; Colaianni, G.; Colucci, S.; Grano, M.; Brunetti, G. Monoclonal antibodies for treating osteoporosis. *Expert Opin. Biol. Ther.* **2018**, *18*, 149–157. [CrossRef]

26. Cosman, F.; de Beur, S.J.; LeBoff, M.S.; Lewiecki, E.M.; Tanner, B.; Randall, S.; Lindsay, R. National Osteoporosis Foundation Clinician's Guide to Prevention and Treatment of Osteoporosis. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **2014**, *25*, 2359–2381. [CrossRef]
27. Forbes, A.; Escher, J.; Hébuterne, X.; Kłęk, S.; Krznaric, Z.; Schneider, S.; Shamir, R.; Stardelova, K.; Wierdsma, N.; Wiskin, A.E.; et al. ESPEN guideline: Clinical nutrition in inflammatory bowel disease. *Clin. Nutr.* **2017**, *36*, 321–347. [CrossRef]
28. Trumbo, P.; Schlicker, S.; Yates, A.; Poos, M. Food and Nutrition Board of the Institute of Medicine; The National Academies Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *J. Am. Diet. Assoc.* **2002**, *102*, 1621–1630. [CrossRef]
29. Jarosz, M. (Ed.) *Normy żywienia dla populacji Polski*; Instytut Żywności i Żywienia im. prof.dra med.Aleksandra Szczygła: Warsaw, Poland, 2017; ISBN 978-83-86060-89-4.
30. Shams-White, M.M.; Chung, M.; Du, M.; Fu, Z.; Insogna, K.L.; Karlsen, M.C.; LeBoff, M.S.; Shapses, S.A.; Sackey, J.; Wallace, T.C.; et al. Dietary protein and bone health: A systematic review and meta-analysis from the National Osteoporosis Foundation. *Am. J. Clin. Nutr.* **2017**, *105*, 1528–1543. [CrossRef]
31. Kim, J.; Kim, B.; Lee, H.; Choi, H.; Won, C. The Relationship between Prevalence of Osteoporosis and Proportion of Daily Protein Intake. *Korean J. Fam. Med.* **2013**, *34*, 43–48. [CrossRef]
32. Nguyen, G.C.; Du, L.; Chong, R.Y.; Jackson, T.D. Hypoalbuminaemia and Postoperative Outcomes in Inflammatory Bowel Disease: The NSQIP Surgical Cohort. *J. Crohns Colitis* **2019**, *13*, 1433–1438. [CrossRef] [PubMed]
33. Nguyen, G.C.; Munsell, M.; Harris, M.L. Nationwide prevalence and prognostic significance of clinically diagnosable protein-calorie malnutrition in hospitalized inflammatory bowel disease patients. *Inflamm. Bowel Dis.* **2008**, *14*, 1105–1111. [CrossRef] [PubMed]
34. Głąbska, D.; Guzek, D.; Lech, G. Analysis of the Nutrients and Food Products Intake of Polish Males with Ulcerative Colitis in Remission. *Nutrients* **2019**, *11*. [CrossRef] [PubMed]
35. Cauley, J.A.; Cawthon, P.M.; Peters, K.E.; Cummings, S.R.; Ensrud, K.E.; Bauer, D.C.; Taylor, B.C.; Shikany, J.M.; Hoffman, A.R.; Lane, N.E.; et al. Risk Factors for Hip Fracture in Older Men: The Osteoporotic Fractures in Men Study (MrOS). *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.* **2016**, *31*, 1810–1819. [CrossRef] [PubMed]
36. Isanejad, M.; Sirola, J.; Mursu, J.; Kröger, H.; Tuppurainen, M.; Erkkilä, A.T. Association of Protein Intake with Bone Mineral Density and Bone Mineral Content among Elderly Women: The OSTPRE Fracture Prevention Study. *J. Nutr. Health Aging* **2017**, *21*, 622–630. [CrossRef] [PubMed]
37. Dong, H.; Hutchins-Wiese, H.; Kleppinger, A.; Annis, K.; Liva, E.; Lammi-Keefe, C.; Durham, H.; Feinn, R.; Kenny, A.M. Effects of Omega-3 Polyunsaturated Fatty Acid Supplementation on Bone Turnover in Older Women. *Int. J. Vitam. Nutr. Res. Int. Z. Vitam.-Ernahrungsforschung J. Int. Vitaminol. Nutr.* **2014**, *84*, 124–132. [CrossRef]
38. Shen, D.; Zhang, X.; Li, Z.; Bai, H.; Chen, L. Effects of omega-3 fatty acids on bone turnover markers in postmenopausal women: Systematic review and meta-analysis. *Climacteric* **2017**, *20*, 522–527. [CrossRef]
39. Tartibian, B.; Hajizadeh Maleki, B.; Kanaley, J.; Sadeghi, K. Long-term aerobic exercise and omega-3 supplementation modulate osteoporosis through inflammatory mechanisms in post-menopausal women: A randomized, repeated measures study. *Nutr. Metab.* **2011**, *8*, 71. [CrossRef]
40. Sugihara, K.; Morhardt, T.L.; Kamada, N. The Role of Dietary Nutrients in Inflammatory Bowel Disease. *Front. Immunol.* **2018**, *9*, 3183. [CrossRef]
41. Zhan, Y.; Zhan, Y.-A.; Dai, S.-X. Is a low FODMAP diet beneficial for patients with inflammatory bowel disease? A meta-analysis and systematic review. *Clin. Nutr. Edinb. Scotl.* **2018**, *37*, 123–129. [CrossRef]
42. Prescha, A.; Pieczyńska, J.; Biernat, K.; Neubauer, K.; Smereka, A.; Ilow, R.; Grajeta, H.; Biernat, J.; Paradowski, L. Nutritional status assessment of patients with inflammatory bowel disease. *Gastroenterol. Pol.* **2010**, *17*, 57–63.
43. Mazidi, M.; Kengne, A.P.; Vatanparast, H. Association of dietary patterns of American adults with bone mineral density and fracture. *Public Health Nutr.* **2018**, *21*, 2417–2423. [CrossRef] [PubMed]
44. Douard, V.; Sabbagh, Y.; Lee, J.; Patel, C.; Kemp, F.W.; Bogden, J.D.; Lin, S.; Ferraris, R.P. Excessive fructose intake causes 1,25-(OH)(2)D(3)-dependent inhibition of intestinal and renal calcium transport in growing rats. *Am. J. Physiol. Endocrinol. Metab.* **2013**, *304*, E1303–E1313. [CrossRef] [PubMed]

45. Goran, M.I.; Martin, A.A.; Alderete, T.L.; Fujiwara, H.; Fields, D.A. Fructose in Breast Milk Is Positively Associated with Infant Body Composition at 6 Months of Age. *Nutrients* **2017**, *9*. [CrossRef] [PubMed]
46. Wyshak, G. Teenaged girls, carbonated beverage consumption, and bone fractures. *Arch. Pediatr. Adolesc. Med.* **2000**, *154*, 610–613. [CrossRef] [PubMed]
47. Vartanian, L.R.; Schwartz, M.B.; Brownell, K.D. Effects of Soft Drink Consumption on Nutrition and Health: A Systematic Review and Meta-Analysis. *Am. J. Public Health* **2007**, *97*, 667–675. [CrossRef] [PubMed]
48. Jakeman, S.A.; Henry, C.N.; Martin, B.R.; McCabe, G.P.; McCabe, L.D.; Jackson, G.S.; Peacock, M.; Weaver, C.M. Soluble corn fiber increases bone calcium retention in postmenopausal women in a dose-dependent manner: A randomized crossover trial. *Am. J. Clin. Nutr.* **2016**, *104*, 837–843. [CrossRef]
49. Krela-Kaźmierczak, I.; Szymczak, A.; Łykowska-Szuber, L.; Eder, P.; Stawczyk-Eder, K.; Klimczak, K.; Linke, K.; Horst-Sikorska, W. The importance of vitamin D in the pathology of bone metabolism in inflammatory bowel diseases. *Arch. Med. Sci. AMS* **2015**, *11*, 1028–1032. [CrossRef]
50. Sunyecz, J.A. The use of calcium and vitamin D in the management of osteoporosis. *Ther. Clin. Risk Manag.* **2008**, *4*, 827–836. [CrossRef]
51. Rusińska, A.; Płudowski, P.; Walczak, M.; Borszewska-Kornacka, M.; Bossowski, A.; Chlebna-Sokół, D.; Czech-Kowalska, J.; Dobrzańska, A.; Franek, E. Zasady suplementacji i leczenia witaminą D- nowelizacja 2018 r. *Postępy Neonatol.* **2018**, *24*, 1–24.
52. Krela-Kaźmierczak, I.; Szymczak, A.; Tomczak, M.; Łykowska-Szuber, L.; Linke, K.; Eder, P. Calcium and phosphate metabolism in patients with inflammatory bowel diseases. *Pol. Arch. Med. Wewn.* **2015**, *125*, 588–590. [CrossRef] [PubMed]
53. Santucci, N.R.; Alkhouri, R.H.; Baker, R.D.; Baker, S.S. Vitamin and zinc status pretreatment and posttreatment in patients with inflammatory bowel disease. *J. Pediatr. Gastroenterol. Nutr.* **2014**, *59*, 455–457. [CrossRef] [PubMed]
54. Aksan, A.; Tugal, D.; Hein, N.; Boettger, K.; Caicedo-Zea, Y.; Diehl, I.; Schumann, C.; Armbruster, F.-P.; Stein, J. Measuring Vitamin D Status in Chronic Inflammatory Disorders: How does Chronic Inflammation Affect the Reliability of Vitamin D Metabolites in Patients with IBD? *J. Clin. Med.* **2020**, *9*. [CrossRef] [PubMed]
55. Kojecký, V.; Matouš, J.; Zádorová, Z.; Gřiva, M.; Kianička, B.; Uher, M. Vitamin D supplementation dose needs to be higher in patients with inflammatory bowel disease: Interventional study. *Vnitrni Lekarstvi* **2019**, *65*, 470–474. [PubMed]
56. Warensjö, E.; Byberg, L.; Melhus, H.; Gedeborg, R.; Mallmin, H.; Wolk, A.; Michaëlsson, K. Dietary calcium intake and risk of fracture and osteoporosis: Prospective longitudinal cohort study. *BMJ* **2011**, *342*, d1473. [CrossRef] [PubMed]
57. Aspray, T.J.; Chadwick, T.; Francis, R.M.; McColl, E.; Stamp, E.; Prentice, A.; von Wilamowitz-Moellendorff, A.; Schoenmakers, I. Randomized controlled trial of vitamin D supplementation in older people to optimize bone health. *Am. J. Clin. Nutr.* **2019**, *109*, 207–217. [CrossRef]
58. Tang, B.M.P.; Eslick, G.D.; Nowson, C.; Smith, C.; Bensoussan, A. Use of calcium or calcium in combination with vitamin D supplementation to prevent fractures and bone loss in people aged 50 years and older: A meta-analysis. *Lancet Lond. Engl.* **2007**, *370*, 657–666. [CrossRef]
59. Burt, L.A.; Billington, E.O.; Rose, M.S.; Raymond, D.A.; Hanley, D.A.; Boyd, S.K. Effect of High-Dose Vitamin D Supplementation on Volumetric Bone Density and Bone Strength. *JAMA* **2019**, *322*, 736–745. [CrossRef]
60. Sahni, S.; Hannan, M.T.; Gagnon, D.; Blumberg, J.; Cupples, L.A.; Kiel, D.P.; Tucker, K.L. Protective effect of total and supplemental vitamin C intake on the risk of hip fracture—A 17-year follow-up from the Framingham Osteoporosis Study. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **2009**, *20*, 1853–1861. [CrossRef]
61. Finck, H.; Hart, A.R.; Jennings, A.; Welch, A.A. Is there a role for vitamin C in preventing osteoporosis and fractures? A review of the potential underlying mechanisms and current epidemiological evidence. *Nutr. Res. Rev.* **2014**, *27*, 268–283. [CrossRef]
62. Chin, K.-Y.; Ima-Nirwana, S. Vitamin C and Bone Health: Evidence from Cell, Animal and Human Studies. *Curr. Drug Targets* **2018**, *19*, 439–450. [CrossRef] [PubMed]
63. Jarmakiewicz, S.; Piątek, D.; Filip, R. Macro and micronutrient deficiency in inflammatory bowel diseases. *Eur. J. Clin. Exp. Med.* **2018**, *15*, 342–348. [CrossRef]

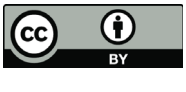
64. Hengstermann, S.; Valentini, L.; Schaper, L.; Buning, C.; Koernicke, T.; Maritschnegg, M.; Buhner, S.; Tillinger, W.; Regano, N.; Guglielmi, F.; et al. Altered status of antioxidant vitamins and fatty acids in patients with inactive inflammatory bowel disease. *Clin. Nutr. Edinb. Scotl.* **2008**, *27*, 571–578. [CrossRef] [PubMed]
65. Vahid, F.; Rashvand, S.; Sadeghi, M.; Hekmatdoost, A. The association between index of nutritional quality and ulcerative colitis: A case-control study. *J. Res. Med. Sci. Off. J. Isfahan Univ. Med. Sci.* **2018**, *23*, 67. [CrossRef]
66. Hartman, C.; Marderfeld, L.; Davidson, K.; Mozer-Glassberg, Y.; Poraz, I.; Silbermintz, A.; Zevit, N.; Shamir, R. Food Intake Adequacy in Children and Adolescents with Inflammatory Bowel Disease. *J. Pediatr. Gastroenterol. Nutr.* **2016**, *63*, 437–444. [CrossRef]
67. Akobeng, A.K.; Richmond, K.; Miller, V.; Thomas, A.G. Effect of exclusive enteral nutritional treatment on plasma antioxidant concentrations in childhood Crohn's disease. *Clin. Nutr. Edinb. Scotl.* **2007**, *26*, 51–56. [CrossRef]
68. Kim, Y.A.; Kim, K.M.; Lim, S.; Choi, S.H.; Moon, J.H.; Kim, J.H.; Kim, S.W.; Jang, H.C.; Shin, C.S. Favorable effect of dietary vitamin C on bone mineral density in postmenopausal women (KNHANES IV, 2009): Discrepancies regarding skeletal sites, age, and vitamin D status. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **2015**, *26*, 2329–2337. [CrossRef]
69. Leveille, S.G.; LaCroix, A.Z.; Koepsell, T.D.; Beresford, S.A.; Van Belle, G.; Buchner, D.M. Dietary vitamin C and bone mineral density in postmenopausal women in Washington State, USA. *J. Epidemiol. Community Health* **1997**, *51*, 479–485. [CrossRef]
70. Chuin, A.; Labonté, M.; Tessier, D.; Khalil, A.; Bobeuf, F.; Doyon, C.Y.; Rieth, N.; Dionne, I.J. Effect of antioxidants combined to resistance training on BMD in elderly women: A pilot study. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **2009**, *20*, 1253–1258. [CrossRef]
71. Kim, M.H.; Lee, H.-J. Osteoporosis, vitamin C intake, and physical activity in Korean adults aged 50 years and over. *J. Phys. Ther. Sci.* **2016**, *28*, 725–730. [CrossRef]
72. Pan, Y.; Liu, Y.; Guo, H.; Jabir, M.S.; Liu, X.; Cui, W.; Li, D. Associations between Folate and Vitamin B12 Levels and Inflammatory Bowel Disease: A Meta-Analysis. *Nutrients* **2017**, *9*. [CrossRef] [PubMed]
73. Enneman, A.; Swart, K.; van Wijngaarden, J.; van Dijk, S.; Ham, A.; Brouwer-Brolsma, E.; Van der Zwaluw, N.; Dhonukshe-Rutten, R.; van der Cammen, T.; Groot, L.; et al. Effect of Vitamin B12 and Folic Acid Supplementation on Bone Mineral Density and Quantitative Ultrasound Parameters in Older People with an Elevated Plasma Homocysteine Level: B-PROOF, a Randomized Controlled Trial. *Calcif. Tissue Int.* **2015**, *96*. [CrossRef] [PubMed]
74. Huang, S.; Ma, J.; Zhu, M.; Ran, Z. Status of serum vitamin B12 and folate in patients with inflammatory bowel disease in China. *Intest. Res.* **2017**, *15*, 103–108. [CrossRef] [PubMed]
75. Kim, J.I.; Moon, J.H.; Chung, H.W.; Kong, M.H.; Kim, H.J. Association between Homocysteine and Bone Mineral Density according to Age and Sex in Healthy Adults. *J. Bone Metab.* **2016**, *23*, 129–134. [CrossRef]
76. Bailey, R.L.; Looker, A.C.; Lu, Z.; Fan, R.; Eicher-Miller, H.A.; Fakhouri, T.H.; Gahche, J.J.; Weaver, C.M.; Mills, J.L. B-vitamin status and bone mineral density and risk of lumbar osteoporosis in older females in the United States. *Am. J. Clin. Nutr.* **2015**, *102*, 687–694. [CrossRef]
77. Gjesdal, C.G.; Vollset, S.E.; Ueland, P.M.; Refsum, H.; Meyer, H.E.; Tell, G.S. Plasma homocysteine, folate, and vitamin B 12 and the risk of hip fracture: The hordaland homocysteine study. *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.* **2007**, *22*, 747–756. [CrossRef]
78. Salari, P.; Abdollahi, M.; Heshmat, R.; Meybodi, H.A.; Razi, F. Effect of folic acid on bone metabolism: A randomized double blind clinical trial in postmenopausal osteoporotic women. *Daru J. Fac. Pharm. Tehran Univ. Med. Sci.* **2014**, *22*, 62. [CrossRef]
79. Keser, I.; Ilich, J.Z.; Vrkić, N.; Giljević, Z.; Colić Barić, I. Folic acid and vitamin B(12) supplementation lowers plasma homocysteine but has no effect on serum bone turnover markers in elderly women: A randomized, double-blind, placebo-controlled trial. *Nutr. Res. N. Y. N.* **2013**, *33*, 211–219. [CrossRef]
80. Roblin, X.; Phelip, J.M.; Genevois, M.; Ducros, V.; Bonaz, B. Hyperhomocysteinaemia is associated with osteoporosis in patients with Crohn's disease. *Aliment. Pharmacol. Ther.* **2007**, *25*, 797–804. [CrossRef]
81. Alkhouri, R.H.; Hashmi, H.; Baker, R.D.; Gelfond, D.; Baker, S.S. Vitamin and Mineral Status in Patients with Inflammatory Bowel Disease. *J. Pediatr. Gastroenterol. Nutr.* **2013**, *56*, 89. [CrossRef]
82. Ambrosini, G.L.; Bremner, A.P.; Reid, A.; Mackerras, D.; Alfonso, H.; Olsen, N.J.; Musk, A.W.; de Klerk, N.H. No dose-dependent increase in fracture risk after long-term exposure to high doses of retinol or beta-carotene.

Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA **2013**, *24*, 1285–1293. [CrossRef] [PubMed]

83. Navarro-Valverde, C.; Caballero-Villarraso, J.; Mata-Granados, J.M.; Casado-Díaz, A.; Sosa-Henríquez, M.; Malouf-Sierra, J.; Nogués-Solán, X.; Rodríguez-Mañas, L.; Cortés-Gil, X.; Delgadillo-Duarte, J.; et al. High Serum Retinol as a Relevant Contributor to Low Bone Mineral Density in Postmenopausal Osteoporotic Women. *Calcif. Tissue Int.* **2018**, *102*, 651–656. [CrossRef] [PubMed]
84. Wu, A.-M.; Huang, C.-Q.; Lin, Z.-K.; Tian, N.-F.; Ni, W.-F.; Wang, X.-Y.; Xu, H.-Z.; Chi, Y.-L. The Relationship Between Vitamin A and Risk of Fracture: Meta-Analysis of Prospective Studies. *J. Bone Miner. Res.* **2014**, *29*, 2032–2039. [CrossRef]
85. Sato, T.; Schurgers, L.J.; Uenishi, K. Comparison of menaquinone-4 and menaquinone-7 bioavailability in healthy women. *Nutr. J.* **2012**, *11*, 93. [CrossRef] [PubMed]
86. Tanaka, S.; Miyazaki, T.; Uemura, Y.; Miyakawa, N.; Gorai, I.; Nakamura, T.; Fukunaga, M.; Ohashi, Y.; Ohta, H.; Mori, S.; et al. Comparison of concurrent treatment with vitamin K_2 and risedronate compared with treatment with risedronate alone in patients with osteoporosis: Japanese Osteoporosis Intervention Trial-03. *J. Bone Miner. Metab.* **2017**, *35*, 385–395. [CrossRef] [PubMed]
87. Inaba, N.; Sato, T.; Yamashita, T. Low-Dose Daily Intake of Vitamin K(2) (Menaquinone-7) Improves Osteocalcin γ-Carboxylation: A Double-Blind, Randomized Controlled Trials. *J. Nutr. Sci. Vitaminol.* **2015**, *61*, 471–480. [CrossRef]
88. Emaus, N.; Gjesdal, C.G.; Almås, B.; Christensen, M.; Grimsgaard, A.S.; Berntsen, G.K.R.; Salomonsen, L.; Fønnebø, V. Vitamin K2 supplementation does not influence bone loss in early menopausal women: A randomised double-blind placebo-controlled trial. *Osteoporos. Int.* **2010**, *21*, 1731–1740. [CrossRef]
89. Park, H.-M.; Heo, J.; Park, Y. Calcium from plant sources is beneficial to lowering the risk of osteoporosis in postmenopausal Korean women. *Nutr. Res. N. Y. N.* **2011**, *31*, 27–32. [CrossRef]
90. Mazierka, A.; Pasternak, K. Calcium and phosphorus in medicine and treatment. *J. Elem.* **2013**, *18*, 529–539. [CrossRef]
91. Hwang, C.; Ross, V.; Mahadevan, U. Micronutrient deficiencies in inflammatory bowel disease: From A to zinc. *Inflamm. Bowel Dis.* **2012**, *18*, 1961–1981. [CrossRef]
92. Pierote, N.R.; Braz, A.F.; Barros, S.L.; Moita Neto, J.M.; Parente, J.M.L.; Silva, M.d.C.M.; Beserra, M.S.; Soares, N.R.M.; Marreiro, D.N.; do Noscimento Nogueira, N. Effect of mineral status and glucocorticoid use on bone mineral density in patients with Crohn's disease. *Nutrition* **2018**, *48*, 13–17. [CrossRef] [PubMed]
93. Bernstein, C.N.; Bector, S.; Leslie, W.D. Lack of relationship of calcium and vitamin D intake to bone mineral density in premenopausal women with inflammatory bowel disease. *Am. J. Gastroenterol.* **2003**, *98*, 2468–2473. [CrossRef] [PubMed]
94. Bischoff-Ferrari, H.A.; Dawson-Hughes, B.; Baron, J.A.; Burckhardt, P.; Li, R.; Spiegelman, D.; Specker, B.; Orav, J.E.; Wong, J.B.; Staehelin, H.B.; et al. Calcium intake and hip fracture risk in men and women: A meta-analysis of prospective cohort studies and randomized controlled trials. *Am. J. Clin. Nutr.* **2007**, *86*, 1780–1790. [CrossRef] [PubMed]
95. Gutiérrez, O.M.; Luzuriaga-McPherson, A.; Lin, Y.; Gilbert, L.C.; Ha, S.-W.; Beck, G.R. Impact of Phosphorus-Based Food Additives on Bone and Mineral Metabolism. *J. Clin. Endocrinol. Metab.* **2015**, *100*, 4264–4271. [CrossRef] [PubMed]
96. Fardellone, P.; Cotté, F.-E.; Roux, C.; Lespessailles, E.; Mercier, F.; Gaudin, A.-F. Calcium intake and the risk of osteoporosis and fractures in French women. *Jt. Bone Spine Rev. Rhum.* **2010**, *77*, 154–158. [CrossRef] [PubMed]
97. Szymczyk, H. Magnesium—Essential trace element for the proper functioning of the body. *Farmacja Współczesna* **2016**, *9*, 217–223.
98. Welch, A.A.; Skinner, J.; Hickson, M. Dietary Magnesium May Be Protective for Aging of Bone and Skeletal Muscle in Middle and Younger Older Age Men and Women: Cross-Sectional Findings from the UK Biobank Cohort. *Nutrients* **2017**, *9*. [CrossRef]
99. Yamamoto, M.; Yamaguchi, T.; Yamauchi, M.; Yano, S.; Sugimoto, T. Acute-onset hypomagnesemia-induced hypocalcemia caused by the refractoriness of bones and renal tubules to parathyroid hormone. *J. Bone Miner. Metab.* **2011**, *29*, 752–755. [CrossRef]

100. Taylor, L.; Almutairdi, A.; Shommu, N.; Fedorak, R.; Ghosh, S.; Reimer, R.A.; Panaccione, R.; Raman, M. Cross-Sectional Analysis of Overall Dietary Intake and Mediterranean Dietary Pattern in Patients with Crohn's Disease. *Nutrients* **2018**, *10*. [CrossRef]
101. Orchard, T.S.; Larson, J.C.; Alghothani, N.; Bout-Tabaku, S.; Cauley, J.A.; Chen, Z.; LaCroix, A.Z.; Wactawski-Wende, J.; Jackson, R.D. Magnesium intake, bone mineral density, and fractures: Results from the Women's Health Initiative Observational Study. *Am. J. Clin. Nutr.* **2014**, *99*, 926–933. [CrossRef]
102. Green, J.H.; Booth, C.; Bunning, R. Impact of supplementary high calcium milk with additional magnesium on parathyroid hormone and biochemical markers of bone turnover in postmenopausal women. *Asia Pac. J. Clin. Nutr.* **2002**, *11*, 268–273. [CrossRef] [PubMed]
103. Farsinejad-Marj, M.; Saneei, P.; Esmaillzadeh, A. Dietary magnesium intake, bone mineral density and risk of fracture: A systematic review and meta-analysis. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **2016**, *27*, 1389–1399. [CrossRef] [PubMed]
104. Barkas, F.; Liberopoulos, E.; Kei, A.; Elisaf, M. Electrolyte and acid-base disorders in inflammatory bowel disease. *Ann. Gastroenterol.* **2013**, *26*, 23–28. [PubMed]
105. Salt Reduction. Available online: https://www.who.int/news-room/fact-sheets/detail/salt-reduction (accessed on 22 January 2020).
106. Fijorek, K.; Püsküllüoğlu, M.; Tomaszewska, D.; Tomaszewski, R.; Glinka, A.; Polak, S. Serum potassium, sodium and calcium levels in healthy individuals-literature review and data analysis. *Folia Med. Cracov.* **2014**, *1*, 53–70.
107. Lim, H.; Kim, H.J.; Hong, S.J.; Kim, S. Nutrient Intake and Bone Mineral Density by Nutritional Status in Patients with Inflammatory Bowel Disease. *J. Bone Metab.* **2014**, *21*, 195–203. [CrossRef]
108. Kim, Y.; Kim, H.-Y.; Kim, J.H. Associations Between Reported Dietary Sodium Intake and Osteoporosis in Korean Postmenopausal Women: The 2008–2011 Korea National Health and Nutrition Examination Survey. *Asia. Pac. J. Public Health* **2017**, *29*, 430–439. [CrossRef]
109. Teucher, B.; Dainty, J.R.; Spinks, C.A.; Majsak-Newman, G.; Berry, D.J.; Hoogewerff, J.A.; Foxall, R.J.; Jakobsen, J.; Cashman, K.D.; Flynn, A.; et al. Sodium and bone health: Impact of moderately high and low salt intakes on calcium metabolism in postmenopausal women. *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.* **2008**, *23*, 1477–1485. [CrossRef]
110. Fatahi, S.; Namazi, N.; Larijani, B.; Azadbakht, L. The Association of Dietary and Urinary Sodium with Bone Mineral Density and Risk of Osteoporosis: A Systematic Review and Meta-Analysis. *J. Am. Coll. Nutr.* **2018**, *37*, 522–532. [CrossRef]
111. Carbone, L.D.; Barrow, K.D.; Bush, A.J.; Boatright, M.D.; Michelson, J.A.; Pitts, K.A.; Pintea, V.N.; Kang, A.H.; Watsky, M.A. Effects of a low sodium diet on bone metabolism. *J. Bone Miner. Metab.* **2005**, *23*, 506–513. [CrossRef]
112. Gür, A.; Colpan, L.; Nas, K.; Cevik, R.; Saraç, J.; Erdoğan, F.; Düz, M.Z. The role of trace minerals in the pathogenesis of postmenopausal osteoporosis and a new effect of calcitonin. *J. Bone Miner. Metab.* **2002**, *20*, 39–43. [CrossRef]
113. Ghishan, F.K.; Kiela, P.R. Vitamins and Minerals in Inflammatory Bowel Disease. *Gastroenterol. Clin. N. Am.* **2017**, *46*, 797–808. [CrossRef] [PubMed]
114. Hill, T.; Meunier, N.; Andriollo-Sanchez, M.; Ciarapica, D.; Hininger-Favier, I.; Polito, A.; O'Connor, J.M.; Coudray, C.; Cashman, K.D. The relationship between the zinc nutritive status and biochemical markers of bone turnover in older European adults: The ZENITH study. *Eur. J. Clin. Nutr.* **2005**, *59*, 73–78. [CrossRef] [PubMed]
115. Baker, A.; Harvey, L.; Majask-Newman, G.; Fairweather-Tait, S.; Flynn, A.; Cashman, K. Effect of dietary copper intakes on biochemical markers of bone metabolism in healthy adult males. *Eur. J. Clin. Nutr.* **1999**, *53*, 408–412. [CrossRef] [PubMed]
116. Kanikowska, A.; Włochal, M.; Mielcarz, M.; Grzymisławski, M.; Kucharski, A.M. Evaluation of zinc and copper concentrations and the total antioxidant capacity of blood plasma in patients with malabsorption syndrome. *J. Elem.* **2015**, *20*, 873–885. [CrossRef]
117. Nangliya, V.; Maksane, S.N.; Sunder, S.; Nijhawan, S.; Sandhya, M. Serum zinc, copper and selenium inflammatory bowel disease patients relation with metabolic bone disease. *Int. J. Recent Trends Sci. Technol.* **2015**, *14*, 1584–1589.

118. Cashman, K.D.; Baker, A.; Ginty, F.; Flynn, A.; Strain, J.J.; Bonham, M.P.; O'Connor, J.M.; Bügel, S.; Sandström, B. No effect of copper supplementation on biochemical markers of bone metabolism in healthy young adult females despite apparently improved copper status. *Eur. J. Clin. Nutr.* **2001**, *55*, 525–531. [CrossRef]
119. Zeng, H.; Cao, J.J.; Combs, G.F. Selenium in bone health: Roles in antioxidant protection and cell proliferation. *Nutrients* **2013**, *5*, 97–110. [CrossRef]
120. Juhászné Tóth, R.; Csapó, J. The role of selenium in nutrition—A review. *Acta Univ. Sapientiae Aliment.* **2018**, *11*, 128–144. [CrossRef]
121. Ringstad, J.; Kildebo, S.; Thomassen, Y. Serum selenium, copper, and zinc concentrations in Crohn's disease and ulcerative colitis. *Scand. J. Gastroenterol.* **1993**, *28*, 605–608. [CrossRef]
122. Wang, Y.; Xie, D.; Li, J.; Long, H.; Wu, J.; Wu, Z.; He, H.; Wang, H.; Yang, T.; Wang, Y. Association between dietary selenium intake and the prevalence of osteoporosis: A cross-sectional study. *BMC Musculoskelet. Disord.* **2019**, *20*, 585. [CrossRef]
123. Beukhof, C.M.; Medici, M.; van den Beld, A.W.; Hollenbach, B.; Hoeg, A.; Visser, W.E.; de Herder, W.W.; Visser, T.J.; Schomburg, L.; Peeters, R.P. Selenium Status Is Positively Associated with Bone Mineral Density in Healthy Aging European Men. *PLoS ONE* **2016**, *11*, e0152748. [CrossRef] [PubMed]
124. Lu, Y.; Zamora-Ros, R.; Chan, S.; Cross, A.J.; Ward, H.; Jakszyn, P.; Luben, R.; Opstelten, J.L.; Oldenburg, B.; Hallmans, G.; et al. Dietary Polyphenols in the Aetiology of Crohn's Disease and Ulcerative Colitis—A Multicenter European Prospective Cohort Study (EPIC). *Inflamm. Bowel Dis.* **2017**, *23*, 2072–2082. [CrossRef] [PubMed]
125. Vezza, T.; Rodríguez-Nogales, A.; Algieri, F.; Utrilla, M.P.; Rodriguez-Cabezas, M.E.; Galvez, J. Flavonoids in Inflammatory Bowel Disease: A Review. *Nutrients* **2016**, *8*. [CrossRef] [PubMed]
126. Hussain, T.; Tan, B.; Yin, Y.; Blachier, F.; Tossou, M.C.B.; Rahu, N. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? *Oxid. Med. Cell. Longev.* **2016**, *2016*. [CrossRef] [PubMed]
127. Salaritabar, A.; Darvishi, B.; Hadjiakhoondi, F.; Manayi, A.; Sureda, A.; Nabavi, S.F.; Fitzpatrick, L.R.; Nabavi, S.M.; Bishayee, A. Therapeutic potential of flavonoids in inflammatory bowel disease: A comprehensive review. *World J. Gastroenterol.* **2017**, *23*, 5097–5114. [CrossRef] [PubMed]
128. Rahman, S.U.; Li, Y.; Huang, Y.; Zhu, L.; Feng, S.; Wu, J.; Wang, X. Treatment of inflammatory bowel disease via green tea polyphenols: Possible application and protective approaches. *Inflammopharmacology* **2018**, *26*, 319–330. [CrossRef] [PubMed]
129. Oz, H.S.; Chen, T.; de Villiers, W.J. Green Tea Polyphenols and Sulfasalazine have Parallel Anti-Inflammatory Properties in Colitis Models. *Front. Immunol.* **2013**, *4*. [CrossRef]
130. Rezaei, N.; Eftekhari, M.H.; Tanideh, N.; Mokhtari, M.; Bagheri, Z. The Protective Effects of Honey and Spirulina platensis on Acetic Acid-Induced Ulcerative Colitis in Rats. Available online: http://sites.kowsarpub.com/articles/62517.html (accessed on 26 May 2020).
131. Samsami-Kor, M.; Daryani, N.E.; Asl, P.R.; Hekmatdoost, A. Anti-Inflammatory Effects of Resveratrol in Patients with Ulcerative Colitis: A Randomized, Double-Blind, Placebo-controlled Pilot Study. *Arch. Med. Res.* **2015**, *46*, 280–285. [CrossRef]
132. Chisari, E.; Shivappa, N.; Vyas, S. Polyphenol-Rich Foods and Osteoporosis. *Curr. Pharm. Des.* **2019**, *25*, 2459–2466. [CrossRef]
133. Welch, A.; MacGregor, A.; Jennings, A.; Fairweather-Tait, S.; Spector, T.; Cassidy, A. Habitual flavonoid intakes are positively associated with bone mineral density in women. *J. Bone Miner. Res.* **2012**, *27*, 1872–1878. [CrossRef]
134. Hardcastle, A.C.; Aucott, L.; Reid, D.M.; Macdonald, H.M. Associations between dietary flavonoid intakes and bone health in a Scottish population. *J. Bone Miner. Res.* **2011**, *26*, 941–947. [CrossRef] [PubMed]



nutrients

MDPI

Article

Satellite Cells and Markers of Muscle Regeneration during Unloading and Reloading: Effects of Treatment with Resveratrol and Curcumin

Laura Mañas-García [1], Maria Guitart [1,2], Xavier Duran [3] and Esther Barreiro [1,2,*]

[1] Pulmonology Department-Muscle Wasting and Cachexia in Chronic Respiratory Diseases and Lung Cancer Research Group, IMIM-Hospital del Mar, Parc de Salut Mar, Health and Experimental Sciences Department (CEXS), Universitat Pompeu Fabra (UPF), Barcelona Biomedical Research Park (PRBB), 08003 Barcelona, Spain; laura.manas@upf.edu (L.M.-G.); mguitart@imim.es (M.G.)

[2] Centro de Investigación en Red de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III (ISCIII), 08003 Barcelona, Spain

[3] Scientific and Technical Department, Hospital del Mar-IMIM, 08003 Barcelona, Spain; xduran@imim.es

* Correspondence: ebarreiro@imim.es; Tel.: +34-93-316-0385; Fax: +34-93-316-0410

Received: 29 April 2020; Accepted: 18 June 2020; Published: 23 June 2020

Abstract: We hypothesized that treatment with pharmacological agents known to increase sirtuin-1 activity (resveratrol and curcumin) may enhance muscle regeneration. In limb muscles of mice (C57BL/6J, 10 weeks) exposed to reloading for seven days following a seven-day period of hindlimb immobilization with/without curcumin or resveratrol treatment, progenitor muscle cell numbers (FACS), satellite cell subtypes (histology), early and late muscle regeneration markers, phenotype and morphometry, sirtuin-1 activity and content, and muscle function were assessed. Treatment with either resveratrol or curcumin in immobilized muscles elicited a significant improvement in numbers of progenitor, activated, quiescent, and total counts of muscle satellite cells, compared to non-treated animals. Treatment with either resveratrol or curcumin in reloaded muscles compared to non-treated mice induced a significant improvement in the CSA of both hybrid (curcumin) and fast-twitch fibers (resveratrol), sirtuin-1 activity (curcumin), sirtuin-1 content (resveratrol), and counts of progenitor muscle cells (resveratrol). Treatment with the pharmacological agents resveratrol and curcumin enhanced the numbers of satellite cells (muscle progenitor, quiescent, activated, and total satellite cells) in the unloaded limb muscles but not in the reloaded muscles. These findings have potential clinical implications as treatment with these phenolic compounds would predominantly be indicated during disuse muscle atrophy to enhance the muscle regeneration process.

Keywords: muscle unloading; muscle reloading; sirtuin-1; muscle progenitor cells; activated satellite cells; quiescent satellite cells; muscle regeneration markers

1. Introduction

Disuse muscle atrophy is an important condition that is the result of progression of other chronic and acute diseases, such as cardiac and respiratory disorders; cancer; prolonged bed rest; and critical illness. Reduced physical activity leading to deconditioning is characterized by the loss of muscle mass and function in the affected muscles in patients [1–5] and in animal models [6–8]. Muscle atrophy resulting from deconditioning may also worsen disease prognosis in patients with chronic and acute diseases regardless of the underlying condition [9,10].

Several pathophysiological and biological mechanisms are involved in the loss of muscle mass and function characteristic of muscle atrophy. In this regard, a great many studies have previously demonstrated that markers of oxidative stress, inflammation, proteolysis, apoptosis, autophagy,

and atrophy signaling pathways were upregulated in the atrophying muscles following periods of disuse in patients [11,12] and animal models [6–8]. Whether regenerative potential is altered in models of disuse muscle atrophy remains to be fully elucidated.

Skeletal muscles are formed as a result of the fusion of progenitor myoblasts during development. Postnatal muscle stem cells replace the muscle turnover resulting from the daily life activity of humans and animals [13]. Thus, regeneration of skeletal muscles is a tightly regulated process [13]. Muscle regeneration relies widely on the interaction between satellite cells and the microenvironment. Their numbers and subtypes may vary according to the underlying condition, such as during muscle atrophy [11,13,14], exposure to cigarette smoking [15], aging [16], and prolonged bed rest [17]. All these conditions may also take place simultaneously within the same individual and may interfere with the process of muscle regeneration in the patients.

Satellite cells are characterized by their heterogeneity, which leads to different functions within the skeletal muscle fibers. The satellite cell reservoir is composed by sublaminar cells that express paired box (Pax)-7 with no expression of myogenic factor (Myf)-5 marker [13]. Importantly, Pax-7+/Myf-5+ satellite cells are the ones that preferentially differentiate into muscle fibers, while Pax-7+/Myf-5- cells do not proliferate, representing the satellite cell reservoir of the muscles. The ability to express Myf-5 determines these two different subtypes of satellite cell populations within the skeletal muscles [13]. In line with this, Pax-7+/Myf-5- satellite cells are identified as the actual stem cells, whereas Pax-7+/Myf-5+ satellite cells are recognized as the committed myogenic progenitors [13].

Resveratrol elicits beneficial effects on tissues including skeletal muscles. It is a natural polyphenol, which is obtained from peanuts, red wine, grapes, and other plants. It is a very popular compound given its effects as a powerful antioxidant [18]. Resveratrol was also shown to improve the lifespan of different animals by attenuating oxidative stress, inflammation, and atherosclerosis [19–22]. Amelioration of injury was also shown in the gastrocnemius of rats in response to treatment with resveratrol [23]. Moreover, muscle regeneration also improved as a result of resveratrol treatment in mice [24,25].

Curcumin is another polyphenolic compound that is obtained from turmeric. Curcumin exerted beneficial effects on several tissues through different mechanisms. For instance, in mouse cells, myocardial-infarction-induced fibrosis was attenuated via sirtuin-1 activation [26]. Senescence of smooth muscle and endothelial cells also improved as a result of curcumin therapy via sirtuin-1 activity [27]. Inhibition of NF-kB elicited by curcumin was shown to attenuate muscle protein degradation in models, such as in sepsis [28,29] and during unloading in mice [30]. Moreover, muscle regeneration was also favored by treatment with the NF-kB inhibitor curcumin [31].

In models of unloading and reloading, it was shown that the acetylation statuses of the transcription factors fork-head box O (FoxO)1 and FoxO3 were relevant mediators of the process of muscle mass loss during unloading of the limb muscles in mice [6,7]. Interestingly, unloading of the gastrocnemius muscles elicited a decline in levels of histone deacetylase sirtuin-1 activity, and the activity level rose up to the control levels following a period of reloading of the hindlimb [6,7]. The beneficial effects observed in the gastrocnemius muscle in those studies were most likely due to the activity of sirtuin-1 enzyme on the transcription factors FoxO1 and FoxO3 [6,7]. Thus, it is plausible to conceive that enhancement of sirtuin-1 activity with compounds such as resveratrol and curcumin may prevent muscles from further muscle loss through attenuation of the activity of atrophy signaling. On the other hand, sirtuin-1 activity may also promote muscle repair and regeneration following disuse muscle atrophy [25].

On this basis, the current hypothesis was that treatment with pharmacological agents (resveratrol and curcumin) known to increase sirtuin-1 activity, among other functions, may enhance muscle regeneration as evaluated by the identification of biological players clearly involved in this process in the limb muscles of mice exposed to muscle reloading following a period of hindlimb unloading. Hence, the objectives were as follows, in the limb muscles of mice exposed to a seven-day period of unloading followed by another seven-day period of reloading treated with either resveratrol or curcumin: (1) progenitor muscle cell numbers (all limb muscles together) were identified using

fluorescent-activated cell sorting (FACS), (2) subtypes of satellite cells in histological preparations of gastrocnemius muscle were counted, (3) markers of early and late muscle regeneration were analyzed, (4) fiber type composition and morphometry of the gastrocnemius muscle were assessed, (5) sirtuin-1 activity and content were explored, and (6) function of the limb muscles was also explored. The experimental model employed in the current investigation has previously been well validated [6–8].

2. Methods

2.1. Study Design and Animal Experiments

Female C57BL/6J mice (10 weeks old, weight ~20 g) were obtained from Harlan Interfauna Ibérica SL (Barcelona, Spain). Female mice were used for practical reasons as previous investigations in our group had also been conducted on this type of animal [6–8,30]. Mice were kept under pathogen-free conditions in the animal house facility at Barcelona Biomedical Research Park (PRBB), with a 12/12 h light–dark cycle.

The entire study protocol is shown in Figure 1. Mice were exposed to unilateral hindlimb immobilization as previously described to reproduce a model of disuse muscle atrophy [6–8,32]. The time-points used in the current investigation have also been validated in previous studies conducted by our group [6–8]. Muscle damage was demonstrated in the limb muscles of the unloaded mice [7]. Reloading of the muscle for another 7-day period elicited an improvement in muscle damage [7].

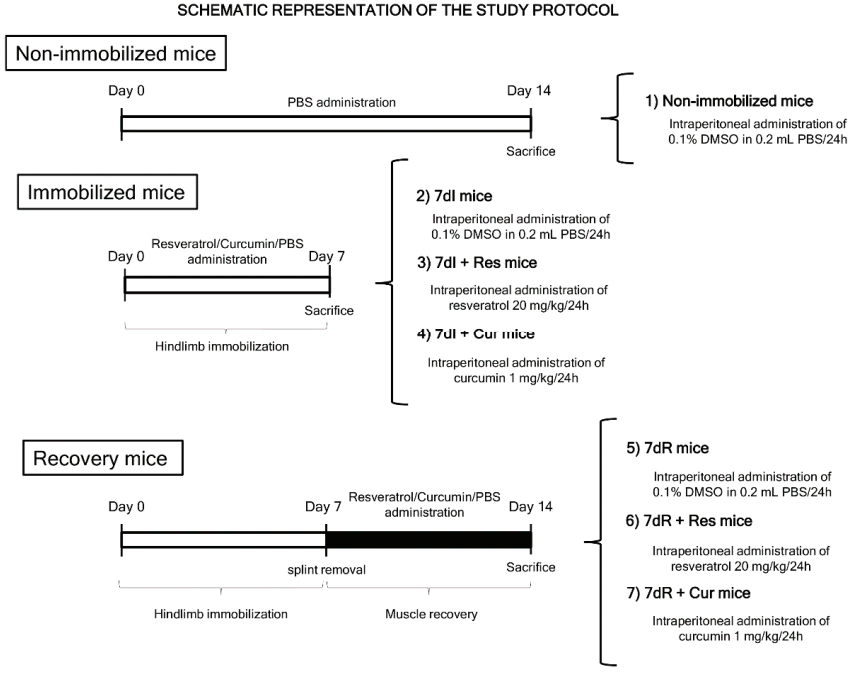


Figure 1. Graphical time-line representation of all the groups and treatments administered to the mice in the study. Definition of abbreviations: PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; mL, millilitre; mg, milligram; kg, kilogram; h, hour; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin.

The left hindlimb was shaved with clippers and was enveloped using surgical tape. The hindlimb was introduced into a 1.5-mL microcentrifuge tube with cover and bottom lids removed, while maintaining the foot in a plantar-flexed position to induce the maximal atrophy of the target limb muscle [6–8,32]. As the weight of the tube was approximately 0.6 g, it did not interfere with the usual mobility of the mice. The following groups of mice were studied (n = 10/group, Figure 1): (1) non-immobilized mice, (2) 7-day-immobilized mice (7dI, left hindlimb immobilized for seven consecutive days), (3) 7dI mice treated with resveratrol (7dI + Res, intraperitoneal administration, 20 mg/kg weight/24 h) [33,34], (4) 7dI mice treated with curcumin (7dI + Cur, intraperitoneal administration, 1 mg/kg weight/24 h) from day 0 to day 7 [35], (5) 7-day-recovery mice (7dR, left hindlimb immobilized for seven consecutive days, when the plastic splint was removed and the animals were moving free in their cages, in order to evaluate muscle recovery), (6) 7dR mice treated with resveratrol (7dR + Res, intraperitoneal administration, 20 mg/kg weight/24 h) from day 7 to day 14 [33,34], and (7) 7dR mice treated with curcumin (7dR + Cur, intraperitoneal administration, 1 mg/kg weight/24 h) from day 7 to day 14 [35].

The half-life of circulating curcumin was previously established to go from 15 to 60 min in animal models and patients [36,37] and from 30 to 60 min for resveratrol [38]. The rationale to administer resveratrol or curcumin intraperitoneally was to ensure that each animal received exactly the same dose of the drug every day. Administration of resveratrol or curcumin using other routes (oral, during food or water administration) would not allow us to ensure an identical dose for each mouse. Furthermore, intraperitoneal administration avoids absorption through the gastrointestinal tract and the first barrier of the hepatic metabolism, as generally happens in oral administration [39,40]. Accordingly, intraperitoneal injection was the selected route due to the fact that it gets into the circulation faster than other routes (oral gavage), to ensure the optimal absorption of the two compounds in view of its short bioavailability in plasma, estimated as 15–60 min in mice [36–38]. In order to control for a potential injection-induced stress response, all four groups (including the non-treated controls) of animals were injected intraperitoneally.

2.2. Ethics

All animal experiments were conducted in the animal facilities at PRBB. This was a controlled study designed in accordance with the ethical regulations on animal experimentation of the European Community Directive 2010/63/EU, Spanish Legislation (*Real Decreto* 53/2013, BOE 34/11370–11421) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (1986). All animal experiments were approved by the Animal Research Committee at PRBB. Ethical approval was obtained by the Animal Research Committee (Animal Welfare Department in Catalonia, Spain, EBP-13-1485).

2.3. In-Vivo Measurements in the Mice

In all the study animals, body weight and food intake were measured at every time point, and food and water were supplied ad libitum for the entire duration of the immobilization or recovery periods. In all mice, limb strength was determined on day 0 and right at the end of each immobilization or recovery time point (as described above) using a grip strength meter (Bioseb, Vitrolles Cedex, France) following previously published methodologies [6–8,30,41] in which grip strength was also the end-point parameter in the different experimental models. Grip strength was assessed in the four limbs at the same time in all mice. In all the animals, limb strength gain was calculated as the percentage of the measurements performed at the end of the study period with respect to the same measurements obtained at baseline (grip strength at the end of the study period − grip strength on day 0)/grip strength on day 0 × 100) [6,7].

2.4. Sacrifice and Sample Collection

Mice from all the experimental groups were sacrificed after the corresponding immobilization or recovery time cohorts (7 or 14 days). Each mouse was previously inoculated intraperitoneally with 0.1 mL sodium pentobarbital (60 mg/Kg). In all cases, the pedal and blink reflexes were evaluated in order to verify total anesthetic depth. Muscles were obtained from all the animals at the time of sacrifice. For isolation of muscle cell progenitor experiments, the following muscles were obtained from all the animals at the time of sacrifice: gastrocnemius, tibialis anterior (TA), extensor digitorum longus (EDL) and quadriceps femoris (QF, entire muscles in all cases). All study muscles from the hindlimb were pooled together by groups of two for each experimental condition: non-immobilized, 7dI and 7dR, with/without treatment with either resveratrol or curcumin. For immunofluorescence experiments, gastrocnemius muscle was obtained at the time of sacrifice in each experimental cohort and was embedded in optimum cutting temperature (OCT, Sakura Finetek, Torrance, CA, USA). For stem cell progenitor isolation experiments, the muscle samples were preserved in cold Dulbecco's Modified Eagle's Medium (DMEM) to be immediately processed as described below. For identification of several muscle regeneration markers, gastrocnemius muscle samples were snap-frozen in liquid nitrogen to be thereafter stored frozen at −80 °C to be further used.

2.5. Tissue Embedding

The gastrocnemius muscles of all study groups were fixed in 4% paraformaldehyde solution, pH 6.9 (EMD Millipore corporation, Billerica, MA, USA) and were embedded progressively with increasing concentrations of sucrose. They were subsequently embedded in tissue-tek OCT compound to be snap-frozen in 2-methyl-butane immersed in liquid nitrogen as previously described [42]. Ten-µm frozen sections were cut using a cryostat microtome (Leica CM3050S, Leica Biosystems, Wetzlar, Germany) at −20 °C and were mounted on glass slides.

2.6. Biological Analyses

Muscle fiber type and morphometry. Slow- and fast-twitch muscle fibers were identified using immunofluorescence procedures with anti-myosin heavy chain (MyHC) I and anti-MyHC II antibodies, respectively. Muscle cross-sections were air-dried for thirty minutes and were rinsed with phosphate-buffered saline (PBS) for another fifteen minutes. PBS was used to rinse sections in the different incubation steps. After rising, the sections were put in cold methanol for six more minutes. The sections were then boiled using a pressure cooker in 10 mM citrate buffer (pH 6.0) for twenty minutes and were then cooled down at room temperature for two hours. Subsequently, sections were incubated with mouse IgG blocking reagent (MOM, Vector Laboratories, Burlingame, CA, USA) for one hour, and in blocking solution (3% bovine serum albumin (BSA), 10% goat serum and 0.5% triton in PBS) for another hour. Afterwards, they were incubated overnight with the mouse monoclonal anti-MyHC I antibody (ab11083, Abcam) and anti-MyHC II antibody (ab51263, Abcam) prepared in blocking solution at 4 °C. Following incubation with the primary antibody and after rising with PBS, the sections were incubated with the corresponding secondary antibody and 4',6-diamino-2-fenilindol (DAPI), which specifically stained deoxyribonucleic acid (DNA) allowing identification of all nuclei for one hour at room temperature: Alexa Fluor® 488 AffiniPure goat anti-mouse IgG, Fcγ Subclass 1 Specific (Jackson Immunoresearch, West Grove, USA), which was also prepared in the blocking solution. The sections were mounted using 70% glycerol in 30% PBS. Myofibers positively stained with the anti-MyHC type I antibody or anti-MyHC type II were fluorescein isothiocyanate (FITC)-stained in green in two consecutives muscle cross-sections. The cross-sectional area (CSA), mean least diameter, and proportions of type I and type II, were assessed using a fluorescence microscope (x20 objective, Nikon Eclipse Ni, Nikon, Tokyo, Japan) coupled with an image-digitizing camera (Zyla 4.2 sCMOS camera, Andor, Belfast, UK) and the Image J software (National Institute of Health, available at http://rsb.info.nih.gov/ij/). In each muscle cross-section, at least 100 fibers were measured and counted,

separately, from all study groups of mice. Fibers that were stained simultaneously for both anti-type I and anti-type II primary antibodies were identified as the hybrid fibers. They were all counted in the histological preparations of all the study groups.

Satellite cell identification using immunofluorescence microscopy. Immunofluorescence staining was used to detect satellite cells in both quiescent and activated states using specific antibodies (see below). Briefly, muscle cross-sections were air-dried for 30 min and were rinsed with PBS for another 15 min. PBS was used to rinse the sections among the different incubation steps. After rinsing, the sections were put in cold methanol for six more minutes. Then, the sections were boiled using a hot bath in 0.1 M citrate buffer (pH 6.0) for 12 min and were then blocked with 10% goat serum in PBS for two hours.

Subsequently, sections were incubated with MOM for 30 min. Afterwards, they were incubated overnight with a mixture of two antibodies: mouse monoclonal anti-Pax-7 antibody (Developmental Studies Hybridoma Bank, Iowa, IA, USA) and rabbit polyclonal anti-Myf-5 antibody (Aviva Systems Biology, San Diego, CA, USA), prepared in an antibody solution (1% goat serum dissolved in PBS, at 4 °C. Anti-Pax-7 antibody alone was used to detect quiescent satellite cells, while the mixture of anti-Pax-7 and anti-Myf-5 antibodies detected committed satellite cells [13]. The addition of quiescent and committed satellite cells corresponded to the total number of satellite cells. Following incubation with the primary antibodies and after rinsing with PBS, the sections were incubated at room temperature with the corresponding secondary antibodies for one hour: Alexa Fluor® 488 AffiniPure goat anti-mouse IgG, Fcγ Subclass 1 Specific and Alexa Fluor® plus 555 goat anti-rabbit IgG (H+L) (Thermo Fisher Scientific, Waltham, USA) also prepared in an antibody solution. Finally, the sections were mounted using the fluorescent mounting medium DAPI G-Fluoromount medium (Southern Biotech, Birmingham, AL, USA), which specifically marks DNA (allowing identification of all nuclei) in the muscle sections. A fluorescence microscope (×40 objective, Nikon Eclipse Ni, Nikon, Tokyo, Japan) coupled with a digitizing camera was used to identify and count the number of satellite cells (10 fields) in each study sample. Results were expressed as Pax-7+/Myf-5- (quiescent) satellite cells, Pax-7+/Myf-5+ (activated) satellite cells or the addition of both (as total satellite cells) to the total number of counted myonuclei in the 10 fields. Additionally, negative control experiments were carried out by omission of the primary antibodies and incubation of the muscle samples only with secondary antibody, to confirm the specificity of each antibody.

Sirtuin-1 activity. Briefly, frozen muscle samples from the gastrocnemius muscle of all study animals were homogenized in a buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 100 mM NaF, 10 mM Na pyrophosphate, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton-X, and no protease inhibitors. Homogenates were centrifuged in a 4 mL buffer containing 30% sucrose, 10 mM Tris HCl (pH 7.5), 10 mM NaCl, and 3 mM $MgCl_2$ at 4 °C and at 1300 *g* for 10 min. The pellets were washed with cold 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl, to be subsequently centrifuged at 4 °C and at 1300 *g* for 10 min. The resultant pellets, which contained the nuclei, were resuspended in 200 μL of extraction buffer containing 50 mM HEPES potassium hydroxide (HEPES KOH, pH 7.5), 420 mM NaCl, 0.5 mM EDTA, 0.1 mM Ethylene glycol tetraacetic acid (EGTA) and 10% glycerol. The nuclei were subsequently sonicated in 15-s cycles. Afterwards, the sonicated nuclei remained on ice for 30 min. Following centrifugation at 4 °C and at 12,000 *g* for 10 min, the supernatants (crude nuclear extracts) were stored at −80 °C until further use [43,44]. Sirtuin-1 activity was evaluated using 50 μg of crude nuclear extracts from the gastrocnemius muscle of all experimental mice.

Satellite cell isolation using fluorescence-activated cell sorting (FACS). A schematic representation of these methodologies is shown in Figure 2. Immediately after dissection, muscles were processed following a modified version of a previously described protocol [45]. Pools of gastrocnemius, TA, EDL and QF muscles were first minced with scissors and secondly with a razor blade (Electron Microscopy Science, Hatfield, PA, USA). The minced muscles were collected in a 50-mL tube containing 40 mL of cold DMEM to be subsequently washed to remove fat tissue. Enzymatic digestion with a DMEM media containing 2.5 U/mL collagenase (Serva Electrophoresis, Heidelberg, Germany) and 2.5 U/mL

dispase (Sigma Aldrich, Sant Louis, MO, USA) was immediately performed at 37 °C in an agitation bath for 10 min.

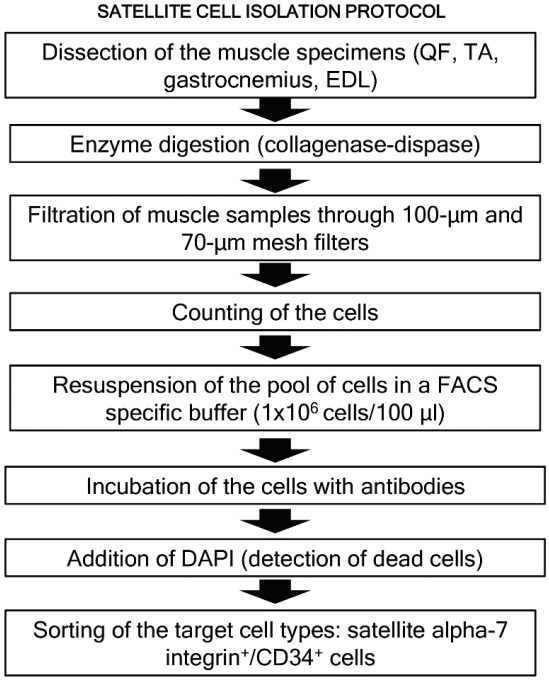


Figure 2. Representative flowchart corresponding to the isolation protocol of the satellite cells using FACS analyses in the mouse limb muscles. Each specific step is described in a box and the flow of the entire protocol is indicated by the dark thick arrows. Definition of abbreviations: QF, quadriceps femoris; TA, tibialis anterior; EDL, extensor digitorum longus; FACS, fluorescence-activated cell sorting; DAPI, 4′,6-diamino-2-fenilindol; *α7*, alpha-7 integrin.

The digestion procedure was repeated four times and the digested muscle solution was then filtered through a 100-µm mesh filter (Corning, New York, NY, USA). Immediately afterwards, the digestion was stopped by adding 2 volumes of 10% fetal bovine serum (FBS) in PBS, and the muscle solution was filtered through a 70-µm mesh filter (Corning). The filtered solution was centrifuged at 300× *g* for 5 min. The pellet was kept and the supernatant was centrifuged again in order to recover the maximum amount of cells. Finally, the two cell pellets were combined in a fresh tube to be re-suspended in FACS buffer ((1 mL PBS solution containing 2.5% goat serum (Sigma Aldrich)). The number of cells was counted using a Neubauer chamber.

Prior to incubation with antibodies, the cells were washed in 20-mL cold DMEM and centrifuged at 1700 rpm for 10 min to recover the cell pellet. Cells were resuspended in 1×10^6 cell/100 µL FACS-specific buffer. All the cells were incubated with antibodies used to specifically identify the satellite cells: Phycoerythrin (PE)-conjugated anti-alpha-7 integrin (Ablab, Vancouver, Canada), a heterodimeric integral membrane protein critical for the modulation of cell–matrix interactions, and allophycocyanin (APC)-conjugated anti-CD34 (BD Pharmigen, San Jose, CA, USA), as a cell surface sialomucin (a mucopolysaccharide molecule containing sialic acid) with reported anti-adhesive, motile, and pre-proliferative properties for 30 min. Additionally, the cells from all study groups of mice were incubated at the same time (30 min) with specific antibodies to exclude other cell type populations that might also have been present in the muscle extracts, namely endothelial cells, leukocytes and

hematopoietic cells: PE-cyanine7 (Cy7)-conjugated anti-CD31 (Biolegend, San Diego, CA, USA), a marker of endothelial cells, both anti-CD11b (Biolegend) and anti-CD45 (Biolegend) as markers of leukocytes, and anti-Sca-1 (Biolegend), a marker of hematopoietic stem cells. The excluded cell populations were named negative-lineage (Lin (−)).

Subsequently, all the study cells were incubated with DAPI in order to exclude the dead cells (cells DAPI+ exclusively) five minutes prior to the start of FACS analyses (FACS Aria II SORP, BD Biosciences, San Jose, CA, USA). Once DAPI+ cells and other cell types (endothelial, leukocytes, and hematopoietic) were excluded, the cells identified using FACS were small and of a round shape, indicating a relatively low complexity. Cells stained for PE-conjugated anti-alpha-7 integrin and APC-conjugated anti-CD34 were sorted and were named alpha-7 integrin^{+}/CD34^{+} muscle progenitors. Muscle progenitor cells were expressed as the percentage of alpha-7 integrin^{+}/CD34^{+} progenitors to the total grams of tissue.

Ribonucleic acid (RNA) extraction. Total RNA was first isolated from the gastrocnemius muscle of mice using Trizol reagent following the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA). Total RNA concentrations were determined spectrophotometrically using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA).

Procedures of messenger (mRNA) reverse transcription (RT). A single RT was performed from which all the target genes of the study were analyzed. First-stranded complementary deoxyribonucleic acid (cDNA) was generated from mRNA using oligo(dT)$_{12–18}$ primers and the Super-Script III reverse transcriptase following the manufacturer's instructions (Life Technologies).

Quantitative real-time polymerase chain reaction *amplification (qRT-PCR).* TaqMan-based qPCR reactions were performed using the ABI PRISM 7900HT Sequence Detector System (Life Technologies, Carlsbad, CA, USA) together with commercially available gene expression assays. The probes corresponding to the following genes involved in muscle regeneration were tested: marker of cell proliferation Ki67 *(Ki67*) (Mm01278617_m1, Life Technologies). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Mm99999915_g1, Life Technologies) served as the endogenous control for the mRNA gene [46,47]. Reactions were run in duplicate, and mRNA data were collected and subsequently analyzed using the Sequence Detection System relative quantification software, version 2.4 (Applied BioSystems), in which the comparative C_T method ($2^{-\Delta\Delta CT}$) for relative quantification was employed [48].

Immunoblotting of 1D electrophoresis. Protein levels of the different molecular markers analyzed in the study were explored by means of immunoblotting procedures as previously described [6,7,30]. Briefly, frozen muscle samples from the gastrocnemius muscle of all mouse experimental groups were homogenized in a buffer containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 0.5% Triton-X, 2 micrograms/mL leupeptin, 100 micrograms/mL phenylmethanesulfonyl fluoride (PMSF), 2 micrograms/mL aprotinin and 10 micrograms/mL pepstatin A. The entire procedure was conducted at 4 °C. Protein levels in crude homogenates were spectrophotometrically determined with the Bradford method using triplicates in each case and BSA as the standard (Bio-Rad protein reagent, Bio-Rad Inc., Hercules, CA, USA). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein (ranging from 5 to 60 micrograms, depending on the antigen and antibody) from crude muscle homogenates were always loaded onto the gels, as well as identical sample volumes/lanes. For the purpose of comparison among the different groups of experimental and control rodents, muscle sample specimens were always run together and kept in the same order. Two independent sets of immunoblots were conducted and four fresh 10-well mini-gels were always simultaneously loaded for each of the antigens. Experiments were confirmed at least twice for all the antigens analyzed in the investigation.

Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with BSA and incubated overnight with selective primary antibodies. Protein levels of sirtuin-1 and myogenic markers Pax-7, myoD and myogein were identified in the gastrocnemius using specific primary antibodies: NAD-dependent protein deacetylase sirtuin-1

(anti-sirtuin-1 antibody, ProteinTech Group Inc., Rosemont, IL, USA), MyoD (anti-MyoD antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), myogenin (anti-myogenin antibody, Santa Cruz Biotechnology), Pax-7 (anti-Pax-7 antibody; Abcam, Cambridge, UK) and GAPDH (anti-GAPDH antibody, Santa Cruz Biotechnology). Antigens from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, samples from the different groups were always detected in the same picture under identical exposure times. The specificity of the different antibodies was confirmed by omission of the primary antibody and incubation of the membranes only with secondary antibodies.

PVDF membranes were scanned with the Alliance Q9 Advanced (Uvitec Cambridge, England, UK). Optical densities of specific bands were quantified using the ImageJ software (National Institute of Health, available at http://rsb.info.nih.gov/ij/). Final optical densities obtained in each specific group of mice corresponded to the mean values of the different samples (lanes) of each of the study antigens. In order to validate equal protein loading among the various lanes, the glycolytic enzyme GAPDH was used as the protein loading control in all the immunoblots [46,47].

2.7. Statistical Analysis

The Shapiro–Wilk test was used to test normality of the study variables. The results are presented as mean values (standard deviation). The variables of food intake and percentage changes in total body weight, limb strength, and muscle structure are represented in Tables 1 and 2. The biological variables are represented in the figures. For each specific treatment (either resveratrol or curcumin), two-way analysis of variance (ANOVA) was performed using STATA (Software for Statistics and Data Science, StataCorp LLC, College Station, TX, USA) independently. For all the study variables, the following effects were analyzed: immobilization/recovery, treatment with either resveratrol or curcumin, and interaction between treatment and condition. Moreover, potential differences between two specific experimental groups were analyzed using post-hoc analysis contrast of marginal linear predictions: (1) comparisons between non-immobilized and immobilized mice, (2) comparisons between recovery and immobilized mice, (3) comparisons between treated and non-treated animals during the unloading period, and (4) comparisons between the treated and non-treated rodents during the recovery period. $p \leq 0.05$ was established as the level of significance.

3. Results

3.1. Physiological Characteristics of the Study Animals

Non-immobilized versus immobilized conditions. Compared with non-immobilized animals, in immobilized mice, total body and gastrocnemius weight and limb strength gain were significantly reduced, while food intake was not modified (Table 1).

Table 1. Physiological parameters in all experimental groups of mice.

	Non-Immobilized (*N* = 10)	7dI (*N* = 10)	7dI + Res (*N* = 10)	7dI + Cur (*N* = 10)	7dR (*N* = 10)	7dR + Res (*N* = 10)	7dR + Cur (*N* = 10)
Food intake (g/24 h)	3.15 (0.10)	3.10 (0.03)	3.17 (0.13)	3.02 (0.56)	3.08 (0.47)	3.11 (0.17)	3.48 (0.65)
Total body weight gain (%)	+7.28 (2.02)	−1.60 (1.82), ***	+0.36 (1.49)	−0.73 (1.20)	+3.23 (1.45), §§	+6.40 (1.92)	+5.94 (1.30)
Gastrocnemius weight (g)	0.115 (0.003)	0.085 (0.05), *	0.091 (0.007)	0.089 (0.003)	0.097 (0.004), §	0.099 (0.004)	0.097 (0.006)
Limb strength gain (%)	+11.44 (5.10)	−5.45 (1.12), *	−0.61 (1.45)	−2.88 (1.81)	+7.00 (2.39), §§	+7.71 (2.08)	+7.60 (1.99)
	Resveratrol			**Curcumin**			
	Immobilization effect *p*-value	**Treatment effect *p*-value**	**Interaction effect *p*-value**	**Immobilization effect *p*-value**	**Treatment effect *p*-value**	**Interaction effect *p*-value**	
Food intake (g/24 h)	0.919	0.454	0.750	0.640	0.426	0.308	
Total body weight gain (%)	**0.0001**	0.052	0.621	**0.0001**	0.126	0.423	
Gastrocnemius weight (g)	**0.0001**	0.078	0.274	**0.0001**	0.270	0.270	
Limb strength gain (%)	**0.048**	0.547	0.637	**0.0171**	0.763	0.890	

Variables are presented as mean (standard deviation). Definition of abbreviations: I, immobilization; R, recovery; g, grams. Statistical significance is represented as follows: *, $p < 0.05$ and ***, $p < 0.001$ between 7dI animals and non-immobilized mice; §, $p < 0.05$ and §§, $p < 0.01$ between the 7dI and 7dR groups of animals. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable; $p < 0.05$ and $p < 0.001$ are presented in bold.

Reloading versus unloading conditions. Compared with unloaded animals, in recovery mice, total body and gastrocnemius weight and limb strength gain significantly increased, while food intake was not modified (Table 1).

Unloading with either resveratrol or curcumin versus unloading. Compared to non-treated unloaded mice, in the gastrocnemius of 7dI + Res and 7dI + Cur mice, food intake, total body and gastrocnemius weight, and limb strength gain did not significantly differ (Table 1).

Reloading with either resveratrol or curcumin versus reloading. Compared to non-treated reloaded animals, in the gastrocnemius of 7dR + Res and 7dI + Cur mice, food intake, total body and gastrocnemius weight, and limb strength gain did not significantly differ (Table 1).

3.2. Structural Phenotypic Characteristics

Non-immobilized versus immobilized conditions. Compared with non-immobilized animals, in the gastrocnemius of immobilized mice, the cross-sectional area of both type I and type II muscle fibers significantly decreased, while the proportions of hybrid fibers increased (Table 2).

Reloading versus unloading conditions. Compared to non-treated unloading animals, in the gastrocnemius of the reloading mice, the CSA of type II fibers significantly increased (Table 2). Fiber type proportions of both slow- and fast-twitch and hybrid fibers did not significantly differ between 7dI and 7dR mice (Table 2). The CSA of slow-twitch and hybrid fibers did not differ between these two groups of animals (Table 2).

Unloading with either resveratrol or curcumin versus unloading. Compared to non-treated unloaded mice, in the gastrocnemius of 7dI + Res or 7dI + Cur mice, no significant differences were detected in either cross-sectional area or muscle fiber type proportions (Table 2).

Reloading with either resveratrol or curcumin versus reloading. Compared to non-treated reloaded animals, in the gastrocnemius of 7dR + Cur mice, the cross-sectional area of the hybrid fibers increased, as almost did the CSA of fast-twitch fibers in the 7dR + Res animals, while no significant differences were observed in the other parameters (Table 2).

Table 2. Structural characteristics of the gastrocnemius muscle in the study groups.

	Non-Immobilized (N = 10)	7dI (N = 10)	7dI + Res (N = 10)	7dI + Cur (N = 10)	7dR (N = 10)	7dR + Res (N = 10)	7dR + Cur (N = 10)
Muscle fiber type, %							
Type I fibers	15.37 (2.99)	16.93 (2.88)	14.68 (2.56)	18.27 (6.00)	16.25 (7.23)	15.21 (8.28)	14.95 (4.84)
Type II fibers	81.96 (5.74)	82.94 (3.09)	85.31 (2.56)	81.72 (6.00)	83.74 (7.23)	84.78 (8.28)	85.04 (4.84)
Muscle fiber size (CSA)							
Cross-sectional area, type I fibers	1246.64 (129.93)	886.56 (115.34), **	982.45 (114.50)	843.59 (133.10)	923.18 (108.64)	1010.70 (111.03)	1035.55 (117.76)
Cross-sectional area, type II fibers	1256.52 (105.47)	914.31 (134.64), *	1002.68 (114.12)	948.48 (115.00)	1079.80 (119.22), §	1256.11 (127.88) (p = 0.075)	1117.72 (120.27)
Muscle hybrid fiber, %	0.66 (0.10)	3.55 (1.80), **	3.14 (1.05)	2.60 (0.67)	3.34 (1.72)	2.11 (1.10)	2.04 (0.99)
Cross-sectional area, hybrid fibers	1014.60 (86.57)	908.03 (150.27)	916.61 (187.89)	787.31 (88.64)	751.68 (167.49)	857.90 (128.07)	971.03 (167.32), #

	Resveratrol			Curcumin		
	Immobilization effect p-value	**Treatment effect p-value**	**Interaction effect p-value**	**Immobilization effect p-value**	**Treatment effect p-value**	**Interaction effect p-value**
Type I fibers, %	0.890	0.275	0.850	0.195	0.726	0.908
Type II fibers, %	0.890	0.275	0.850	0.325	0.926	0.423
Cross-sectional area, type I fibers	0.750	0.156	0.832	0.210	0.770	0.470
Cross-sectional area, type II fibers	**0.010**	0.147	0.713	**0.0140**	0.563	0.911
Muscle hybrid fiber, %	0.810	0.352	0.990	0.455	0.587	0.674
Cross-sectional area, hybrid fibers	0.515	0.694	0.576	0.283	0.621	**0.0187**

Variables are presented as mean (standard deviation). Definition of abbreviations: I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin; CSA, cross-sectional area. Statistical significance is represented as follows: *, $p < 0.05$ and **, $p < 0.01$ between 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals; #, $p < 0.05$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective experimental group (7dI or 7dR mice). $p = 0.075$: statistical significance between 7dR + Res versus 7dR. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable; $p < 0.05$ and $p < 0.01$ are presented in bold.

3.3. Sirtuin-1 Content and Activity

Non-immobilized versus immobilized conditions. Compared with non-immobilized animals, in the gastrocnemius of immobilized mice, sirtuin-1 activity did not differ, while sirtuin-1 protein levels significantly decreased (Figure 3A–C, respectively).

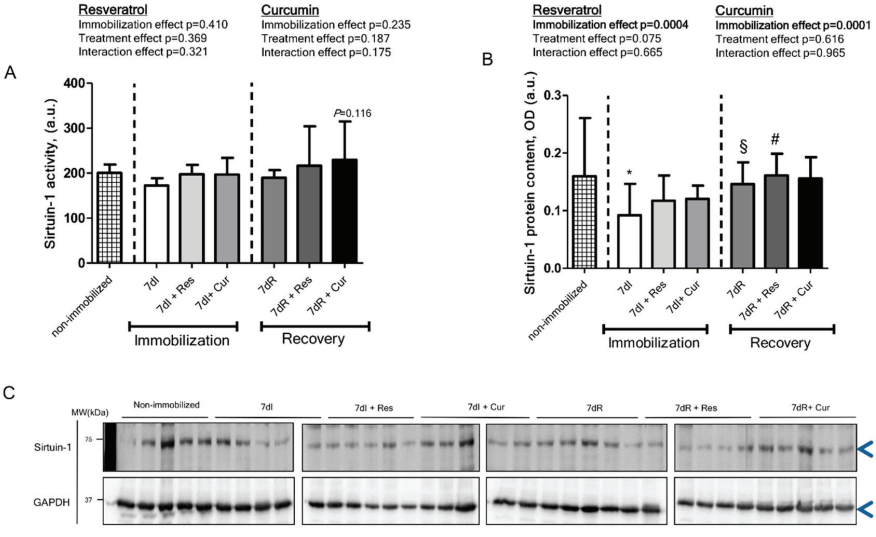


Figure 3. (**A**) Mean values and standard deviation of sirtuin-1 activity levels in the gastrocnemius muscle of the different study groups of mice, as measured by fluorescence in arbitrary units (a.u.). Definition of abbreviations: a.u., arbitrary units; I, immobilization; R, recovery; Res, resveratrol; Cur, Curcumin. $p = 0.116$: statistical significance between 7dR + Cur versus 7dR. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**B**) Mean values and standard deviation of sirtuin-1 protein content in the gastrocnemius muscle of the different study groups of mice, as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: OD, optical densities; a.u., arbitrary units; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: *, $p < 0.05$ between 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals; $^{\#}$, $p < 0.05$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective group (7dI or 7dR mice). The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**C**) Representative immunoblots of sirtuin-1 and GAPDH proteins in the gastrocnemius muscle of all study groups of mice. Arrowheads indicate the corresponding analyzed band. Definition of abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight; kDa, kilodalton; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin.

Reloading versus unloading conditions. Compared to non-treated unloading animals, in the muscles of the reloading mice, sirtuin-1 activity levels did not differ, while those of sirtuin-1 protein levels significantly increased (Figure 3A–C, respectively).

Unloading with either resveratrol or curcumin versus unloading. Compared to non-treated unloaded mice, in the gastrocnemius of 7dI + Res or 7dI + Cur mice, no significant differences were detected in either sirtuin-1 protein content or activity (Figure 3A–C, respectively).

Reloading with either resveratrol or curcumin versus reloading. Compared to non-treated reloaded animals, in the gastrocnemius of 7dR + Cur mice a significant rise in sirtuin-1 activity was almost ($p = 0.116$) observed, while sirtuin-1 protein levels showed a significant increase in 7dR + Res animals (Figure 3A–C, respectively).

3.4. Satellite Cell Counts

Non-immobilized versus immobilized conditions. A significant decline in α7-integrin $^{+}$/CD34^{+} cells was detected in the limb muscles of the unloaded mice compared to non-immobilized animals (Figure 4A). Moreover, activated (Pax-7+ and Myf-5+) satellite cells increased in the gastrocnemius of the immobilized mice compared to the non-immobilized mice, while quiescent/regenerative potential (Pax-7+ and Myf-5-) cells decreased, and total numbers of satellite cells did not significantly differ in muscles between the two experimental groups (Figure 4B–D and Figure 5A, respectively).

Reloading versus unloading conditions. A significant increase in α7-integrin^{+}/CD34^{+} cells was seen in the limb muscles of the reloading mice compared to immobilized animals (Figure 4A). Activated (Pax-7+ and Myf-5+) satellite cell numbers did not differ in the gastrocnemius of the recovery mice compared to unloaded muscles, while quiescent/regenerative potential (Pax-7+ and Myf-5-) cells and total numbers of satellite cells increased (Figure 4B–D and Figure 5A,B, respectively).

Unloading with either resveratrol or curcumin versus unloading. A significant increase in α7-integrin^{+}/CD34^{+} cells was observed in the limb muscles of the unloading mice treated with either resveratrol or curcumin compared to immobilized animals (Figure 4A). A significant rise in activated (Pax-7+ and Myf-5+) satellite cells, quiescent/regenerative potential (Pax-7+ and Myf-5-) cells, and in total satellite cell numbers was observed in the gastrocnemius of the immobilized mice treated with either resveratrol or curcumin compared to immobilized animals (Figure 4B–D and Figure 5A, respectively).

Reloading with either resveratrol or curcumin versus reloading. A significant increase in α7-integrin^{+}/CD34^{+} cells was observed in the limb muscles of the reloading mice treated with resveratrol compared to recovery animals, whereas curcumin elicited no significant modifications (Figure 4A). No significant differences in activated (Pax-7+ and Myf-5+) satellite cells, quiescent/regenerative potential (Pax-7+ and Myf-5-) cells, or total satellite cell numbers were detected in the gastrocnemius between recovery mice treated with either resveratrol or curcumin and non-treated recovery animals (Figure 4B–D and Figure 5B, respectively).

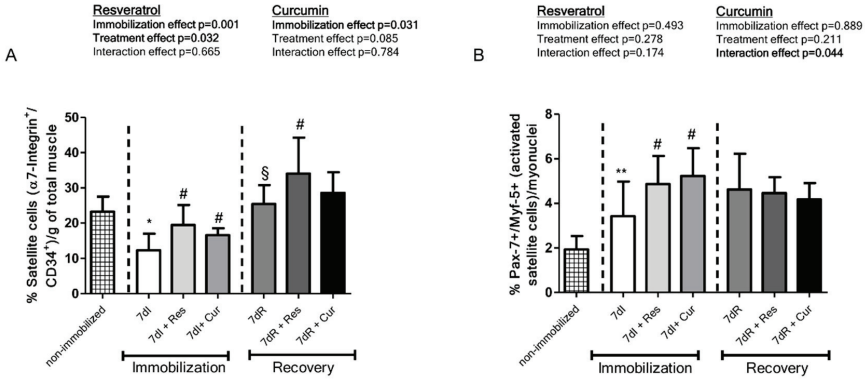


Figure 4. *Cont.*

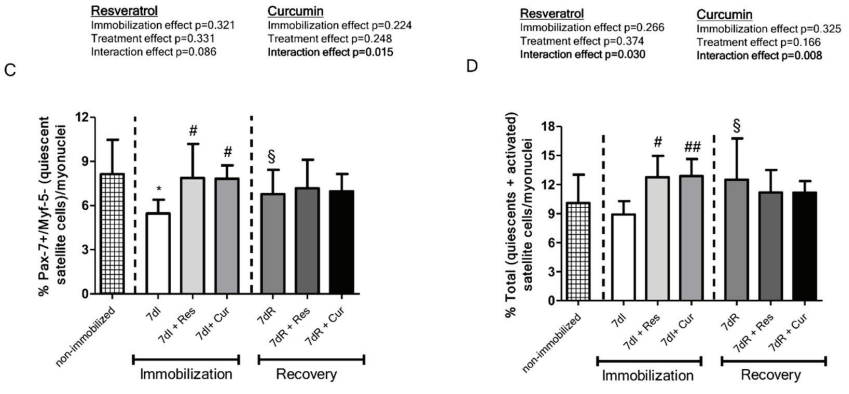


Figure 4. (**A**) Mean values and standard deviation of the percentage of satellite cells (alpha-7 integrin$^+$/CD34$^+$) measured by FACS analyses in the limb muscles of the different study groups of mice. Definition of abbreviations: α7, alpha-7 integrin; g, grams; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: *, $p < 0.05$ between the 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals; $^{\#}$, $p < 0.05$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective group (7dI or 7dR mice). The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**B**) Mean values and standard deviation of the percentage of activated satellite cell counts as identified by the number of Pax-7/Myf-5-positive cells in the gastrocnemius muscle of the different study groups of mice. *Definition of abbreviations*: Pax-7, paired box-7; Myf-5, myogenic factor 5; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: **, $p < 0.01$ between 7dI animals and non-immobilized mice; $^{\#}$, $p < 0.05$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective group (7dI or 7dR mice). The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**C**) Mean values and standard deviation of the percentage of quiescent satellite cell counts as identified by the number of Pax7-positive cells (Myf-5-negative) in the gastrocnemius muscle of the different study groups of mice. Definition of abbreviations: Pax-7, paired box-7; Myf-5, myogenic factor 5; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: *, $p < 0.05$ between 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals; $^{\#}$, $p < 0.05$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective group (7dI or 7dR mice). The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**D**) Mean values and standard deviation of the percentage of total satellite cell counts as identified by the number of quiescent and activated satellite cells in the gastrocnemius muscle of the different study groups of mice. Definition of abbreviations: I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: §, $p < 0.05$ between the 7dI and 7dR groups of animals; $^{\#}$, $p < 0.05$ and $^{\#\#}$, $p < 0.01$ for any group of pharmacologically treated mice (resveratrol or curcumin) compared to their respective group (7dI or 7dR mice). The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable.

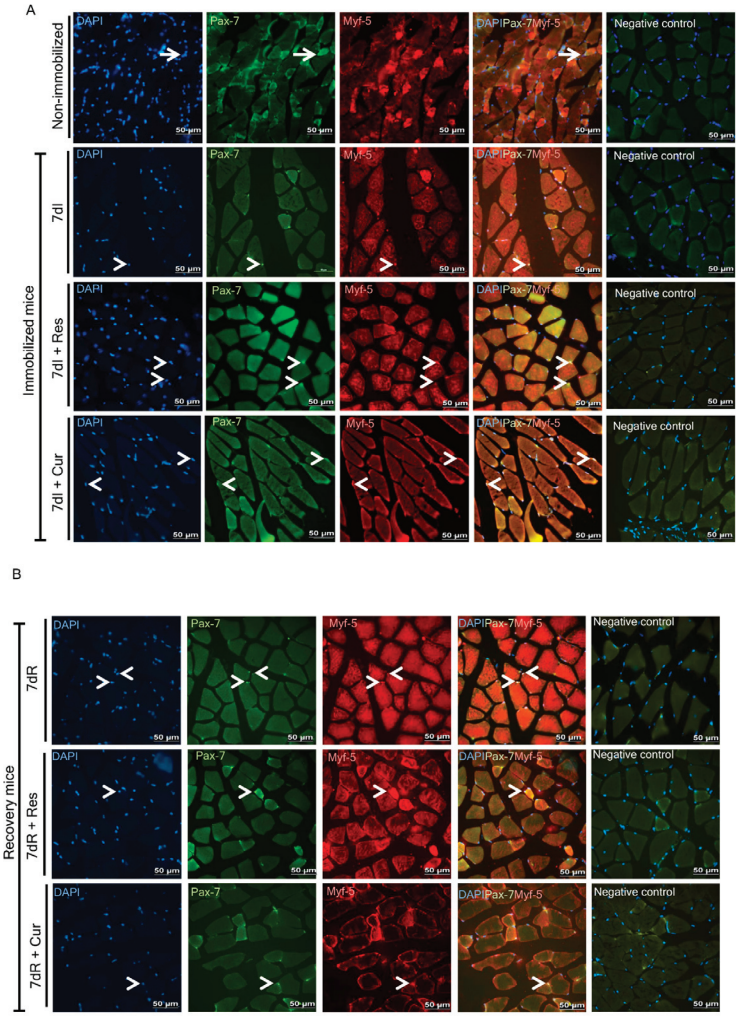


Figure 5. (**A**) Representative images of immunofluorescence staining of DAPI (left panels), Pax-7 (middle-left panels), Myf-5 (middle panels) and cells positively stained for both Pax-7 and Myf-5 markers (middle-right panels), and negative control (right panels) in the gastrocnemius muscle of the non-immobilized mice and immobilized mice with and without treatment (7dI, 7dI + Res and 7dI + Cur groups of mice). Thin arrows indicate Pax-7-positive cells, and arrowheads indicate double-stained nuclei for both Pax-7- and Myf-5-positive cells (activated satellite cells). Definition of abbreviations: Pax-7, paired box-7; Myf-5, myogenic factor 5; DAPI, 4′,6-diamino-2-fenilindol; I, immobilization; Res, resveratrol; Cur, curcumin. (**B**) Representative images of immunofluorescence staining of DAPI (left panels), Pax-7 (middle-left panels), Myf-5 ((middle panels) and cells positively stained for both Pax-7 and Myf-5 markers (middle-right panels) and negative control (right panels) in the gastrocnemius muscle of the recovery mice with and without treatment (7dR, 7dR + Res and 7dR + Cur groups of mice). Thin arrows indicate Pax-7-positive cells, and arrowheads indicate double-stained nuclei for both Pax-7- and Myf-5-positive cells (activated satellite cells). Definition of abbreviations: Pax-7, paired box-7; Myf-5, myogenic factor 5; DAPI, 4′,6-diamino-2-fenilindol; R, recovery; Res, resveratrol; Cur, curcumin.

3.5. Myogenic Markers of Muscle Regeneration

Non-immobilized versus immobilized conditions. In the gastrocnemius muscle of immobilized rodents compared to non-immobilized mice, gene expression of *Ki67* did not differ (Figure 6A). In the gastrocnemius muscle of immobilized mice compared to non-immobilized animals, protein expression levels of Pax-7 significantly improved, those of myogenin did not differ, while those of MyoD increased (Figure 6B–E, respectively).

Reloading versus unloading conditions. In the gastrocnemius muscle of recovery mice compared to immobilized animals, gene expression of *Ki67* increased (Figure 6A). In the gastrocnemius muscle of recovery mice compared to immobilized animals, protein expression of Pax-7 significantly declined, myogenin increased, and MyoD significantly decreased (Figure 6B–E, respectively).

Unloading with either resveratrol or curcumin versus unloading. In the gastrocnemius of unloading mice treated with either resveratrol or curcumin compared to non-treated unloaded muscles, no differences were observed in *Ki67* expression levels (Figure 6A). In the gastrocnemius of unloading mice treated with either resveratrol or curcumin compared to non-treated unloaded muscles, protein expression of Pax-7, myogenin and MyoD did not differ (Figure 6B–E, respectively).

Reloading with either resveratrol or curcumin versus reloading. In the gastrocnemius of reloading mice treated with curcumin or resveratrol, compared to non-treated reloaded muscles, gene expression of *Ki67* did not differ (Figure 6A). In the gastrocnemius of reloading mice treated with either resveratrol or curcumin compared to non-treated reloaded muscles, protein expression of Pax-7, myogenin and MyoD did not differ (Figure 6B–E, respectively).

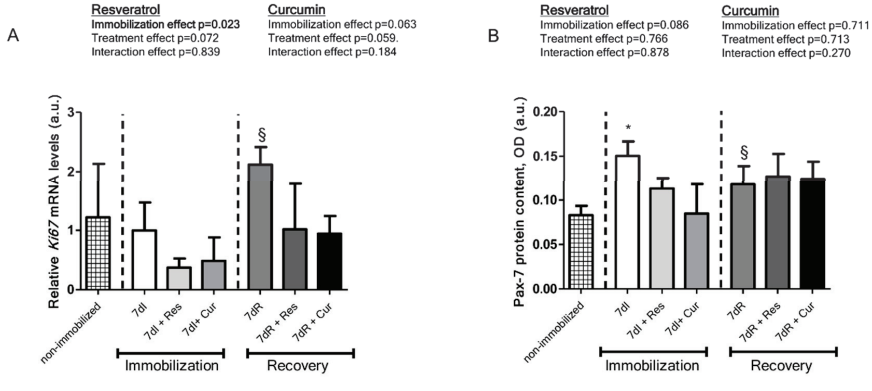


Figure 6. *Cont.*

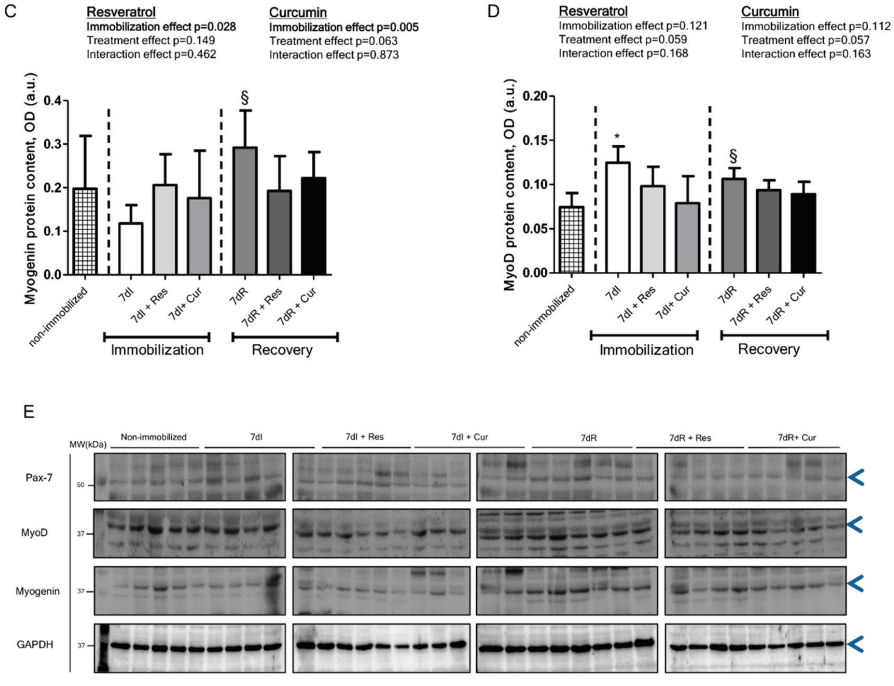


Figure 6. (**A**) Mean values and standard deviation of gene expression of *Ki67* in the gastrocnemius muscle of the different study groups of mice. Definition of abbreviations: Ki67, cell proliferation Ki-67; mRNA, messenger ribonucleic acid; a.u., arbitrary units; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: §, $p < 0.05$ between the 7dI and 7dR groups of animals. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**B**) Mean values and standard deviation of Pax-7 protein content, as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: OD, optical densities; a.u., arbitrary units; Pax-7, paired box-7; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: *, $p < 0.05$ between the 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**C**) Mean values and standard deviation of myogenin protein content, as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: OD, optical densities; a.u., arbitrary units; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: §, $p < 0.05$ between the 7dI and 7dR groups of animals. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**D**) Mean values and standard deviation of MyoD protein content, as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: OD, optical densities; a.u., arbitrary units; MyoD, myogenic differentiation 1; I, immobilization; R, recovery; Res, resveratrol; Cur, curcumin. Statistical significance is represented as follows: *, $p < 0.05$ between 7dI animals and non-immobilized mice; §, $p < 0.05$ between the 7dI and 7dR groups of animals. The effect of immobilization and treatment and interaction effects are also indicated as actual p values for each variable. (**E**) Representative immunoblots of Pax-7, MyoD, myogenin and GAPDH proteins in the gastrocnemius muscle of all study groups of mice. Arrowheads indicate the corresponding analyzed band. Definition of abbreviations: Pax-7, paired box-7; MyoD, myogenic differentiation 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight; kDa, kilodalton; I, immobilization; R, recovery; Res, resveratrol, Cur, curcumin.

4. Discussion

The main findings of the current investigation were that in immobilized mice compared to non-immobilized mice total body and gastrocnemius weight, limb strength gain, CSA of both slow- and fast-twitch fibers, sirtuin-1 protein levels, numbers of muscle progenitor cells (α7-integrin$^+$/CD34$^+$), and numbers of quiescent (Pax-7+ and Myf-5-) satellite cells significantly decreased, while proportions of hybrid fibers, activated (Pax-7+ and Myf-5+) satellite cell counts, and both Pax-7 and MyoD protein levels significantly increased.

In the muscles of the reloading animals compared to unloaded mice, total body and gastrocnemius weight, limb strength gain, CSA of fast-twitch fibers, sirtuin-1 protein levels, numbers of α7-integrin$^+$/CD34$^+$ satellite cells, numbers of quiescent (Pax-7+ and Myf-5-) satellite cells, total counts of satellite cells, *Ki67* levels (gene expression), and myogenin protein levels significantly improved.

In muscles of the immobilized mice treated with either resveratrol or curcumin compared to non-treated immobilized animals, counts of satellite α7-integrin$^+$/CD34$^+$ cells, activated (Pax-7+ and Myf-5+) satellite cells, quiescent (Pax-7+ and Myf-5-) satellite cells, and the total counts of satellite cells improved.

In muscles of the reloaded animals treated with either resveratrol or curcumin compared to non-treated reloaded mice, the CSA of both hybrid (only in curcumin-treated mice) and fast-twitch fibers (only in resveratrol-treated animals), sirtuin-1 activity levels (only in curcumin-treated animals), sirtuin-1 protein levels (only in resveratrol-treated mice), and counts of α7-integrin$^+$/CD34$^+$ satellite cells (only in the resveratrol-treated mice) significantly increased.

Unloading of the limb muscles induced a significant decline in the numbers of muscle progenitor cells and quiescent satellite cells, while a rise in activated satellite cell counts was observed. These are interesting findings that put research forward on the impact of a seven-day period of unloading on the initial steps of the muscle regeneration process.

In different experimental models, treatment with resveratrol has been shown to improve muscle mass and phenotype as a result of sirtuin-1 activity [49] and attenuation of apoptosis [25]. Increased sirtuin-1 during reloading of previously unloaded muscles also induced an improvement in muscle mass and function [6]. In the current study, CSA of fast-twitch myofibers also improved in response to treatment with resveratrol during reloading of hindlimb muscles. These findings are in line with previous results obtained in our group (unpublished observations). On the other hand, curcumin administration during reloading also elicited an improvement in muscle phenotype and function through attenuation of apoptosis and proteolysis (ubiquitin–proteasome system) in experimental models [30,35]. Furthermore, NF-kB-dependent muscle wasting was also alleviated as a result of curcumin treatment in septic rats [28,29,50].

In the present study, treatment of immobilized mice with either resveratrol or curcumin induced a significant improvement in the numbers of muscle progenitor cells, quiescent, activated and total satellite cell counts in the limb muscles compared to non-treated immobilized animals. In line with this, it was previously shown [51] that sirtuin-1 maintained satellite cells in a stem-like state. This probably accounted for the rise in the numbers of quiescent and total satellite cells seen in the muscles of the unloaded mice treated with either curcumin or resveratrol. Furthermore, in trained elderly subjects treated with resveratrol, satellite cell numbers also increased in their limb muscles [52]. Collectively, these findings suggest that resveratrol and curcumin favor muscle regenerative potential following unloading.

Importantly, treatment with resveratrol was also shown to favor the process of muscle regeneration through several mechanisms. In fact, similar levels of muscle progenitor cells to those encountered in the current investigation were also reported in a former study [25]. Interestingly, a modest enhancement of myogenic precursor cell proliferation was seen in resveratrol-treated muscles following reloading [25]. Furthermore, recovery of muscle mass and of the size of fast-twitch fibers in the rat hindlimb muscles was also mediated by the action of proapoptotic proteins including cleaved caspase-3 [25]. Another mechanism whereby resveratrol may favor muscle regeneration was its ability to attenuate

muscle damage (contusion model) in mice [24]. Furthermore, local and systemic markers of muscle regeneration were attenuated in response to resveratrol treatment in mice [24].

Specifically, muscle regenerative markers and recovery of normal tissue architecture was favored by the systemic administration of curcumin in mice in response to freeze injury [31]. The beneficial effects seen in muscles were due to blocking of NF-kB activity [31]. In a previous study from our group [30], curcumin also elicited an improvement in muscle phenotype and function via attenuation of NF-kB activity (lower levels of acetylation) in mice exposed to the same experimental conditions as in the present study. Taken together, these findings suggest that NF-kB is a probably a major regulator of the muscle regeneration process detected in the hindlimb muscles of mice in the present investigation.

Protein levels of early markers of muscle regeneration (Pax-7 and MyoD) significantly increased in the gastrocnemius of the immobilized mice compared to non-immobilized animals. These findings are in accordance with previous reports [8,17,53] and suggest that the process of muscle regeneration has been triggered. On the other hand, protein levels of the early markers Pax-7 and MyoD significantly declined in the gastrocnemius of the reloaded muscles, while those of the late marker myogenin significantly rose. These results are also consistent with previous reports in which late markers of muscle regeneration were upregulated during recovery in limb muscles [8,17,53].

Importantly, treatment with either curcumin or resveratrol in the reloading periods elicited an increase in sirtuin-1 activity and sirtuin-1 protein levels, while it did not induce any significant modifications in levels of any of the analyzed markers of muscle regeneration, cells or myogenic markers (ki67, pax-7, myoD, and myogenin) in the gastrocnemius of any of the reloaded animals. In keeping with this, a potential role of sirtuin-1 activity was established as a negative regulator of early myogenic markers (proliferation of muscle precursor cells) of muscle regeneration [54]. Another plausible explanation relies on the fact that the process of muscle regeneration would be almost entirely complete during the reloading phase, and treatment with any of the phenolic compounds would not be able to exert additional beneficial effects. Thus, treatment with either resveratrol or curcumin did not elicit any significant modification of the markers of muscle regeneration beyond the physiological effects elicited by the experimental conditions of unloading or reloading.

In line with this, treatment with resveratrol during recovery for two weeks of aged rats exposed to tail suspension also induced moderate therapeutic benefits as identified by muscle mass recovery and increased size of the fast-twitch fibers of limb muscles, probably resulting from the attenuation of apoptosis [25]. Another study [55] also demonstrated that the beneficial effects induced by resveratrol on the limb muscles of type I diabetic mice was not dependent on sirtuin-1 activity. In that investigation, mitochondrial membrane potential was restored in muscle fibers without the interference of sirtuin-1 activity in the mouse muscles [55].

5. Conclusions

Unloading of the limb muscles triggered a program of muscle regeneration characterized by the activation of satellite cells and the upregulation of early myogenic factors. Treatment with the pharmacological agents resveratrol and curcumin enhanced the numbers of the identified subtypes of satellite cells (muscle progenitor, quiescent, activated, and total satellite cells) in the unloaded limb muscles but not in the reloaded muscles. These findings have potential clinical implications as treatment with these phenolic compounds would predominantly be indicated during disuse muscle atrophy to enhance the muscle regeneration process. Treatment with either curcumin or resveratrol would not elicit as many beneficial effects during reloading.

Author Contributions: L.M.-G.: animal experiments, molecular biology, data analyses, results preparation including graphical and tabular representation, and manuscript draft writing; M.G.: animal experiments, molecular biology, data analyses; X.D.: statistical analyses of all the study results; E.B.: study design, data analyses and interpretation, results preparation, and manuscript writing—final version. All authors have read and agreed to the published version of the manuscript.

Funding: Instituto de Salud Carlos III: FIS 18/00075 (FEDER), Instituto de Salud Carlos III: CIBERES 2019. Sociedad Española de Neumología y Cirugía Torácica: SEPAR 2018.

Acknowledgments: Laura Mañas-García was a recipient of a predoctoral fellowship from the Department of Experimental and Health Sciences of Pompeu Fabra University (DCEXS-UPF). We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The current research has been supported by Instituto de Salud Carlos-III, contract grant numbers, CIBERES, FIS 18/00075 (FEDER), Spanish Ministry of Science and Innovation, Spanish Respiratory Society (SEPAR), contract grant numbers, SEPAR 2018.

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

Editorial Support: None to declare.

References

1. Shrikrishna, D.; Patel, M.; Tanner, R.; Seymour, J.M.; Connolly, B.; Puthucheary, Z.; Walsh, S.L.; Bloch, S.; Sidhu, P.; Hart, N.; et al. Quadriceps wasting and physical inactivity in patients with COPD. *Eur. Respir. J.* **2012**, *40*, 1115–1122. [CrossRef] [PubMed]
2. Schmidt, S.F.; Rohm, M.; Herzig, S.; Diaz, M.B. Cancer Cachexia: More Than Skeletal Muscle Wasting. *Trends Cancer* **2018**, *4*, 849–860. [CrossRef] [PubMed]
3. Barreiro, E. Impact of Physical Activity and Exercise on Chronic Obstructive Pulmonary Disease Phenotypes: The Relevance of Muscle Adaptation. *Arch. Bronconeumol.* **2019**, *55*, 613–614. [CrossRef] [PubMed]
4. Gea, J.; Pascual, S.; Castro-Acosta, A.; Hernández-Carcereny, C.; Castelo, R.; Márquez-Martín, E.; Montón, C.; Palou, A.; Faner, R.; Furlong, L.I.; et al. The Biomepoc Project: Personalized Biomarkers and Clinical Profiles in Chronic Obstructive Pulmonary Disease. *Archivos de Bronconeumología (Engl. Ed.)* **2019**, *55*, 93–99. [CrossRef]
5. Gea, J.; Martínez-Llorens, J. Muscle Dysfunction in Chronic Obstructive Pulmonary Disease: Latest Developments. *Archivos de Bronconeumología (Engl. Ed.)* **2019**, *55*, 237–238. [CrossRef]
6. Chacon-Cabrera, A.; Gea, J.; Barreiro, E. Short- and Long-Term Hindlimb Immobilization and Reloading: Profile of Epigenetic Events in Gastrocnemius. *J. Cell. Physiol.* **2016**, *232*, 1415–1427. [CrossRef]
7. Chacon-Cabrera, A.; Lund-Palau, H.; Gea, J.; Barreiro, E. Time-Course of Muscle Mass Loss, Damage, and Proteolysis in Gastrocnemius following Unloading and Reloading: Implications in Chronic Diseases. *PLoS ONE* **2016**, *11*, e0164951. [CrossRef]
8. Guitart, M.; Lloreta, J.; García, L.M.; Barreiro, E. Muscle regeneration potential and satellite cell activation profile during recovery following hindlimb immobilization in mice. *J. Cell. Physiol.* **2018**, *233*, 4360–4372. [CrossRef]
9. Marquis, K.; Debigaré, R.; Lacasse, Y.; Leblanc, P.; Jobin, J.; Carrier, G.; Maltais, F. Midthigh Muscle Cross-Sectional Area Is a Better Predictor of Mortality than Body Mass Index in Patients with Chronic Obstructive Pulmonary Disease. *Am. J. Respir. Crit. Care Med.* **2002**, *166*, 809–813. [CrossRef]
10. Maltais, F.; Decramer, M.; Casaburi, R.; Barreiro, E.; Burelle, Y.; Debigare, R.; Dekhuijzen, P.N.R.; Franssen, F.; Gayan-Ramirez, G.; Gea, J.; et al. An official American Thoracic Society/European Respiratory Society statement: Update on limb muscle dysfunction in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **2014**, *189*, e15–e62. [CrossRef]
11. Wall, B.T.; Dirks, M.L.; Snijders, T.; Senden, J.M.G.; Dolmans, J.; Van Loon, L. Substantial skeletal muscle loss occurs during only 5 days of disuse. *Acta Physiol.* **2013**, *210*, 600–611. [CrossRef] [PubMed]
12. Simpson, J.A.; Labugger, R.; Collier, C.; Brison, R.J.; Iscoe, S.; Van Eyk, J.E. Fast and Slow Skeletal Troponin I in Serum from Patients with Various Skeletal Muscle Disorders: A Pilot Study. *Clin. Chem.* **2005**, *51*, 966–972. [CrossRef] [PubMed]
13. Kuang, S.; Kuroda, K.; Le Grand, F.; Rudnicki, M.A. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. *Cell* **2007**, *129*, 999–1010. [CrossRef] [PubMed]
14. Mozdziak, P.; Pulvermacher, P.M.; Schultz, E. Muscle regeneration during hindlimb unloading results in a reduction in muscle size after reloading. *J. Appl. Physiol.* **2001**, *91*, 183–190. [CrossRef] [PubMed]
15. Nogueira, L.; Trisko, B.M.; Lima-Rosa, F.L.; Jackson, J.; Lund-Palau, H.; Yamaguchi, M.; Breen, E.C. Cigarette smoke directly impairs skeletal muscle function through capillary regression and altered myofibre calcium kinetics in mice fatigue resistance and myofibre calcium handling, and these changes ultimately affect contractile efficiency of locomotor muscles independent of a change in lung function. *J. Physiol.* **2018**, *596*, 14.

16. Suetta, C.; Frandsen, U.; Mackey, A.L.; Jensen, L.; Hvid, L.G.; Bayer, M.L.; Petersson, S.J.; Schrøder, H.D.; Andersen, J.L.; Aagaard, P.; et al. Ageing is associated with diminished muscle re-growth and myogenic precursor cell expansion early after immobility-induced atrophy in human skeletal muscle. *J. Physiol.* **2013**, *591*, 3789–3804. [CrossRef]
17. Arentson-Lantz, E.J.; English, K.L.; Paddon-Jones, D.; Fry, C.S. Fourteen days of bed rest induces a decline in satellite cell content and robust atrophy of skeletal muscle fibers in middle-aged adults. *J. Appl. Physiol.* **2016**, *120*, 965–975. [CrossRef]
18. Jackson, J.R.; Ryan, M.J.; Hao, Y.; E Alway, S. Mediation of endogenous antioxidant enzymes and apoptotic signaling by resveratrol following muscle disuse in the gastrocnemius muscles of young and old rats. *Am. J. Physiol. Integr. Comp. Physiol.* **2010**, *299*, 1572–1581. [CrossRef]
19. Donnelly, L.; Newton, R.; Kennedy, G.E.; Fenwick, P.S.; Leung, R.H.F.; Ito, K.; Russell, R.E.; Barnes, P.J. Anti-inflammatory effects of resveratrol in lung epithelial cells: Molecular mechanisms. *Am. J. Physiol. Cell. Mol. Physiol.* **2004**, *287*, L774–L783. [CrossRef]
20. Jarolim, S.; Millen, J.; Heeren, G.; Laun, P.; Goldfarb, D.S.; Breitenbach, M. A novel assay for replicative lifespan in Saccharomyces cerevisiae. *FEMS Yeast Res.* **2004**, *5*, 169–177. [CrossRef]
21. Jiang, Q.; Cheng, X.; Cui, Y.; Xia, Q.; Yan, X.; Zhang, M.; Lan, G.; Liu, J.; Shan, T.; Huang, Y. Resveratrol regulates skeletal muscle fibers switching through the AdipoR1-AMPK-PGC-1α pathway. *Food Funct.* **2019**, *10*, 3334–3343. [CrossRef] [PubMed]
22. Zhu, W.; Chen, S.; Li, Z.; Zhao, X.; Li, W.; Sun, Y.; Zhang, Z.; Ling, W.; Feng, X. Effects and mechanisms of resveratrol on the amelioration of oxidative stress and hepatic steatosis in KKAy mice. *Nutr. Metab.* **2014**, *11*, 35. [CrossRef]
23. Feng, Y.; He, Z.; Mao, C.; Shui, X.; Cai, L. Therapeutic Effects of Resveratrol Liposome on Muscle Injury in Rats. *Med. Sci. Monit.* **2019**, *25*, 2377–2385. [CrossRef]
24. Hsu, Y.-J.; Ho, C.-S.; Lee, M.-C.; Ho, C.-S.; Huang, C.-C.; Kan, N.-W. Protective Effects of Resveratrol Supplementation on Contusion Induced Muscle Injury. *Int. J. Med. Sci.* **2020**, *17*, 53–62. [CrossRef]
25. Bennett, B.T.; Mohamed, J.S.; E Alway, S. Effects of Resveratrol on the Recovery of Muscle Mass Following Disuse in the Plantaris Muscle of Aged Rats. *PLoS ONE* **2013**, *8*, e83518. [CrossRef]
26. Ji, X.; Xiao, J.; Sheng, X.; Zhang, X.; Guo, M. Curcumin protects against myocardial infarction-induced cardiac fibrosis via SIRT1 activation in vivo and in vitro. *Drug Des. Dev. Ther.* **2016**, *10*, 1267–1277. [CrossRef] [PubMed]
27. Grabowska, W.; Suszek, M.; Wnuk, M.; Lewinska, A.; Wasiak, E.; Sikora, E.; Bielak-Zmijewska, A. Curcumin elevates sirtuin level but does not postpone in vitro senescence of human cells building the vasculature. *Oncotarget* **2016**, *7*, 19201–19213. [CrossRef] [PubMed]
28. Poylin, V.; Fareed, M.U.; O'Neal, P.; Alamdari, N.; Reilly, N.; Menconi, M.; Hasselgren, P.-O. The NF-kappaB inhibitor curcumin blocks sepsis-induced muscle proteolysis. *Mediat. Inflamm.* **2008**, *2008*, 317851. [CrossRef]
29. Jin, B.; Li, Y.-P. Curcumin prevents lipopolysaccharide-induced atrogin-1/MAFbx upregulation and muscle mass loss. *J. Cell. Biochem.* **2007**, *100*, 960–969. [CrossRef]
30. Mañas-García, L.; Bargalló, N.; Gea, J.; Barreiro, E. Muscle Phenotype, Proteolysis, and Atrophy Signaling During Reloading in Mice: Effects of Curcumin on the Gastrocnemius. *Nutrients* **2020**, *12*, 388. [CrossRef]
31. Thaloor, D.; Miller, K.J.; Gephart, J.; Mitchell, P.O.; Pavlath, G.K. Systemic administration of the NF-κB inhibitor curcumin stimulates muscle regeneration after traumatic injury. *Am. J. Physiol. Cell Physiol.* **1999**, *277*, C320–C329. [CrossRef]
32. Lang, S.M.; Kazi, A.A.; Hong-Brown, L.; Lang, C.H. Delayed Recovery of Skeletal Muscle Mass following Hindlimb Immobilization in mTOR Heterozygous Mice. *PLoS ONE* **2012**, *7*, e38910. [CrossRef] [PubMed]
33. Park, S.-J.; Ahmad, F.; Philp, A.; Baar, K.; Williams, T.; Luo, H.; Ke, H.; Rehmann, H.; Taussig, R.; Brown, A.L.; et al. Resveratrol Ameliorates Aging-Related Metabolic Phenotypes by Inhibiting cAMP Phosphodiesterases. *Cell* **2012**, *148*, 421–433. [CrossRef]
34. Chang, C.-C.; Yang, M.-H.; Tung, H.-C.; Chang, C.-Y.; Tsai, Y.-L.; Huang, J.-P.; Yen, T.-H.; Hung, L.-M. Resveratrol exhibits differential protective effects on fast- and slow-twitch muscles in streptozotocin-induced diabetic rats *J. Diabetes* **2014**, *6*, 60–67. [CrossRef] [PubMed]

35. Vazeille, E.; Slimani, L.; Claustre, A.; Magne, H.; Labas, R.; Bechet, D.; Taillandier, D.; Dardevet, M.; Astruc, T.; Attaix, D.; et al. Curcumin treatment prevents increased proteasome and apoptosome activities in rat skeletal muscle during reloading and improves subsequent recovery. *J. Nutr. Biochem.* **2012**, *23*, 245–251. [CrossRef] [PubMed]
36. Anand, P.; Kunnumakkara, A.B.; Newman, R.A.; Aggarwal, B.B. Bioavailability of Curcumin: Problems and Promises. *Mol. Pharm.* **2007**, *4*, 807–818. [CrossRef]
37. Gutierres, V.O.; Campos, M.L.; Arcaro, C.A.; Assis, R.P.; Baldan-Cimatti, H.M.; Peccinini, R.G.; Paula-Gomes, S.; Kettelhut, I.C.; Baviera, A.M.; Brunetti, I.L. Curcumin Pharmacokinetic and Pharmacodynamic Evidences in Streptozotocin-Diabetic Rats Support the Antidiabetic Activity to Be via Metabolite(s). *Evid. Based Complement. Altern. Med.* **2015**, *2015*, 1–13. [CrossRef] [PubMed]
38. Baltaci, S.B.; Mogulkoc, R.; Baltaci, A.K. Resveratrol and exercise (review). *Biomed. Rep.* **2016**, *5*, 525–530. [CrossRef] [PubMed]
39. Liu, H.-W.; Su, Y.-K.; Bamodu, O.A.; Hueng, D.-Y.; Lee, W.-H.; Huang, C.-C.; Deng, L.; Hsiao, M.; Chien, M.; Yeh, C.-T.; et al. The Disruption of the β-Catenin/TCF-1/STAT3 Signaling Axis by 4-Acetylantroquinonol B Inhibits the Tumorigenesis and Cancer Stem-Cell-Like Properties of Glioblastoma Cells, In Vitro and In Vivo. *Cancers* **2018**, *10*, 491. [CrossRef]
40. Turner, P.V.; Brabb, T.; Pekow, C.; Vasbinder, M.A. Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. *J. Am. Assoc. Lab. Anim. Sci.* **2011**, *50*, 600–613.
41. Barreiro, E.; Puig-Vilanova, E.; Marin-Corral, J.; Chacon-Cabrera, A.; Salazar-Degracia, A.; Mateu, X.; Maestu, L.P.; Garcia-Arumi, E.; Andreu, A.L.; Molina, L. Therapeutic Approaches in Mitochondrial Dysfunction, Proteolysis, and Structural Alterations of Diaphragm and Gastrocnemius in Rats with Chronic Heart Failure. *J. Cell. Physiol.* **2015**, *231*, 1495–1513. [CrossRef] [PubMed]
42. Barthel, L.K.; Raymond, P.A. Improved method for obtaining 3-microns cryosections for immunocytochemistry. *J. Histochem. Cytochem.* **1990**, *38*, 1383–1388. [CrossRef] [PubMed]
43. Wilkie, G.S.; Schirmer, E.C. Purification of Nuclei and Preparation of Nuclear Envelopes from Skeletal Muscle. *Adv. Struct. Saf. Stud.* **2008**, *463*, 23–41. [CrossRef]
44. Vilà, L.; Elias, I.; Roca, C.; Ribera, A.; Ferre, T.; Casellas, A.; Lage, R.; Franckhauser, S.; Bosch, F. AAV8-mediated Sirt1 gene transfer to the liver prevents high carbohydrate diet-induced nonalcoholic fatty liver disease. *Mol. Ther. Methods Clin. Dev.* **2014**, *1*, 14039. [CrossRef] [PubMed]
45. Pasut, A.; Oleynik, P.; Rudnicki, M.A. Isolation of Muscle Stem Cells by Fluorescence Activated Cell Sorting Cytometry. *Adv. Struct. Saf. Stud.* **2011**, *798*, 53–64. [CrossRef]
46. Kuang, J.; Yan, X.; Genders, A.; Granata, C.; Bishop, D.J. An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. *PLoS ONE* **2018**, *13*, e0196438. [CrossRef] [PubMed]
47. Touchberry, C.D.; Wacker, M.J.; Richmond, S.R.; Whitman, S.A.; Godard, M.P. Age-Related Changes in Relative Expression of Real-Time PCR Housekeeping Genes in Human Skeletal Muscle. *J. Biomol. Tech.* **2006**, *17*, 157–162. [PubMed]
48. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
49. Kuno, A.; Tanno, M.; Horio, Y. The effects of resveratrol and SIRT1 activation on dystrophic cardiomyopathy. *Ann. N. Y. Acad. Sci.* **2015**, *1348*, 46–54. [CrossRef]
50. He, J.; Xie, H.; Wu, S. Dietary supplementation of curcumin alleviates NF-κB-dependent skeletal muscle wasting in rat. *Endocr. Metab. Immune Disord. Drug Targets* **2016**, *16*, 1. [CrossRef]
51. Ryall, J.; Dell'Orso, S.; Derfoul, A.; Juan, A.; Zare, H.; Feng, X.; Clermont, D.; Koulnis, M.; Gutierrez-Cruz, G.; Fulco, M.; et al. The NAD (+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell* **2015**, *16*, 171–183. [CrossRef]
52. Alway, S.E.; McCrory, J.L.; Kearcher, K.; Vickers, A.; Frear, B.; Gilleland, D.L.; Bonner, D.E.; Thomas, J.M.; Donley, D.A.; Lively, M.W.; et al. Resveratrol Enhances Exercise-Induced Cellular and Functional Adaptations of Skeletal Muscle in Older Men and Women. *J. Gerontol. Ser. A Boil. Sci. Med. Sci.* **2017**, *72*, 1595–1606. [CrossRef] [PubMed]
53. Mackey, A.L.; Kjaer, M.; Charifi, N.; Henriksson, J.; Bojsen-Møller, J.; Holm, L.; Kadi, F. Assessment of satellite cell number and activity status in human skeletal muscle biopsies. *Muscle Nerve* **2009**, *40*, 455–465. [CrossRef]

54. Pardo, P.S.; Boriek, A.M. The physiological roles of Sirt1 in skeletal muscle. *Aging* **2011**, *3*, 430–437. [CrossRef] [PubMed]
55. Jeong, J.; Conboy, M.J.; Conboy, I.M. Sirt1-Independent Rescue of Muscle Regeneration by Resveratrol in Type I Diabetes. *J. Diabetes Metab.* **2013**, *4*. [CrossRef]

nutrients

MDPI

Article

Effects of a Fat-Rich Diet on the Spontaneous Release of Acetylcholine in the Neuromuscular Junction of Mice

Carlos Gimenez-Donoso [1], Marc Bosque [2], Anna Vila [2], Gemma Vilalta [2] and Manel M Santafe [2,*]

[1] Centre de Fisioteràpia Inspira, Carrer Muntaner num 200, 2º, 2ª, 08036 Barcelona, Spain; carlos.gimenez@centroinspira.es

[2] Unit of Histology and Neurobiology, Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Rovira i Virgili University, Carrer St Llorenç num 21, 43201 Reus, Spain; fisioterapia.marc@gmail.com (M.B.); annaviimo@gmail.com (A.V.); gemmaspirit1@gmail.com (G.V.)

* Correspondence: manuel.santafe@urv.cat; Tel.: +34-97775-9300 (ext. 9343)

Received: 11 September 2020; Accepted: 16 October 2020; Published: 21 October 2020

Abstract: Western societies are facing a clear increase in the rate of obesity and overweight which are responsible for musculoskeletal pain. Some of the substances described in the environment of myofascial trigger points (MTrPs) are the same as those found in the skeletal muscle of obese people, such as cytokines. Furthermore, elevated neuromuscular neurotransmission has been associated with MTrPs. The main objective of this study is to assess whether obesity or overweight may be a facilitator of myofascial pain. The experiments were performed on male Swiss mice. One experimental group was given a typical "cafeteria" diet and another group a commercial high-fat diet for six weeks. Intramuscular adipocytes were assessed with Sudan III. The functional study was performed with electromyographic recording to determine the plaque noise and intracellular recording of miniature endplate potentials (MEPPs). The intake of a cafeteria diet showed the presence of more adipocytes in muscle tissue, but not with the fat-supplemented diet. Both experimental groups showed an increase in the plaque noise and an increase in the frequency of MEPPs that lasted several weeks after interrupting diets. In summary, the supply of a hypercaloric diet for six weeks in mice increases spontaneous neurotransmission, thus facilitating the development of MTrPs.

Keywords: electromyography; high-fat diet; myofascial pain syndrome; obesity; spontaneous neurotransmission

1. Introduction

At present, Western societies are having a clear increase in the rate of obesity and overweight [1]. From 1975 to 2016, the global obesity rate has tripled [2]. By 2030, over 38% of the world's adult population will be overweight and 20% will be obese [3]. Obesity and overweight are considered an epidemic related to the development of various pathologies such as diabetes, cardiovascular disease, metabolic syndrome and musculoskeletal pain [3].

There seems to be a strong relationship between obesity and pain [4]. Usually, the association between overweight and musculoskeletal pain has been attributed to an increase in the mechanical stress caused by overweight in load bearing joints. However, the literature also shows an association between pain and overweight in joints that do not support load [3,5–9]. For example, associations between overweight and symptomatic osteoarthritis of the hand [5], shoulder and neck pain [6], the number of episodes and intensity of migraine attacks [7,8], even with neuropathic pain [9] have also been described. Thus, the relationship between overweight and musculoskeletal pain appears to be at least, in part, independent of the mechanical overload and probably involves systemic phenomena.

Obesity is accompanied by a chronic inflammatory response with high production of adipokines (IL-6, TNF-α, adiponectin, leptin, and resistine) and macrophage infiltration into the adipose tissue [10]. This chronic inflammatory response has been linked to metabolic syndrome and musculoskeletal pain [11]. Moreover, musculoskeletal studies demonstrate the impact of these cytokines on muscle metabolism [12]. However, there are no studies that analyze the impact that obesity may have on other muscle functions or on muscle pain.

Myofascial pain syndrome (MPS) is the set of sensory (local and referred pain), motor (limited range of motion, weakness) and autonomic signs and symptoms caused by myofascial trigger points (MTrPs) [13]. The prevalence in the general population ranges from 20% to 90% and it is suggested that practically, all adults will suffer at least one episode of myofascial pain in their lifetime [14].

MTrPs have been proposed to be a neuromuscular dysfunction in which abnormal motor end plate function results in an excessive concentration of acetylcholine (ACh) in the synaptic cleft [15]. This excess ACh causes a localized contraction of the sarcomeres below the neuromuscular junction. Thereupon, a cascade of events that cause cellular suffering, local acidic pH, and the release of sensitizing substances from the nociceptive nerve terminals occur [16]. Altogether these changes are responsible for local pain. In 2005, Shah identified a high presence of several of these substances (pH, SP, IL-6, TNF-α, CGRP. . .) in the environment of MTrPs [16]. On the other hand, in our laboratory, we experimentally cause the appearance of contracted subsynaptic sarcomere by increasing local ACh with anticholinesterase drugs [17]. This increase in ACh release can be recorded by needle electromyography as spontaneous low-voltage electrical activity (30–60 mV) like endplate noise [14,17,18].

Some of the substances that Shah [16] found in the environment of active MTrPs are the same as those found in the skeletal muscle of obese people: IL-6, IL-1B, TNF-α [12]. No one has ever established a relationship between obesity and MPS before. However, given that in both clinical situations, a similar inflammatory profile surrounding muscle tissue is found, we hypothesized that muscle neurotransmission in obese or overweight individuals is increased, thus constituting a predisposing factor for the development of MTrPs.

In the present study, intracellular recordings and electromyography were performed at the end of the diet exposure period and several weeks after. An increase in the body weight of the mice was paralleled by a significant increase in the spontaneous release of acetylcholine that lasts for several weeks after diet manipulations. All the results obtained suggest that obesity and overweight can cause myofascial muscle pain.

2. Materials and Methods

The mice were cared for in accordance with the guidelines of the European Community's Council Directive (2010/63/EU) and the Spanish Royal Decree 53/2013 for the humane treatment of laboratory animals. The Animal Research Committee of the Universitat Rovirai Virgili (Reference number: 0233) reviewed and approved all experiments on animals. The experiments were performed on young (45–50 days) adult Swiss male mice (Charles River, L'Arbresle, France). Mice were habituated to the facility for at least 1 week prior to studies and were housed in groups of four, with sawdust bedding and ad libitum access to water and food throughout the study. The animals' rooms were maintained at a temperature of 22 ± 2 °C, a relative humidity of 50 ± 10%, and a 12 h light/dark automatic light cycle.

2.1. Animals and Dietary Protocol

Mice were randomly divided into cages of 2 animals. The cages were randomly grouped into three groups (see Figure 1): a control group (CTR; N = 10 cages, 20 animals), a "cafeteria diet" group (CAD; N = 10 cages, 20 animals) and a high-fat diet group (HFD; N = 10 cages, 20 animals). During the experiment, the CTR group received a normal laboratory diet (SAFE Diets: 230 HF Rat & Mouse Diet, Augy, France). A "cafeteria diet" model (detailed below) was added to the CAD group and a fat-supplemented diet (230 HF Rat & Mouse Diet, SAFE, Augy, France) was added to the HFD group [19]. Exposure to this type of diet was carried out during a period of 6 weeks. After 6 weeks,

both the cafeteria diet and the high-fat diet were withdrawn and all mice were fed exclusively with the regular rodent chow for 3 extra weeks. At the end of the diet exposure (6 weeks), 4 animals were sacrificed for histological studies and 4 animals for electrophysiological and electromyographical recordings. Then, during the next three weeks after diet exposure, 4 animals were sacrificed each week for electrophysiological and electromyographical recordings (Figure 1).

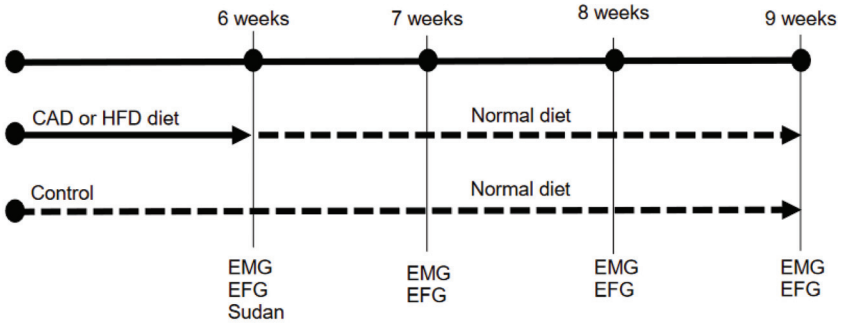


Figure 1. Timeline of the experimental procedure. CAD, cafeteria diet; HFD, high-fat diet; EMG, electromyography (endplate noise recordings); EFG, electrophysiology (intracellular recordings).

The diets supplied were:

All animals were provided with regular rodent chow (SAFE A04 diet, Panlab, Barcelona, Spain) ad libitum. The composition of this diet was shown in Table 1.

Table 1. Nutritional facts of the diets used.

	CAD	HFD	Regular Chow Diet
Calories (kcal)	459	532	397
Total Fat	23	60.6	6.9
Saturated Fat	11	21.7	-
Total Carbohydrate	56	26.3	68
Sugars	24	9.7	-
Dietary Fiber	2.5	-	-
Protein	5.3	13.1	25
Sodium	0.65	0.23	0.3

The nutritional data are expressed per 100 g. Cafeteria diet (CAD). High-fat diet (HFD).

The cafeteria diet (CAD) used in this study consisted of industrial pastries rich in saturated fat (cakes and cookies filled with chocolate) and fried peanuts [19–23]. The average composition is shown in Table 1. Every 2 days, the chopped pastries and peanuts were introduced together in the cage for the animals to eat *ad libitum*. Each time, the old cafeteria diet leftovers were removed and a new ration supplied. At all times, the animals had free access to their usual feed, so the animals continued to ingest the necessary nutrients so as not to suffer any nutritional deficiency. To ensure the amount of cafeteria diet eaten by the mice, the food introduced into the cage was weighed and the food debris was reweighed when removed. Each cage of 2 animals consumed 56 g of the CAD diet per week.

The high-fat diet (HFD), the other type of diet used in this study to induce overweight in animals was a diet enriched in fat (SAFE Diets: 230 HF Rat & Mouse Diet, Augy, France). Unlike the cafeteria diet, this diet is free of additives, colorants, stabilizers and flavorings that could interfere with the results [24–26]. The composition of this diet is shown in Table 1. This type of diet was placed inside

the cage to facilitate its availability. As with the cafeteria diet, the food was weighed before placing it in the cage and when removing the remains in the next supply. At all times, the animals also had free access to their usual feed to ensure that they continued to ingest the necessary nutrients and not incur any nutritional deficiencies. Each cage of 2 animals consumed 60 g of the HFD diet per week.

2.2. Muscles

Animals were deeply anesthetized with isoflurane before being euthanized by exsanguination. The *levator auris longus* (LAL) was excised and dissected on a Sylgard-coated Petri dish containing normal Ringer solution (containing (in mM): 135 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 15 $NaHCO_3$ and 11 glucose) continuously bubbled with 95% O_2/5% CO_2. The LAL muscles were used for methylene blue staining and immunologically labeled. The gastrocnemius muscles were used for electromyographic recordings. LAL muscles were used for Sudan III fat staining. The LAL is a small, flat muscle located immediately under the skin of the murine skull and is extremely useful for intracellular recording techniques (to visualize the muscle fibers and localize the possible synapses requires flat, thin, and transparent muscles). It is also useful for histological techniques since it does not require microtomy, thus minimizing the appearance of the artifacts.

2.3. Sudan III

This histological classical staining was performed in the LAL muscle of all the experimental groups and the controls at the end of the period of exposure (6 weeks). Sudan III stains lipids orange-red [27]. The LAL muscles were extracted and fixed in formalin.

The LAL muscle is a flat, thin muscle that does not require microtomy. Whole LAL muscles were immersed in the Sudan III preparation (50 mL of 50% alcohol, 50 mL acetone, 1 g Sudan III—Sigma-Aldrich, Steinheim, Germany) for 5 min. After cleaning the excess dye with 50% alcohol, a methylene blue contrast stain was performed (1 min). After washing off the excess under tap water, it was mounted on glycerin for visualization.

2.4. Endplate Noise Recordings

Electromyography (EMG) recordings were obtained from an anesthetized animal at controlled room temperature (22 °C–25.8 °C). The muscle used for this study was the gastrocnemius because of its ease of access and suitability. Recordings were obtained with an electromyography system (MedelecMystro plus, GR20) using a monopolar EMG needle (Natus Manufacturing Limited, London, UK) [17]. The needle was slowly inserted into the muscle and once inside, it was moved in order to enable recording in all directions. The muscle was divided into twelve areas to cover both the entire muscle and avoid recording the same endplate noise twice [17]. The recording needle was introduced into the gastrocnemius until an audible change was heard. The electromyography screen was then checked and if correct (without an alternating current, artifacts, etc.), the endplate noise was recorded. The number of areas with endplate noise (maximum twelve) and the frequency (number of potentials per second that appeared, expressed in Hz) were recorded.

2.5. Electrophysiology: Intracellular Recordings

Spontaneous miniature endplate potentials (MEPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (20–40 MΩ). Records were rejected if the membrane potential was <−50 mV or if it fell by more than 5 mV during the recording period.

The recording electrodes were connected to an amplifier (Tecktronics, AMS02, Tektronix, Inc., Beaverton, OR, US). A distant Ag–AgCl electrode connected to the bath solution via an Agar bridge (Agar 3.5% in 137 mMNaCl) was used as a reference. The MEPPs were digitized (DIGIDATA 1200 Interface, Axon Instruments Inc, San Jose, CA, USA), stored, and analyzed using a computer. The Axoscope 10.2 was used (Axon Instruments Inc.) for data acquisition and analysis. The MEPP frequency was recorded for 100 s from at least 15 different neuromuscular junctions and the mean values

were determined. The mean amplitude (mV) per fiber was calculated and corrected for non-linear summation [28], assuming a membrane potential of –80 mV.

2.6. Statistical Procedure

Values are expressed as the mean ± SEM. In some instances, the values are expressed as "percentage of change". This is defined as: (experimental value/control value) × 100. We used the two-tailed Welch's *t*-test for unpaired values because our variances were not equal. This test was chosen as it is more conservative than the ordinary *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Body Weight Evolution

All the mice in each of the three groups increased their body weight during the first 6 weeks. However, the two groups supplemented with the hypercaloric diets (CAD and HFD) increased their weight over the values of the control group (Table 2). At the end of the exposure, at 6 weeks, the group of mice subjected to a cafeteria diet increased their body weight by 52% more than the controls and the group of mice subjected to a high-fat diet increased their weight by 45% over the weight of the controls.

Table 2. Weight evolution.

Procedure	Age	Control	CAD	HFD
1: 6 weeks of age; **2**: 6 weeks with diet	12 weeks	38.00 ± 2.85 (n = 20)	58.03 ± 4.04 * (n = 20)	55.15 ± 1.39 * (n = 20)
1: 6 weeks of age; **2**: 6 weeks with diet; **3**: 1 week without diet	13 weeks	43.73 ± 0.88 (n = 12)	45.53 ± 1.03 (n = 12)	45.11 ± 2.11 (n = 12)
1: 6 weeks of age; **2**: 6 weeks with diet; **3**: 2 weeks without diet	14 weeks	43.24 ± 0.86 (n = 8)	45.50 ± 0.98 (n = 8)	43.97 ± 3.09 (n = 8)
1: 6 weeks of age; **2**: 6 weeks with diet; **3**: 3 weeks without diet	15 weeks	42.17 ± 2.85 (n = 4)	44.67 ± 1.22 (n = 4)	43.25 ± 2.57 (n = 4)

Values are expressed as the mean ± SEM. Cafeteria diet group, CAD. High-fat diet group, HFD. *, $p < 0.05$ with respect to the weight of the control animals.

By suppressing the supplementation of the cafeteria and the high-fat diets and maintaining the usual rodent chow, both groups reduced their weight from the first week to match the weight of the controls of the same age (Table 2).

It is confirmed that exposing the mice to 6 weeks of either a cafeteria diet or a high-fat diet causes overweight. By eliminating the hypercaloric diet supplementation, the animals reduced the previously acquired overweight by the first week.

3.2. Muscle Fat

In the group subjected to a cafeteria diet, a greater amount of adipocytes appeared between the muscle fibers of the LAL (Figure 2B) than in the controls (Figure 2A) and the HFD group (Figure 2C). This technique was applied to four animals per group (control, CAD and HFD).

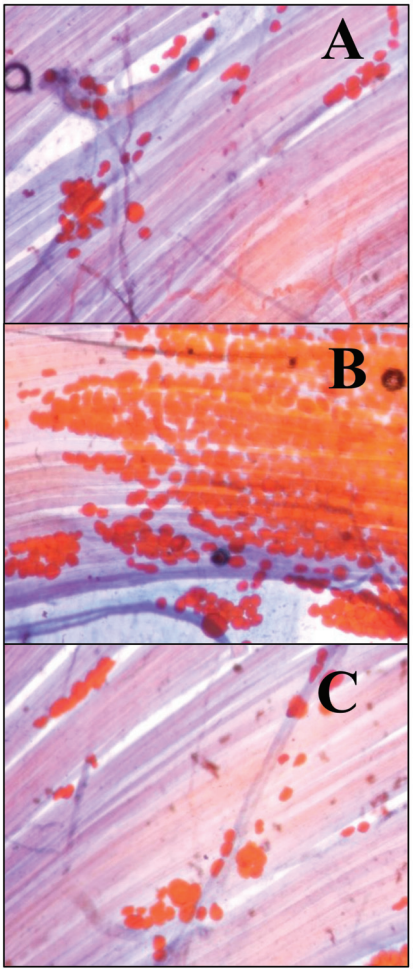


Figure 2. Intramuscular adipocytes. The adipocytes were stained with Sudan III. Fat looks orange. Methylene blue has been used as a contrast dye. (**A**) *Levator auris longus* (LAL) muscle from a control animal. (**B**) LAL muscle from an animal exposed to a cafeteria diet for 6 weeks. (**C**) LAL muscle from an animal exposed to a high-fat diet for 6 weeks. Initial magnification 400×.

3.3. Electrophysiology: Intracellular Recording

As shown in Figure 3, at the end of the 6 weeks of exposure to the cafeteria diet, a significant increase in the frequency of MEPPs was observed, which is maintained in the following three weeks after withdrawing from the cafeteria diet. On the other hand, at the end of the 6 weeks of exposure to the high-fat diet, a potent increase in the frequency of MEPPs was also observed, greater than in the CAD group (Figure 3). This increase in spontaneous neurotransmission decreased immediately from the first week after withdrawing from the high-fat diet, but remained high for the next 3 weeks.

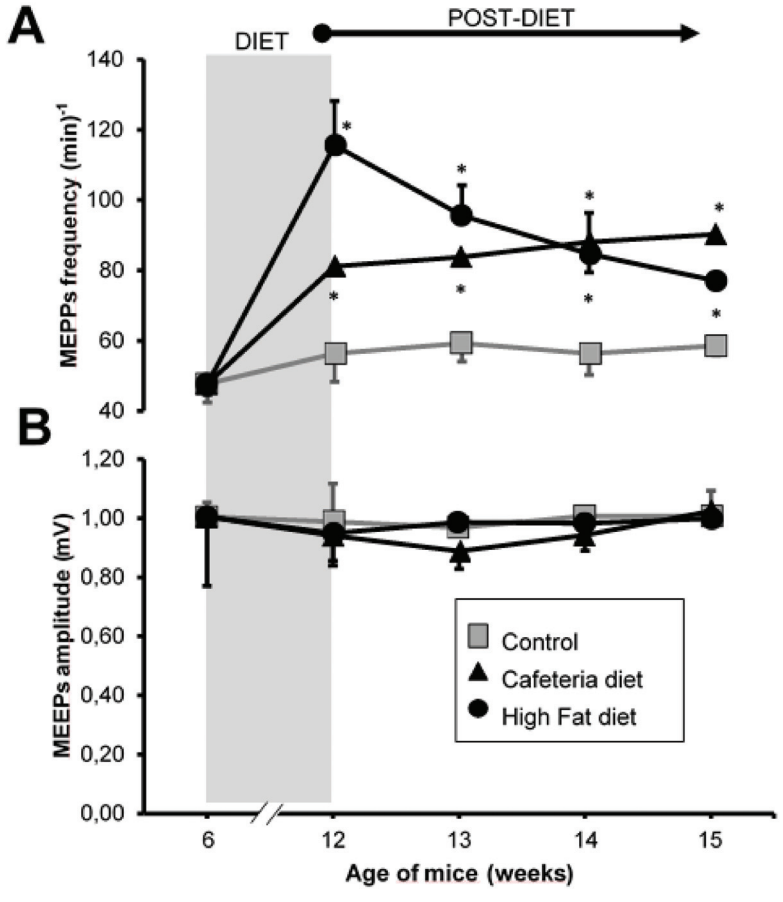


Figure 3. Intracellular recordings. (**A**) Frequency of miniature endplate potentials (MEPPs) expressed as number of events per minute. (**B**) Mean amplitude of the MEPPs expressed in mV. Grey area, period in which the animals were exposed to the CAD or HFD diets. Values are expressed as the mean ± SEM. For each experimental series, $N = 4$ animals. * $p < 0.05$ with respect to control values. Cafeteria diet group, CAD. High-fat diet group, HFD.

The size of the MEPPs did not change at any time in any of the experimental groups (Figure 3B). The data in Figure 3 are included in Supplementary Materials (Table S1).

3.4. Electromyography

Upon the hypercaloric diets, the number of areas with plaque noise increased similarly in both the CAD and HFD groups (Figure 4A). When the diet was withdrawn, the number of areas with plaque noise in the CAD group remain elevated for 2 weeks but in the HFD group, it was elevated for only one week.

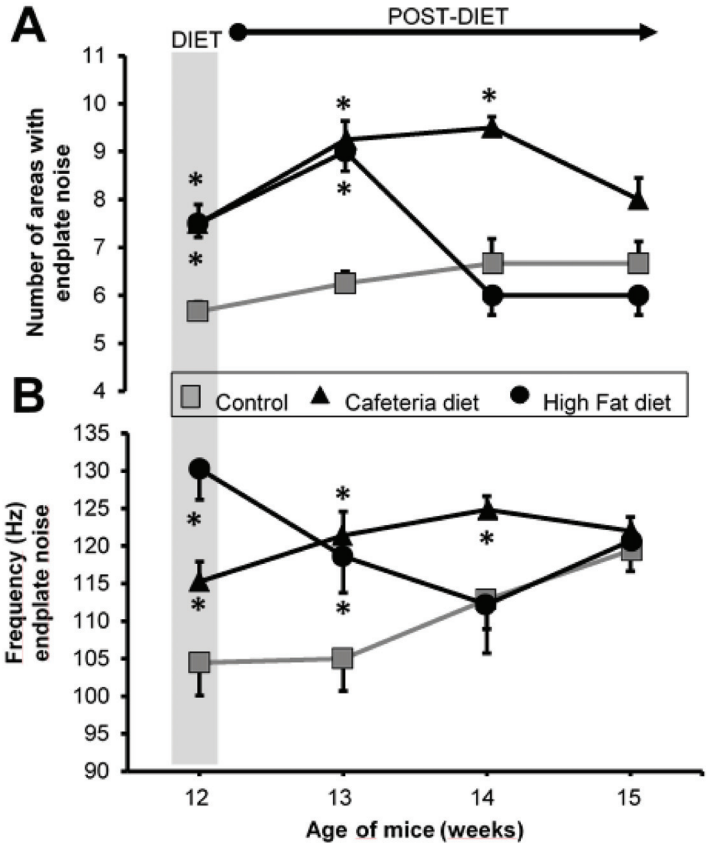


Figure 4. Electromyography. (**A**) Number of average areas with plate noise. (**B**) Each area with endplate noise was analyzed by quantifying the number of events/s (Hz). Grey area, end of the period in which the animals were exposed to the diets. Values are expressed as the mean ± SEM. For each experimental series, $N = 8$ gastrocnemius muscles from 4 animals. * $p < 0.05$ with respect to control values.

Regarding the number of events in each area with plaque noise, a similar increase was obtained in the MEPPs record (Figure 4B): it was more powerful in the HFD group than in the CAD group. When withdrawing the dietary supplementation, as with the MEPPs recording, the HFD group returned to the control values faster than the CAD group. However, at 3 weeks, the two groups achieved control values. The data in Figure 4 are included in Supplementary Materials (Table S2).

In summary, the increase in the release of ACh caused by the CAD and HFD diets tends to last longer in time once the supplementation is withdrawn.

4. Discussion

The main hypothesis of this study is that the accumulation of fat in the skeletal muscle of overweight individuals causes an increase in the spontaneous neurotransmission at the neuromuscular junction.

4.1. Overweight

Different types of diets useful in achieving overweight rodents have been described [19]. Initially, in the present study, a hypercaloric CAD was used. This type of diet has been shown to produce overweight mice by increasing the accumulation of fat in the different tissues [20]. However, this type of

diet contains several substances such as preservatives, colorings, salt, and processed sugars that could have an effect beyond the induction of overweight. In a bid to isolate the effects of being overweight, a second intervention group was used, which was administered as HFD, which has also been shown to induce overweight in mice [24], but which is free of the remaining substances that could interfere with the results.

Our results confirm that 6 weeks of exposure to both a CAD as used in our study, as well as a HFD, are sufficient to achieve a significant increase in the weight of the mice (50%) compared to individuals of the same age on a normal diet. Therefore, exposure to these diets is a suitable model for studying overweight.

Similarly, the weight gain achieved with both diets is quickly reversed to a normal diet from the first week. In this sense, other studies such as the one by Reynés et al. [21] have shown that it is possible to reverse the overweight caused by the administration of a cafeteria diet for 1.5 months following a normal diet for 1.5 months in rats.

Furthermore, the rapid weight reduction obtained in the first week of withdrawal from the cafeteria diet in the present study was consistent with the results obtained by other authors such as Lalanza et al. [22] These authors described that after one week of withdrawal from the cafeteria diet, the overweight caused in rats subjected to 2 months of the cafeteria diet was reversed. This rapid decrease in the weight of the animals was justified by Rogers in 1985 [23] by which the change from a more "tasty" diet to a diet based on feed causes severe hypophagia, especially in the first days after the change.

4.2. Muscle Fat

Once the weight gain of the mice in both groups was confirmed, it is interesting to see if this weight gain was accompanied by a greater presence of fat in the muscle tissue. The Sudan III technique allows identifying a greater number of adipocytes between the muscle fibers only in the group subjected to a cafeteria diet. However, in the group subjected to the fat diet, despite suffering the same weight gain, no differences were observed in the accumulation of adipocytes in the muscle tissue compared to the control group. Little variations in the diet composition are an important issue to take into consideration as the Cafeteria diet is higher in carbohydrates and levels of proteins are lower than those in the HFD.

There are previous studies that compare the cafeteria diet with a diet rich in fat for rats and describe, in addition to an increase in weight, an increase in visceral and subcutaneous fat. These studies showed that the changes caused by the cafeteria diet are greater than those caused by the high-fat diet [19,21]. It has been proposed that the cafeteria diet induces a greater hyperphagia in animals than the high-fat diets that cause a stabilization in the amount of daily intake after the first weeks [21]. In the present study, only muscle fat was studied and no assessment of other fat deposits was made. However, both groups significantly increased their weight, which suggests that HFD also caused accumulations of visceral fat, although, it did not infiltrate the muscle tissue.

Kahn et al. in 2015 [29] described an increased presence of intramuscular fat in mice which are subjected to a high-fat diet. However, these authors maintained the exposure to a high-fat diet for a much longer time—24 weeks—than in the current study. It is possible that to obtain an increase in muscle fat, the HFD group needs a longer exposure time to the diet. Note that in that study [29] an increase in macrophages in the muscle was demonstrated before the muscle fat was increased. In the present study, the study of the presence of fat 3 weeks after stopping the cafeteria diet was not carried out since weight is normalized.

4.3. Spontaneous Neurotransmission

There is an increase in spontaneous neurotransmission in mice subjected to both diets, this increase was more important in the HFD group than in CAD. When the diet was withdrawn, the HFD group maintained this increase for more weeks than the CAD group. The alterations registered intracellularly

and those registered with EMG do not behave the same since the intracellular recording is a more sensitive technique than EMG and therefore detects more subtle changes.

In the intracellular registry, the amplitude of the MEPPs was not modified in any experiment. This suggests that the possible accumulation of intramuscular fat does not affect the functional integrity of muscle fiber. In this sense, Garcia et al. [30], exposing muscles to anti-motor neuron IgG, injured the axons, but the muscle remains preserved, in such a way as to obtain a pathological increase in MEPPs without any variation of amplitude.

The supplementation of both diets causes an increase in spontaneous neurotransmission at the neuromuscular junction that persists for a time longer than the exposure to the diet. This result confirms only part of the hypothesis in the present study, which states that high-calorie diets cause an alteration in spontaneous muscle neurotransmission. However, the results obtained do not corroborate a correlation of this increase in neurotransmission with being overweight and the accumulation of muscle fat, since an increase in neurotransmission was found in the HFD despite no accumulation of muscle fat. These results open new questions regarding the mechanism by which an increase in spontaneous muscle neurotransmission is brought about by this type of diet. However, the fact that coherent changes appear in muscles as disparate functional strengthens the hypothesis that the studied effects of these diets on neurotransmission must be systemic in nature.

A possible factor that may explain these results is the involvement of the sympathetic nervous system. In a 2012 review, Smith and Minson [31] stated that there is sufficient evidence to show that increased fat deposits correlate with increased activity of the sympathetic nervous system in certain tissues such as the kidneys or skeletal muscle. Previously, in 1994, Scherrer [32] demonstrated that individuals with a BMI > 27 have a rate of sympathetic discharge to skeletal muscle that is twice as high as in lean individuals. On the other hand, Chen et al. [33] demonstrated that the activity of the sympathetic nervous system blocked by phentolamine may decrease the spontaneous neurotransmission recorded in a PGM. In addition, McNultty and colleagues [34] demonstrated that psychological stress can increase the spontaneous neurotransmission recorded in a PGM. It should be remembered that the neuromuscular junction of skeletal muscle in mice is innervated by the sympathetic nervous system [35]. Based on these data, it can be suggested that the mechanism by which the CAD is capable of increasing spontaneous neurotransmission is due to an excitation of the sympathetic nervous system caused more by the accumulation of fat than by the inflammatory state of the muscle secondary to fat. On the other hand, the spontaneous neurotransmission elevated and maintained for several weeks after stopping the diet and normalizing the weight of the mice could also be due to the increased activity of the sympathetic nervous system, in this case, it is secondary to the stress suffered by the animals when being deprived of the hypercaloric diet. This last situation has been described by Lalanza and co-authors [22], who observed that by suppressing a cafeteria diet in rats, an increase in their anxiety levels is caused. In this sense, it is known that the following diets based on tasty foods rich in fat can produce an addictive effect and its suppression generates a withdrawal syndrome that partly explains this increased anxiety [36,37].

On the other hand, the increase in neurotransmission may be due to the development of a pro-inflammatory state in the muscle because of diet. In this sense, Khan et al. [29] demonstrated that after only 2 weeks of HFD, macrophages already appear in the muscle fat tissue of mice. Furthermore, Fink et al. [25] showed that for mice exposed to a diet rich in fat, abundant pro-inflammatory macrophages and neutrophils appear early and that it increases with time. Taken together, these data suggest that from the first weeks of exposure to high-fat diets, a pro-inflammatory state begins to develop in muscle tissue, even before significant weight gains are achieved. In other words, changes could occur in the release of ACh without accumulations of fat in the muscle, as it occurs for the mice exposed to HFD in the present study.

A common factor for the two types of diet used in this study is their high content of saturated fat. It is well known that saturated fats have a direct effect on the immune system by activating the release of pro-inflammatory cytokines [38]. In a recent study, Song et al. [26] found that subjecting rats to a diet

rich in fat produced an increase in the postoperative pain in them. This fact coincides with other similar studies evaluating other types of pain models such as inflammatory or neuropathic [39,40]. In addition, Song et al. [26] demonstrated that a single week of a high-fat diet was not capable of producing obesity, but an increase in the pain response, although of lesser magnitude. That is, the increase in pain seems to be more related to the diet itself than to obesity. In addition, we also observed that the changes caused by an HFD returned faster to normality than the changes produced by cafeteria diet, which are maintained for one more week. This can be related to fat accumulation in muscle. All the changes observed between the two diets deserve more investigation and provides a model to investigate systemic vs. local effects of fat accumulation.

Within the possible relationship between the nervous system and the increase in spontaneous neurotransmission, some authors have proposed that some of the persistent changes in obese individuals after losing weight may be due to phenomena related to synaptic neuroplasticity [40]. In the results obtained in the present study, this phenomenon could intervene in the maintenance of elevated spontaneous neurotransmission after stopping the diet.

Several studies correlate the administration of high-fat diets or cafeteria diets with being overweight and with significant increases in the pain response of different pain models: neuropathic, postoperative, and inflammatory [26,39,40]. Currently, there are no studies that assess whether this type of diet has any influence on myofascial pain. The central factor of MPS are the MTrPs and their essential characteristic is an increased spontaneous neurotransmission [18]. The present study suggests that this type of hypercaloric diets could facilitate the development of MTrPs by increasing the spontaneous neurotransmission they generate. However, it is not clear if the effects come exclusively from diet or if being overweight and accumulating fat may also play a relevant role.

5. Conclusions

Exposure for six weeks to a hypercaloric diet (cafeteria or highfat) causes overweight in mice, an increase in adipocytes in muscle tissues and an increase in spontaneous neuromuscular neurotransmission. Upon abandoning the diets, the mice recover their weight rapidly, but spontaneous neuromuscular neurotransmission remains elevated. In other words, the alteration of spontaneous neurotransmission is not exclusively related to being overweight or to an increase in muscle fat. Overall, it can be concluded that exposure to a hypercaloric diet for 6 weeks in mice may be a predisposing factor for the development of MPS and other muscle alterations aggravated by a maintained increase in spontaneous neuromuscular neurotransmission.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/10/3216/s1, Table S1: Figure 3 data, Table S2: Figure 4 data.

Author Contributions: Methodology, investigation: C.G.-D.; conceptualization, formal analysis: M.B.; investigation, resources, roles/writing—original draft: A.V.; validation, visualization: G.V.; supervision, writing—review and editing: M.M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We greatly appreciate the suggestions of M.T. Colomina in the draft of this article.

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

References

1. Hruby, A.; Hu, F.B. The epidemiology of obesity: A Big Picture. *Pharmacoeconomics* **2015**, *33*, 673–689. [CrossRef] [PubMed]
2. Obesidad y Sobrepeso. Available online: https://www.who.int/es/news-room/fact-sheets/detail/obesity-and-overweight (accessed on 26 July 2020).
3. Kelly, T.; Yang, W.; Chen, C.S.; Reynolds, K.; He, J. Global burden of obesity in 2005 and projections to 2030. *Int. J. Obes.* **2008**, *32*, 1431–1437. [CrossRef] [PubMed]
4. Okifuji, A.; Hare, B. The association between chronic pain and obesity. *J. Pain Res.* **2015**, *8*, 399. [CrossRef]

5. Grotle, M.; Hagen, K.B.; Natvig, B.; Dahl, F.A.; Kvien, T.K. Obesity and osteoarthritis in knee, hipand/or hand: An epidemiological study in the general population with years follow-up. *BMC Musculoskelet. Disord.* **2008**, *9*, 132. [CrossRef] [PubMed]
6. Luime, J.J.; Kuiper, J.I.; Koes, B.W.; Verhaar, J.A.; Miedema, H.S.; Burdorf, A. Work-related risk factorsfor the incidence and recurrence of shoulder and neck complaints among nursing-home andelderly-care workers. *Scand. J. Work Environ. Health* **2004**, *30*, 279–286. [CrossRef] [PubMed]
7. Bigal, M.E.; Lipton, R.B. Obesity is a risk factor for transformed migraine but not chronictension-type headache. *Neurology* **2006**, *67*, 252–257. [CrossRef] [PubMed]
8. Bigal, M.E. Body Mass Index and Episodic Headaches: A Population-Based Study. *Arch. Intern. Med.* **2007**, *167*, 1964–1970. [CrossRef]
9. Hozumi, J.; Sumitani, M.; Matsubayashi, Y.; Abe, H.; Oshima, Y.; Chikuda, H.; Takeshita, K.; Yamada, Y. Relationship between Neuropathic Pain and Obesity. *Pain Res. Manag.* **2016**, *2016*, 2487924. [CrossRef]
10. Bastard, J.P.; Maachi, M.; Lagathu, C.; Kim, M.J.; Caron, M.; Vidal, H.; Capeau, J.; Feve, B. Recent advances in therelationship between obesity, inflammation, and insulin resistance. *Eur. Cytokine Netw.* **2006**, *17*, 4–12.
11. Gandhi, R.; Perruccio, A.V.; Rizek, R.; Dessouki, O.; Evans, H.M.K.; Mahomed, N.N. Obesity-Related Adipokines Predict Patient-Reported Shoulder Pain. *Obes. Facts* **2013**, *6*, 536–541. [CrossRef]
12. Wu, H.; Ballantyne, C.M. Skeletal muscle inflammation and insulin resistance in obesity. *J. Clin. Investig.* **2017**, *127*, 43–54. [CrossRef]
13. Simons, D.G.; Travell, J.G.; Fernández de las Peñas, C.; Finnegan, M.; Freeman, J.L.; Donnelly, J.M. *Travell, Simons & Simons' Myofascial Pain and Dysfunction: The Trigger Point Manual*, 3rd ed.; Wolters Kluwer: Philadelphia, PA, USA, 2019.
14. Mayoral, O.; Salvat, I. *Fisioterapia Invasiva del Síndrome de Dolor Myofascial*, 1st ed.; Médica Panamericana: Madrid, Spain, 2017.
15. Gerwin, R.D.; Dommerholt, J.; Shah, J.P. An expansion of Simons' integrated hypothesis of trigger point formation. *Curr. Pain Headache Rep.* **2004**, *8*, 468–475. [CrossRef] [PubMed]
16. Shah, J.P.; Phillips, T.M.; Danoff, J.V.; Gerber, L.H. An in vivo microanalytical technique for measuring the local biochemical milieu of human skeletal muscle. *J. Appl. Physiol.* **2005**, *99*, 1977–1984. [CrossRef] [PubMed]
17. Margalef, R.; Sisquella, M.; Bosque, M.; Romeu, C.; Mayoral, O.; Monterde, S.; Priego, M.; Guerra-Perez, R.; Ortiz, N.; Tomàs, J.; et al. Experimental myofascial trigger point creation in rodents. *J. Appl. Physiol.* **2019**, *126*, 160–169. [CrossRef]
18. Simons, D.G.; Hong, C.Z.; Simons, L.S. Endplate Potentials Are Common to Midfiber Myofacial Trigger Points. *Am. J. Phys. Med. Rehabil.* **2002**, *81*, 212–222. [CrossRef]
19. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome With Liver and Adipose Inflammation: Comparison to High-Fat Diet. *Obesity* **2011**, *19*, 1109–1117. [CrossRef] [PubMed]
20. Zeeni, N.; Dagher-Hamalian, C.; Dimassi, H.; Faour, W.H. Cafeteria diet-fed mice is a pertinent model of obesity-induced organ damage: A potential role of inflammation. *Inflamm. Res.* **2015**, *64*, 501–512. [CrossRef] [PubMed]
21. Reynés, B.; García-Ruiz, E.; Díaz-Rúa, R.; Palou, A.; Oliver, P. Reversion to a control balanced diet is able to restore body weight and to recover altered metabolic parameters in adult rats long-term fed on a cafeteria diet. *Food Res. Int.* **2014**, *64*, 839–848. [CrossRef]
22. Lalanza, J.F.; Caimari, A.; del Bas, J.M.; Torregrosa, D.; Cigarroa, I.; Pallàs, M. Effects Of A Post-Weaning Cafeteria Diet In Young Rats: Metabolic Syndrome, Reduced Activity And Low Anxiety-Like Behaviour. *PLoS ONE* **2014**, *9*, e85049. [CrossRef]
23. Rogers, P.J. Returning "cafeteria-fed" rats to a chow diet: Negative contrast and effects of obesity on feeding behaviour. *Physiol. Behav.* **1985**, *35*, 493–499. [CrossRef]
24. Winzell, M.S.; Ahren, B. The High-Fat Diet-Fed Mouse: A Model for Studying Mechanisms and Treatment of Impaired Glucose Tolerance and Type 2 Diabetes. *Diabetes* **2004**, *53*, S215–S219. [CrossRef] [PubMed]
25. Fink, L.N.; Costford, S.R.; Lee, Y.S.; Jensen, T.E.; Bilan, P.J.; Oberbach, A.; Blüher, M.; Olefsky, J.M.; Sams, A.; Klip, A. Pro-Inflammatory macrophages increase in skeletal muscle of high fat-Fed mice and correlate with metabolic risk markers in humans: Muscle Macrophages in Obesity and Diabetes. *Obesity* **2014**, *22*, 747–757. [CrossRef]

26. Song, Z.; Xie, W.; Chen, S.; Strong, J.A.; Print, M.S.; Wang, J.I.; Shareef, A.F.; Ulrich-Lai, Y.M.; Zhang, J.M. High-fat diet increases pain behaviors in rats with or without obesity. *Sci. Rep.* **2017**, *7*, 1–14. [CrossRef]
27. Gasparin, F.R.S.; Carreño, F.O.; Mewes, J.M.; Gilglioni, E.H.; Pagadigorria, C.L.S.; Natali, M.R.M.; Utsunomiya, K.S.; Constantin, R.P.; Ouchida, A.T.; Curti, C.; et al. Sex differences in the development of hepatic steatosis in cafeteria diet-induced obesity in young mice. *Biochim. Biophys. Acta Mol. Basis Dis.* **2018**, *1864*, 2495–2509. [CrossRef] [PubMed]
28. McLachlan, E.M.; Martin, A.R. Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol.* **1981**, *311*, 307–324. [CrossRef]
29. Khan, I.M.; Perrard, X.Y.; Brunner, G.; Lui, H.; Sparks, L.M.; Smith, S.R.; Wang, X.; Shi, Z.Z.; Lewis, D.E.; Wu, H.; et al. Intermuscular and perimuscular fat expansion in obesity correlates with skeletal muscle T cell and macrophage infiltration and insulin resistance. *Int. J. Obes.* **2015**, *39*, 1607–1618. [CrossRef] [PubMed]
30. García, J.; Engelhardt, J.I.; Appel, S.H.; Stefani, E. Increased MEPP frequency as an early sign of experimental immune-mediated motoneuron disease: Early Sign of Experimental MND. *Ann. Neurol.* **1990**, *28*, 329–334. [CrossRef]
31. Smith, M.M.; Minson, C.T. Obesity and adipokines: Effects on sympathetic overactivity: Adipokines and sympathetic outflow. *J. Physiol.* **2012**, *590*, 1787–1801. [CrossRef]
32. Scherrer, U.; Randin, D.; Tappy, L.; Vollenweider, P.; Jéquier, E.; Nicod, P. Body fat and sympathetic nerve activity in healthy subjects. *Circulation* **1994**, *89*, 2634–2640. [CrossRef]
33. Chen, J.T.; Chen, S.M.; Kuan, T.S.; Chung, K.C.; Hong, C.Z. Phentolamine effect on the spontaneous electrical activity of active loci in a myofascial trigger spot of rabbit skeletal muscle. *Arch. Phys. Med. Rehabil.* **1998**, *79*, 790–794. [CrossRef]
34. McNulty, W.H.; Gevirtz, R.N.; Hubbard, D.R.; Berkoff, G.M. Needle electromyographic evaluation of trigger point response to a psychological stressor. *Psychophysiology* **1994**, *31*, 313–316. [CrossRef] [PubMed]
35. Khan, M.M.; Lustrino, D.; Silveira, W.A.; Wild, F.; Straka, T.; Issop, Y.; O'Connor, E.; Cox, D.; Reischl, M.; Marquardt, T.; et al. Sympathetic innervation controls homeostasis of neuromuscular junctions in health and disease. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 746–750. [CrossRef] [PubMed]
36. Coccurello, R.; Maccarrone, M. Hedonic Eating and the "Delicious Circle": From Lipid-Derived Mediators to Brain Dopamine and Back. *Front. Neurosci.* **2018**, *12*, 271. [CrossRef]
37. Stice, E.; Figlewicz, D.P.; Gosnell, B.A.; Levine, A.S.; Pratt, W.E. The contribution of brain reward circuits to the obesity epidemic. *Neurosci. Biobehav. Rev.* **2013**, *37*, 2047–2058. [CrossRef]
38. Totsch, S.K.; Waite, M.E.; Sorge, R.E. Dietary Influence on Pain via the Immune System. *Prog. Mol. Biol. Transl. Sci.* **2015**, *131*, 435–469. [PubMed]
39. Totsch, S.K.; Quinn, T.L.; Strath, L.J.; McMeekin, L.J.; Cowell, R.M.; Gower, B.A.; Sorge, R.E. The impact of the Standard American Diet in rats: Effects on behavior, physiology and recovery from inflammatory injury. *Scand. J. Pain* **2017**, *17*, 316–324. [CrossRef]
40. Matikainen-Ankney, B.A.; Kravitz, A.V. Persistent effects of obesity: A neuroplasticity hypothesis: Neuroplasticity hypothesis of obesity. *Ann. N. Y. Acad. Sci.* **2018**, *1428*, 221–239. [CrossRef]

nutrients

MDPI

Article

L-Cysteine and Vitamin D Co-Supplementation Alleviates Markers of Musculoskeletal Disorders in Vitamin D-Deficient High-Fat Diet-Fed Mice

Rajesh Parsanathan, Arunkumar E. Achari, Prasenjit Manna and Sushil K. Jain *

Department of Pediatrics and Center for Cardiovascular Diseases and Sciences, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130, USA; rparsa@lsuhsc.edu or rajesh.uom@gmail.com (R.P.); bioarun1985@gmail.com (A.E.A.); pmanna2012@gmail.com (P.M.)
* Correspondence: sjain@lsuhsc.edu; Tel.: +1-318-675-6086

Received: 24 September 2020; Accepted: 3 November 2020; Published: 6 November 2020

Abstract: Vitamin D (VD) deficiency is associated with musculoskeletal disorders. This study examines whether co-supplementation of L-cysteine (LC) and VD is better than monotherapy with LC or VD at alleviating musculoskeletal dyshomeostasis in the skeletal muscle of VD-deficient high-fat diet (HFD-VD-) fed mice. Mice were fed a healthy diet or an HFD; for VD-deficient animals, the mice were maintained on a HFD-VD-diet (16 weeks); after the first 8 weeks, the HFD-VD-diet-fed mice were supplemented for another 8 weeks with LC, VD-alone, or the same doses of LC + VD by oral gavage. Saline and olive oil served as controls. Myotubes were exposed with high-glucose, palmitate, Monocyte Chemoattractant Protein 1 (MCP-1), and Tumor Necrosis Factor (TNF), to mimic the in vivo microenvironment. In vitro deficiencies of glutathione and hydrogen sulfide were induced by knockdown of GCLC and CSE genes. Relative gene expression of biomarkers (myogenic: MyoD, Mef2c, Csrp3; muscle dystrophy: Atrogin1, Murf1, and Myostatin; bone modeling and remodeling: RANK, RANKL, OPG) were analyzed using qRT-PCR. Co-supplementatoin with LC + VD showed beneficial effects on gene expression of myogenic markers and OPG but reduced markers of dystrophy, RANK/RANKL in comparison to LC or VD alone-supplementation. In vitro myotubes treated with glutathione (GSH) precursors also showed a positive effect on OPG and the myogenesis genes, and inhibited RANK/RANKL and muscle-dystrophy markers. This study reveals that the co-supplementation of LC with VD significantly alleviates the markers of musculoskeletal disorders in the skeletal muscle better than monotherapy with LC or VD in HFD-VD-fed mice.

Keywords: vitamin D deficiency; L-cysteine; glutathione; myogenic markers; dystrophy markers; skeletal muscle

1. Introduction

Vitamin D (VD) deficiency or insufficiency is associated with diseases affecting muscle and bone health [1–3]. Low blood levels of both 25-hydroxyvitamin D (25(OH)D) and glutathione (GSH) are positively associated with metabolic syndrome in human subjects [4–8]. Antioxidant GSH deficiency increases the oxidative stress that may favor endogenous protein oxidative modification, impairs cellular physiology, and leads to the disease's manifestation. Supplementation with GSH or its precursor, the sulfur-containing amino acid L-cysteine (LC), has been successfully used to improve the GSH status in blood and tissues, reducing immune-metabolic syndrome [4–6,8].

Skeletal muscle is the largest tissue in the body, and any loss of function or regenerative properties debilitates the musculoskeletal system [9]. Myogenic markers such as myoblast determination protein 1 (MyoD), myocyte enhancer factor 2C (Mef2c), and cysteine and glycine-rich protein 3 (Csrp3) are positive regulators and promote myogenesis, regeneration, and play an essential role in muscle

function [10,11]. Conversely, skeletal muscle-specific F-box protein (Atrogin1), muscle RING-finger protein-1 (Murf1), and Myostatin (Mstn) are critical molecules involved in muscle atrophy [12,13]. Skeletal muscle dystrophy/atrophy is a debilitating consequence of many pathological conditions and diseases [14]. Receptor activator of nuclear factor-kB (RANK), its ligand RANKL, and the soluble decoy receptor osteoprotegerin (OPG) pathway control bone remodeling and homeostasis [15,16]. The effects of RANK/RANKL/OPG extend well beyond its classical functions; in skeletal muscle, interaction with RANKL/RANK causes atrophy and dysfunction, whereas OPG provides significant protection against muscle damage [15].

Studies have shown that high-fat diet (HFD)-induced obesity leads to skeletal muscle oxidative stress, inflammation, and muscle mass loss by decreasing myogenic markers such as MyoD, Mef2c, and Csrp3, increasing muscle dystrophy markers such as Atrogin1, Murf1, and Myostatin [17–19]. Supplementation with a cysteine/thiol-based antioxidant delays or attenuates muscular dysfunction [20–22]. Similarly, supplementation with N-acetyl cysteine decreases osteoclast differentiation and increases bone mass in obese diabetic mice [23]. VD also maintains a normal bone resorption rate and formation through the RANKL/OPG signal [24]. VD deficiency is detrimental to muscle function, independent of alterations in phosphate and calcium levels [25]. Observational studies of VD-deficiency also associate reduced muscle mass and weakness [1–3]. However, interventional trials and meta-analyses of VD deficiency have yielded contradictory findings [25]. Our previous preclinical studies demonstrate that GSH epigenetically regulates VD metabolism genes. Supplementation with the VD + LC combination was more successful at boosting 25(OH)D levels by improving the status of VD metabolism genes in the liver, kidney, and muscle [4–8,26]. However, as far as we know, no previous study has examined the effect of co-supplementation with LC+VD on musculoskeletal markers in the muscle of HFD-VD-mice.

This study reports that LC (a GSH precursor) co-supplementation with VD significantly alleviates dyshomeostasis of the skeletal muscle in VD-deficient high-fat diet-fed mice, suggesting that combined supplementation with the nutraceuticals LC + VD could be a better option for musculoskeletal system disorders rather than supraphysiological monotherapy with VD alone.

2. Materials and Methods

The reagents used in the study, and all other chemicals were of analytical and molecular grade unless otherwise mentioned, and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Animal Experimental Design and Treatment

Male C57BL/6J mice (5 weeks old, 20–24 g) were procured from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were given access to food and water ad libitum and housed for acclimation (1 week) in a temperature-controlled room (22 ± 2 °C) with light/dark cycles (12/12 h). They were maintained under standard housing conditions throughout the experiment. After receiving approval (P-15-006) from the Institutional Animal Committee, according to the guidelines of the institution's ethical standards, all the procedures were performed.

Mice were randomized, labeled in individual cages, and divided into various groups. The mice were fasted overnight and tested for hyperglycemia by measuring their blood glucose concentrations before starting the treatment plan. Fasting blood glucose was analyzed by the tail prick method using a glucometer (Accu-Chek, Boehringer Mannheim Corp., Indianapolis, IN, USA).

Control animals were fed a healthy diet (Ctrl; lower in fat), while animals in the high-fat diet group were fed a high-fat diet (HFD) for a total of 16 weeks. The mice were maintained on a VD-deficient HFD (HFD-VD-) for 16 weeks (to mimic the VD-deficient condition). After the first 8 weeks, the mice were supplemented by oral gavage for another 8 weeks with either 5 mg LC/kg BW daily (LC), 67 IU VD/kg BW (VD) alone, or the same doses of LC + VD co-supplemented to HFD-VD-mice. Additionally, two control groups of HFD-VD-mice were maintained on oral gavage with either saline (S-Ctrl) or the same dose of the vehicle olive oil used for dissolving cholecalciferol

(OO-Ctrl). These diets—(healthy diet, HFD, and HFD-VD-) composition details and dose justification for LC and vitamin D—are given in our recent publication [4].

Sex steroids, estrous cycle, and hormonal impacts (sexual dimorphism) influence musculoskeletal markers [27–31]; therefore, we chose only male mice for this pilot study. Furthermore, choosing a 22 ± 2 °C housing temperature better represents humans living in colder regions that also lack environmental temperature controls (to mimic human thermal relation) [32,33].

As mentioned above, at the end of the supplementation, the animals were isoflurane euthanized and then perfused with cold saline. Skeletal muscle (gastrocnemius) was collected immediately, quickly diced, and frozen in liquid nitrogen at −80 °C. This model (HFD and HFD-VD-) of dietary-induced insulin resistance created fasting hyperglycemia, hyperinsulinemia, elevated proinflammatory cytokines, decreased glutathione, and VD deficiency, thus representing a reasonable model of the human condition [4–6,34].

2.2. Cell Culture, Treatments, and RNA Interference of GCLC and CSE

Mouse C_2C_{12} myoblasts (ATCC® CRL-1772™, Manassas, VA, USA) were cultured and brought to myotubes differentiation with appropriate conditions following the methods used in our previous published studies [5,6,35,36]. High glucose (HG; 25 mM), palmitate (0.6 mM) for 24 h; MCP-1 (2.5 ng/mL) and TNF (250 pg/mL) for 6 h treated to the myotubes, the doses and time points justifications given in our previous published studies [5,6,35,36]. GCLC or CSE and Control siRNA-A (50 and 100 nM siRNA), transiently transfected as per the method described earlier [4–6,35,36]. GSH precursor (L-cysteine; 300 μM) or an H_2S donor (NaHS; 20 μM) were treated following the methods used in our previous published studies [5,6,35,36] to boost the level of GSH cellular content and H_2S production. Cell viability was determined using the Alamar Blue reduction bioassay in all the experimental conditions [5,6,35,36].

2.3. Relative Gene Expression

RNA isolated from cells or tissues, quality and concentration determined, and 1 μg of RNA samples were reverse transcribed, and the qPCR performed as per our previously published studies [5,6,35,36]. The following primer/probe sets from Applied Biosystems™ TaqMan™ Gene Expression Assays were used for qPCR; MyoD (Mm00440387_m1), Mef2c (Mm01340842_m1), Csrp3 (Mm00443379_m1), Atrogin1/Fbxo32 (Mm00499523_m1), MuRF1/Trim63 (Mm01185221_m1), Mstn/Myostatin (Mm01254559_m1), Rank/Tnfrsf11a (Mm00437132_m1), RankL/Tnfsf11 (Mm00441906_m1), Opg/Tnfrsf11b (Mm01205928_m1) and Gapdh (Mm99999915_g1). The results were expressed as the relative quantification (RQ, the fold of control).

2.4. Statistical Analyses

Data were generated from multiple repeats of different biological experiments to obtain the mean values and standard errors of the mean. Significance was set at $p < 0.05$, and statistical differences were evaluated using two-way ANOVA followed by Dunnett's multiple comparisons test between groups were conducted using GraphPad Prism 8.2.1 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Effect of HFD, VD-Deficient HFD, and L-Cysteine and Vitamin D Co-Supplementation on Gene Expression of Musculoskeletal Markers in Mouse Skeletal Muscle

The skeletal muscle of HFD-fed mice showed attenuated myogenic markers (MyoD, Mef2c, and Csrp3) (Figure 1a), but there were no significant alterations in muscle dystrophy markers such as Atrogin1, Murf1, and Myostatin (Figure 1b). Only osteoprotegerin was downregulated in the RANK/RANKL/OPG system (Figure 1c). VD-deficient HFD-fed mice's skeletal muscle showed downregulation of myogenic markers similar to those seen in the HFD-fed mice (Figure 1a). However, muscle dystrophy markers increased significantly in the skeletal muscle of the HFD-VD- group

compared to those in the HFD group (Figure 1b). Compared to skeletal muscle in HFD-fed mice, the mRNA level of RANK/RANKL increased significantly in the HFD-VD- group, but the level of OPG was significantly downregulated in HFD-VD- group (Figure 1c).

Groups supplemented with L-cysteine or vitamin D alone showed a partially significant beneficial effect on markers such as MyoD, Mef2c, and OPG (Figure 1a,c). However, supplementation with LC or VD alone, or co-supplementation, significantly suppressed muscle the dystrophy markers, RANK, and RANKL in mouse skeletal muscle compared to results in the HFD-VD- group (Figure 1b). LC and VD co-supplementation more significantly alleviated myogenic markers and OPG (Figure 1a,c) in mouse skeletal muscle compared to results in the HFD and HFD-VD- groups, including those supplemented with LC or VD alone.

These findings indicated that co-supplementing LC with VD enhanced the beneficial effects against musculoskeletal disorder marker gene expression in skeletal muscle compared to monotherapy supplementation with LC or VD.

3.2. Impact of High Glucose, Palmitate, and Inflammatory Cytokines on Musculoskeletal Markers

Myotubes were exposed to high glucose-mediated glucotoxicity, palmitate-mediated lipotoxicity, and inflammatory cytokines to mimic low-grade inflammation models, which is observed in both obesity and diabetes. Glucolipotoxicity significantly downregulated the mRNA levels of the myogenic markers (MyoD, Mef2c, and Csrp3), and OPG, but the levels of dystrophy markers (Atrogin1, Murf1, and Myostatin), RANK, and RANKL were elevated (Figure 2a) compared to the control group. Inflammatory cytokines did not alter the level of myogenic markers. Proinflammatory cytokines such as MCP-1 and TNF elevated the expression of dystrophy markers, RANK, and RANKL (Figure 2b) compared to that in the control group. Collectively, this result indicates that glucolipotoxicity negatively affects the myogenic markers, dystrophy markers, and RANK/RANKL/OPG system, while inflammatory cytokines in vitro induce dystrophy markers, RANK, and RANKL. They may also contribute to musculoskeletal disorders.

3.3. The Deficiency of Transsulfuration Pathway Key Genes GCLC and CSE (Knockdown) in Myotubes Affects Musculoskeletal Markers

The expression of myogenic markers and OPG was attenuated in the GCLC, and CSE siRNA treated myotubes (Figure 3a,b), but the levels of dystrophy markers, RANK, and RANKL increased significantly compared to those of the control group (Figure 3a,b). Altogether, these data demonstrate that inhibited flow in the rate-limiting sulfur-containing amino acid (L-cysteine) pathway leads to a deficiency in the physiological antioxidant glutathione (GSH) or hydrogen sulfide (H_2S), which alone or synergistically, may alter the expression of the musculoskeletal marker genes.

3.4. GSH and H_2S Inhibit Muscle Dystrophy Markers and Positively Induce Myogenic Markers Genes

The possible beneficial effect of H_2S or GSH on the expression of genes involved in myogenesis, muscle dystrophy, and the RANK/RAKL/OPG system was explored with the antioxidant precursors L-cysteine (a GSH/H_2S precursor) or NaHS (an H_2S donor) following the methods used in previous publications [4,6]. Results showed that compared to levels in the control group, the mRNA levels of myogenic genes and OPG significantly increased following LC or NaHS treatment, which also decreased dystrophy markers, RANK, and RANKL (Figure 4a). These responses to treatment with LC and NaHS indicate that GSH and H_2S may directly or indirectly affect these genes and suggest that H_2S and GSH may have a beneficial effect on muscle physiology.

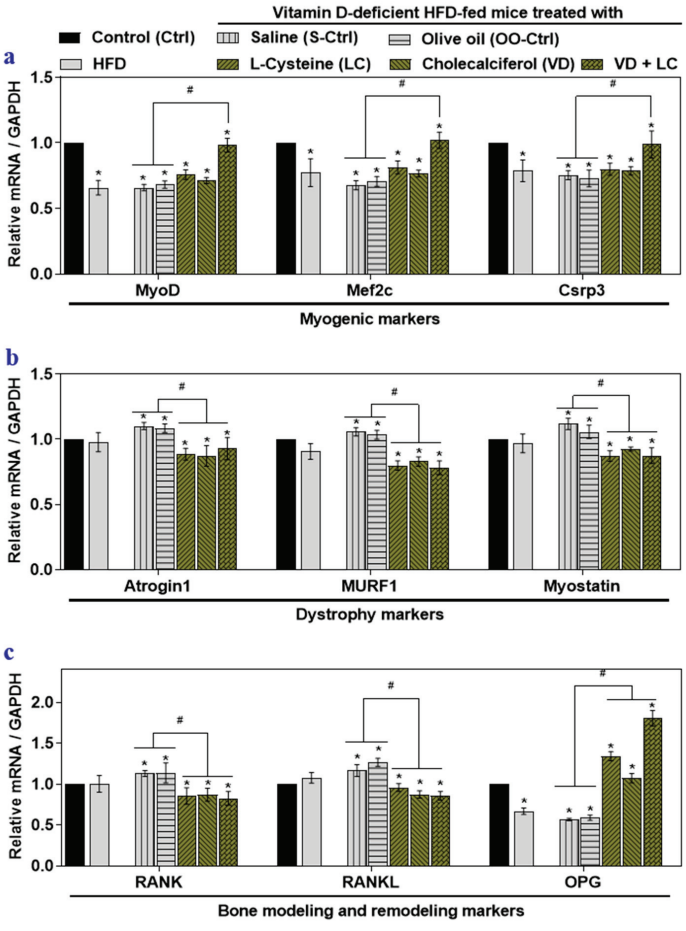


Figure 1. The effects of high-fat diet (HFD), vitamin D (VD)-deficient HFD (HFD-VD-), and L-cysteine and vitamin D co-supplementation on gene expression musculoskeletal markers in mouse skeletal muscle. Male C57BL/6J mice (5 weeks old) were fed with standard chow diet (Control; Ctrl), a high-fat diet (HFD), or a VD-deficient HFD for 16 weeks. Mice were gavaged with saline (S-Ctrl), Olive oil (OO-Ctrl), L-Cysteine (LC), Cholecalciferol (VD), or VD + LC during the last 8 weeks. The mRNA levels of myogenic marker genes: myoblast determination protein 1, myocyte enhancer factor 2C, and cysteine and glycine-rich protein 3 (MyoD, Mef2c, and Csrp3) (**a**); dystrophy marker genes: skeletal muscle-specific F-box protein, muscle RING-finger protein-1, and Myostatin (Atrogin1, Murf1, and Myostatin) (**b**); bone modeling and remodeling genes: receptor activator of nuclear factor-kB, receptor activator of nuclear factor-kB ligand, and osteoprotegerin (RANK, RANKL, and OPG) (**c**) were analyzed using qRT-PCR. Results are mean ± SEM (n = 4). Two-way ANOVA, followed by Dunnett's multiple comparisons test, was performed between groups. Significance at $p < 0.05$: Asterisk symbol (*) represents a comparison between control (Ctrl) with all other groups, whereas the hash symbol (#) represents a comparison between HFD-VD- saline and olive oil control (S-Ctrl and OO-Ctrl) with LC, VD, VD + LC co-supplementation groups. MyoD: Myoblast determination protein 1, Mef2c: Myocyte enhancer factor 2C, Csrp3: Cysteine and glycine-rich protein 3, Atrogin1: skeletal muscle-specific F-box protein, Murf1: Muscle RING-finger protein-1, RANK: Receptor activator of nuclear factor-kB, RANKL: Receptor activator of nuclear factor-kB ligand, OPG: Osteoprotegerin.

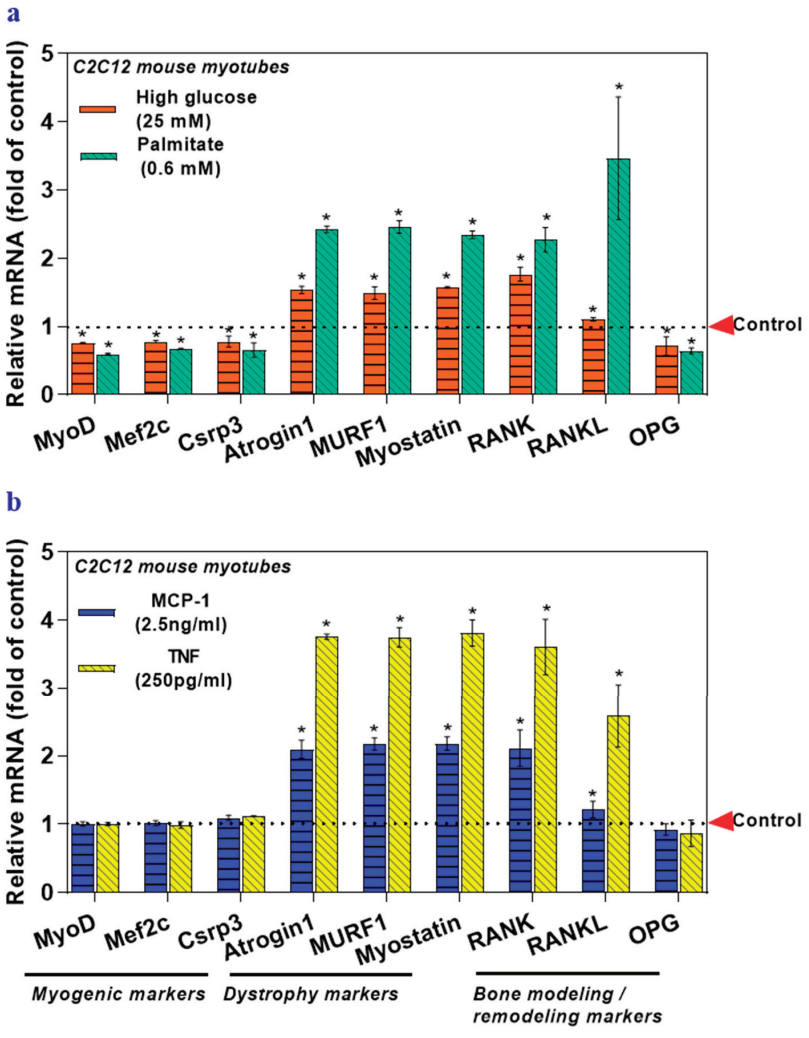


Figure 2. Glucolipotoxicity and inflammatory cytokines (MCP-1 and TNF) affect musculoskeletal markers in myotubes. Myotubes were treated with high glucose (25 mM) or palmitate (0.6 mM) for 24 h. Mannitol was used as an osmolality control (**a**). In another set of experiments, myotubes were treated with MCP-1 (2.5 ng/mL) or TNF (250 pg/mL) for 6 h (**b**). The mRNA levels of target genes responsible for myogenesis, muscle dystrophy, bone modeling, and remodeling (MyoD, Mef2c, Csrp3, Atrogin1, Murf1, Myostatin, RANK, RANKL, and OPG) were analyzed using qRT-PCR (**a**,**b**). Results are mean ± SEM (n = 3). Two-way ANOVA, followed by Dunnett's multiple comparisons test, was performed between groups. A p-value of <0.05 for a statistical test was considered significant and represented as an asterisk symbol (*) compared with the control group. MCP-1: Monocyte Chemoattractant Protein 1, TNF: Tumor Necrosis Factor.

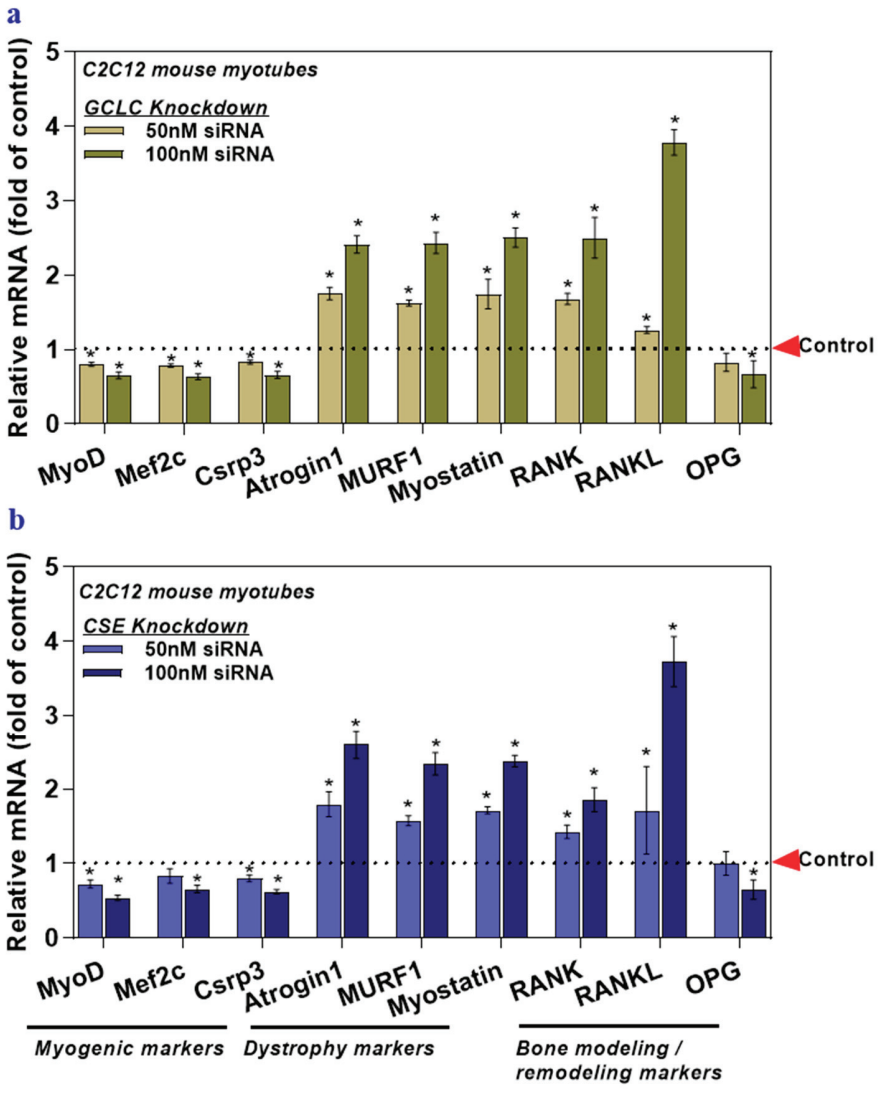


Figure 3. The deficiency of transsulfuration pathway key genes (GCLC and CSE knockdown) in myotubes affects musculoskeletal markers. Myotubes were transfected with GCLC siRNA (GSH deficient) (**a**) or CSE siRNA (H_2S deficient) (**b**). Scrambled siRNA, served as a control. The mRNA levels of target genes responsible for myogenesis, muscle dystrophy, bone modeling, and remodeling (MyoD, Mef2c, Csrp3, Atrogin1, Murf1, Myostatin, RANK, RANKL, and OPG) were analyzed using qRT-PCR (**a**,**b**). Results are mean ± SEM (n = 3). Two-way ANOVA, followed by Dunnett's multiple comparisons test, was performed between groups. A p-value of <0.05 for a statistical test was considered significant and represented as an asterisk symbol (*) compared with the control group.

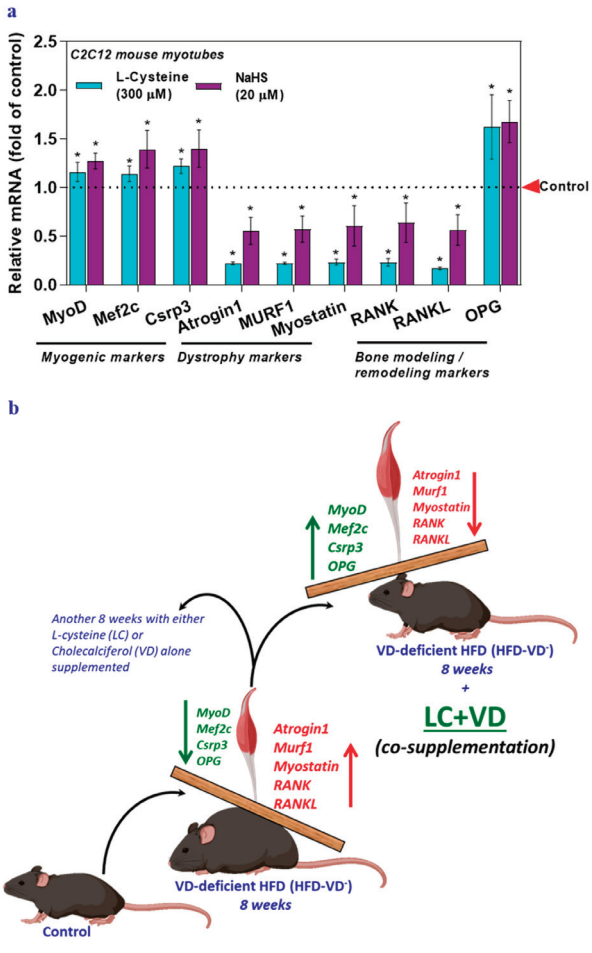


Figure 4. Glutathione (L-cysteine) or hydrogen sulfide (sodium hydrosulfide) supplementation alters musculoskeletal markers in myotubes. Myotubes were treated with either L-cysteine (LC; 300 μM) or sodium hydrosulfide (NaHS; 20 μM) for 6 h. The mRNA levels of target genes responsible for myogenesis, muscle dystrophy, bone modeling, and remodeling (MyoD, Mef2c, Csrp3, Atrogin1, Murf1, Myostatin, RANK, RANKL, and OPG) were analyzed using qRT-PCR (**a**). Results are mean ± SEM (n = 3). Two-way ANOVA, followed by Dunnett's multiple comparisons test, was performed between groups. A p-value of <0.05 for a statistical test was considered significant and represented as an asterisk symbol (*) compared with the control group. Control animals were fed a healthy diet (Ctrl; lower in fat), while animals in the high-fat diet group were fed a high-fat diet (HFD) for a total of 16 weeks (not shown in scheme). The mice were maintained on a VD-deficient HFD (HFD-VD-) for 16 weeks (to mimic the VD-deficient condition). After the first 8 weeks, the mice were supplemented by oral gavage for another 8 weeks with either 5 mg LC/kg BW daily (LC), 67 IU VD/kg BW (VD) alone, or the same doses of LC + VD co-supplemented to HFD-VD-mice. The markers of Myogenic: MyoD, Mef2c, and Csrp3; Muscle dystrophy: Atrogin1, Murf1, and Myostatin; Bone modeling and remodeling: RANK, RANKL, and OPG were analyzed. Myogenic markers and OPG decreased in HFD-VD-mice muscle, whereas muscle dystrophy markers increased significantly. LC + VD co-supplementation to HFD-VD-mice ameliorate partially or entirely all the markers mentioned above at par with control groups (**b**).

4. Discussion

Vitamin D (VD) is a nutrient essential for maintaining good bone health and improving muscle strength [1–3,37,38]. VD deficiency or insufficiency is associated with various musculoskeletal disorders [39–41]. After multivitamins, vitamin D, by itself, is the second-highest vitamin supplement consumed by the public for better health and delay or prevent musculoskeletal disorders [41–44]. However, controlled clinical studies show that VD alone supplementations' have limited therapeutical benefits, despite the clinical association between VD deficiency and disease outcome [4,7,26,45]. This study examined the hypothesis that the co-supplementation of L-cysteine (LC) with VD is better compared to monotherapies with LC or VD at alleviating dyshomeostasis in the skeletal muscle of VD-deficient high-fat diet-fed (HFD-VD-) mice.

This study reports that LC+VD co-supplementation showed significant beneficial effects on vital myogenic markers such as MyoD, Mef2c, and Csrp3 in an animal model of HFD-VD-. Further, in vitro studies carried out in mouse myotubes demonstrated that, while H_2S/GSH deficiency (oxidative stress or antioxidant deficient condition), high glucose, and palmitate (metabolic insults) decreased myogenic markers, inflammatory cytokines, such as TNF and MCP-1, did not affect the markers of myogenesis. Previously it has been shown that low physiological concentrations (a deficient state) of $1,25(OH)_2D_3$ (active VD) induces transdifferentiation of muscle cells into adipose cells (adipogenesis), whereas higher (physiological and supraphysiological) concentrations attenuate this effect and promote myogenic cell differentiation [46,47]. Further, VD ameliorates fat accumulation with AMPK/SIRT1 pathway activation in myotubes [48,49]. Moreover, GSH depletion and chronic inflammation impair myogenic differentiation through redox-dependent and independent pathways, while these effects are reversible following NAC or GSH replenishment [50]. GSH and H_2S levels affect the intracellular redox state. In vitro supplementation of LC and NaHS shows a positive effect on myogenic markers. Additionally, preclinical studies have shown that co-supplementation of LC + VD significantly reduced oxidative stress by boosting GSH and positively upregulating the VD-regulatory genes (VDBP/VD-25-hydroxylase/VDR) epigenetically in the liver of mice in a 25(OH)D deficiency mouse model [4–6]. GSH optimization, along with VD is a better approach to alleviate myogenic genes. Therefore, reduction or prevention of oxidative imbalance and VD in muscle is of vital importance for the maintenance of the myogenic pathway.

GSH is a critical redox factor that mitigates oxidative stress and oxidative damage to endogenous proteins, impairs cellular physiology and leads to the manifestation of the disease. Supplementation with the GSH rate-limiting amino acid precursor L-cysteine has been used successfully to improve the GSH status, VD metabolism genes, and lower the incidence of immune-metabolic syndrome [4–8]. In the present study, supplementation with LC, along with VD, suppressed skeletal muscle dystrophy markers such as Atrogin1, Murf1, and Myostatin and RANK and its ligand (RANKL). VD deficiency is associated with muscle atrophy. In vitro metabolic insults, exposure to inflammatory cytokines, and antioxidant deficient conditions (H_2S/GSH deficiency) induced muscle dystrophy markers along with RANK/RANKL.

Conversely, OPG showed an inverse relationship, unlike RANK/RANKL. Previous studies have shown that the muscle content of Atrogin1 was the highest in patients deficient in VD and lowest in patients sufficient in it, whereas VD supplementation seemed to repel atrophic changes and systemic inflammatory markers [51]. Antioxidants maintain muscle homeostasis, whereas disturbed redox status, known as the major contributing factor towards atrophy. N-Acetyl cysteine (NAC) treatment reduces muscle atrophy through beneficial antioxidant, anti-inflammatory, and anti-fibrotic effects [52]. Further, GSH/NAC inhibits RANK-L induced osteoclastogenesis both in vitro and in vivo [53]. VD also maintains a regular rate of bone resorption and formation through the receptor activator of the nuclear factor κB (RANK)/RANK ligand (RANKL)/osteoprotegerin axis [54]. Our study shows that antioxidant supplementation with LC and NaHS suppresses muscle dystrophy markers, RANK/RANKL, and boosts the levels of OPG. Hence, LC + VD co-supplementation treatment was extremely effective against the myopathic changes contributing to pathophysiology.

5. Conclusions

In VD insufficiency/deficiency, muscle function and physical function may be impaired before clinical or biochemical signs of musculoskeletal disease are evident. Low circulating levels of both glutathione (GSH) and 25(OH)D are positively associated with metabolic syndrome and poor health in human subjects [4]. A possible explanation for the limited success of clinical trials with VD-alone could be the need to simultaneously optimize deficiencies in essential antioxidant nutrients such as LC along with VD. Therefore, an efficient novel therapeutic strategy would be using the combined nutraceuticals LC + VD, which could simultaneously antagonize cellular oxidative stress and inflammation and thus provide a better option for musculoskeletal system disorders than supraphysiological monotherapy with VD [7]. Current data obtained from preclinical studies in a 25(OH)D deficient mouse model suggest that LC + VD more effectively boosted the actions/efficacy of VD on musculoskeletal markers than monotherapies with LC or VD (Figure 4b). Our data support the need for a clinical trial of co-supplementation of LC with VD to achieve better health outcomes, including skeletal muscle functions.

Author Contributions: Conceptualization, R.P. and S.K.J.; methodology, R.P.; software, R.P.; validation, R.P. and S.K.J.; formal analysis, R.P. and S.K.J.; investigation, R.P., A.E.A. and P.M.; resources, S.K.J.; data curation, R.P., A.E.A. and P.M.; writing—original draft preparation, R.P.; writing—review and editing, R.P. and S.K.J.; visualization, R.P.; supervision, S.K.J.; project administration, R.P. and S.K.J.; funding acquisition, R.P. and S.K.J. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Malcolm W. Feist Cardiovascular Research Fellowship to R.P. and the Endowed Chair in Diabetes to S.K.J. from the Center for Cardiovascular Diseases and Sciences (CCDS), LSUHSC-Shreveport, as well as grants to S.K.J. from the National Institutes of Health/National Center for Complementary and Integrative Health (RO1 AT007442, 2013-16 and 1 R33 AT010637-01A1, 2020-2023).

Acknowledgments: We thank Paula Polk, Manager and Wiola Luszczek, Research Specialist at the Research Core Facility at LSUHSC-Shreveport for their expert technical assistance. We also thank William E. McLean and Christopher M. Stevens for lab assistance. The authors thank Georgia Morgan for excellent editing.

Conflicts of Interest: The authors declare 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

Atrogin1/Fbxo32	skeletal muscle-specific F-box protein
CSE/CTH	Cystathionine Gamma-Lyase
GCLC/ γ-GCSc	Glutamate-Cysteine Ligase Catalytic Subunit
GSH	Glutathione
H_2S	Hydrogen sulfide
LC	L-cysteine
HFD-VD-	VD-deficient high-fat diet-fed
MCP-1	Monocyte Chemoattractant Protein 1
TNF	Tumor Necrosis Factor
MuRF1/Trim63	Muscle RING-finger protein-1
Mstn	Myostatin
MyoD	Myoblast determination protein 1
Mef2c	Myocyte enhancer factor 2C
Csrp3	Cysteine and glycine-rich protein 3
Rank/Tnfrsf11a	Receptor activator of nuclear factor-kB
RANKL	Receptor activator of nuclear factor-kB ligand
OPG	Osteoprotegerin
VD	Vitamin D

References

1. Holick, M.F. Sunlight "D"ilemma: Risk of skin cancer or bone disease and muscle weakness. *Lancet* **2001**, *357*, 4–6. [CrossRef]
2. Holick, M.F.; Grant, W.B. Vitamin D status and ill health. *Lancet Diabetes Endocrinol.* **2014**, *2*, 273–274. [CrossRef]
3. Holick, M.F. Vitamin D: Importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *Am. J. Clin. Nutr.* **2004**, *79*, 362–371. [CrossRef] [PubMed]
4. Jain, S.K.; Parsanathan, R.; Achari, A.E.; Kanikarla-Marie, P.; Bocchini, J.A., Jr. Glutathione stimulates vitamin D regulatory and glucose-metabolism genes, lowers oxidative stress and inflammation, and increases 25-hydroxy-vitamin D levels in blood: A novel approach to treat 25-hydroxyvitamin D deficiency. *Antioxid. Redox Signal.* **2018**, *29*, 1792–1807. [CrossRef] [PubMed]
5. Parsanathan, R.; Jain, S.K. Glutathione deficiency induces epigenetic alterations of vitamin D metabolism genes in the livers of high-fat diet-fed obese mice. *Sci. Rep.* **2019**, *9*, 14784. [CrossRef] [PubMed]
6. Parsanathan, R.; Jain, S.K. Glutathione deficiency alters the vitamin D-metabolizing enzymes CYP27B1 and CYP24A1 in human renal proximal tubule epithelial cells and kidney of HFD-fed mice. *Free Radic. Biol. Med.* **2019**, *131*, 376–381. [CrossRef]
7. Jain, S.K.; Parsanathan, R. Can vitamin D and L-Cysteine co-supplementation reduce 25(OH)-vitamin D deficiency and the mortality associated with COVID-19 in African Americans? *J. Am. Coll. Nutr.* **2020**. [CrossRef]
8. Jain, S.K.; Kanikarla-Marie, P.; Warden, C.; Micinski, D. L-Cysteine supplementation upregulates glutathione (GSH) and vitamin D binding protein (VDBP) in hepatocytes cultured in high glucose and in vivo in liver, and increases blood levels of GSH, VDBP, and 25-hydroxy-vitamin D in Zucker diabetic fatty rats. *Mol. Nutr. Food Res.* **2016**, *60*, 1090–1098. [CrossRef]
9. Dunn, A.; Talovic, M.; Patel, K.; Patel, A.; Marcinczyk, M.; Garg, K. Biomaterial and stem cell-based strategies for skeletal muscle regeneration. *J. Orthop. Res.* **2019**, *37*, 1246–1262. [CrossRef]
10. Hernandez-Hernandez, J.M.; Garcia-Gonzalez, E.G.; Brun, C.E.; Rudnicki, M.A. The myogenic regulatory factors, determinants of muscle development, cell identity and regeneration. *Semin. Cell Dev. Biol.* **2017**, *72*, 10–18. [CrossRef]
11. Kong, Y.; Flick, M.J.; Kudla, A.J.; Konieczny, S.F. Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. *Mol. Cell Biol.* **1997**, *17*, 4750–4760. [CrossRef] [PubMed]
12. Bodine, S.C.; Baehr, L.M. Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1. *Am. J. Physiol. Endocrinol. Metab.* **2014**, *307*, E469–E484. [CrossRef]
13. Lokireddy, S.; McFarlane, C.; Ge, X.; Zhang, H.; Sze, S.K.; Sharma, M.; Kambadur, R. Myostatin induces degradation of sarcomeric proteins through a Smad3 signaling mechanism during skeletal muscle wasting. *Mol. Endocrinol.* **2011**, *25*, 1936–1949. [CrossRef]
14. Powers, S.K.; Lynch, G.S.; Murphy, K.T.; Reid, M.B.; Zijdewind, I. Disease-induced skeletal muscle atrophy and fatigue. *Med. Sci. Sports Exerc.* **2016**, *48*, 2307–2319. [CrossRef] [PubMed]
15. Dufresne, S.S.; Boulanger-Piette, A.; Bosse, S.; Frenette, J. Physiological role of receptor activator nuclear factor-kB (RANK) in denervation-induced muscle atrophy and dysfunction. *Receptors Clin. Investig.* **2016**, *3*, e13231–e13236. [CrossRef]
16. Grimaud, E.; Soubigou, L.; Couillaud, S.; Coipeau, P.; Moreau, A.; Passuti, N.; Gouin, F.; Redini, F.; Heymann, D. Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am. J. Pathol.* **2003**, *163*, 2021–2031. [CrossRef]
17. Collins, K.H.; Paul, H.A.; Hart, D.A.; Reimer, R.A.; Smith, I.C.; Rios, J.L.; Seerattan, R.A.; Herzog, W. A high-fat high-sucrose diet rapidly alters muscle integrity, inflammation and gut microbiota in male rats. *Sci. Rep.* **2016**, *6*, 37278. [CrossRef]
18. Zhu, S.; Tian, Z.; Torigoe, D.; Zhao, J.; Xie, P.; Sugizaki, T.; Sato, M.; Horiguchi, H.; Terada, K.; Kadomatsu, T.; et al. Aging- and obesity-related peri-muscular adipose tissue accelerates muscle atrophy. *PLoS ONE* **2019**, *14*, e0221366. [CrossRef]
19. Peris-Moreno, D.; Taillandier, D.; Polge, C. MuRF1/TRIM63, master regulator of muscle mass. *Int. J. Mol. Sci.* **2020**, *21*, 6663. [CrossRef]

20. McLeay, Y.; Stannard, S.; Houltham, S.; Starck, C. Dietary thiols in exercise: Oxidative stress defence, exercise performance, and adaptation. *J. Int. Soc. Sports Nutr.* **2017**, *14*, 12. [CrossRef] [PubMed]
21. Michailidis, Y.; Karagounis, L.G.; Terzis, G.; Jamurtas, A.Z.; Spengos, K.; Tsoukas, D.; Chatzinikolaou, A.; Mandalidis, D.; Stefanetti, R.J.; Papassotiriou, I.; et al. Thiol-based antioxidant supplementation alters human skeletal muscle signaling and attenuates its inflammatory response and recovery after intense eccentric exercise. *Am. J. Clin. Nutr.* **2013**, *98*, 233–245. [CrossRef]
22. Lands, L.C.; Grey, V.L.; Smountas, A.A. Effect of supplementation with a cysteine donor on muscular performance. *J. Appl. Physiol. (1985)* **1999**, *87*, 1381–1385. [CrossRef]
23. Cao, J.J.; Picklo, M.J. N-acetylcysteine supplementation decreases osteoclast differentiation and increases bone mass in mice fed a high-fat diet. *J. Nutr.* **2014**, *144*, 289–296. [CrossRef]
24. Takahashi, N.; Udagawa, N.; Suda, T. Vitamin D endocrine system and osteoclasts. *Bonekey Rep.* **2014**, *3*, 495. [CrossRef] [PubMed]
25. Gunton, J.E.; Girgis, C.M. Vitamin D and muscle. *Bone Rep.* **2018**, *8*, 163–167. [CrossRef]
26. Jain, S.K.; Parsanathan, R.; Levine, S.N.; Bocchini, J.A.; Holick, M.F.; Vanchiere, J.A. The potential link between inherited G6PD deficiency, oxidative stress, and vitamin D deficiency and the racial inequities in mortality associated with COVID-19. *Free Radical Biol. Med.* **2020**, *161*, 84–91. [CrossRef]
27. Aguiar, A.S., Jr.; Speck, A.E.; Amaral, I.M.; Canas, P.M.; Cunha, R.A. The exercise sex gap and the impact of the estrous cycle on exercise performance in mice. *Sci. Rep.* **2018**, *8*, 10742. [CrossRef]
28. Carson, J.A.; Manolagas, S.C. Effects of sex steroids on bones and muscles: Similarities, parallels, and putative interactions in health and disease. *Bone* **2015**, *80*, 67–78. [CrossRef]
29. Vanderschueren, D.; Laurent, M.R.; Claessens, F.; Gielen, E.; Lagerquist, M.K.; Vandenput, L.; Borjesson, A.E.; Ohlsson, C. Sex steroid actions in male bone. *Endocr. Rev.* **2014**, *35*, 906–960. [CrossRef]
30. Galhardo, A.P.M.; Mukai, M.K.; Mori, M.; Carvalho, K.C.; Baracat, M.C.P.; Simoes, M.J.; Soares, J.M., Jr.; Baracat, E.C. Influence of age and gender on sex steroid receptors in rat masticatory muscles. *Sci. Rep.* **2019**, *9*, 18403. [CrossRef]
31. Brown, M. Skeletal muscle and bone: Effect of sex steroids and aging. *Adv. Physiol. Educ.* **2008**, *32*, 120–126. [CrossRef]
32. Keijer, J.; Li, M.; Speakman, J.R. What is the best housing temperature to translate mouse experiments to humans? *Mol. Metab.* **2019**, *25*, 168–176. [CrossRef]
33. Fischer, A.W.; Cannon, B.; Nedergaard, J. The answer to the question "What is the best housing temperature to translate mouse experiments to humans?" is: Thermoneutrality. *Mol. Metab.* **2019**, *26*, 1–3. [CrossRef] [PubMed]
34. Della Vedova, M.C.; Munoz, M.D.; Santillan, L.D.; Plateo-Pignatari, M.G.; Germano, M.J.; Rinaldi Tosi, M.E.; Garcia, S.; Gomez, N.N.; Fornes, M.W.; Gomez Mejiba, S.E.; et al. A mouse model of diet-induced obesity resembling most features of human metabolic syndrome. *Nutr. Metab. Insights* **2016**, *9*, 93–102. [CrossRef]
35. Parsanathan, R.; Jain, S.K. Hydrogen sulfide increases glutathione biosynthesis, and glucose uptake and utilisation in C2C12 mouse myotubes. *Free Radic. Res.* **2018**, *52*, 288–303. [CrossRef]
36. Parsanathan, R.; Jain, S.K. Hydrogen sulfide regulates circadian-clock genes in C2C12 myotubes and the muscle of high-fat-diet-fed mice. *Arch. Biochem. Biophys.* **2019**, *672*, 108054. [CrossRef]
37. Grober, U.; Spitz, J.; Reichrath, J.; Kisters, K.; Holick, M.F. Vitamin D: Update 2013: From rickets prophylaxis to general preventive healthcare. *Dermatoendocrinol.* **2013**, *5*, 331–347. [CrossRef]
38. Holick, M.F. The vitamin D deficiency pandemic and consequences for nonskeletal health: Mechanisms of action. *Mol. Aspects Med.* **2008**, *29*, 361–368. [CrossRef]
39. Laird, E.; Ward, M.; McSorley, E.; Strain, J.J.; Wallace, J. Vitamin D and bone health: Potential mechanisms. *Nutrients* **2010**, *2*, 693–724. [CrossRef]
40. Wacker, M.; Holick, M.F. Vitamin D—Effects on skeletal and extraskeletal health and the need for supplementation. *Nutrients* **2013**, *5*, 111–148. [CrossRef]
41. Wintermeyer, E.; Ihle, C.; Ehnert, S.; Stockle, U.; Ochs, G.; de Zwart, P.; Flesch, I.; Bahrs, C.; Nussler, A.K. Crucial role of vitamin D in the musculoskeletal system. *Nutrients* **2016**, *8*, 319. [CrossRef]
42. National Institutes of Health, O.o.D.S. Vitamin D: Fact Sheet for Health Professionals. Available online: https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/ (accessed on 30 October 2020).
43. National Institutes of Health, O.o.D.S. Vitamin D: Fact Sheet for Consumers. Available online: https://ods.od.nih.gov/factsheets/VitaminD-Consumer/ (accessed on 30 October 2020).

44. National Institutes of Health, O.o.D.S. Multivitamin/mineral Supplements: Fact Sheet for Health Professionals. Available online: https://ods.od.nih.gov/factsheets/MVMS-HealthProfessional/ (accessed on 30 October 2020).
45. Amrein, K.; Scherkl, M.; Hoffmann, M.; Neuwersch-Sommeregger, S.; Kostenberger, M.; Tmava Berisha, A.; Martucci, G.; Pilz, S.; Malle, O. Vitamin D deficiency 2.0: An update on the current status worldwide. *Eur. J. Clin. Nutr.* **2020**. [CrossRef]
46. Ryan, K.J.; Daniel, Z.C.; Craggs, L.J.; Parr, T.; Brameld, J.M. Dose-dependent effects of vitamin D on transdifferentiation of skeletal muscle cells to adipose cells. *J. Endocrinol.* **2013**, *217*, 45–58. [CrossRef]
47. Hosoyama, T.; Iida, H.; Kawai-Takaishi, M.; Watanabe, K. Vitamin D inhibits myogenic cell fusion and expression of fusogenic genes. *Nutrients* **2020**, *12*, 2192. [CrossRef]
48. Chang, E.; Kim, Y. Vitamin D ameliorates fat accumulation with AMPK/SIRT1 activity in C2C12 skeletal muscle cells. *Nutrients* **2019**, *11*, 2806. [CrossRef]
49. Manna, P.; Achari, A.E.; Jain, S.K. 1,25(OH)2-vitamin D3 upregulates glucose uptake mediated by SIRT1/IRS1/GLUT4 signaling cascade in C2C12 myotubes. *Mol. Cell Biochem.* **2018**, *444*, 103–108. [CrossRef]
50. Ardite, E.; Barbera, J.A.; Roca, J.; Fernandez-Checa, J.C. Glutathione depletion impairs myogenic differentiation of murine skeletal muscle C2C12 cells through sustained NF-kappaB activation. *Am. J. Pathol.* **2004**, *165*, 719–728. [CrossRef]
51. Dzik, K.P.; Skrobot, W.; Kaczor, K.B.; Flis, D.J.; Karnia, M.J.; Libionka, W.; Antosiewicz, J.; Kloc, W.; Kaczor, J.J. Vitamin D deficiency is associated with muscle atrophy and reduced mitochondrial function in patients with chronic low back pain. *Oxid. Med. Cell Longev.* **2019**, *2019*. [CrossRef]
52. Pinniger, G.J.; Terrill, J.R.; Assan, E.B.; Grounds, M.D.; Arthur, P.G. Preclinical evaluation of N-acetylcysteine reveals side effects in the mdx mouse model of Duchenne muscular dystrophy. *J. Physiol.* **2017**, *595*, 7093–7107. [CrossRef] [PubMed]
53. Yan, G.; Guo, Y.; Guo, J.; Wang, Q.; Wang, C.; Wang, X. N-Acetylcysteine attenuates lipopolysaccharide-induced osteolysis by restoring bone remodeling balance via reduction of reactive oxygen species formation during osteoclastogenesis. *Inflammation* **2020**. [CrossRef]
54. Shymanskyi, I.; Lisakovska, O.; Mazanova, A.; Labudzynskyi, D.; Veliky, M. Vitamin D3 modulates impaired crosstalk between RANK and glucocorticoid receptor signaling in bone marrow cells after chronic prednisolone administration. *Front. Endocrinol. (Lausanne)* **2018**, *9*, 303. [CrossRef]

nutrients

MDPI

Review

Metabolic and Nutritional Issues Associated with Spinal Muscular Atrophy

Yang-Jean Li [1], Tai-Heng Chen [2,3,*], Yan-Zhang Wu [2] and Yung-Hao Tseng [2]

[1] Department of Pediatrics, Kaohsiung Municipal United Hospital, Kaohsiung 80455, Taiwan; ptstyle1986@gmail.com

[2] Department of Pediatrics, Division of Pediatric Emergency, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung 80708, Taiwan; ijw168@yahoo.com.tw (Y.-Z.W.); smapten@gmail.com (Y.-H.T.)

[3] School of Post-Baccalaureate Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

* Correspondence: taihen@kmu.edu.tw; Tel.: +886-7-312-1101; Fax: +886-7-321-2062

Received: 31 October 2020; Accepted: 15 December 2020; Published: 16 December 2020

Abstract: Spinal muscular atrophy (SMA), the main genetic cause of infant death, is a neurodegenerative disease characterized by the selective loss of motor neurons in the anterior horn of the spinal cord, accompanied by muscle wasting. Pathomechanically, SMA is caused by low levels of the survival motor neuron protein (SMN) resulting from the loss of the *SMN1* gene. However, emerging research extends the pathogenic effect of SMN deficiency beyond motor neurons. A variety of metabolic abnormalities, especially altered fatty acid metabolism and impaired glucose tolerance, has been described in isolated cases of SMA; therefore, the impact of SMN deficiency in metabolic abnormalities has been speculated. Although the life expectancy of these patients has increased due to novel disease-modifying therapies and standardization of care, understanding of the involvement of metabolism and nutrition in SMA is still limited. Optimal nutrition support and metabolic monitoring are essential for patients with SMA, and a comprehensive nutritional assessment can guide personalized nutritional therapy for this vulnerable population. It has recently been suggested that metabolomics studies before and after the onset of SMA in patients can provide valuable information about the direct or indirect effects of SMN deficiency on metabolic abnormalities. Furthermore, identifying and quantifying the specific metabolites in SMA patients may serve as an authentic biomarker or therapeutic target for SMA. Here, we review the main epidemiological and mechanistic findings that link metabolic changes to SMA and further discuss the principles of metabolomics as a novel approach to seek biomarkers and therapeutic insights in SMA.

Keywords: spinal muscular atrophy; metabolomics; nutrition; therapeutics; biomarkers

1. Introduction

Spinal muscular atrophy (SMA) is a congenital neuromuscular disease characterized by progressive muscle weakness resulting from the degeneration of motor neurons (MN) in the spinal cord [1]. Although SMA is considered a rare disease and the global incidence of live births is estimated to be about 1/10,000, SMA is still the second most common autosomal recessive genetic disease and the most common monogenic disorder that causes early infant death [2]. The carrier frequency varies from 1 in 38 to 1 in 72 among different ethnic groups, with a pan-ethnic average of 1 in 54 [3,4].

In a pathological view, SMA is resulted from an insufficient level of a 38 kDa protein, called the survival motor neuron (SMN), as a result of homologous deletion or mutation of the *Survival of Motor Neuron 1* (*SMN1*) gene [5]. Subsequent studies showed that two genes encode SMN protein in humans: *SMN1* and a 99% identical copy in sequence, known as *SMN2*. Indeed, *SMN2* mainly differs from

SMN1 by a single nucleotide (C-to-T) substitution in the exon 7 [6]. Such a critical variant results in exon 7 exclusion in most transcripts (90%) of *SMN2*, SMNΔ7. Unlike the *SMN1* gene, *SMN2* can only produce 10 % full-length (FL) SMN [7]. Given that the residual FL-*SMN2* transcripts can compensate for defect *SMN1* to a limited extent, the SMA severity is partially rescued by *SMN2* copy numbers [8]. However, the correlation between this phenotype and genotype is not absolute, and recent studies have pointed out that other potential cellular mechanisms may also be involved in modifying the clinical severity of SMA [9].

It is still unclear whether the pathogenesis of SMA is caused by a specific pattern or a combination of dysregulated effects. The cell-autonomous effects due to SMN deficiency are the main causes of MN degeneration; however, it cannot be explained for the full SMA phenotype, implicating not only dysregulated neural networks but other non-neuronal cell types involved in the SMA pathology [10,11]. Emerging research extends the pathogenic effect of SMN deficiency beyond the MN, including other cells inside and outside the central nervous system, so that many peripheral organs and non-neural tissues show pathological changes in preclinical SMA models and diseased patients (Figure 1) [12–14]. Furthermore, increasing evidence suggests metabolic abnormalities in patients with SMA, such as altered fatty acid metabolism, impaired glucose tolerance, and muscle mitochondria defects [15–17]. Recent studies also indicate that many SMA patients are either undernourished, underfed, or overfed [18]. Notably, in some SMA patients, metabolic dysregulations may even present before their first neuromuscular signs [19]. These findings suggest that SMN is essential for the survival of motor neurons and affects certain enzyme production in the metabolism.

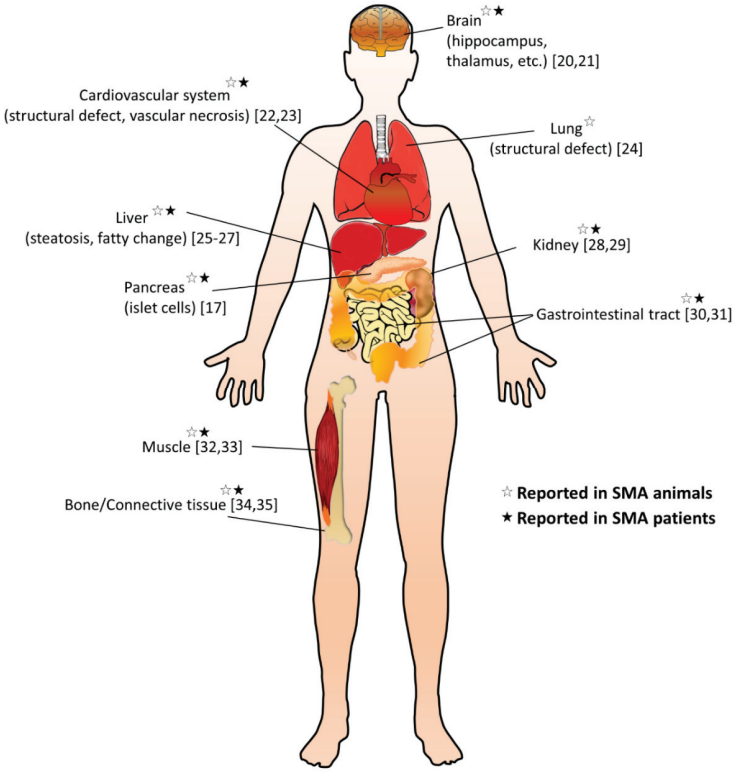


Figure 1. Overview of non-neuromuscular systemic pathology in spinal muscular atrophy (SMA). A summary of multi-organ involvement has been reported in SMA animal models and/or patients [17,20–35].

Over the last few years, the increased life expectancy of SMA patients has been achieved through the invention of novel therapies and the standardization of clinical care. However, knowledge of the altered metabolism and nutrition in SMA remains limited. The impact of SMN deficiency on metabolic abnormalities has been recently proposed. Before and after the onset of the disease, metabolomics studies in SMA patients can provide valuable information about the direct or indirect effects of SMN deficiency on metabolic abnormalities [13]. The present review will discuss the current knowledge regarding the metabolic involvement in SMA and the role of metabolomics in pursuing potential biomarkers and therapeutic insights for SMA.

2. Lipid Metabolic Abnormalities in SMA

Abnormalities of lipid metabolism have been described in different motor neuron diseases, including SMA [36]. As shown in Table 1, dysregulated lipid metabolism is the first and most studied nutritional problem in SMA [37,38]. Compared with healthy controls and non-SMA motor neuron diseases with equally debilitating statuses, the abnormal lipid metabolism found in patients and animal models appears unique to SMA [39,40]. Abnormal levels of fatty acid oxidation metabolites, especially dicarboxylic aciduria and esterified carnitine, were first reported in several studies of patients with severe SMA type [38,41,42]. Subsequently, an increasing number of studies suggest that patients with SMA are likely to have metabolic defects involving fatty acid metabolism. Of note, increased fat mass, even though relatively low caloric consumption has been repeatedly reported in patients with SMA [40,43]. Several serum fatty acids and lipids have been found correlated to the motor function of patients with SMA, suggesting potential biomarker candidates for SMA [44]. It has recently been implicated that defects in fatty acid transport and mitochondrial β-oxidation may also contribute to muscle wasting in patients with a severe SMA phenotype [32]. Nevertheless, the exact mechanism of this lipid metabolism abnormality in SMA is still unclear, but it is suspected to be related to the absence of the SMN gene product, defects in neighboring genes, or the loss of a neural "trophic factor" [31,42,45].

Although abnormal levels of fatty acid metabolites have been reported, no direct evidence has substantiated a specific defect of mitochondrial β-oxidation in SMA patients. There are several differences in metabolomics between patients with SMA and patients with a genetic defect of fatty acid β-oxidation. SMA patients usually had a normal acylcarnitine profile [42], contrary to an increased acylcarnitine level always found in mitochondrial β-oxidation defects. Moreover, fasting patients with impaired fatty acid β-oxidation always have markedly decreased ketone bodies. However, patients with SMA usually present with a normal or even a high ketone body level (increased ketosis), especially under stress [45,46]. The ability to mount fasting ketosis means that the liver can utilize fatty acids normally, but it does not rule out that it may be caused by muscle-specific mitochondrial defects in β-oxidation [32]. Therefore, it is postulated that dysregulated fatty acid metabolism in SMA patients might be directly related to SMN deficiency but is not attributed to the consequence of major enzyme block of mitochondrial β-oxidation, disuse muscle atrophy, or denervation [13,42,47].

Table 1. Altered Lipid Metabolism in Patients with Spinal Muscular Atrophy.

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Lipid (fatty acid), carnitine	Kelley et al. (1986) [38]	Case report	To describe an SMA infant with elevated certain urinary organic acids, suggesting a defect of fatty acid metabolism.	SMA: 1	SMA type 1; age 9 months old	• Increased urine dicarboxylic acids in both fed and fasting states, especially in the longer-chain (C10 and C22) 3-hydroxydicarboxylic acids. • Serum carnitine concentration slightly decreased (24 μM/L, free; 37, total). • Mild to moderate macrovesicular fatty vacuolization was found in the postmortem liver. • Findings could be events not specific to SMA.
Lipid (fatty acid), carnitine, acylcarnitine	Harpey et al. (1990) [37]	Cross-sectional	assess the metabolic defects of fatty acids and carnitine/acylcarnitine among patients with SMA	SMA: 14	SMA type 2: 100%; age range 1–11.5 years old	• Urine organic acids showed an abnormal excretion of ethylmalonic acid in all 14 children. • Reduced carnitine level in serum of 10 patients and muscles of 6 patients. • Increased excretion of urinary acylcarnitines in all examined patients ($n = 8$). • Possible multiple Flavin adenine dinucleotide (FAD)-linked acyl-CoA dehydrogenase deficiencies. • Carnitine deficiency may be due to intramitochondrial accumulation of acylcarnitines, followed by renal excretion. • Findings could be events not specific to SMA.

Table 1. *Cont.*

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Lipid (fatty acid), carnitine, acylcarnitine	Tein et al. (1995) [41]	Cross-sectional	To identify and quantify the FA oxidation abnormalities in SMA and to correlate these with disease severity and to identify specific underlying defects.	SMA: 15	SMA type 1: 20%, type 2: 53%, type 3: 27%; age range 2 months old–20 years old	• Serum carnitine total/free ratios tend toward an increased esterified fraction ranging 35–58% of total carnitine in children with SMA type 1 and 2. • SMA Patients > 23 months old showed normal esterified carnitine levels. • Urinary organic acid analysis: abnormalities in SMA type 1 and 2. Mostly normal in SMA type 3. • Impaired β-oxidation noted in 5 children (two type 1, two type 2, and one type 3) with a significant reduction in the activities of short-chain L-3-hydroxyacyl-CoA dehydrogenase, long-chain L-3-hydroxyacyl-CoA dehydrogenase, acetoacetyl-CoA thiolase, and 3-ketoacyl-CoA thiolase. • All cases had normal crotonase activity. • Marked increase in crotonase activity ratios to L-3-hydroxyacyl-CoA dehydrogenase and thiolase activities with short- and long-chain substrates. • Findings could be events specific to SMA.
Lipid (fatty acid), carnitine	Crawford et al. (1999) [42]	• Plasma studies: cross-sectional • Urine studies: two or more single-arm study	To evaluate fasting and non-fasting lipid profiles in urine and plasma in infants and children with SMA.	SMA: 50 healthy controls: 22 disease controls: 6 SMA: 13 healthy control: 23	SMA type 1: 66%, type 2/3: 34% Disease controls: non-SMA denervation disorders ($n = 6$) Healthy controls: age 8–11 months old ($n = 4$), age 1–6 years old ($n = 19$)	• Plasma concentration of dodecanoic acid increased in severe SMA. • Normal plasma acylcarnitine profiles in 10 infants with severe SMA. • The ratio of molar quantities of dodecanoic to tetradecanoic acid differed significantly between severe SMA, age-matched controls, disease controls, and milder SMA type. • All severe SMA patients evaluated in the fasting state developed marked dicarboxylic aciduria, including saturated, unsaturated, and 3-hydroxy forms, comparable in magnitude with that of children with primary fatty acid defects β-oxidation. • Findings could be events specific to SMA.

Table 1. *Cont.*

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Lipid (fatty acid), carnitine, acylcarnitine, ketone, glucose	Zolkipli et al. (2012) [48]	Case report	Describing a type 2 SMA children with catabolic crisis related to possibly impaired intramitochondrial β-oxidation.	SMA: 1	SMA type 2; age 15 years old	• Catabolic crisis onset 4 days after surgery, associated with hypoketotic hypoglycemia, lactic acidemia, hyperammonemia and liver failure. • No ketonuria. • Urine organic acids revealed moderate lactic acid. • Low plasma free and total carnitine with a raised esterified fraction. • Increase in C6 and C140H serum acylcarnitines after liver transplantation. • Liver pathology showed diffuse macro- and micro-vesicular steatosis. • The crisis responded in part to mitochondrial therapy and anabolic rescue. • Findings could be events specific to SMA.
Lipid (fatty acid), ketone, glucose	Mulroy et al. (2016) [45]	Case report	Describing a type 2 SMA adult presented with severe ketoacidosis with mild hypoglycemia	SMA: 1	SMA type 2; age: 50 years old; BMI: 16.4 kg/m^2	• Reductions in muscle mass, physical stress, and defects in fatty acid metabolism may cause hypoglycemia and non-diabetic ketoacidosis. • Findings could be events not specific to SMA.
Lipid (fatty acid), ketone	Lakkis et al. (2018) [46]	Case report	Describing a type 3 SMA adult presented with severe ketoacidosis with normal serum glucose	SMA: 1	SMA type 3; age: 36 years old; BMI: 23 kg/m^2	• Presented with ketoacidosis related to moderate fasting. • Denervation or SMN deficiency may affect metabolism and response to hormones, resulting in decreased uptake and fatty acid utilization by muscles. • The influence of denervation on muscle β-oxidation may elevate acetyl-CoA, a ketone precursor, in the liver. • Findings could be events not specific to SMA.

Table 1. *Cont.*

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Lipid (fatty acid), glucose	Deguise et al. (2019) [27]	Cross-sectional	• To investigate the lipid profile, including total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), non-HDL, and triglycerides to assess abnormalities in fatty acid metabolism • To check glucose dysregulation by HbA1C	SMA: 72	SMA type 1 20%, type 2 72%, type 3 8%; median age 3.8 years old	• 37.5% of SMA patients, most commonly type 1 and 2, had dyslipidemia • HbA1C trended lower in most SMA patients, with 57% having an abnormally low readout (HbA1C < 5%) • Dyslipidemia and low glucose levels align well with clinical findings in enrolled SMA patients. • Findings could be events specific to SMA.

SMA: spinal muscular atrophy.

Fatty vacuolization with macro- or micro-vesicular steatosis of the liver has been found in early studies of SMA patients [38,41,42]. Of note, liver failure and Reye-like syndrome with diffuse vesicular steatosis have been recently reported in patients with type 1 or 2 SMA [48,49]. An updated study further reports an increased susceptibility to develop dyslipidemia in 37% of SMA patients, with evidence of liver steatosis in their pathological specimens [27]. Similarly, these human findings are reproduced in different SMA mouse models, of which a specific Smn $^{2B/-}$ mice model developed the non-alcoholic fatty liver disease (NAFLD) before denervation. Hyperglucagonemia might also contribute to dyslipidemia and hepatic steatosis, possibly through the pancreas–liver axis, leading to peripheral lipolysis of white adipose tissue and an increase in circulating lipids. These findings imply that the liver-intrinsic SMN deficiency might also cause dysregulated metabolism of the hepatocytes [26,50], which could predispose the cells to fat accumulation. Noteworthily, subacute liver failure was recently reported in two patients with type 1 SMA following gene replacement therapy [49]. It is postulated that increased susceptibility to dyslipidemia and associated fatty liver disease could predispose the SMA patient to liver injury, which might be induced or exacerbated after the gene therapy. A thorough investigation of the lipid content in the liver of SMA patients and mouse models, before and after the onset of the disease [47], may provide further evidence for whether the direct or indirect effects of SMN deficiency affect metabolic abnormalities.

Since carnitine and its acyl esters (acylcarnitines) are cofactors for β-oxidation, abnormal lipid metabolism may also be reflected in their production, fractions, and transportation. Because carnitine is essential for intramitochondrial β-oxidation, reduced carnitine would limit β-oxidation. Acylcarnitines are known to play a crucial role in stabilizing neuronal membranes and neurotransmission [51]. Supplementation of acylcarnitine has shown beneficial effects in treating chronic degenerative diseases [52,53]. However, there are still controversies regarding the dysregulation of production, synthesis, and carnitine/acylcarnitine extraction in SMA patients. Early studies suggested that the integrity of nerve and motor neurons might influence carnitine transportation and lipid β-oxidation in muscles. Reduced muscle carnitine and decreased activity of β-oxidation have been observed in animal models after denervation [54,55]. Similarly, reduced carnitine and acylcarnitine levels in plasma and muscles and increased urine excretion of acylcarnitine have been reported in SMA patients [37,56]. However, normal or mild-to-moderate elevated serum acylcarnitines, particularly C5-OH acylcarnitine and C3 propionylcarnitine, were found in the following studies of SMA patients with a severe phenotype [41,42]. In contrast, an updated article reported an adolescent with type 2 SMA who showed a dramatically low serum carnitine/acylcarnitine level at a catabolic state [48]. This finding suggests impaired intramitochondrial β-oxidation associated with dysregulated carnitine metabolism in SMA would become more prominent, especially under stress.

In the fat metabolism of healthy individuals, longer-chain fatty acids are transported into the mitochondria for β-oxidation. Carnitine palmitoyltransferase 1 (CPT1) is an enzyme that converts long-chain acyl-CoA into long-chain acylcarnitine, thereby transporting long-chain fatty acids to the mitochondria. Decreased CPT activity has been reported in muscles of severe type 1 SMA patients, compared with aged-matched infants [56]. Recently, reduced CPT1 activity was also found in an SMA (Smn $^{2B/-}$) mice model [25]. Of note, an isoform of CPT1, called CPT1c, which mainly expresses in neurons, including motor neurons, shows biosynthetic activity in neuron-specific acyl-CoA. Reduced activity of CPT1c leads to motor function impairment and muscle weakness [57]. Interestingly, an updated study indicates that MN-specific CPT1C can interact with atlastin-1 encoded by the ATL1 gene, which is mutated in hereditary spastic paraplegia, a kind of motor neuron degenerative disorder [58].

Acylcarnitines can also interact with different proteins to influence signaling pathways of neuronal cells [52]. Growth-associated protein 43 (GAP43), a protein involved in neuronal development, neurotransmission, and neuroplasticity, is modified post-translationally by a long-chain acylcarnitine, possibly through the acylation pathway [59]. Interestingly, a recent study found that motor neurons from SMA mouse models showed reduced GAP43 protein levels in axons and growth cones [60,61]. SMN seems to be responsible for regulating the localization and translation of GAP43 mRNA in these

axons, and the restoration of GAP43 mRNA and protein levels rescued the defect of axon growth in SMA mice. Therefore, dysregulated acylcarnitine might also affect SMA phenotypes, possibly through the post-translational regulation of motor neuron-specific protein GAP43. Acylcarnitine plays a role in GAP43-related axon growth/repair pathways and may represent a promising SMA treatment target.

Nevertheless, the inconsistent findings of carnitine/acylcarnitine metabolites in SMA patients argue the pathomechanism of the impaired β-oxidation in SMA. Applying modern techniques for quantitative analysis of carnitine and acylcarnitine of various lengths in different samples (e.g., plasma, urine, and muscle) may help decipher this ambiguity [62,63]. However, similar studies in SMA patients are scarce, and the findings of changed carnitine/acylcarnitine levels in SMA patients with different *SMN2* copies have not been updated. The discovery of plasma and urinary metabolite patterns, specifically reflective of fatty acid catabolism, can help clarify biochemical pathways that link lipid metabolism and provide potential biomarkers monitoring disease progression.

3. Glucose Metabolic Abnormalities in SMA

The concern about glucose metabolism abnormalities was initially raised through clinical observations in mild-to-intermediate phenotypes of SMA patients (Table 2). Two studies of type 2 SMA patients suggested they might be more likely to experience hypoglycemia following fasting [64,65]. A recent study in type 1 SMA patients also showed a similar finding of hypoglycemia even after a short-term fasting (>4 h but <6 h) [66]. The presence of hypoglycemia after fasting has been postulated to have an association with reduced gluconeogenesis. Because skeletal muscle is an important source of gluconeogenic substrates during fasting, hypoglycemia must be considered for SMA patients with severe muscle wasting, especially during surgery and fever [65]. Therefore, it is recommended that patients with recurrent hypoglycemia episodes should be provided with regular meals based on carbohydrates and protein, including late-night meals.

In contrast, other studies have reported hyperglycemia during fasting in patients with type 2 and type 3 SMA, some of whom were diagnosed with diabetes and ketoacidosis (Table 2) [17,67]. The metabolic syndrome features of increased fat mass and decreased lean mass have been reported in patients with type 2 and type 3 SMA [40]. A recent study also indicated that, in a good state, obese children with SMA type 2 were at increased risk of insulin resistance and impaired glucose tolerance, with 50% of participants showing urinary ketones [16]. It has been postulated that as the skeletal muscle is a major target of insulin action, muscle wasting (sarcopenia) promotes insulin resistance with increased risk of hyperglycemia [68,69]. Additionally, hyperleptinemia has been observed in patients with SMA types 1 to 3, which implies an indirect link to insulin resistance [70]. Nevertheless, even if glucose and insulin metabolism show an increased risk of insulin resistance, HbA1c levels are usually normal in most SMA patients examined [16,69,70].

Table 2. Altered Glucose Metabolism in Patients with Spinal Muscular Atrophy.

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Glucose, ketone	Bruce et al. (1995) [64]	Case study	To describe a phenomenon of hypoglycemia in patients with SMA.	SMA: 2	SMA type 2 100%; age 14 years old and 20 years old, respectively	• Repeated episodes of hypoglycemia in both cases. • One associated with acidosis and ketonuria. • Hypoglycemia was explained by reducing muscle protein, leading to lower availability of amino acids (alanine) as substrates for gluconeogenesis in the liver. • Regular meals and a late evening meal were recommended in SMA patients with recurrent hypoglycemia. • Findings could be events not specific to SMA.
Glucose	Orngreen et al. (2003) [65]	Two or more single-arm study	To investigate the effect of 23 h of fasting on plasma glucose and other metabolites, glucose turnover, and hormonal changes in NMD patients with low muscle mass.	SMA: 4 Healthy controls: 6	SMA type 2 100%; mean age 25 years old; average body weight 29.8 kg Controls: mean age 24 years old; average body weight 69.5 kg	• All SMA patients developed hypoglycemia with continued fasting compared to nil hypoglycemia in healthy controls. • Patients experienced frequent attacks of headache, nausea, and dizziness that could be alleviated by food intake. • Findings could be events specific to SMA.
Glucose, insulin	Bowerman et al. (2012) [17]	Cross-sectional	To describe glucose metabolism and pancreatic developmental defects in SMA.	SMA: 6	SMA type 1 100%; age range 7–35 months old. Control: age range 4–36 months old.	• Pancreatic islets from SMA type 1 patients had significantly more alpha cells and fewer beta cells vs. control islets. • Islets appeared disorganized in appearance. • Pancreatic islet compositions were similar among all patients independent of age. • Findings could be events specific to SMA.
Glucose, ketone	Lamarca et al. (2013) [67]	Case study	To describe a phenomenon of DM and diabetic ketoacidosis in a patient with type 2 SMA.	SMA: 1	SMA typ 2, age 29 years old, BMI: 10.2 kg/m^2	• New onset DM presenting with blood sugar: 591 mg/dL), ketonuria (4+) and acidosis (anion gap: 31) • Positive family history of type 2 DM (3 patients onset at 60–70 years old) • Autoimmune markers for type 1 DM were negative • Findings could be events not specific to SMA.

Table 2. *Cont.*

Altered Metabolic Aspect	Reference	Study Design	Study Aim	Enrollment	Patient Features	Main Findings
Glucose, insulin, ketone	Davies et al. (2015) [16]	Case series	To examine the impact of fasting and glucose tolerance in an SMA type 2 population.	SMA: 6	SMA type 2 100%; mean age 8.9 ± 1.7 years old (range 7–10 years old)	• Five of the six patients demonstrated normal HbA1c, and IGF-1 and one participant had a slightly elevated HbA1c, considered prediabetic. • During a 20 h fast, no participant experienced hypoglycemia. • At the end of fasting, insulin, alanine, phenylalanine, and branched-chain amino acids were significantly decreased, whereas free fatty acids were increased considerably, and urine ketones were detected in 50% of participants. • During an oral glucose tolerance test, 100% of participants showed hyperinsulinemia, and 50% showed impaired glucose tolerance, and 83% showed insulin resistance. • Findings could be events specific to SMA.
Glucose	Berti et al. (2020) [66]	Cross-sectional	To describe the incidence of hypoglycemia in type 1 SMA patients after short-term fasting (> 4 h but <6 h)	SMA:45	SMA type 1: 100%; median age: 42 months old (hypoglycemic) vs. 21.5 months old (non-hypoglycemic); BMI: −2.19 kg/m^2	• Hypoglycemia in 17 of 45 patients (5 with fasting for acute illness and 12 with fasting for planned procedure). • Main presentations associated with hypoglycemia are hyperhidrosis and tachycardia • All symptomatic cases improved after intravenous glucose. • Conclude that despite type 1 patients fasting for less than 6 h, hypoglycemia was still common. • Findings could be events not specific to SMA.
Glucose, insulin, IGF-1	Brener et al. (2020) [69]	Cross-sectional	To determine the IGF-1 status in SMA patients and its association with insulin resistance.	SMA: 34	SMA type 1: 47%, type 2: 29%, type 3: 24%; mean age: 7.1 years old; mean BMI: −1.60 kg/m^2	• Insulin-resistant patients (n = 20) had higher IGF-1 levels compared to insulin-sensitive patients (n = 14). • Insulin-resistant SMA patients had normal lipid profile and normal glycemic control (HbA1c levels). • IGF-1 status is associated with insulin resistance in SMA patients with early-onset sarcopenia. • Findings could be events specific to SMA.

SMA: spinal muscular atrophy, DM: diabetes mellitus (DM), IGF-1: insulin-like growth factor-1 (IGF-1).

Similarly, perturbations of glucose metabolism affecting glucose sensitivity and pancreatic defects have been observed in the SMA mice model [17,39,71]. In particular, the metabolic defects in the SMA Smn $^{2B/-}$ mice model were characterized by fasting hyperglycemia, glucose intolerance, hypersensitivity to insulin, and hyperglucagonemia [17]. In the same study, analysis of pancreatic tissue from infants with SMA type 1 has recapitulated similar pancreatic development defects. Reduced SMN protein levels may also affect the insulin-like growth factor 1 (IGF-1) pathway in the liver of SMA mouse model [72]. IGF-1 is an anabolic hormone with a molecular structure comparable to insulin, which shows myotrophic effects on muscle tissue. Dysregulation of the IGF-1 signaling pathway has also been reported in biopsies from patients with type 1 SMA [73]. A recent study further indicated that IGF1 status is associated with insulin resistance in young SMA patients with early-onset sarcopenia [69]. However, the authors concluded that the myotrophic effect of IGF-1 might be adversely affected by insulin resistance, so therapeutic interventions for dysregulated glucose metabolism in SMA should target insulin resistance.

Nevertheless, it has been suggested that SMA patients receiving partial SMN restoration therapy may increase the risk of having pancreatic and glucose metabolism defects [71]. Meticulous monitoring of glucose homeostasis in SMA patients is essential to clarify the role of SMN in glucose metabolism and pancreatic function.

4. Altered Vitamin Level in SMA

A previous study indicates that the activity of SMN depends on folic acid and vitamin B12, both of which are necessary for protein methylation [74]. SMN binds to certain proteins with arginine- and glycine-rich domains, which are modified to dimethylarginine. The binding of other proteins that interact with SMN can also be greatly enhanced by methylation. Inadequate intake of folic acid and vitamin B12 may lead to protein hypomethylation [75], and subsequently may affect the clinical severity of SMA.

The SMN protein may play an active role in bone remodeling or uptake of vitamin D and calcium [35]; therefore, patients with SMA are often accompanied by osteopenia and may contend with fractures due to minor injuries. Compared with other neuromuscular disorders, reduced bone mineral density seems more significant in patients with SMA, especially in those losing ambulatory function [76]. Suboptimal vitamin D intake is frequently observed in patients with all SMA types [18,77]. Low serum levels of vitamin D and 25-OH vitamin D have been reported in patients with type 2 or 3 SMA [34]. However, in a small group of patients with type 1 SMA, the corresponding serum vitamin D levels did not reflect insufficient consumption [78]. Low bone mineral density (BMD) and femur fractures are highly prevalent in all SMA subtypes from a young age; however, few patients met osteoporosis criteria [79]. Adequate bone health assessment and intervention may be an unmet medical need for patients with SMA. It is imperative to determine the natural trajectory of BMD changes at different skeletal sites, especially in adolescent and young adult patients with SMA, and determine if low BMD and propensity to fracture are related to immobility and muscle weakness or direct action of SMN on bone turnover. More work is required to identify effective interventions to delay the decline in BMD and prevent fractures in patients with SMA.

Besides vitamin D and calcium, vitamin E, vitamin K, and folate intakes have been reported below values of Recommended Dietary Allowance (RDA) in half the cohort of patients with SMA [77]. Further research is needed to determine the appropriate intake of vitamin D and other macro- and micro-nutrients in this population.

5. Dietary Issues in SMA

Patients with SMA are at higher risk of suboptimal nutrition intake, and nearly half of the cohort demonstrated either undernutrition (underweight) or overnutrition (overweight) over time [18,77]. Changes in body composition, especially the loss of lean body mass, can be particularly harmful to

SMA patients because it can impair the respiratory strength of already weak muscles [43]. Therefore, nutrition support is considered a core component of multidisciplinary care for SMA patients [15,80].

However, the specific nutritional challenges in this population are not well described, and a particular diet has not been scientifically evaluated. An early study showed that when the mother was fed a lipid-rich diet, the pups of SMA mice could have a longer survival period and improved motor function [39]. These findings suggested that higher fat content may confer protective benefits during motor neuron loss. However, an updated study reported that low-fat diets could nearly double survival in Smn $^{2B/-}$ mice, independent of changes in SMN levels, liver steatosis, or enhanced hepatic functions [81]. Although both studies are in the preclinical phase, such controversies suggest a need to establish clinical nutrition guidance from evidence-based research to provide better care for SMA patients.

The advances in therapy for SMA have improved survival and quality of life, which poses new challenges. The survival of patients with severe SMA has generated new phenotypes, and long-term outcomes are unknown [82]. Noteworthily, nutritional management may have a significant impact on the clinical course and even prognosis. For example, previous studies indicated that nutritional support could affect the therapeutic effects of trial agents on different SMA mice models [83,84]. Although it is difficult to show a clear association between metabolic effects in SMA patients who received therapies at this time, it has been emphasized that nutritional care must also be revised and monitored according to individual needs, especially in the SMA therapeutic era [15]. Optimal nutritional management for patients with SMA includes longitudinal evaluation of weight and length and dietary analysis. Recent studies have demonstrated that a modified diet based on measured energy expenditure and optimized protein can improve ventilation and lean body mass in patients with SMA [18,85]. In the future, non-invasive approaches for body composition assessment, e.g., bioelectrical impedance analysis, can be used to evaluate the nutritional status of children with SMA. Further research is needed to assess the use of elemental and semi-elemental formulas in SMA management, including the optimal intake of macronutrients and micronutrients for nutritional support and the ideal fat content and composition.

6. Conclusions

Active nutrition support and metabolism surveillance are crucial for patients with SMA, and a comprehensive nutritional assessment could guide individualized nutrition therapy for this vulnerable population. With the emergence of new gene-targeted and disease-modifying therapies, which may affect the metabolism of SMA patients, personalized nutritional optimization may become particularly important. Metabolomics study in SMA patients, before and after the disease onset, may provide valuable information regarding the direct or indirect effect of SMN deficiency on metabolic abnormalities. Furthermore, identifying and quantifying the specific metabolites in biofluids of SMA patients may serve as an authentic biomarker or therapeutic target for SMA.

Author Contributions: Y.-J.L. contributed conceptualization, original draft preparation, and manuscript writing. T.-H.C. contributed conceptualization, data curation, original draft preparation, manuscript writing and final approval to this work. Y.-Z.W. contributed conceptualization, data curation, original draft preparation to this work. Y.-H.T. contributed conceptualization, data curation, original draft preparation to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Kaohsiung Medical University Hospital in Kaohsiung, Taiwan, grant number KMUH-108-8R47.

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

References

1. Dubowitz, V. Ramblings in the history of spinal muscular atrophy. *Neuromuscul. Disord.* **2009**, *19*, 69–73. [CrossRef] [PubMed]
2. Darras, B.T. Spinal muscular atrophies. *Pediatric Clin. N. Am.* **2015**, *62*, 743–766. [CrossRef] [PubMed]
3. Lunn, M.R.; Wang, C.H. Spinal muscular atrophy. *Lancet* **2008**, *371*, 2120–2133. [CrossRef]

4. Farrar, M.A.; Park, S.B.; Vucic, S.; Carey, K.A.; Turner, B.J.; Gillingwater, T.H.; Swoboda, K.J.; Kiernan, M.C. Emerging therapies and challenges in spinal muscular atrophy. *Ann. Neurol.* **2017**, *81*, 355–368. [CrossRef] [PubMed]
5. Lefebvre, S.; Burglen, L.; Reboullet, S.; Clermont, O.; Burlet, P.; Viollet, L.; Benichou, B.; Cruaud, C.; Millasseau, P.; Zeviani, M.; et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **1995**, *80*, 155–165. [CrossRef]
6. Burghes, A.H. When is a deletion not a deletion? When it is converted. *Am. J. Hum. Genet.* **1997**, *61*, 9–15. [CrossRef]
7. Burghes, A.H.; Beattie, C.E. Spinal muscular atrophy: Why do low levels of survival motor neuron protein make motor neurons sick? *Nat. Rev. Neurosci.* **2009**, *10*, 597–609. [CrossRef]
8. Tisdale, S.; Pellizzoni, L. Disease mechanisms and therapeutic approaches in spinal muscular atrophy. *J. Neurosci.* **2015**, *35*, 8691–8700. [CrossRef]
9. Wirth, B.; Karakaya, M.; Kye, M.J.; Mendoza-Ferreira, N. Twenty-five years of spinal muscular atrophy research: From phenotype to genotype to therapy, and what comes next. *Annu. Rev. Genom. Hum. Genet.* **2020**, *21*, 231–261. [CrossRef]
10. Tu, W.Y.; Simpson, J.E.; Highley, J.R.; Heath, P.R. Spinal muscular atrophy: Factors that modulate motor neurone vulnerability. *Neurobiol. Dis.* **2017**, *102*, 11–20. [CrossRef]
11. Chen, T.H. New and developing therapies in spinal muscular atrophy: From genotype to phenotype to treatment and where do we stand? *Int. J. Mol. Sci.* **2020**, *21*, 3297. [CrossRef]
12. Nash, L.A.; Burns, J.K.; Chardon, J.W.; Kothary, R.; Parks, R.J. Spinal muscular atrophy: More than a disease of motor neurons? *Curr. Mol. Med.* **2016**, *16*, 779–792. [CrossRef] [PubMed]
13. Simone, C.; Ramirez, A.; Bucchia, M.; Rinchetti, P.; Rideout, H.; Papadimitriou, D.; Re, D.B.; Corti, S. Is spinal muscular atrophy a disease of the motor neurons only: Pathogenesis and therapeutic implications? *Cell Mol. Life Sci.* **2016**, *73*, 1003–1020. [CrossRef] [PubMed]
14. Yeo, C.J.J.; Darras, B.T. Overturning the paradigm of spinal muscular atrophy as just a motor neuron disease. *Pediatr. Neurol.* **2020**, *109*, 12–19. [CrossRef] [PubMed]
15. Mercuri, E.; Finkel, R.S.; Muntoni, F.; Wirth, B.; Montes, J.; Main, M.; Mazzone, E.S.; Vitale, M.; Snyder, B.; Quijano-Roy, S.; et al. Diagnosis and management of spinal muscular atrophy: Part 1: Recommendations for diagnosis, rehabilitation, orthopedic and nutritional care. *Neuromuscul. Disord.* **2018**, *28*, 103–115. [CrossRef]
16. Davis, R.H.; Miller, E.A.; Zhang, R.Z.; Swoboda, K.J. Responses to fasting and glucose loading in a cohort of well children with spinal muscular atrophy type ii. *J. Pediatrics* **2015**, *167*, 1362–1368. [CrossRef] [PubMed]
17. Bowerman, M.; Swoboda, K.J.; Michalski, J.P.; Wang, G.S.; Reeks, C.; Beauvais, A.; Murphy, K.; Woulfe, J.; Screaton, R.A.; Scott, F.W.; et al. Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann. Neurol.* **2012**, *72*, 256–268. [CrossRef]
18. Mehta, N.M.; Newman, H.; Tarrant, S.; Graham, R.J. Nutritional status and nutrient intake challenges in children with spinal muscular atrophy. *Pediatric Neurol.* **2016**, *57*, 80–83. [CrossRef]
19. Lipnick, S.L.; Agniel, D.M.; Aggarwal, R.; Makhortova, N.R.; Finlayson, S.G.; Brocato, A.; Palmer, N.; Darras, B.T.; Kohane, I.; Rubin, L.L. Systemic nature of spinal muscular atrophy revealed by studying insurance claims. *PLoS ONE* **2019**, *14*, e0213680. [CrossRef]
20. Wishart, T.M.; Huang, J.P.; Murray, L.M.; Lamont, D.J.; Mutsaers, C.A.; Ross, J.; Geldsetzer, P.; Ansorge, O.; Talbot, K.; Parson, S.H.; et al. Smn deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy. *Hum. Mol. Genet.* **2010**, *19*, 4216–4228. [CrossRef]
21. Mendonca, R.H.; Rocha, A.J.; Lozano-Arango, A.; Diaz, A.B.; Castiglioni, C.; Silva, A.M.S.; Reed, U.C.; Kulikowski, L.; Paramonov, I.; Cusco, I.; et al. Severe brain involvement in 5q spinal muscular atrophy type 0. *Ann. Neurol.* **2019**, *86*, 458–462. [CrossRef]
22. Somers, E.; Lees, R.D.; Hoban, K.; Sleigh, J.N.; Zhou, H.; Muntoni, F.; Talbot, K.; Gillingwater, T.H.; Parson, S.H. Vascular defects and spinal cord hypoxia in spinal muscular atrophy. *Ann. Neurol.* **2016**, *79*, 217–230. [CrossRef] [PubMed]
23. Wijngaarde, C.A.; Blank, A.C.; Stam, M.; Wadman, R.I.; van den Berg, L.H.; van der Pol, W.L. Cardiac pathology in spinal muscular atrophy: A systematic review. *Orphanet J. Rare Dis.* **2017**, *12*, 67. [CrossRef] [PubMed]

24. Schreml, J.; Riessland, M.; Paterno, M.; Garbes, L.; Rossbach, K.; Ackermann, B.; Kramer, J.; Somers, E.; Parson, S.H.; Heller, R.; et al. Severe sma mice show organ impairment that cannot be rescued by therapy with the hdaci jnj-26481585. *Eur. J. Hum. Genet.* **2013**, *21*, 643–652. [CrossRef] [PubMed]
25. Deguise, M.-O.; Pileggi, C.; Beauvais, A.; Tierney, A.; Chehade, L.; De Repentigny, Y.; Michaud, J.; Llavero-Hurtado, M.; Lamont, D.; Atrih, A.J.B. A mouse model for spinal muscular atrophy provides insights into non-alcoholic fatty liver disease pathogenesis. *bioRxiv* **2020**. [CrossRef]
26. Szunyogova, E.; Zhou, H.; Maxwell, G.K.; Powis, R.A.; Muntoni, F.; Gillingwater, T.H.; Parson, S.H. Survival motor neuron (smn) protein is required for normal mouse liver development. *Sci. Rep.* **2016**, *6*, 34635. [CrossRef]
27. Deguise, M.O.; Baranello, G.; Mastella, C.; Beauvais, A.; Michaud, J.; Leone, A.; De Amicis, R.; Battezzati, A.; Dunham, C.; Selby, K.; et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. *Ann. Clin. Transl. Neurol.* **2019**, *6*, 1519–1532. [CrossRef]
28. Nery, F.C.; Siranosian, J.J.; Rosales, I.; Deguise, M.O.; Sharma, A.; Muhtaseb, A.W.; Nwe, P.; Johnstone, A.J.; Zhang, R.; Fatouraei, M.; et al. Impaired kidney structure and function in spinal muscular atrophy. *Neurol. Genet.* **2019**, *5*, e353. [CrossRef]
29. Allardyce, H.; Kuhn, D.; Hernandez-Gerez, E.; Hensel, N.; Huang, Y.T.; Faller, K.; Gillingwater, T.H.; Quondamatteo, F.; Claus, P.; Parson, S.H. Renal pathology in a mouse model of severe spinal muscular atrophy is associated with downregulation of glial cell-line derived neurotrophic factor (gdnf). *Hum. Mol. Genet.* **2020**, *29*, 2365–2378. [CrossRef]
30. Sintusek, P.; Catapano, F.; Angkathunkayul, N.; Marrosu, E.; Parson, S.H.; Morgan, J.E.; Muntoni, F.; Zhou, H. Histopathological defects in intestine in severe spinal muscular atrophy mice are improved by systemic antisense oligonucleotide treatment. *PLoS ONE* **2016**, *11*, e0155032. [CrossRef]
31. Davis, R.H.; Godshall, B.J.; Seffrood, E.; Marcus, M.; LaSalle, B.A.; Wong, B.; Schroth, M.K.; Swoboda, K.J. Nutritional practices at a glance: Spinal muscular atrophy type i nutrition survey findings. *J. Child. Neurol.* **2014**, *29*, 1467–1472. [CrossRef] [PubMed]
32. Ripolone, M.; Ronchi, D.; Violano, R.; Vallejo, D.; Fagiolari, G.; Barca, E.; Lucchini, V.; Colombo, I.; Villa, L.; Berardinelli, A.; et al. Impaired muscle mitochondrial biogenesis and myogenesis in spinal muscular atrophy. *JAMA Neurol.* **2015**, *72*, 666–675. [CrossRef]
33. Deguise, M.O.; Boyer, J.G.; McFall, E.R.; Yazdani, A.; De Repentigny, Y.; Kothary, R. Differential induction of muscle atrophy pathways in two mouse models of spinal muscular atrophy. *Sci. Rep.* **2016**, *6*, 28846. [CrossRef] [PubMed]
34. Vai, S.; Bianchi, M.L.; Moroni, I.; Mastella, C.; Broggi, F.; Morandi, L.; Arnoldi, M.T.; Bussolino, C.; Baranello, G. Bone and spinal muscular atrophy. *Bone* **2015**, *79*, 116–120. [CrossRef] [PubMed]
35. Shanmugarajan, S.; Tsuruga, E.; Swoboda, K.J.; Maria, B.L.; Ries, W.L.; Reddy, S.V. Bone loss in survival motor neuron (smn(−/−) smn2) genetic mouse model of spinal muscular atrophy. *J. Pathol.* **2009**, *219*, 52–60. [CrossRef] [PubMed]
36. Schmitt, F.; Hussain, G.; Dupuis, L.; Loeffler, J.P.; Henriques, A. A plural role for lipids in motor neuron diseases: Energy, signaling and structure. *Front. Cell Neurosci.* **2014**, *8*, 25. [CrossRef] [PubMed]
37. Harpey, J.P.; Charpentier, C.; Paturneau-Jouas, M.; Renault, F.; Romero, N.; Fardeau, M. Secondary metabolic defects in spinal muscular atrophy type ii. *Lancet* **1990**, *336*, 629–630. [CrossRef]
38. Kelley, R.I.; Sladky, J.T. Dicarboxylic aciduria in an infant with spinal muscular atrophy. *Ann. Neurol.* **1986**, *20*, 734–736. [CrossRef]
39. Butchbach, M.E.; Rose, F.F., Jr.; Rhoades, S.; Marston, J.; McCrone, J.T.; Sinnott, R.; Lorson, C.L. Effect of diet on the survival and phenotype of a mouse model for spinal muscular atrophy. *Biochem. Biophys. Res. Commun.* **2010**, *391*, 835–840. [CrossRef]
40. Sproule, D.M.; Montes, J.; Montgomery, M.; Battista, V.; Koenigsberger, D.; Shen, W.; Punyanitya, M.; De Vivo, D.C.; Kaufmann, P. Increased fat mass and high incidence of overweight despite low body mass index in patients with spinal muscular atrophy. *Neuromuscul. Disord. Nmd* **2009**, *19*, 391–396. [CrossRef]
41. Tein, I.; Sloane, A.E.; Donner, E.J.; Lehotay, D.C.; Millington, D.S.; Kelley, R.I. Fatty acid oxidation abnormalities in childhood-onset spinal muscular atrophy: Primary or secondary defect(s)? *Pediatric Neurol.* **1995**, *12*, 21–30. [CrossRef]
42. Crawford, T.O.; Sladky, J.T.; Hurko, O.; Besner-Johnston, A.; Kelley, R.I. Abnormal fatty acid metabolism in childhood spinal muscular atrophy. *Ann. Neurol.* **1999**, *45*, 337–343. [CrossRef]

43. Poruk, K.E.; Davis, R.H.; Smart, A.L.; Chisum, B.S.; Lasalle, B.A.; Chan, G.M.; Gill, G.; Reyna, S.P.; Swoboda, K.J. Observational study of caloric and nutrient intake, bone density, and body composition in infants and children with spinal muscular atrophy type i. *Neuromuscul. Disord.* **2012**, *22*, 966–973. [CrossRef] [PubMed]
44. Finkel, R.S.; Crawford, T.O.; Swoboda, K.J.; Kaufmann, P.; Juhasz, P.; Li, X.; Guo, Y.; Li, R.H.; Trachtenberg, F.; Forrest, S.J.; et al. Candidate proteins, metabolites and transcripts in the biomarkers for spinal muscular atrophy (bforsma) clinical study. *PLoS ONE* **2012**, *7*, e35462. [CrossRef] [PubMed]
45. Mulroy, E.; Gleeson, S.; Furlong, M.J. Stress-induced ketoacidosis in spinal muscular atrophy: An under-recognized complication. *J. Neuromuscul. Dis.* **2016**, *3*, 419–423. [CrossRef]
46. Lakkis, B.; El Chediak, A.; Hashash, J.G.; Koubar, S.H. Severe ketoacidosis in a patient with spinal muscular atrophy. *Cen Case Rep.* **2018**, *7*, 292–295. [CrossRef]
47. Shababi, M.; Lorson, C.L.; Rudnik-Schoneborn, S.S. Spinal muscular atrophy: A motor neuron disorder or a multi-organ disease? *J. Anat.* **2014**, *224*, 15–28. [CrossRef]
48. Zolkipli, Z.; Sherlock, M.; Biggar, W.D.; Taylor, G.; Hutchison, J.S.; Peliowski, A.; Alman, B.A.; Ling, S.C.; Tein, I. Abnormal fatty acid metabolism in spinal muscular atrophy may predispose to perioperative risks. *Eur. J. Paediatr. Neurol.* **2012**, *16*, 549–553. [CrossRef]
49. Feldman, A.G.; Parsons, J.A.; Dutmer, C.M.; Veerapandiyan, A.; Hafberg, E.; Maloney, N.; Mack, C.L. Subacute liver failure following gene replacement therapy for spinal muscular atrophy type 1. *J. Pediatr.* **2020**, *225*, 252–258.e1. [CrossRef]
50. Hua, Y.; Sahashi, K.; Rigo, F.; Hung, G.; Horev, G.; Bennett, C.F.; Krainer, A.R. Peripheral smn restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **2011**, *478*, 123–126. [CrossRef]
51. Ferreira, G.C.; McKenna, M.C. L-carnitine and acetyl-l-carnitine roles and neuroprotection in developing brain. *Neurochem. Res.* **2017**, *42*, 1661–1675. [CrossRef] [PubMed]
52. Jones, L.L.; McDonald, D.A.; Borum, P.R. Acylcarnitines: Role in brain. *Prog. Lipid Res.* **2010**, *49*, 61–75. [CrossRef] [PubMed]
53. Pennisi, M.; Lanza, G.; Cantone, M.; D'Amico, E.; Fisicaro, F.; Puglisi, V.; Vinciguerra, L.; Bella, R.; Vicari, E.; Malaguarnera, G. Acetyl-l-carnitine in dementia and other cognitive disorders: A critical update. *Nutrients* **2020**, *12*, 1389. [CrossRef] [PubMed]
54. Czyzewski, K.; Stern, L.Z.; Sadeh, M.; Bahl, J.J. Changes in muscle carnitine during regeneration. *Exp. Neurol.* **1984**, *86*, 73–80. [CrossRef]
55. Czyzewski, K.; Stern, L.Z.; Sadeh, M.; Bahl, J.J. Altered rat skeletal muscle carnitine with age and after denervation. *Muscle Nerve* **1985**, *8*, 34–37. [CrossRef]
56. Bresolin, N.; Freddo, L.; Tegazzin, V.; Bet, L.; Armani, M.; Angelini, C. Carnitine and acyltransferase in experimental neurogenic atrophies: Changes with treatment. *J. Neurol.* **1984**, *231*, 170–175. [CrossRef]
57. Carrasco, P.; Jacas, J.; Sahun, I.; Muley, H.; Ramirez, S.; Puisac, B.; Mezquita, P.; Pie, J.; Dierssen, M.; Casals, N. Carnitine palmitoyltransferase 1c deficiency causes motor impairment and hypoactivity. *Behav. Brain Res.* **2013**, *256*, 291–297. [CrossRef]
58. Rinaldi, C.; Schmidt, T.; Situ, A.J.; Johnson, J.O.; Lee, P.R.; Chen, K.L.; Bott, L.C.; Fado, R.; Harmison, G.H.; Parodi, S.; et al. Mutation in cpt1c associated with pure autosomal dominant spastic paraplegia. *JAMA Neurol.* **2015**, *72*, 561–570. [CrossRef]
59. Liang, X.; Lu, Y.; Neubert, T.A.; Resh, M.D. Mass spectrometric analysis of gap-43/neuromodulin reveals the presence of a variety of fatty acylated species. *J. Biol. Chem.* **2002**, *277*, 33032–33040. [CrossRef]
60. Fallini, C.; Donlin-Asp, P.G.; Rouanet, J.P.; Bassell, G.J.; Rossoll, W. Deficiency of the survival of motor neuron protein impairs mrna localization and local translation in the growth cone of motor neurons. *J. Neurosci.* **2016**, *36*, 3811–3820. [CrossRef]
61. Fuller, H.R.; Gillingwater, T.H.; Wishart, T.M. Commonality amid diversity: Multi-study proteomic identification of conserved disease mechanisms in spinal muscular atrophy. *Neuromuscul Disord.* **2016**, *26*, 560–569. [CrossRef] [PubMed]
62. Okun, J.G.; Kolker, S.; Schulze, A.; Kohlmuller, D.; Olgemoller, K.; Lindner, M.; Hoffmann, G.F.; Wanders, R.J.; Mayatepek, E. A method for quantitative acylcarnitine profiling in human skin fibroblasts using unlabelled palmitic acid: Diagnosis of fatty acid oxidation disorders and differentiation between biochemical phenotypes of mcad deficiency. *Biochim. Biophys. Acta* **2002**, *1584*, 91–98. [CrossRef]

63. Aguer, C.; McCoin, C.S.; Knotts, T.A.; Thrush, A.B.; Ono-Moore, K.; McPherson, R.; Dent, R.; Hwang, D.H.; Adams, S.H.; Harper, M.E. Acylcarnitines: Potential implications for skeletal muscle insulin resistance. *FASEB J.* **2015**, *29*, 336–345. [CrossRef]
64. Bruce, A.K.; Jacobsen, E.; Dossing, H.; Kondrup, J. Hypoglycaemia in spinal muscular atrophy. *Lancet* **1995**, *346*, 609–610. [PubMed]
65. Orngreen, M.C.; Zacho, M.; Hebert, A.; Laub, M.; Vissing, J. Patients with severe muscle wasting are prone to develop hypoglycemia during fasting. *Neurology* **2003**, *61*, 997–1000. [CrossRef] [PubMed]
66. Berti, B.; Onesimo, R.; Leone, D.; Palermo, C.; Giorgio, V.; Buonsenso, D.; Pane, M.; Mercuri, E. Hypoglycaemia in patients with type 1 sma: An underdiagnosed problem? *Arch. Dis. Child.* **2020**, *105*, 707. [CrossRef] [PubMed]
67. Lamarca, N.H.; Golden, L.; John, R.M.; Naini, A.; Vivo, D.C.; Sproule, D.M. Diabetic ketoacidosis in an adult patient with spinal muscular atrophy type ii: Further evidence of extraneural pathology due to survival motor neuron 1 mutation? *J. Child. Neurol.* **2013**, *28*, 1517–1520. [CrossRef]
68. Moon, S.S. Low skeletal muscle mass is associated with insulin resistance, diabetes, and metabolic syndrome in the korean population: The korea national health and nutrition examination survey (knhanes) 2009–2010. *Endocr. J.* **2014**, *61*, 61–70. [CrossRef]
69. Brener, A.; Sagi, L.; Shtamler, A.; Levy, S.; Fattal-Valevski, A.; Lebenthal, Y. Insulin-like growth factor-1 status is associated with insulin resistance in young patients with spinal muscular atrophy. *Neuromuscul Disord.* **2020**, *30*, 888–896. [CrossRef]
70. Kolbel, H.; Hauffa, B.P.; Wudy, S.A.; Bouikidis, A.; Della Marina, A.; Schara, U. Hyperleptinemia in children with autosomal recessive spinal muscular atrophy type i-iii. *PLoS ONE* **2017**, *12*, e0173144.
71. Bowerman, M.; Michalski, J.P.; Beauvais, A.; Murray, L.M.; DeRepentigny, Y.; Kothary, R. Defects in pancreatic development and glucose metabolism in smn-depleted mice independent of canonical spinal muscular atrophy neuromuscular pathology. *Hum. Mol. Genet.* **2014**, *23*, 3432–3444. [CrossRef]
72. Tsai, L.K.; Chen, C.L.; Ting, C.H.; Lin-Chao, S.; Hwu, W.L.; Dodge, J.C.; Passini, M.A.; Cheng, S.H. Systemic administration of a recombinant aav1 vector encoding igf-1 improves disease manifestations in sma mice. *Mol. Ther.* **2014**, *22*, 1450–1459. [CrossRef]
73. Millino, C.; Fanin, M.; Vettori, A.; Laveder, P.; Mostacciuolo, M.L.; Angelini, C.; Lanfranchi, G. Different atrophy-hypertrophy transcription pathways in muscles affected by severe and mild spinal muscular atrophy. *BMC Med.* **2009**, *7*, 14. [CrossRef]
74. Friesen, W.J.; Massenet, S.; Paushkin, S.; Wyce, A.; Dreyfuss, G. Smn, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Mol. Cell* **2001**, *7*, 1111–1117. [CrossRef]
75. Majumder, A.; Behera, J.; Jeremic, N.; Tyagi, S.C. Hypermethylation: Causes and consequences in skeletal muscle myopathy. *J. Cell Biochem.* **2017**, *118*, 2108–2117. [CrossRef]
76. Khatri, I.A.; Chaudhry, U.S.; Seikaly, M.G.; Browne, R.H.; Iannaccone, S.T. Low bone mineral density in spinal muscular atrophy. *J. Clin. Neuromuscul. Dis.* **2008**, *10*, 11–17. [CrossRef]
77. Martinez, E.E.; Quinn, N.; Arouchon, K.; Anzaldi, R.; Tarrant, S.; Ma, N.S.; Griffin, J.; Darras, B.T.; Graham, R.J.; Mehta, N.M. Comprehensive nutritional and metabolic assessment in patients with spinal muscular atrophy: Opportunity for an individualized approach. *Neuromuscul. Disord.* **2018**, *28*, 512–519. [CrossRef]
78. Aton, J.; Davis, R.H.; Jordan, K.C.; Scott, C.B.; Swoboda, K.J. Vitamin d intake is inadequate in spinal muscular atrophy type i cohort: Correlations with bone health. *J. Child. Neurol.* **2014**, *29*, 374–380. [CrossRef]
79. Wasserman, H.M.; Hornung, L.N.; Stenger, P.J.; Rutter, M.M.; Wong, B.L.; Rybalsky, I.; Khoury, J.C.; Kalkwarf, H.J. Low bone mineral density and fractures are highly prevalent in pediatric patients with spinal muscular atrophy regardless of disease severity. *Neuromuscul. Disord.* **2017**, *27*, 331–337. [CrossRef]
80. Chen, Y.S.; Shih, H.H.; Chen, T.H.; Kuo, C.H.; Jong, Y.J. Prevalence and risk factors for feeding and swallowing difficulties in spinal muscular atrophy types ii and iii. *J. Pediatrics* **2012**, *160*, 447–451. [CrossRef]
81. Deguise, M.O.; Chehade, L.; Tierney, A.; Beauvais, A.; Kothary, R. Low fat diets increase survival of a mouse model of spinal muscular atrophy. *Ann. Clin. Transl. Neurol.* **2019**, *6*, 2340–2346. [CrossRef] [PubMed]
82. Mercuri, E.; Pera, M.C.; Scoto, M.; Finkel, R.; Muntoni, F. Spinal muscular atrophy—Insights and challenges in the treatment era. *Nat. Rev. Neurol.* **2020**, *16*, 706–715. [CrossRef] [PubMed]

83. Butchbach, M.E.; Singh, J.; Gurney, M.E.; Burghes, A.H. The effect of diet on the protective action of d156844 observed in spinal muscular atrophy mice. *Exp. Neurol.* **2014**, *256*, 1–6. [CrossRef] [PubMed]
84. Narver, H.L.; Kong, L.; Burnett, B.G.; Choe, D.W.; Bosch-Marce, M.; Taye, A.A.; Eckhaus, M.A.; Sumner, C.J. Sustained improvement of spinal muscular atrophy mice treated with trichostatin a plus nutrition. *Ann. Neurol.* **2008**, *64*, 465–470. [CrossRef]
85. Bertoli, S.; De Amicis, R.; Mastella, C.; Pieri, G.; Giaquinto, E.; Battezzati, A.; Leone, A.; Baranello, G. Spinal muscular atrophy, types i and ii: What are the differences in body composition and resting energy expenditure? *Clin. Nutr.* **2017**, *36*, 1674–1680. [CrossRef]

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Nutrients Editorial Office
E-mail: nutrients@mdpi.com
www.mdpi.com/journal/nutrients

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-0365-3352-0