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Feature Papers 2021

Edited by
Peter Clifton

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Editor

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Editorial

Editorial: Diabetology: Feature Papers 2021

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We begin this editorial with a discussion about insulin. Stuart Brink [1] has been involved with paediatric patients with type 1 diabetes for many years and is clearly very knowledgeable and passionate about the subject. He begins with a short history of the idea of pancreatic secretion being involved in the control of glucose, before discussing the Toronto laboratories extraction and their first treatment of Leonard Thompson. He moves from beef and porcine insulin to human insulin, into the modern era of modified insulin molecules to make them short acting or long acting, as well as the demonstration that better glucose control with multiple injections leads to reduced complications both during the trial as well as 20–30 years later. The DCCT was a landmark trial, which pushed clinicians and patients to tighten control, and with that, obtain better measures of control, from sensors to pumps. Technology has expanded dramatically to help with this, has along with newer insulins to gain better control with fewer hypos. Stuart describes how to manage patients transitioning from older insulins to newer analogues or how to start new patients on these regimes, and this provides practical guidance for new clinicians. He also provides some guidance on the use of data analysis from CGMS and how best to use it. Common CGM devices are well described, both freestanding and linked to insulin pumps. He describes advances in inhaled insulin and ultrafast and ultraslow insulins, all of which await full development and approval. Finally, he discusses the thorny issues of availability of insulin for financially distressed parts of the world and what current solutions are available.

Immune Mechanisms in Type 1 Diabetes

Sanjay Rathod [2] has written an elegant review of the immune mechanisms of destruction of beta cells and potential methods of keeping destructive autoimmunity in check. The key point is that the immune system is hardwired to recognise self-antigens and to develop an immune response to them, which is kept under control by T-reg cells—i.e., this immunity is in a non-reactive tolerant state. Thymic tolerance eliminates lymphocytes producing high-affinity antibodies, as well as those not reacting to self-antigens, but it also tolerates lymphocytes producing medium-affinity antibodies. The presence of infection, inflammation or tissue damage may allow the medium-affinity antibodies to become destructive.

Although immune modulation studies have been pursued for over 35 years, no single study has demonstrated clinical effectiveness with an acceptable safety profile, but there have been several hopeful studies that have led to a better understanding of the mechanisms of beta cell destruction.

For T-cell modulation, two anti-CD3 agents (Teplizumab, Otelexizumab) have shown modest clinical benefits by reducing T-cell activation and reducing T-effector cell numbers. Rituximab, an CD20 B cell blocker, also has clinical benefits. Therapies involving CTLA-4-IgG1 chimeric proteins acting as decoy receptors for CD80/86 reduce T-cell activation, as do LFA-3 -IgG1 chimeric proteins. TNF antagonism and combination cyclophosphamide for immune suppression along with ATG and GCSF for T-reg stimulation also have clinical benefits.

A single 14-day regimen of Teplizumab (anti-CD3, a pan T cell marker) delayed the onset of autoimmune T1D by 24.4 months when compared to a placebo-treated group, and

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29 percent of treated patients had HbA1c levels less than 7% and insulin dose requirements of less than 0.5 U/kg per day. This drug now has FDA-expedited status.

Many other potential pathways are discussed, including modulating IL17 cytokine levels and increasing IL2 levels to enhance T-reg cells to damp down the cytotoxic T cells. A new technique currently in clinical trials is to generate cytolytic CD4 T cells that specifically target antigen-presenting cells (APC) with Beta-cell-specific antigens. This destroys the APC cells as well as T cells activated by the APCs.

The other five papers in this special edition covered a wide variety of topics in people with type 2 diabetes, from focus groups in rural Pakistan to the use of the FINDRISC questionnaire in young people in Tanzania [3–7], the use of ultrasound to measure median nerve size, a meta-analysis of studies of the renal tubular marker N-Acetyl-β-D-glucosaminidase to assess early diabetic nephropathy, as well as a study of COVID-19-associated mucormycosis in patients with diabetes. Ultrasound was not helpful as a diagnostic tool, nor was FINDRISC in young people. Urine NAG was best at differentiating normal people from those with normo and microalbuminuria.

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Review

Insulin Past, Present, and Future: 100 Years from Leonard Thompson

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Abstract: Before the discovery of insulin and the critical role of the pancreas vis-à-vis diabetes mellitus pathophysiology, childhood diabetes or what we now call type 1 or autoimmune diabetes mellitus was almost universally fatal. In limited-resource countries (LRC) around the world, this remains sadly true because of the expense and unavailability of medical care, medical information, and/or medications. In 1889, Minkowski and Mering identified the pancreas as the likely source of the problem in pancreatectomized dog experiments, and Langerhans, working with Virchow, identified the islands of pancreatic tissue now named after Langerhans as the likely source of the problem. Prior to that, Cawley, Boucherdat, Zuelzer, Gley, de Meyer, Schafer, Scott, Kleiner, and Paulescu all worked on this problem with varying results until Banting, Best, MacLeod, and Collip in Toronto in 1921 successfully treated pancreatectomized dogs with an alcohol-based pancreatic extract and then were the first to do the same with children and adults with diabetes, starting with Leonard Thompson in early 1922. Urinary and blood glucose levels were reduced, and clinical symptoms decreased concurrently. The magnificent medical historical work by Professor Michael Bliss, also from Toronto, as well as an excellent US NPR Television documentary, describes the trials and tribulations of this event that culminated in the "fastest Nobel Prize" awarded. Progressive biopharmaceutical advances have modified insulin from pigs and cows and then genetically engineered insulin to work much faster and also much slower to provide more modernized ways of providing insulin. Insulin pens then replaced vial and syringe administration, and then insulin pumps coupled with continuous blood glucose sensors have made delivery more physiologic in addition to more attention paid to nutrition advice, education, and psychosocial support around the world. Programs to assist delivery of expensive insulin to LRC administered by Insulin for Life, Life for a Child (LFAC), Changing Diabetes in Children (CDIC) coupled with support by ISPAD (International Society for Pediatric and Adolescent Diabetes) have continued to make such advances available thorough wonderful philanthropy in insulin manufacturers and manufacturers of blood glucose monitoring equipment and insulin pump/sensor suppliers.

Keywords: insulin; banting; best; MacLeod; collip; limited-resource countries (LRC); insulin pumps; blood glucose monitoring; continuous blood glucose sensors

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1. The Pancreas, the "Internal Secretions", and the Discovery of Insulin

In the pre-1900s, what we now call type 1 or autoimmune diabetes mellitus was almost universally fatal. Most presented in childhood or adolescence with classic symptoms of diabetes became cachectic and emaciated from significant glycosuria as well as catabolic changes causing muscle wasting, weakness, and loss of body fat, severe weight loss reflecting acute insulin deficiency, and often, there was diabetic ketoacidosis, decompensation, coma, and death. Diabetes was recognized as the "wasting" disease or the "honey wasting" disease, and hence, its Latin and Greek nomenclature, diabetes mellitus. In financially distressed parts of the world where medical care is minimally available or just too expensive or too far away, not much has changed about diabetes mellitus for children and adolescents until programs such as Changing Diabetes in Children and Life for a Child as well as Insulin for Life offered hope and insulin—sometimes also offering trained diabetes

staff, glucose meters, test strips, and lancets—in recent years [1]. Instead of dying at rates exceeding 95%, such children now are living relatively normal lives with their families and friends, attending schools, and thriving.

It took a very long time, however, for medical specialists to determine that it was the pancreas that was the site of the problem [2]. Oskar Minkowski, working with Joseph Von Mering in 1889 in Strasbourg, identified that the pancreas was the likely source of the problem after pancreatectomized dogs in experiments concerning digestive effects of the pancreas became diabetic and died in their laboratories. Prior to that, Paul Langerhans, a medical student working with Rudolph Virchow in Berlin, had identified the islands of pancreatic tissue that seemed different from the rest of the pancreas, but there was no identification of these "islands" as having anything to do with diabetes until many years later when Gustave-Edourt Laguesse later named them the "islets of Langerhans" and there appeared suspicions, without proof, that there was some type of pancreatic secretion involved with diabetes. In fact, TA Cawley, in 1788, was the first to propose in writing that the pancreas was the culprit anatomically involved with diabetes. In 1845, Boucherdat suspected pancreatic "juices", and in 1883, Claudio Ulesko in Russia wrote about "internal pancreas secretions", but none had been identified.

Michael Bliss, in his amazing historical research (Figure 1) published his book, "The Discovery of Insulin" [3], suggests about 400 citations in the medical literature that he has uncovered talking about the forerunner of insulin and the pancreatic secretions with various degrees of scientific guess-work, research, lectures, and written work to back up these proponents before the awarding of the Nobel Prize in Medicine to Macleod and Banting in 1923, 18 months after the first successful treatment of a human for more than a few hours with what they called insulin. Fourteen-year-old Leonard Thompson was dying at Toronto General Hospital of diabetes, cachectic but not in acute diabetic ketoacidosis or coma when he was brought "back to life" with first relatively unpure beef insulin extract and then with a more purified ethanol extract following the success of Collip, Banting, Best, and Macleod experiments with dogs the previous 6 months. However, the search for the elusive "extract" from the pancreas did not begin in Toronto or Bucharest but perhaps more than 50 years before the work that led to manufacturing success and production of insulin in Canada. For instance, in 1900, George Zuelzer in Berlin patented pancreatic extraction procedures after treating several dogs and then five humans, but complications with fever and excessive hypoglycemia, as well as his described inability to remove the "impurities" from his preparations, caused him to cease his research. Before doing so, he enlisted the resources of several large German pharmaceutical companies, notably Hoechst, Schering, and Hoffmann-La Roche, and the work was discontinued. In 1905, Eugen Gley presented a paper at the French Society of Biology in which he described extracts of degenerated pancreas given to pancreatectomized dogs, in whom decreased urinary sugar and alleviation of diabetes symptoms were noted. Once again, follow-up research was discontinued for lack of support. De Meyer in Belgium in 1906 and then, independently and without apparent knowledge of de Meyer's proposal, Schafer in 1916 proposed "insuline" as the name for the internal pancreatic secretions still remaining elusive but felt to be the source of the problem with diabetes. Ernest Scott wrote his master's thesis that was published in 1912 [4], and in 1919, Israel Kleiner, working at the Rockefeller Institute in New York City, published his work on pancreas extracts abler to decrease both blood sugar and urinary sugar readings [5].

In the late 1800s and the early 1900s, treatment of diabetes was very primitive by today's standards. Understanding was limited, urinary glucose measurements were tedious and not always available, and blood glucose measurements were imprecise, plus required large amounts of blood to be processed in specialized research facilities. JR Macleod, a Scottish physician who had moved to Toronto, Canada, to lead the Department of Physiology at the Medical School, was one of the world's experts on carbohydrate metabolism and had helped create such a way for measuring blood glucose in relatively small quantities of blood. Clinical treatment, however, was promoted by many, such as renowned American

physicians Elliot Joslin in Boston and Frederick Allen in Morristown, New Jersey, to include strict avoidance of sugars and carbohydrates with a diet loaded with fat to attempt to provide calories to youngsters starving from the caloric losses of high volumes of glycosuria and accompanied by protein and fat catabolism, ketosis, and general cachexia. For those who did not succumb to diabetic ketoacidosis and its attendant metabolic decompensation, coma, and death, life could sometimes be prolonged for several months with such a starvation regimen.

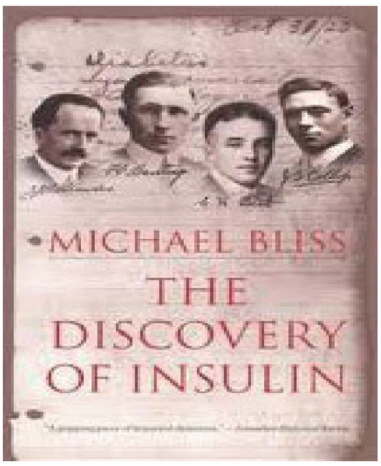

Figure 1. The Discovery of Insulin by Michael Bliss.

During this same time period, a Romanian physician and physiologist Nicolae Paulescu (Figure 2) worked and studied in France and rose to become Professor of Medicine in Bucharest. In the late 1890s, he conducted studies on diabetes that he continued off and on with resumed efforts around 1916 and thereafter. He became convinced that the diabetes factor from the pancreas, which he called either "pancreine" or "pancreatine", could be produced as an extract, but his experiments were inconclusive and not always reproducible [6]. He had some success and read a paper at an international meeting in France, which was published in 1921 [7] with follow-up research that same year [8], but the events in Toronto coupled with the success of Collip, Connaught, and eventually, Eli Lilly and Company overshadowed his work until years later.

Figure 2. Nicolae Paulescu Romanian postage stamp.

At about the same time, and presumably without a good deal of preparation and reading of the available medical literature (but perhaps having heard Paulescu's paper in France), an unemployed young Canadian surgeon Frederick Grant Banting (Figure 3) had an idea about ligating the ducts of the pancreas in dogs so that he could either then transplant the remaining pancreatic tissue back to the dogs to "cure" their surgically induced diabetes or, alternatively, if that did not work, he could figure out a way to produce the elusive pancreatic secretions from the remnant pancreases and then give this extract to reverse diabetes [3]. He went to Macleod to present his idea, and Macleod generously provided free laboratory space for the summer of 1921 to Banting, a medical student,

Charles Best, to serve as his laboratory assistant and several dogs for the experiments. Macleod presumably also made available his methodology for measuring blood glucose from relatively small quantities of blood to complement the known measurements of glycosuria and ketonuria so that the biochemical effects of these experiments could be undertaken. Macleod then went on vacation back to Scotland, while Banting and Best began their work otherwise unsupervised. Attempts at pancreatectomy killed several of their dogs, and those that did not die from the transplant or the surgery/anesthesia probably died from the second surgical attempt at transplantation of residual pancreatic tissue post-pancreatic duct ligation. While there are different descriptions of these events from the four main participants from 1921 to 1922, Banting, Best, [9] Macleod [10], and J.B. Collip [11], something along those lines took place, and the surgical approach with ligation of the pancreatic ducts was abandoned in favor of attempting to produce a pancreatic extract with the internal secretions. These were first recorded as being called "isletin" and then only later changed to insulin.

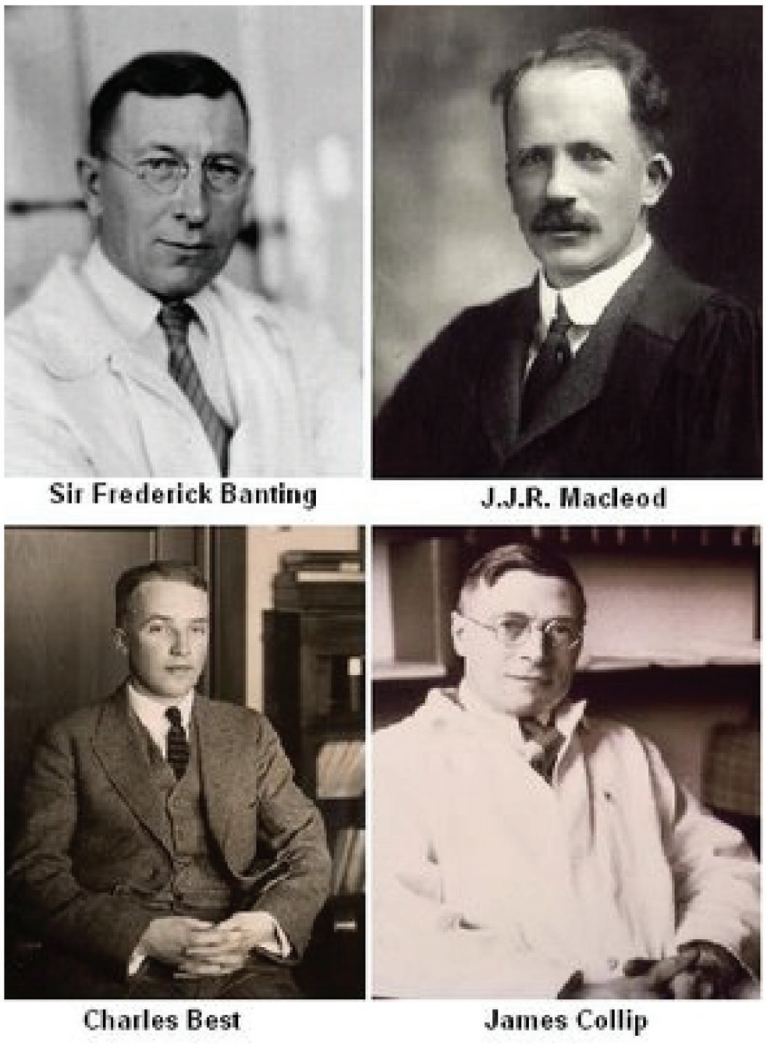

Figure 3. Frederick Banting, Charles Best, J.J.R. Macleod, and James Collip photographs.

With some success in bringing the blood glucose, urine glucose, and urinary ketone levels down in some of their dog, and after some trial and error with various methods of extracts, attempts at purification to remove fever-producing infections in their animals, abscess formation, and death, J.B. Collip was brought in as a Visiting Professor of Pharmacology to assist with the extraction procedures and their success with the dogs was

presented 14 November 1921 at the University of Toronto Physiological Journal Club and in a paper published in February 1922 [12].

Eventually, high-dose ethanol was used as an extraction processor with some success. Fourteen-year-old Leonard Thompson, a patient at Toronto General Hospital, was near death; the team prepared the pancreatic extracts and injected Leonard Thompson on 21 January 1922—a point in time when his blood glucose was approximately 580 mg/dL (approximately 32 mmol), and his glucose levels dropped about 100 mg/dL (approximately 5.5 mmol) (Figures 4 and 5). Soon afterward, a sterile abscess occurred, and the extract was halted. Sugar levels rose, and several days later, a higher potency pancreas extract was prepared so that on 23–25 January 1922, Leonard Thompson's glucose levels normalized, his glycosuria decreased significantly, and his ketonuria cleared. This was well documented in a paper presented by Macleod on 3 May 1922 in Washington DC and published that same year [13].

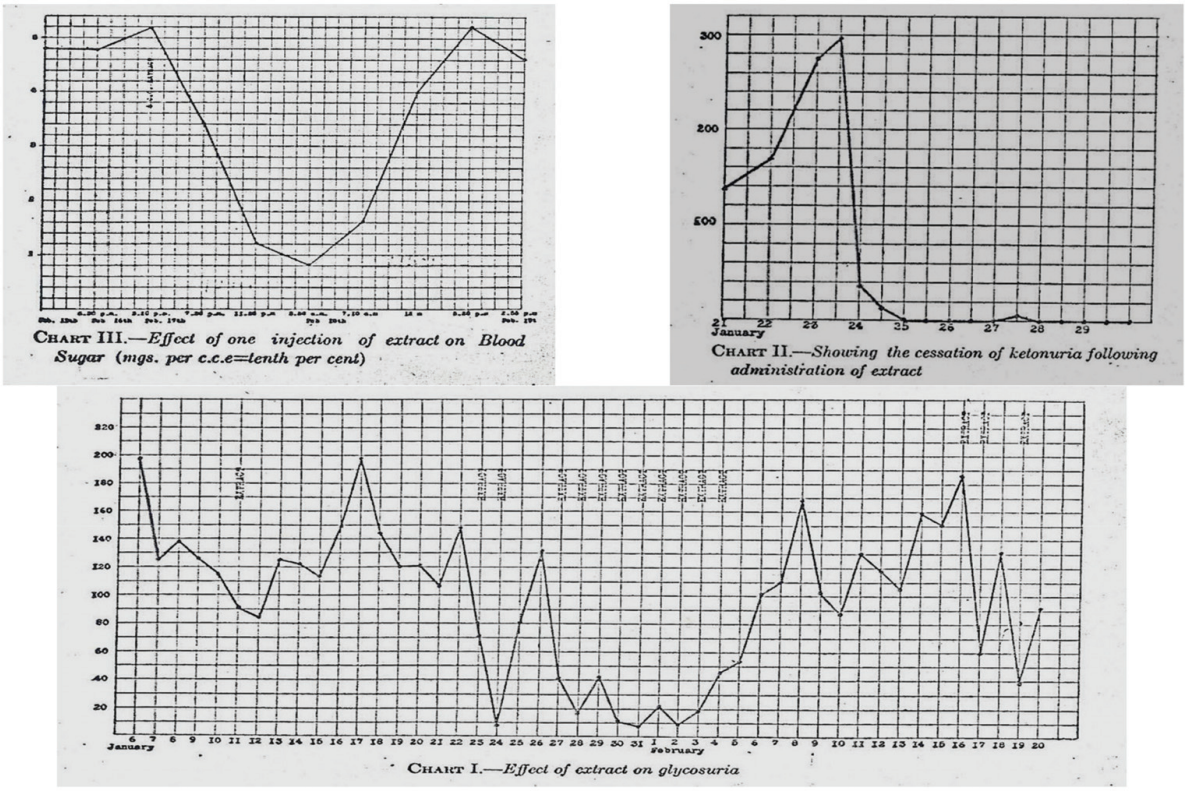

Figure 4. Banting and Best's experimental data.

Figure 5. Old insulin vials.

Patents were submitted, and Connaught Laboratories in Toronto was authorized to begin production of what would later be called "Toronto insulin" or regular insulin elsewhere in the world. Production problems and removal of impurities continued to be problematic, and Eli Lilly, an American pharmaceutical company in Indianapolis, became involved in the problem, solving it successfully in 1922 so that insulin in sufficient volume to treat not only the patient in Toronto but also elsewhere became possible. Lilly officials agreed to pay royalties to the University of Toronto to support research in exchange for manufacturing rights for North and South America, while Connaught retained manufacturing rights for Canada. Lilly's insulin was named Iletin, and beef, as well as pig pancreas, was collected for processing.

In 1922, August Krogh (Figure 6), a Danish physician and physiologist, having heard about their discoveries, visited Macleod and Banting. With a physician wife who also had diabetes, he returned home to Copenhavn with manufacturing rights to produce insulin at the Nordisk Laboratories, working closely with his wife, Marie Krogh, and another physician pharmacologist, Hans Christian (HC) Hagedorn (Figure 7). The Medical Research Council in the UK (eventually Wellcome) obtained rights for British manufacturing, as did Hoechst in Germany. In 1925, Novo Laboratories, started by two former employees of Krogh and Hagedorn, also began producing insulin in direct competition with Nordisk; Nordisk and Novo remained in highly competitive mode through the next several decades [14] until finally merging and ending "hostilities" as Novo Nordisk Laboratories in 1989.

Figure 6. August and Marie Krogh's photograph.

Figure 7. Hans Christian Hagedorn's photograph.

Stories of the miracle of insulin were published in the Toronto newspapers and in other newspapers around the world. Physicians from around the world heard of these successes, and patients came to Toronto to receive insulin until it became more widely available throughout Canada, the USA, and Western Europe. Within 18 months of this discovery, an incredibly short time, Banting and Macleod were awarded the Nobel Prize in Medicine and Physiology in 1923 principally for bringing the extraction work to fruition with sufficient production to allow numerous pharmaceutical companies around the world as well as the Connaught Laboratories in Toronto to make insulin available for the treatment of humans on a larger scale than ever before. Adding to an already tumultuous partnership

among the four principles, Banting immediately sent a telegram to Dr. Elliot Joslin, then hosting Charles Best in a meeting in Boston, that Banting would share his portion of the Nobel Prize with Best. Macleod responded in kind and announced that he would share his portion of the prize with Collip. It would take many years before the animosities of the four Canadian investigators would be resolved, and now, some 90+ years after this Nobel Prize was awarded, there still remain many who would have favored other insulin investigators for the pioneering work that they did to make it all happen, such as Zuelzer, Gley, Scott, Kleiner, Paulescu, etc.

Figure 8 shows now world-famous photographs of before and after pictures of several patients representing the face of pediatric diabetes and the miracle of insulin in bringing them back to life as photographed by Dr. Ralph Major in Kansas City and Dr. H. Rawle Geyelin in New York City in Figure 9.

Figure 8. Normal young child prior to diagnosis, same child extremely emaciated at diagnosis of diabetes before insulin treatment and then several months after successful insulin treatment by Dr. Ralph Major in Missouri.

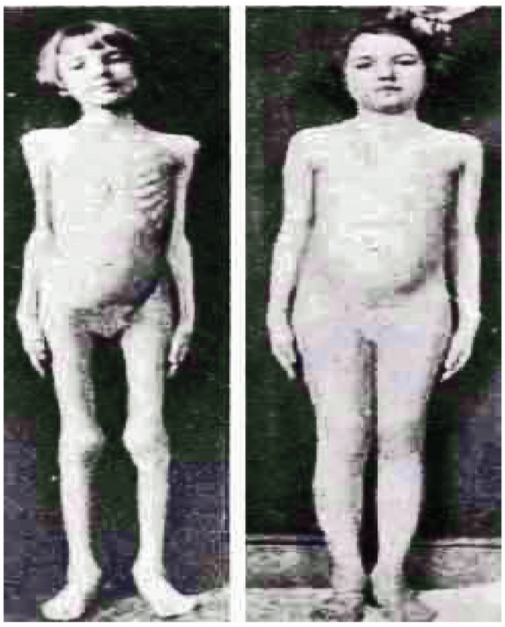

Figure 9. Emaciated school-age young girl pre-diagnosis and the same child several months after successful insulin treatment for diagnosis of diabetes by Dr. H. Rawle Geyelin in New York.

In the years that followed, further improvements in removing impurities made the multiple insulin injections easier to tolerate for patients. Research competition between Nordisk and Novo as well as Lilly and others helped develop protamine zinc long-acting insulin (PZI) in or around 1936 and then what became known as NPH, neutral protamine Hagedorn as an intermediate-acting insulin in or around 1946. PZI and NPH were used in

addition to regular insulin based on the patient's urine glucose readings. In 1954, different formulations of zinc were used to change the time course of insulin into an alternative intermediate-acting insulin called lente insulin, combining proportions of a shorter acting semilente insulin with the longer-acting ultralente insulin (Figures 10 and 11). In or around 1973, more purified preparations, especially those purely extracted from pig pancreas, became available to help with those allergic still to the impurities of insulin.

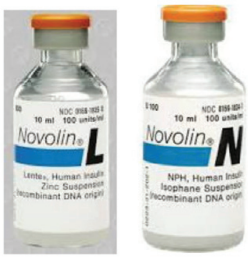

Figure 10. Novolin Lente and Novolin NPH insulin vials.

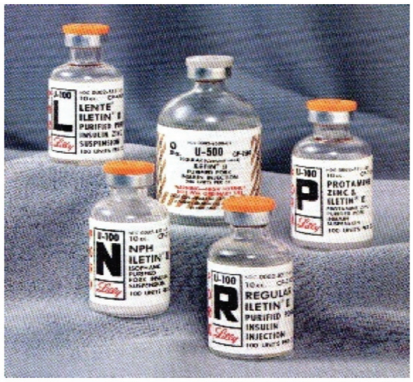

Figure 11. Lilly Lente, NPH, Regular, and PZI insulin u100 vials as well as u500 vial.

In the 1970s, scientific progress helped researchers create semi-synthetic insulin starting from animal-source insulin and modifying some of the amino acids in the insulin molecule itself for the first "human" insulins available. In the 1980s, bioengineering had harnessed the knowledge of genetics using modified E.coli or yeast cells to become "factories" to produce insulin, and thus bioengineered human insulin became available from the major manufacturers: insulin analogs (Figure 12). With each progressive step, impurities were reduced, and some of the allergic problems, as well as most—but not all—of the lipoatrophy and lipohypertrophy, also believed to be related to such impurities, diminished.

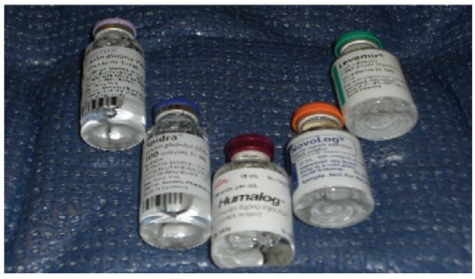

Figure 12. Insulin analog vials.

In 1985, insulin pens (Figure 13) became available, first in Denmark, then in the rest of Europe, and afterward in America and around the world. Such pens used smaller needles, were therefore less painful than syringes, easier to use, and easier to carry around as multiple injection therapy was becoming more popular and attempts at improving

overall glycemic control in conjunction with self-monitoring of blood glucose became more available.

Figure 13. Insulin pens.

During these years, research studies such as the KROC study pointed to the long-term complications of diabetes as likely being directly related to short- and long-term glycemic control so that efforts were directed more at lowering glycemic targets safely. Insulin pumps were introduced but were initially cumbersome and very expensive. The multicentered Diabetes Control and Complications Trial (DCCT) (Figure 14) began in 1982, recruited its first patients in 1983, and reported its first final formal study results—a year earlier than originally anticipated because the results were so dramatically conclusive—at the American Diabetes Association annual scientific meetings in Las Vegas in 1993 to an enormous audience [15]. The small cohort of adolescents recruited for the DCCT was more difficult to manage, as expected, than the large adult study cohort but showed the same patterns of metabolic memory as did the adult cohort [16,17]. DCCT follow-up studies, EDIC, continue to highlight the importance of "glucose memory" and its role in all these known long-term complications associated with type 1 diabetes even 20–30 years after initiation of improved glycemia as part of the original DCCT study cohort [18].

Figure 14. DCCT investigator's hat (photographed by Dr. Stuart Brink).

2. Insulin Analogs: Fast-Acting Lispro, Aspart, and Glulisine

During the 1980s and 1990s, insulin analogs became available as molecular scientists deciphered the actual alpha and beta chain molecular structure of insulin and its connecting peptide from the proinsulin molecule. Thereafter, bioengineering maneuvers allowed pharmacologists to create the genetic sequencing to produce human insulin by inserting these newly identified sequences into genetically modified bacteria or yeast. Rapid-acting insulin analogs began to replace bioengineered human regular insulin to provide faster initial absorption subcutaneously, more physiologic postprandial glycemic coverage, shorter overall duration of insulin effect, and improved flexibility of treatment at the expense of more daily injections of prandial insulin. The potential for reducing oxidative stress with reductions in postprandial glycemic excursions has also been documented with all three such analogs [19]. Lilly produced the first available such rapid-acting insulin analog, lispro, (Humalog®) (Figure 15) in 1996 [20], followed thereafter by aspart insulin (Novolog® or Novorapid®) by Novo Nordisk in 1999 and glulisine (Apidra®) by Sanofi-Aventis. All three were available in pen or syringe and vial format coupled with improvements in pen mechanics and use.

Figure 15. Humalog pen.

Lispro insulin (see Figures 16–18) is biosynthetic human insulin produced by modified E.coli with lysine and proline amino acids 28 and 29 switched in their positions on the beta chain of the insulin molecule. With this modification, the regular insulin was less likely to form hexamers (see Figure 17) as its three-dimensional configuration was altered and thus absorption characteristics were changed, and speedier uptake occurred as there were more dimers and monomers being picked up in the subcutaneous space [21]. A shorter total duration of activity was also noticed so that there was significantly less nocturnal and late-onset hypoglycemia in the following 3–12 h time periods [22]. Because of the earlier fast-acting insulin uptake, better postprandial glycemia was noted [23], and patients reported 60% improved ease of use, 58% improved meal timing and preparation, and 30% improvement in daily activities [24], and significant treatment satisfaction [25].

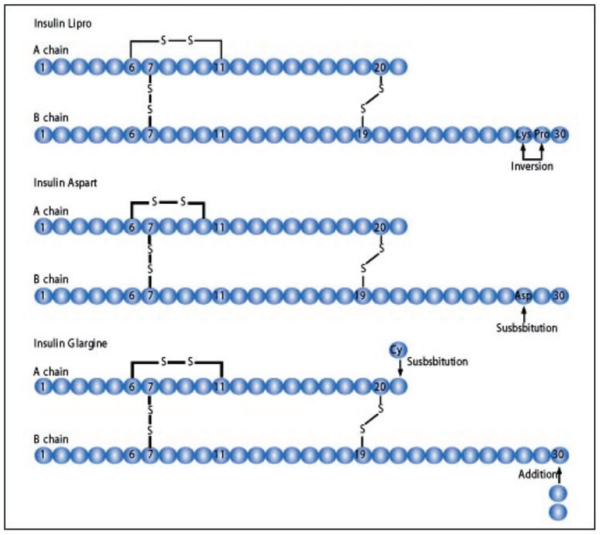

Figure 16. Molecular substitution of insulin molecules to produce insulin lispro, insulin aspart, and insulin glargine.

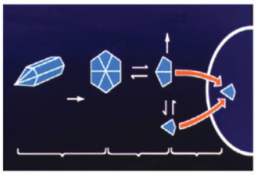

Figure 17. Insulin lispro producing fewer hexamers and more dimers and monomers.

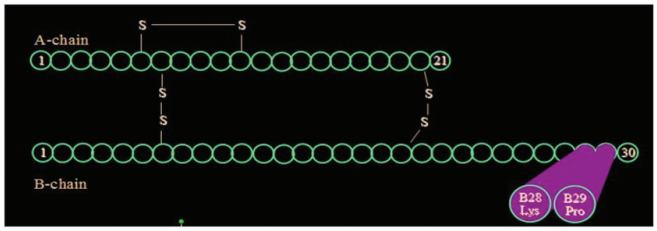

Figure 18. Insulin lispro beta chain aminoacid substitutions.

Prepubertal lispro studies in which we participated [26] using home blood glucose monitoring profiles during routine activities in comparison with regular insulin coupled with then available basal NPH dosage showed that pre- and post-meal lispro insulin could be utilized with good therapeutic results, although pre-meal dosage was slightly better than post-meal dosage and both lispro dose strategies were equal or better than regular insulin for prandial dosing so that the proof-of-concept for this new form of insulin was verified per protocol intent.

In the peripubertal period as well as in the pubertal and post-pubertal age groups, the same results were published in multidose insulin protocols with an emphasis in many such studies on the decrease in nocturnal hypoglycemia (Figure S1), presumably reflecting the shorter "tail-effect" of the faster analog compared to regular insulin [27]. Life-table analysis in the same studies confirmed this relative decrease of overnight hypoglycemia in the adolescents participating (Figure S2). In a separate study published the same year where twice-a-day basal NPH was provided in addition to prandial lispro insulin and efforts were made to optimize the NPH dosage according to blood glucose self-report, comparisons of glycosylated hemoglobin results and frequency of even mild hypoglycemia showed improvement (Figure S3). This was particularly significant since the DCCT studies showed the importance of such lowering of GHb results but at the expense of more frequent hypoglycemia; the lispro studies showed that the overall glycemic exposure (i.e., GHb) could be lowered in a safer fashion with the rapid-acting analogs as prandial performers [28], and this was true at all GHb levels achieved.

Aspart insulin (Novolog® or Novorapid®), a modification of the B-chain 28th amino acid with aspartic acid (Figure 19), was introduced a few years later by Novo Nordisk also in pen and vial and syringe format (Figure 20). In their studies (Figure 21), improvements in postprandial glycemic excursions were documented for all three meals in young patients compared to regular insulin used prandially. In addition, the same improvement in avoiding nocturnal hypoglycemia was documented as with the lispro studies [29]. Fructosamine and glycemia area under the curve both showed very statistically significant improvements in these somewhat shorter studies. These studies were designed to not only show equivalent efficacy with animal source and semisynthetic human regular insulin but succeeded in showing better results postprandially (i.e., lower glucose levels) at the same time that they decreased late-onset hypoglycemia after the meals and in the middle-of-the-night [30].

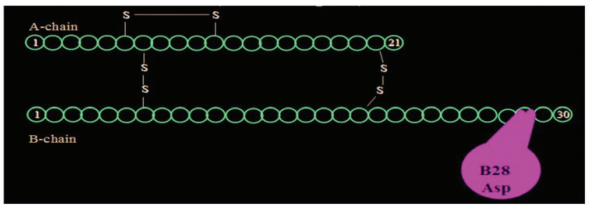

Figure 19. Aspart (Novolog®) aminoacid substitution scheme.

Figure 20. Novolog pen.

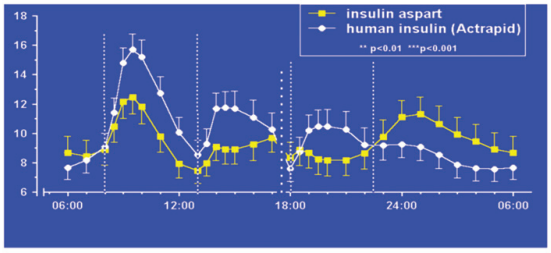

Figure 21. Aspart (Novolog®) vs. regular Actrapid study results showing less hypoglycemia.

The third rapid-acting insulin available was created by Sanofi-Aventis, glulisine insulin (Apidra®) (Figure 22, pen format) and involves substation of lysine for asparagine in the B3 position and lysine replaced by glutamic acid in the B29 position (Figure 23) to modify the action of the insulin molecule in the subcutaneous space. It has similar beneficial characteristics, acts in an improved manner as a prandial insulin, and decreases postprandial hypoglycemia and nocturnal hypoglycemia hours after the meals in a similar fashion clinically to lispro and aspart insulins [31].

Figure 22. Apidra® glulisine pen.

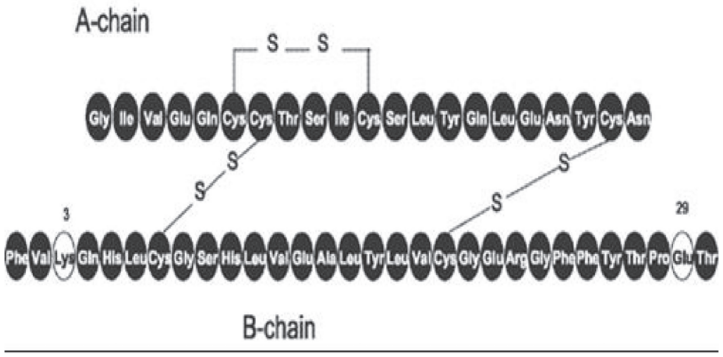

Figure 23. Aspart (Novolog®) insulin schematic diagram showing B3 substitution of lysine for asparagine and B29 with glutamic acid substitution.

In practical terms, all three of the rapid-acting insulins cost about the same but are more expensive than the previous animal source as well as biosynthetic human regular insulin preparations. All three can be used in infants, children, adolescents, adults, and the elderly without any difficulties understanding that they would ideally be provided about 15 min ahead of food to try to match glycemic excursions of most foods and snacks and would need to be coupled with some type of basal/background insulin for between-meal glycemic control. All three fast-acting insulin analogs have the same potential benefits and have minimal differences in terms of clinical performance, lessening of nocturnal and postprandial hypoglycemia, improvement in coverage for postprandial glycemic excursions, and equal or improved A1c results. Because of their fast uptake, in the very young child who does not always eat a full meal or in those where this is not possible psychologically, postprandial dosing still works quite well as a "back-up" plan [32].

With insulin pumps, the three fast-acting insulin analogs, lispro, aspart, and glulisine, are all available, and all work quite well. As with an MDI regimen, with CSII [33] use, there is less daytime as well as nocturnal hypoglycemia occurring and specifically far less nocturnal severe episodes of hypoglycemia-related loss of consciousness or seizures, the most severe types of hypoglycemia. The actual bolus effect seems to diminish by about 30% per hour after the bolus is administered, so after the first hour, about 70% of the insulin action occurs; after the second hour, about 40% occurs; after the third hour, this tapers to about 10% with very little, if any, effect on the blood glucose readings by about the fourth hour after bolus pump administration.

3. Insulin Analogs: Basal Slow-Acting Glargine and Detemir

Hoechst began clinical trials in 1998 with what was then called HOE 901, later glargine insulin. Glargine (Lantus®) (Figures 24 and 25) was genetically modified human insulin with an A-21 glycine substitution for the A-chain end terminal amino acid ASN in addition to an arginine-arginine addition to the end of the B-chain of the insulin molecule. Glargine had a flatter action curve compared to NPH, lente, or ultralente insulin at the

same time; it was "relatively peakless", and this was especially important for their success in documenting less nocturnal hypoglycemia [34]. Several different zinc formulations of glargine were studied, and all showed the same therapeutic benefits with lowered fasting glucose levels; at the same time, there were fewer nocturnal hypoglycemic episodes [35]. The comparison data (see Figure 26) were demonstrated [36] using glucose levels achieved as well as glucose infusion rates and the expected earlier NPH peak effect compared to the lower, relatively peakless, and more prolonged glargine effects. In some patients with glargine, there is a mild peak effect between 12 and 16 h after injection, while in many patients using relatively small doses of glargine, the initial 24+ hour duration of glargine waned somewhat. This was especially seen in the younger patients using smaller overall dosage (compared to the adults or more overweight patients) so that while it is typical to start with a bedtime-only glargine dose, depending upon actual blood glucose profile data analysis, it is not uncommon for pediatric, adolescent and young adult patients to require glargine twice-a-day with a ratio of about 70–80% glargine at bedtime and 20–30% glargine with breakfast in the morning. Some even have a reverse need for the larger morning glargine dose and the smaller bedtime dose of glargine. Others have a different time action course with the mild peak occurring post-breakfast or prelaunch so that a suppertime rather than bedtime glargine dose works better. These decisions can be recognized with frequent blood glucose monitoring and data analysis [37].

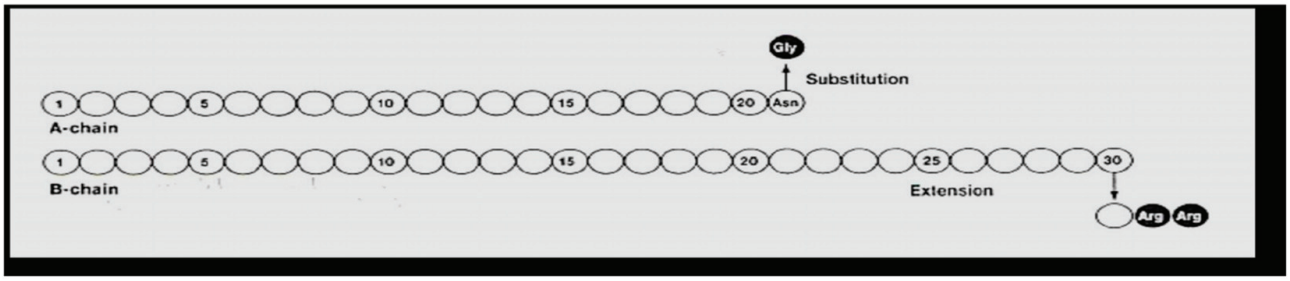

Figure 24. Lantus® (insulin glargine) glycine–arginine–arginine schematic.

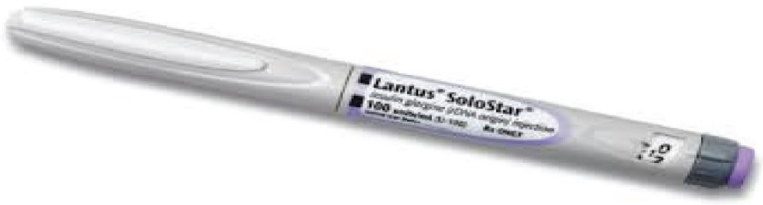

Figure 25. Lantus® pen.

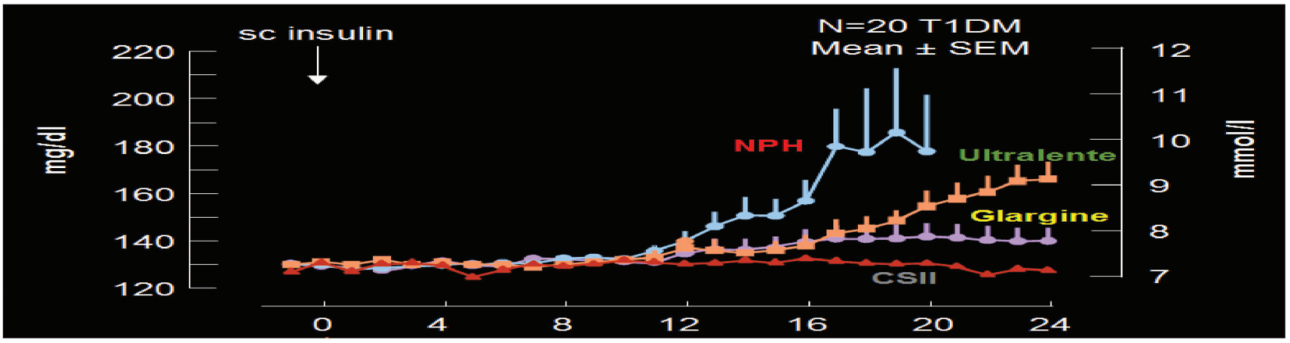

Figure 26. Study results comparing earlier NPH peak and shorter duration effects on glucose levels compared to continuous subcutaneous insulin infusion (CSII; insulin pump) vs. glargine insulin (more similar to CSII results) vs. ultralente insulin (longer effects than NPH but shorter duration than glargine or CSII).

Novo Nordisk (see Figure 27) entered the insulin market with their basal insulin, detemir, in 2005. Detemir (Levemir®) substituted a threonine for the terminal B29 lysine amino acid and attached a C14 fatty acid chain (myristic acid) to the threonine (Figure 28). Through this process, the detemir insulin molecule binds to albumin in tissue and blood and is therefore very slowly released to the general circulation [38]. Detemir became the second basal bioengineered insulin to be made available with similar favorable clinical results [39] plus it had "less binding" to the IGF-1 receptors [40], and so there was potentially less mitogenicity associated with detemir compared to glargine (Figure 29); clinically, there has not been any long-term studies to answer this question scientifically. There were some initial worries that glargine had some association with cancer risk increases, but these turned out to be statically incorrect, and further longer studies have not drawn these same conclusions. Detemir also had a relatively flat effect on glucose levels, but its duration was a bit shorter than glargine, and this difference was magnified a bit in the younger patients using smaller doses than the more overweight adult population studied. For most practical purposes, while detemir can be started as a single night-time basal insulin, it is most frequently utilized as a twice-a-day, pre-breakfast plus bedtime overlapping basal insulin, with the same caveats of utilizing blood glucose monitoring profiles to identify those who need higher or lower doses either in the evening or morning, more equal doses for the twice-a-day detemir treatment or even reversed distribution as mentioned with glargine insulin provision. Detemir, like glargine, is associated with improved fasting glucose levels (i.e., lower); at the same time, there is less nocturnal and between-meal hypoglycemia compared to NPH or other intermediate or longer-lasting insulin products [41].

Figure 27. Levemir® (detemir) pen.

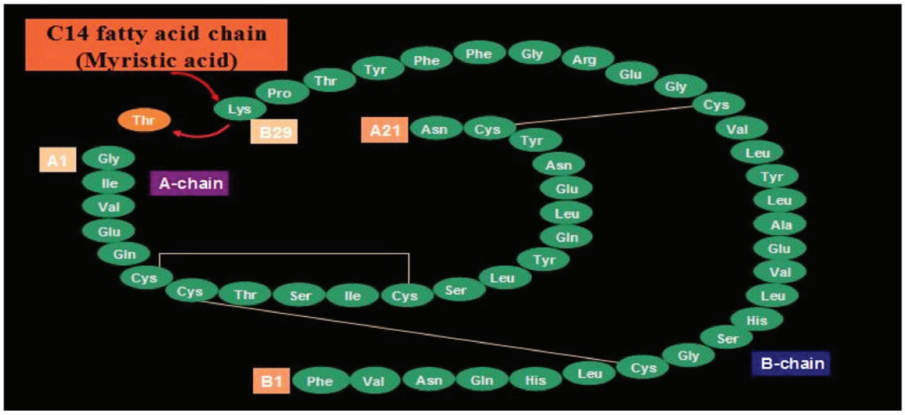

Figure 28. Levemir® (detemir) B29 and C14 substitutions on insulin molecule.

At our center, NEDEC, we utilize all three rapid-acting analogs as long as they have equal availability and insurance company coverage. Insulin decisions are individualized in terms of doses required for the three major meals of the day, and when there is little activity or large snacking, then sometimes snacks are also covered in prandial fashion using a model of insulin–carb ratios created for that person as well as correction factors in a style similar to what we teach for insulin pump treatment. None of this is rigid and dogmatic, and all is aimed to provide maximum flexibility for day-to-day variability, especially with changes in toddlers, children, and adolescents as well as young adult lifestyles. We teach the importance of waiting about 15 min prior to food intake to allow the subcutaneous rapid-acting insulins to begin to reach peak insulin efficacy about the same time as the food intake is reaching peak glycemic load. In this fashion, we believe that we maximize opportunities for improved insulin coverage of food peaks and allow the rapid-acting

insulin analogs to begin to decrease their effects and concentrations in the body at about the same time that the food effects are waning. In comparison with the Hvidore study reports of A1c in a multicenter analysis [42], NEDEC A1c results are in the "excellent, green" range without excessive or severe hypoglycemia reported either during the daytime hours or overnight time period (Figure 30). Nevertheless, despite maximizing educational and re-educational efforts [43], this remains a challenge to optimize such insulin–food timing for many of our patients and their families [44]. In keeping with the message of the DCCT, we would summarize the NEDEC Type 1 Diabetes Treatment Goals as aiming for blood glucose as close as possible to those without diabetes, but without excessive or severe episodes of hypoglycemia. The education approach is a psychosocially oriented approach wherein the entire team of health care professionals, physicians, nurses, nurse-educators, nutrition specialists, exercise specialists, psychologists, and social workers work with the same philosophy and with the same goals individualized for the patient and his or her family to optimize such treatment. This usually involves a multi-dose basal-bolus model with one of the three fast-acting analogs given at times when food is eaten coupled with one of the two longer-lasting basal analogs (see below). The NEDEC philosophy was spelled out in some detail in several summary clinical articles [45]. When the transition from beef and pork insulin to human insulin took place, it was an easy change because this meant fewer instances of localized lipoatrophy and lipohypertrophy were seen. The rare case of allergic reactions locally or systemically to beef or pork insulin products also became even rarer. With the advent of the synthetic insulin analogs, both the fast-acting and the slower basal insulin analogs, the transition continued since not only did such local problems became even more rare, but the improved physiological balance of insulin to food helped decrease hypoglycemia while at the same time made the quality-of-life improvements because the timing of insulin administration and the use of pens over syringes were easier for our patients and their families. This all happened at the expense of more daily injections and was seen in many other centers as well. Insulin pump use also increased from about 10% of the NEDEC patient population in the 1990s to now about 60% using pumps, with about 45% using combinations of insulin pumps and continuous glucose sensors. An example of the improvements in A1c outcomes [46] is presented from the Barbara Davis Diabetes Center at the University of Colorado in Denver when one of the insulins being used was changed to a faster-acting analog prandial lispro (Figure 31).

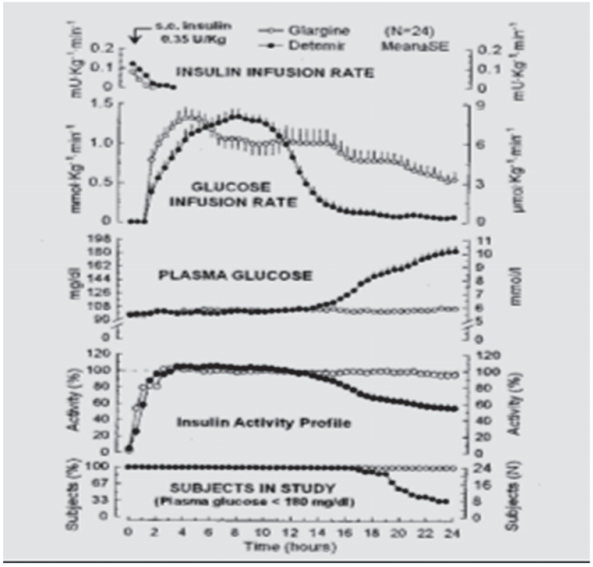

Figure 29. Glargine vs. detemir insulin comparison effects.

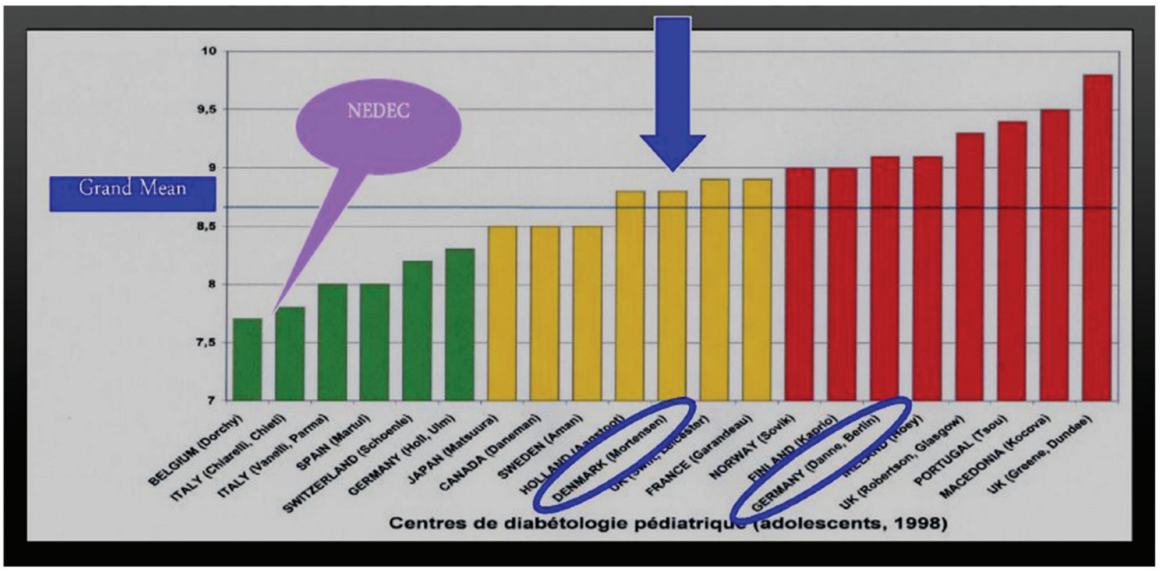

Figure 30. MDI treatment regimens: prandial lispro, aspart or glulisine plus basal glargine or detemir insulins comparing glycohemoglobin average results in participating pediatric and adolescent diabetes specialty centers in Hvidore Study vs. NEDEC results.

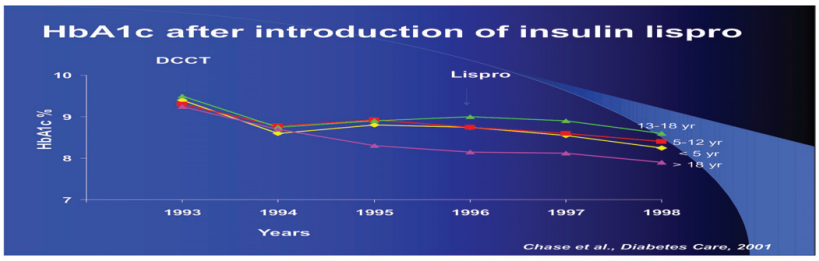

Figure 31. Lispro A1c improvement.

When eating away from home at school or at a restaurant, the fast-acting analogs could be better timed with the food consumed. If food flexibility was desirable, decisions to cover extra snacks could easily be accommodated with these fast-acting analogs (Figure 32). For the preschoolers whose parents could not always guarantee that a certain amount of food would actually be eaten, fast-acting insulins could be provided after the meal rather than before with almost equal coverage of the food. When changes in activities or exercise occurred without advance warning, adaptations with food and insulin could also be realized easier than with the earlier insulin preparations using the basal-bolus model. What might be called the "Professor Harry Dorchy" model of flexibility and adaptation [47,48] was possible with the prior insulin preparations but more easily facilitated with the analogs and their physiological adaptations and time-course of action based on frequent blood glucose monitoring and analysis of such monitoring results. Detailed logbooks help in this learning process, as do newer computer downloading of meters, which make it easier to graph patterns, identify trends, and allow proactive as well as reactive responses. Insulin–carbohydrate ratios for foods and glycemic index of foods both are more ideally treated at NEDEC with insulin pumps because square waves and dual waves, as well as temporary basal adjustments, make such adaptations easy to be taught and accommodated. Similar concepts are also taught with multi-dose insulin regimens (MDI), which makes the transition from MDI to CSII much easier to accomplish since the same terminology is utilized by the educational team.

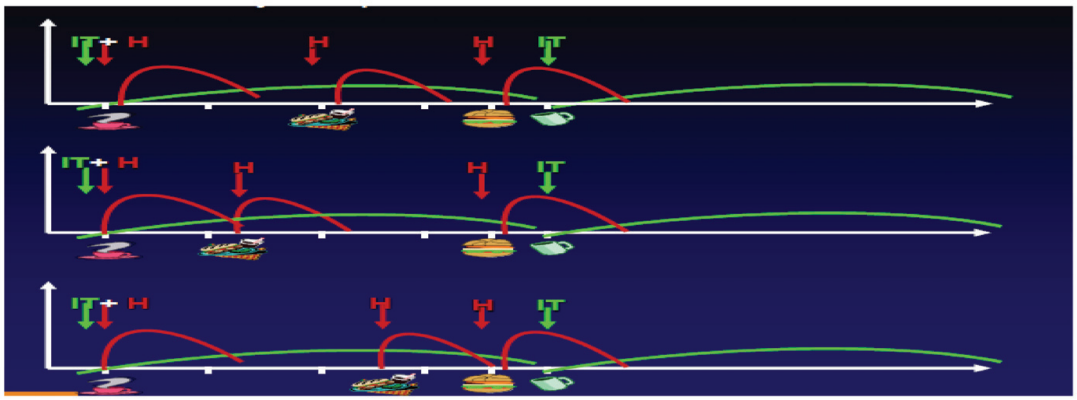

Figure 32. Basal-bolus flexible meal options.

4. NEDEC Transition from Previous Insulin Regimens to Basal-Bolus MDI with Analogs

Coupled with the fast-acting meal-time and snack-time analogs are either of the two readily available longer-lasting "basal" insulin analogs that replaced NPH, lente, and ultralente insulin: glargine and detemir insulin. With type 2 diabetes patients, bedtime glargine or detemir can often be utilized just once a day, but with smaller doses required for children and also in growing adolescents, usually twice-a-day glargine and twice-a-day detemir seem to be needed to provide smoother basal insulin coverage. This, too, is not done dogmatically but rather started with a single night-time basal insulin dose of glargine or detemir once a day. Thereafter, blood glucose levels throughout the day and the night guide the treatment team, patient, and family as to the necessity of once- or twice-a-day basal insulin analog provision. The smaller the dose of insulin, usually the need for two smaller overlapping basal insulin analog doses each day. Occasionally, a reverse pattern is required wherein only morning glargine or detemir seems to work better than bedtime or twice-a-day basal insulin, and this too is decided based upon actual BG results and pattern analysis of food and activity.

At NEDEC, when the new basal analogs became available, most of our patients were using MDI with overlapping three or four doses of NPH; in an attempt to provide optimal basal insulin at the same time, there were lower doses at any moment, thus decreasing hypoglycemia risks between meals and especially overnight. This proved to be very successful and assisted the safe achievement of DCCT-level GHb results; at the same time, there were minimal severe episodes of hypoglycemia taking place. When the new analogs showed improvement in this same fashion, but with even fewer injections needed and even lowered nocturnal hypoglycemia, the patients were slowly but nearly universally changed to either detemir or glargine basal insulin preparations. The more common use of analogs has been documented in many studies for these similar reasons [49].

The NEDEC changeover protocol that was successfully developed was as follows:

1. Add up the total cloudy insulin doses for the entire day (i.e., all three or four NPH doses being used);
2. Start with eliminating all the NPH doses the next day but providing pre- and postprandial blood glucose testing and coverage with either lispro, aspart, or glulisine prandially;
3. Give the first basal insulin dose as glargine or detemir at 80% of the total basal insulin dose previously used and give it at bedtime;
4. Keep this dose steady for 2–3 days with some 3–4 am blood glucose checks to prove that there is no nocturnal hypoglycemia occurring;
5. Titrate the prandial bolus insulin analog dose according to pre and postprandial paired BG readings;
6. Titrate the bedtime glargine or detemir dose based upon the next morning's pre- and post-breakfast as well as prelaunch BG readings aiming for a target of fasting BG of 100 mg/dL (~5.5 mmol);
7. With prebreakfast hypoglycemia, decrease the bedtime glargine or detemir;

8. With prebreakfast hyperglycemia, increase the bedtime glargine or detemir;
9. With the expectation that only 10–20% of pediatric and adolescent patients provided bedtime glargine or detemir alone will have sufficient basal insulin effect to last a full 24 h, expect that some morning glargine or detemir will be added based on late afternoon–evening hyperglycemia;
10. When the morning basal insulin is added, expect the evening glargine or detemir dose to be decreased by approximately an equal amount;
11. Understand that sometimes the reverse pattern occurs and more morning glargine or detemir is needed than evening basal analog;
12. Similarly, in some individuals, the morning and bedtime long-lasting analog doses are equal, but this is a smaller portion of patients;
13. Once the overall pattern of basal insulins is established, go back and fine-tune to adjust the prandial fast-acting analogs by looking at pre and postprandial glycemic excursions with reminders about appropriate pre-meal timing of analog doses to actual timing of when food is ingested (usually about 15 min);
14. Sometimes there is also a need to change the bedtime basal insulin to suppertime when there is a more prolonged peak from the glargine or detemir;
15. If there is a large morning or afternoon snack, these may also need prandial bolus insulin analog coverage according to activity and BG results;
16. Many no longer need mid-morning, mid-afternoon, or even bedtime snacks because of the relatively peakless nature of the basal insulin compared to the stronger NPH peaks used previously, and so calories can be cut back, and these snacks eliminated.

A major benefit of the NEDEC approach [50] with analog MDI insulins is a reduction in frequency and severity of hypoglycemia at the same time that overall improvement in glycemic control. This produced better GHb results and less glycemic variability using a flexible carbohydrate counting approach coupled with frequent monitoring of blood glucose levels for analysis either in a color-coded logbook or by downloading blood glucose meters for computer graphic review. The analogs offer more predictable effects than the beef-pork, purified animal insulins, or semisynthetic human insulin preparations. Pen use has increased for ease of provision since there is a separate decision needed for prandial bolus dosing with this MDI approach. Pens are also available for both glargine and detemir basal insulins. Syringes can be used in those who prefer them or for cost considerations, with vials of analog insulin also available per individual circumstances. The MDI basal-bolus insulin approach is easier to teach than with premixed insulins, allows for dose adjustments for activity and food changes as well. Usually, glycemic excursions are decreased, and carbohydrate counting benefits become more apparent when using this educational approach to insulin delivery coupled with more daily pre and postprandial BG checking. Some detemir studies have suggested that detemir was somewhat more predictable than glargine, but these have not been conclusive. Within-subject variability remains rather large from day-to-day with old as well as new insulin systems because of vagaries of measurement, dose administration, subcutaneous uptake, differences in glycemic food excursions and food variability, carbohydrate counting expertise, and perhaps also with inconsistencies of activity and stress effects on day to day management of type 1 diabetes inherent in the human condition: gym or no gym, after-school sports or homework, video gaming stress, family dynamics, and friend-to-friend relationships, exam-taking, sleeping late, etc. A unique Novo Nordisk clamp study using NPH, glargine, and detemir documented the day-to-day variabilities with these three subjects' clamp results shown as an example in Figure 33.

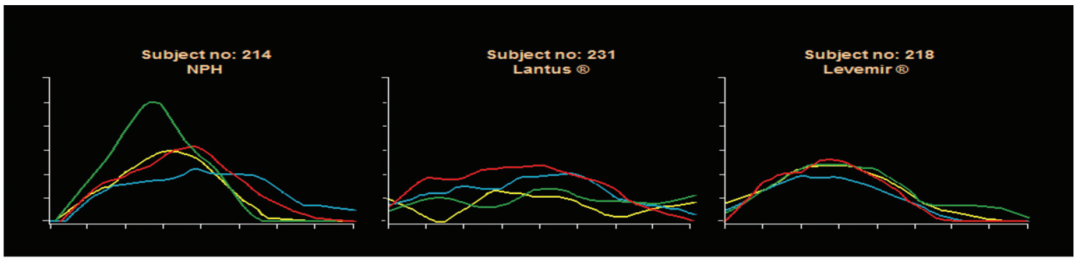

Figure 33. Different subjects in clinical study comparing NPH, Lantus, and Levemir basal insulin regimens with each of four days represented in a different color and Lantus showing significantly less day-to-day variability than NPH and Levemir showing more consistency than either NPH or Lantus.

As Sperling elegantly has written [51], the difficulties of type 1 diabetes remain with the inherent variability of food ingested, changes in activity from day-to-day, vagaries of insulin absorption and effect metabolically, counter-regulatory responses, and the ongoing potential dangers and fears of hypoglycemia; what he has called the Scylla and Charybdis of blood glucose control in children but this is somewhat lessened with the newest analogs compared to earlier preparations.

5. NEDEC New Patients Using MDI Basal-Bolus Analog Insulins

New patients can be started with a pump-like basal-bolus educational treatment protocol either as an outpatient in the ambulatory clinic or in the hospital, depending upon the set-up and functioning of the diabetes team. At NEDEC, our preference if patients are not in acute DKA at diagnosis is to keep patients at home with their families, but this requires mother and father to be available for teaching on a daily basis for the first few days, requires that they have adequate transportation to and from the clinic/office, and requires that they have access to a telephone to communicate for dose adjustments and questions as the teaching ensues. This has been extremely successful, cost-saving, and psychologically beneficial since it provides a very positive message that diabetes will be manageable, that the team will be available for questions and problem solving, and that life will proceed [52].

New type 1 diabetes patients can be treated with the following protocol:

1. Teach self-monitoring of blood glucose to the patient if old enough and to both parents, sometimes to older siblings as well;
2. Teach home blood or urine ketone monitoring to all;
3. Start with preprandial rapid-acting analogs, either lispro, aspart, or glulisine, unless one or another is mandated by an individual insurance company at that moment;
4. Start with an initial guestimate dose of glargine or detemir for the first day;
5. Sometimes only give rapid-acting analog as an initial dose and wait for bedtime to give first basal glargine or detemir and sometimes give a small basal dose as well as the first prandial dose just to get started;
6. Pre and 2 h postprandial BG levels in the color-coded logbook for home analysis;
7. Begin simple carbohydrate restrictions for the first day, figure out the first "home" meal to be able to estimate initial meal dosage;
8. A phone call to learn first home blood glucose and ketone results;
9. Review what will be eaten;
10. Estimate of next prandial lispro, aspart, or glulisine dosage based on what was learned from the initial pre and postprandial glucose levels;
11. A phone call several hours later with next meal and review of what will be eaten once again for the second meal at home and for ongoing encouragement to drink sufficient salty liquids to correct any dehydration at diagnosis;
12. Decisions about prandial lispro, aspart, or glulisine dose for the second meal based on whether initial guestimates of prandial dosing were correcting for hyperglycemia appropriately or not;
13. If no evening basal insulin given yet, give first evening basal insulin at bedtime;

14. Often see patient and family for prebreakfast BG testing, second teaching session, and creation of first formal meal plan the next morning according to schedule demands;
15. Bring breakfast from home;
16. Review techniques of blood glucose and ketone testing once again together;
17. Administer and teach some basics of deciding to go up or down with initial breakfast prandial analog dosing based on initial day's responses;
18. Review administration of prandial analog together;
19. Wait 15 min and allow breakfast to be eaten while some meal plan teaching begins;
20. Answer questions and encourage some reading at home with continued preprandial telephone communication to have health care professionals decide prandial dose, review BG pre and postprandially, adjust food choices, etc.;
21. Continue four times/day communications for several days until some pattern exists to either start second basal insulin in the morning, switch to a supper vs. bedtime basal insulin choice, and/or family and patient begin to gain some comfort in initial choices about insulin dosing.

Usually, by day 3–4, phone calls can be spaced out to one a day depending upon how often in-office teaching will take place, reading and math skills and documented learning is taking place, etc. The same can be done in an inpatient model, although with the much higher expense for overnight hospital stay vs. returning patient and family to home. If patients travel far distances to come to the hospital/clinic because of expense or distance, the ambulatory approach to new diagnosis must be modified. As more comfort and learning are established, downloading blood glucose meters can also be taught for home use in addition to color-coding logbooks for reactive as well as proactive treatment decisions and maximizing day-to-day flexibility. We also usually start with relatively simple pamphlets that talk about insulin, insulin administration and pen use, blood glucose monitoring, and family participation, then move to more kid-friendly books such as those available by Disney (multiple languages), Lilly, Novo Nordisk, sanofi-aventis, Ragnar Hanas Type 1 Diabetes Manual (available in numerous languages), American Diabetes Association Wizdom series, or the Barbara Davis Centers' Understanding Diabetes manual by Peter Chase and David Maahs. Many others are available from local diabetes teams and/or diabetes associations or can be made available online depending upon circumstances and resources. Home learning should complement hospital and/or in-office ambulatory teaching and be individualized according to patient and family caregivers' needs and skills.

6. Insulin Pumps

Over the last several decades, insulin pumps (continuous insulin infusion, CSII), initially without continuous glucose monitoring sensors (CGMS) attached to them but more recently integrated with pumps and sensors that communicate with each other, have become available. These have become smaller, more sophisticated, and all deliver basal fast-acting or more rapid-acting insulin automatically programmed and changeable on an hour-by-hour basis if necessary. These basal insulins replace the earlier intermediate- and longer-acting insulin preparations but in a more adaptable fashion so that individuals can change different parts of the day and night accordingly based on blood glucose monitoring patterns identified. When food is eaten or if there is any obvious change in either hyperglycemia or hypoglycemia, further adaptations can be provided with bolus changes manually. More recent innovations connect with CGMS and are beginning to predict and adapt in advance of actual heights and nadirs of glycemia in an effort to avoid some of the extreme glycemic variability that often still occurs with injectable regimens. As computer algorithms and sensors improve and as insulin is further adapted for more predictable responses, such innovative combinations of pumps and sensors coupled with newer insulins should continue to evolve and move toward fully mechanical artificial pancreases, which started as manually operated insulin pumps but with computer-driven artificial intelligence moving them to more and more automatic bidirectionality able to respond to glycemic variability in a predictive as well as responsive fashion. This should hopefully

help reduce extreme hypoglycemia as well as hyperglycemic bursts that all too frequently still occur with manually provided insulin injections, even with the newer analogs.

7. Blood Glucose Monitoring with AI, Artificial Pancreas Hybrid Closed Loops, and Closed Loops: Decision Support Systems for Insulin Adjustment

Cell phones, computers, blue tooth, wi-fi, and the internet have all contributed to amazing interactivity [53], and this type of technology has been increasingly incorporated into diabetes technology management in an effort to assist day to day management hurdles. Continuous glucose monitoring systems have begun replacing self-blood glucose measurement, allowing better analysis of glucose data information as well as more identification of patterns for both hyperglycemia and hypoglycemia, particularly overnight and post-meal glycemic excursions [54]. Insulin data can also be recorded with these same systems with more technological advancements and data processing capabilities not only with pumps but also with insulin pens facilitating such processing capacity. Medical integration with other technologies such as smart watches and GPS connectivity allow more and more of such data to become available for health care providers as well as families and patients themselves. Enormous amounts of such data require sophisticated data processing, which frequently increases the psychological burden of analyzing the data, the time needed by the health care team for analysis, and, of course, also home processing between follow-up visitation [55]. Clearly, there is also stress and anxiety in the use of such equipment that also needs to be recognized and addressed.

Availability of such new technologies is expensive, and systems for incorporation for reimbursement also need to be developed as well as acknowledging that this does not automatically improve glycemic control without support services vis-à-vis education, strategies for safe use, and efforts to promote continued and ongoing usage as well as integration so that anxiety and monitoring stresses are also reduced at the same time overall glycemic improvement occurs. This has been demonstrated [56] in younger children (and their parents), school-agers, and adolescents as well as young and older adults, and even in type 2 diabetes patients, it is now being evaluated for such technological advanced usage.

However, there remains a data paradox that having more information and more sophisticated methods of delivering insulin, as well as ways to counter-balance food and activity as well as stress effects on glucose levels per se, sometimes also correlates with worse health outcomes, and this is thought to reflect the increased financial and emotional burden of such systems. Time spent during health care visits also may become more face-time with computers than actual health care professional: patient/family time during visits as well as between visits, especially in the beginning phase of such treatment processes—and even with ongoing support and encouragement. Health care professionals have to learn to use such tools in somewhat different ways compared to more traditional approaches and, of course, in areas where there are limited or only medium resources available, such tools remain impractical under such circumstances (see section below). Nevertheless, with more availability of cellular technologies, even in LRC or MRC areas of the world, electronic reminders and ways to communicate are being facilitated increasingly. Reminders to health care providers also can be incorporated into such systems. Focused patient reports and ways to highlight important aspects of such results can also facilitate improving care.

Such decisions involve food and snack choices, carbohydrate counting, and ways to more optimally balance insulin delivery to improve glucose time in range at the same time as decreasing hypoglycemia and hyperglycemia occurrences. Initial efforts in recent years have focused on reducing hypoglycemic events, especially severe hypoglycemia associated with loss of consciousness and/or seizures, while attempting to improve time in range instead of merely shifting the glycemic results into hyperglycemic ranges with raised A1c results and known long-term complications risks. This has been demonstrable with numerous insulin delivery systems and different types of pumps and sensor systems [57]. The person with diabetes (PWD) needs to cope and make decisions about insulin when food changes, amounts of food or types of food change, the timing of eating changes, and

also when activity or illness, as well as emotional stresses of normal life, occur and this can actually be documented to involve 1–2 h every single day. As stated by Dr. Nimri: "Both the complexity and intensity of diabetes management justify an overwhelming need for further decision-support that is becoming available". With the recent COVID-19 worldwide pandemic and the associated decreases in face-to-face office and clinic consultations, such digital diabetes data applications and reviews have been taken place in many parts of the world where teleconferencing and connectivity are possible. Dr. Nimri has further documented more than 1000 diabetes-related applications currently available on the Apple App Store and Google Play to assist health care providers as well as PWD and their families. These help to collect data, summarize and highlight important aspects of such data, and/or remind/encourage healthier choices with increasingly available digitized systems beginning to use medical-based artificial intelligence to help with actual decision making. Automatic downloading has been shown to facilitate such systems actually being used when time can be saved in the process. Not only pump and sensor systems but also blood glucose meters can be connected in such systems for automatic or semi-automatic analysis. Insulin dose titration algorithms can also be recommended in many of these systems with the goal of more consistent application, better pattern recognition, and treatment recommendations to consider. Medtronic Carelink, Abbott Libre, Tandem t:connect, and Dexcom Clarity are some of the available systems involving pumps and sensors. Blood glucose meters by Johnson and Johnson, Ascencia, Abbott and Roche, and others also have similar systems, and independent downloading systems such as Glooko and Tidepool have also become available for sharing such data.

8. Data Analysis

One of the more important graphs for home review as well as in-office/clinic review is the standard or modal day pattern. This can be obtained from the usual blood glucose stand-alone systems downloaded to most of the home, office/clinic/hospital computerized programs currently available and can generate many types of graphic displays, pie charts, and numerical summaries depending on individual preferences and circumstances. This allows home data analysis of blood glucose information on a daily, weekly, or monthly basis and helps facilitate potential changes in food, activity, and/or insulin choices accordingly. Not all PWD will have access to such electronics, and not all PWD will have the mathematical skills to use these on their own. Appropriate educational and psychosocial support should be an integrated part of the health care team's responsibilities and ongoing discussions to help meet the needs of the PWD and his or her family members [58].

Examples of such data analysis are presented in the next figure, which shows overall patterns as well as color-coded day-by-day comparative analysis.

For this author, one of the more potent graphs for home review as well as in-office review is the standard or modal day pattern to see if there are outliers. If these can be explained and learned, then it should be possible to utilize such information to be proactive and therefore prevent future occurrences more and more often. In Figure 34, for instance, there are several days of post-dinner hyperglycemia that would suggest either poor carbohydrate potion control not being covered by sufficient prandial insulin, lack of use of the extended bolus function for high-fat foods at this meal, or just not timing the insulin bolus correctly without allowing sufficient time prior to insulin administration to adequately cover the meal quantities. Because of the 24 h time period for these several days, the "correct" answer in this instance was not utilizing the square wave bolus function for high fat-carbohydrate foods on those three days; just making this correction "solved" the problem on future evenings quite readily.

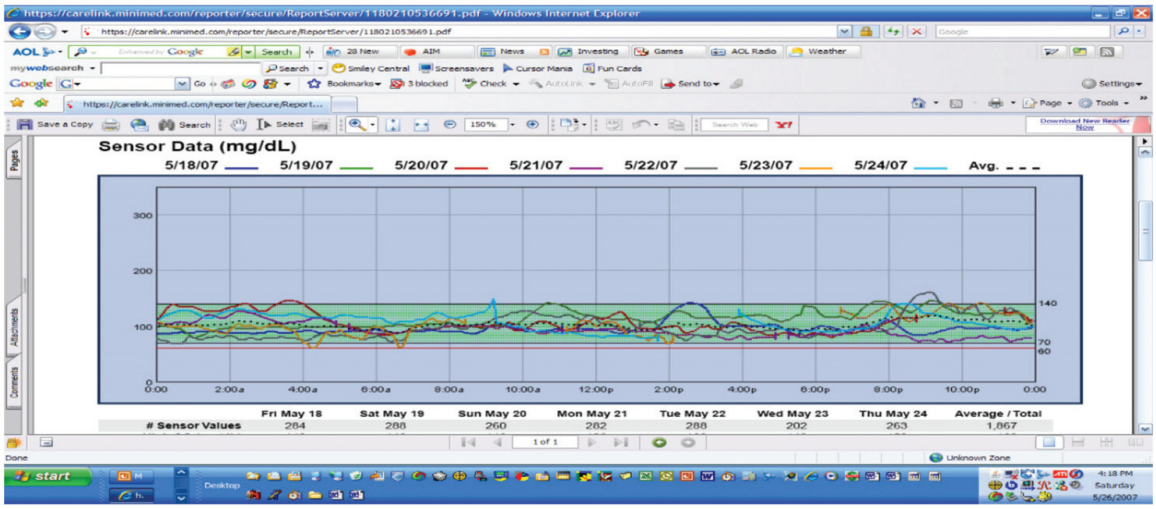

Figure 34. Carelink sensor data.

Alternative programs (Figure 35) have alternative graphic displays, and some are easier to use for individual patients so that whatever works is the program that should be utilized. Some will identify averages but not individual day-by-day readings of either home blood glucose readings or CGMS data. Newer pump statistical downloads allow identification of hypoglycemic episodes, meal-time analysis vs. overnight analysis, as well as more detailed statistics including averages, percentage hyperglycemia vs. hypoglycemia, total average insulin dose, basal–bolus ratios, and differences between sensor glycemia and blood glucose capillary data.

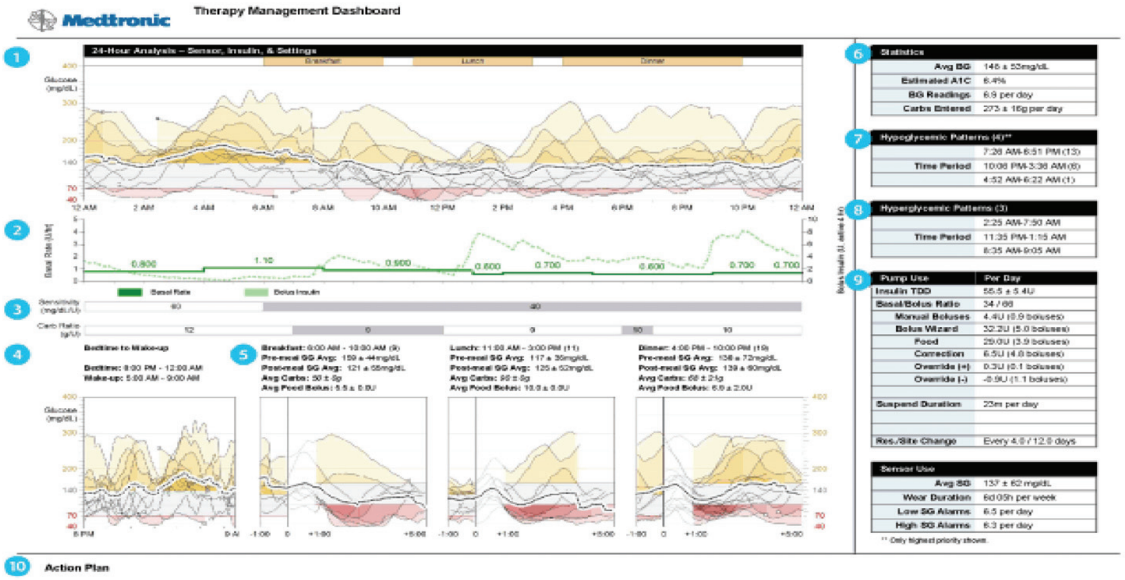

Figure 35. Pump CGMS data analysis graphics.

A key issue for the health care team is to have a unified approach to recommendations for how often downloading at home should occur, whether or not it should take place only at the office visits or between visits with this author recommending optimal downloading twice a week so that specific aberrations or variance can be identified and remembered in an effort to become more proactive and less reactive. It remains extremely important to have nurses, dieticians, social workers/psychologists, students, and physicians making consistent recommendations and approaches to minimize confusion for the PWD or family members. This also facilitates improved compliance while still emphasizing individual

needs and differences. Both reactivity to high and low values as well as being more preventive (proactivity) are very important to learn and practice so that improved glycemic control, lowered glycemic variability, and ultimately better quality of life, and fewer short- and long-term complications may occur. Some patients can learn this quite effectively, and others who have math or language difficulties may take longer to learn to respond, if at all. Sometimes other members of the family must be called to assist under such circumstances. Practice in the office and practice at home between visits need to be encouraged, and assistance with computer problems from meters, pumps, and CGMS manufacturers also may be required to help with home systems.

In the past few years, the CGMS have improved dramatically [59] so that less effort is needed for daily calibration, and more and more of the newest CGMS will be factory calibrated in the near future. Three major sensors now are available DexCom®, Enlite® and Libre®. Most provide round-the-clock every 5 min automatic subcutaneous glucose data on a continuous basis, and most sensors last from 4 to 14 days depending upon the particular system. The DexCom® sensors transmit their information to their own receivers as well as download it to computers and phones. More recently, they also transmit such information to several insulin pump systems, with the pumps beginning to also have the capability of "responding" to such data automatically. The PWD or family members can still manually respond and make basal and/or bolus dose adjustments as well. Improvements of systems using these DexCom CGMS using better and more sophisticated artificial intelligence algorithms have worked well for reducing hypoglycemic events, reducing the severity of hypoglycemia, and even reducing hyperglycemia as well as glycemic variability [60]. This is true for Omnipod®, Roche®, Animas®, and T:Slim pump systems working with DexCom sensors [61].

With the initial Medtronic pump systems coupled with Enlite® sensors, there was some more variability, and the DexCom sensors seemed to be more reliable and more accurate; over recent years, the Enlite sensors have improved dramatically so that there is virtually no difference in accuracy or specificity any longer with virtually identically MARD values of 0.4 [62]. The biggest advantage currently of the Medtronic pumps is not only that the Enlite sensors are more accurate and reliable but also that the Enlite sensors send information to mobile phones and home computers as well as directly to Medtronic insulin pumps acting as information receivers. The screens of the Medtronic 530G insulin pump (Figure 36) and more recently the 630, 640, and 670G Medtronic pumps, in color (Figure 37), now show every 5 min actual blood glucose readings from the sensor, graphic analysis (24 h, 12 h, 6 h, and 3 h updated curves) looking at trend analysis and arrows that highlight upward or downward trends for easier identification. Information about the connectivity of the sensors as well as battery power and insulin reserve is also provided on most pump screens.

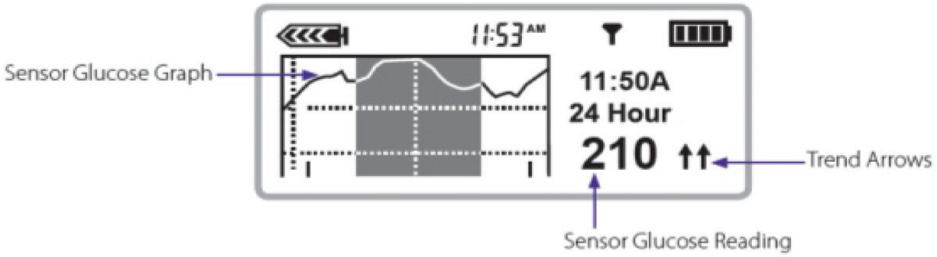

Figure 36. Medtronic 530G® screenshot.

Figure 37. Medtronic 630G® screenshot.

Other systems are available for those who prefer raw mathematical data identifying amount of time in range, above or below individually identified target glycemic goals and pie-charts for graphic representation looking at overall time periods as well as specifically identified hours of the day or night. Figure 38 shows one such pie chart analysis.

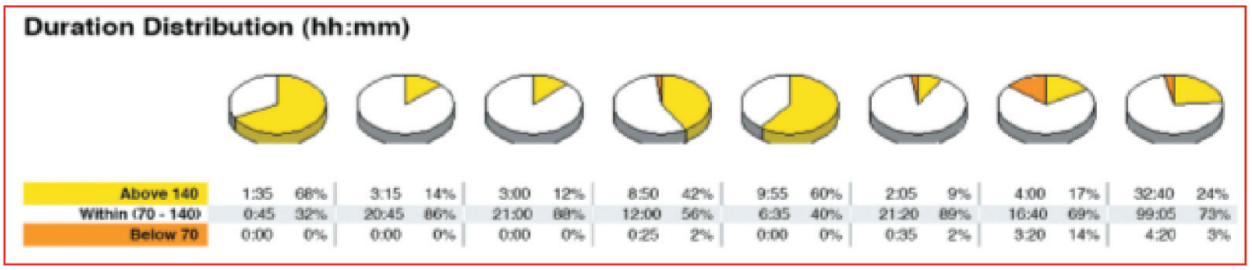

Figure 38. Pie chart CGMS downloaded data analysis.

As older PWD and family members get more comfortable with analysis of their own pump and CGMS data, not only the modal day and summary data are important but better problem solving can take place when reviewing the individual daily summary downloads. These provide specific information to verify the accuracy or inaccuracy of the sensor graphs compared to capillary blood glucose calibration information since the graphic display overlays this quite easily for review. Insulin delivery and use (or lack of use of square and dual waves), as well as manual or automatic low glucose suspension features of the pumps, is also demonstrable. Carbohydrate counting errors, missed information, exercise, and any other events so code also can help assess glycemic responses. These daily response graphs are most dramatic when people believe that the sensors are inaccurate, yet their own data can help convince them under what circumstances this is valid or misconstrued information. Figure 39 shows such a daily summary download chart showing such features.

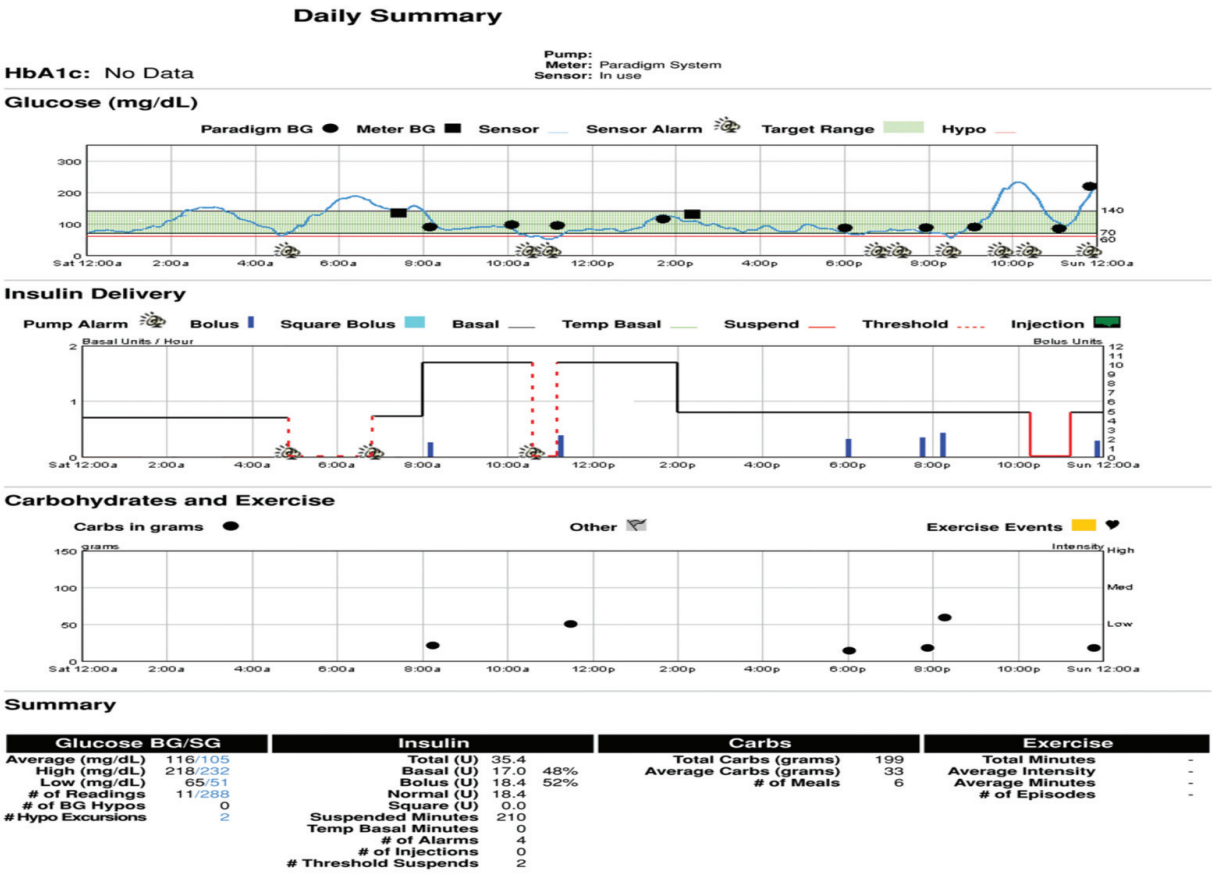

Figure 39. Carelink® daily combination CSII and CGMS daily summary sheet.

A demonstration of the DexCom G4 sending information to the DexCom receiver and the newer DexCom G4 sending information to a mobile phone app are presented in Figure 40 demonstration of two weeks of DexCom sensor data and the superb response of the PWD showing remarkable improvement in glycemic variability on the second week of DexCom sensor use (Figure 41).

Figure 40. DexCom G4® sensor with receiver, DexCom G5 sensor®, and phone app.

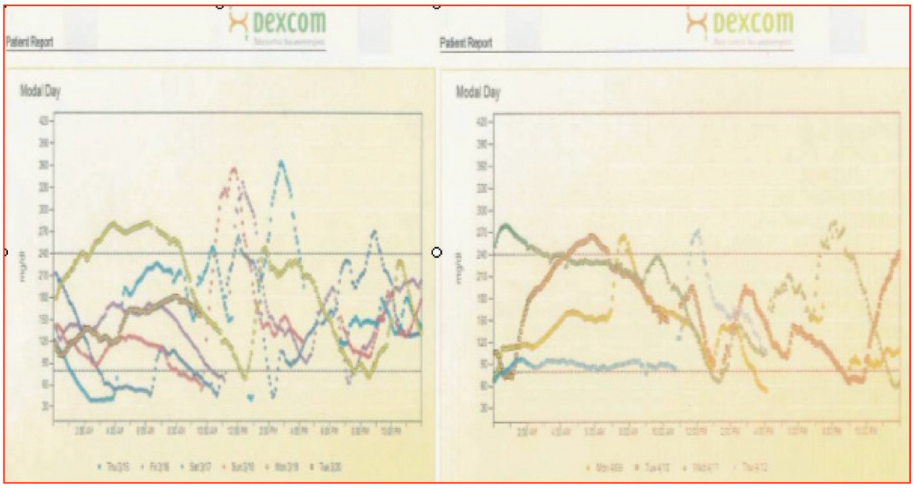

Figure 41. DexCom® week 1 data analysis and then follow-up week 2 data response.

Figure 42 demonstrates the Abbott FreeStyle Libre Pro® results pages which is a stand-alone sensor that can be used with multidose insulin regimens so does not require an insulin pump and shows time in range, averages, percentage time above target and below target, as well as low glucose events and their duration. Estimated glycohemoglobin results are also provided mathematically. Data scanning increased throughout the day, and routine fingerstick BGs were eliminated by 91% of the participants since no calibration was required (Figures 43–45).

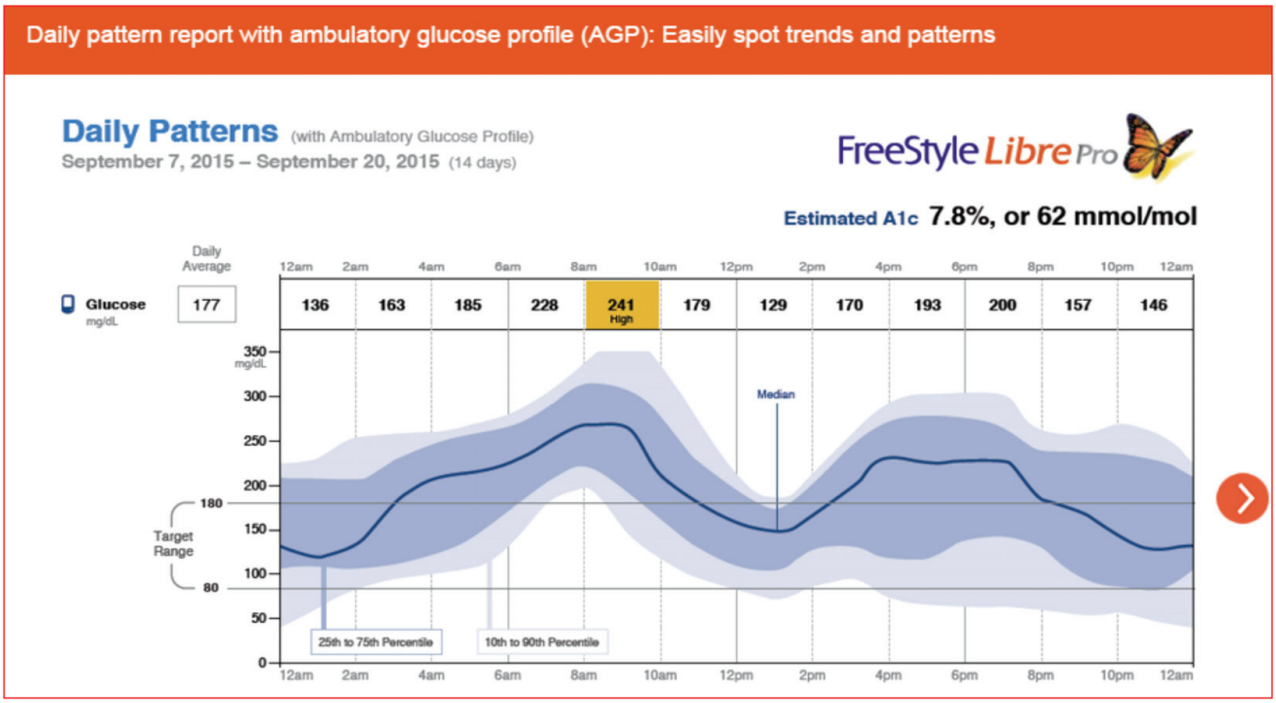

Figure 42. FreeStyle Libre Pro® ambulatory glucose daily pattern profile.

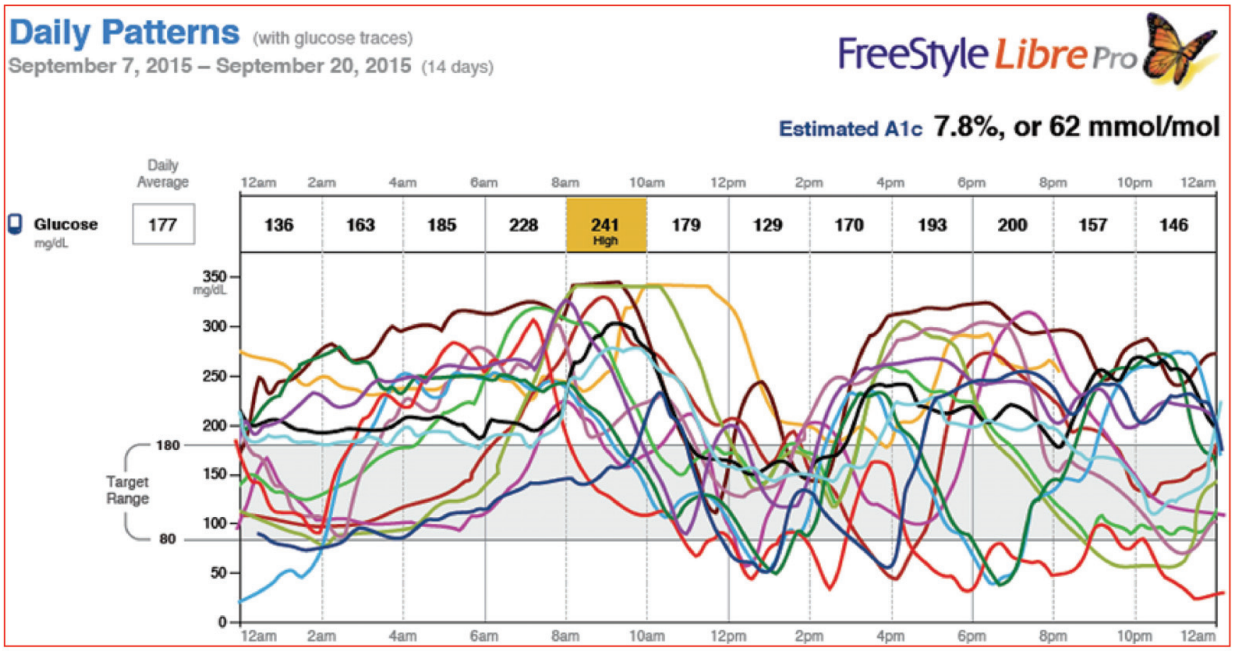

Figure 43. FreeStyle Libre Pro® 14-day daily patterns with glucose traces.

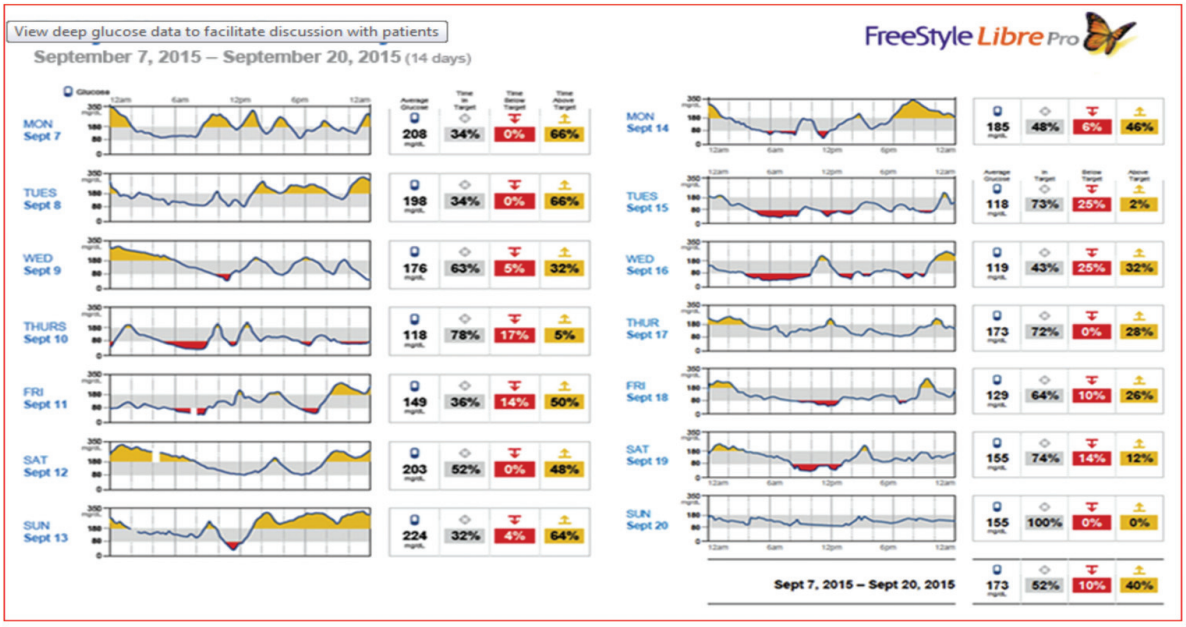

Figure 44. FreeStyle Libre Pro® daily glucose averages and % time at, below, and above target each day.

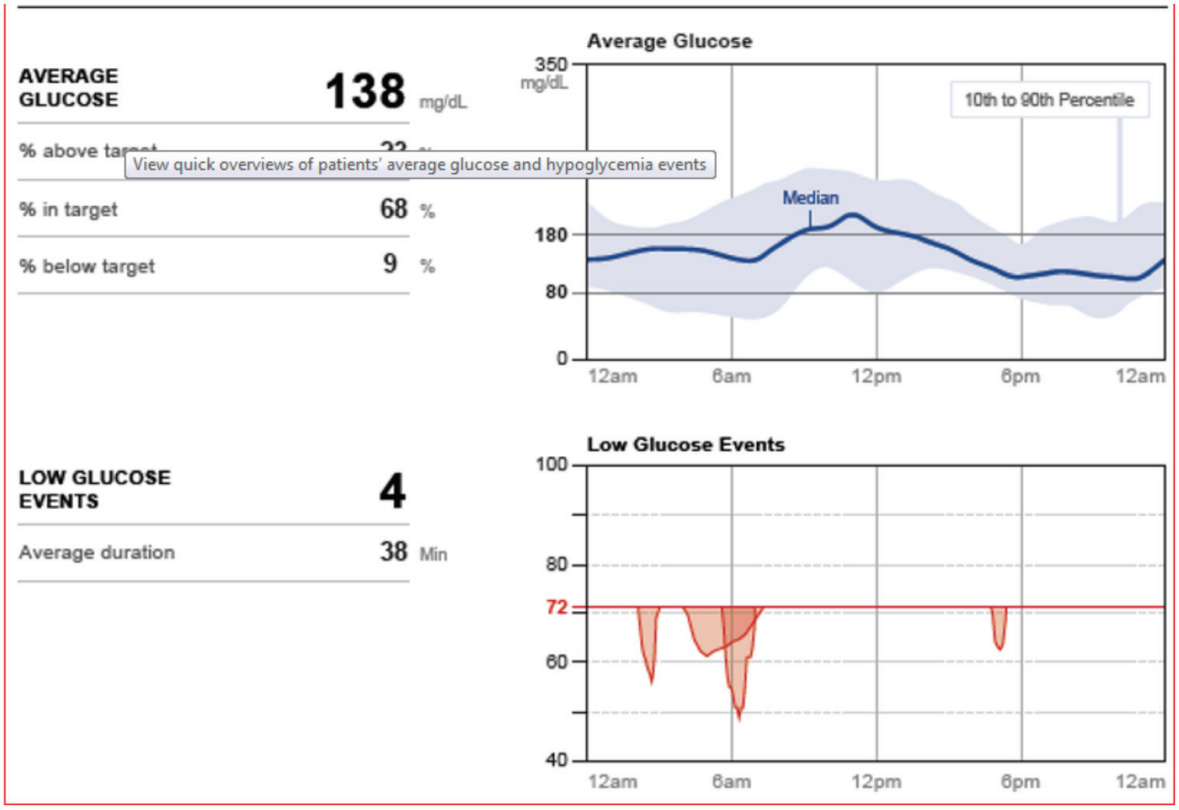

Figure 45. FreeStyle Libre Pro® snapshot modal day averages and low glucose events.

The latest advance in the march towards the artificial pancreas was approved by the US FDA in September 2016 and is called the "hybrid" artificial pancreas as presented by Medtronic utilizing the Medtronic 670G pump and, more recently, upgrades to 780G system. It involves a color screen, is waterproof, and includes the LGS (low glucose suspension) automatic features of earlier models. In addition, it keeps the Guardian® CGMS integrated with bidirectional communication to the newest pumps with a "hybrid closed loop" (HCL) [63] that automatically adjusts basal rates trending upwards if there is a pattern of increasing glycemia and, in addition, automatically also adjusts basal rates trending downward—before LGS initiates, to attempt to prevent more severe hypoglycemia. Two levels of personalization with this 670G "hybrid" artificial pancreas: suspend before low option and auto mode option, which adjusted basal insulin delivery every 5 min based on defined sugar levels to maintain improved target range glycemia day and night without operator activity [64], as shown in Figure 46.

The 780G CSII systems with Guardian Connect® assisted hybrid closed-loop (AHCL) pediatric and adolescent studies were presented at the 2021 Annual (virtual) Meeting of ISPAD [65]. Such systems not only improve time in range but continue to minimize hypoglycemic events. Such systems allow connection to mobile cellular telephones, including access to programed alerts for patients and their family members to take action. Cloud data analysis for health care professionals is also readily available. Such shared data are available with DexCom systems as well, and efforts to make more consistent downloaded glucose data available have been forthcoming from many groups working around the world in close collaboration with diabetes teams and diabetes supply manufacturers.

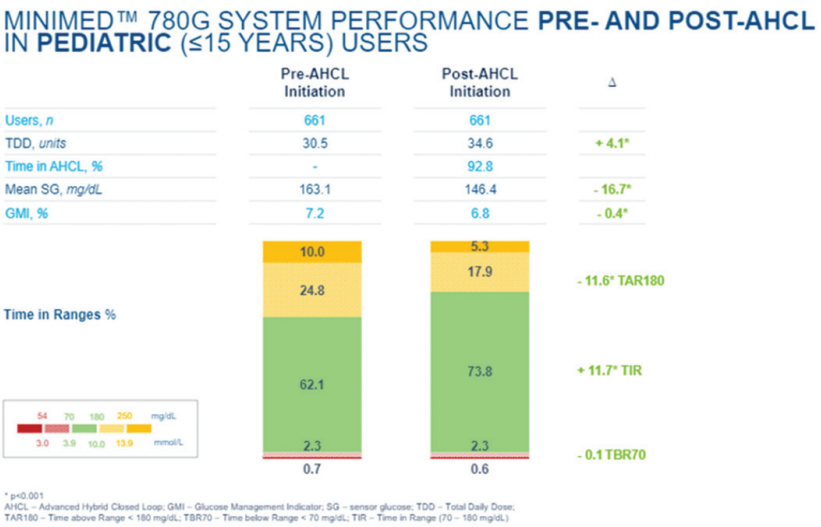

Figure 46. Advanced hybrid closed-loop (AI) pump and sensor.

In an e-poster presented at the 2021 Annual (virtual) Meeting of ISPAD [66], improvements in A1c as well as time in the range were confirmed with such hybrid closed-loop systems while maintaining minimal overall hypoglycemia and completely avoiding all severe daytime and nocturnal hypoglycemic events (Figures 47–49).

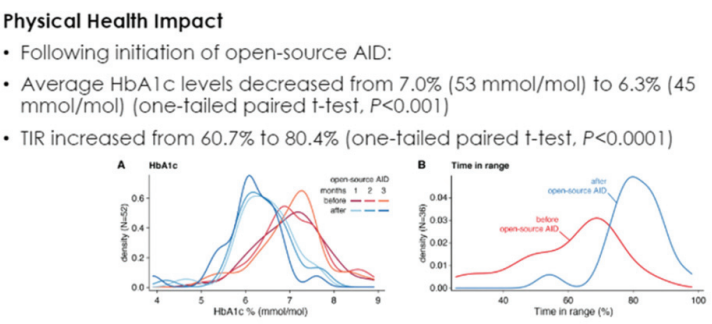

Figure 47. A1c (**A**) and TIR (**B**) changes pre and post AI-HCL.

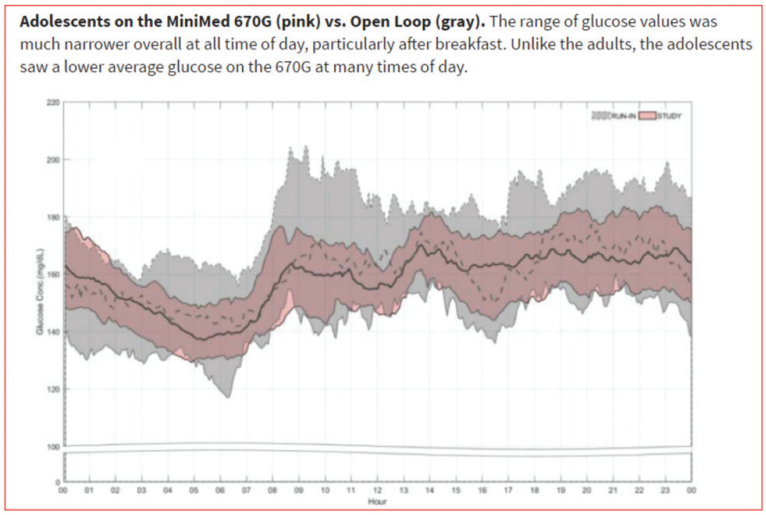

Figure 48. *Cont.*

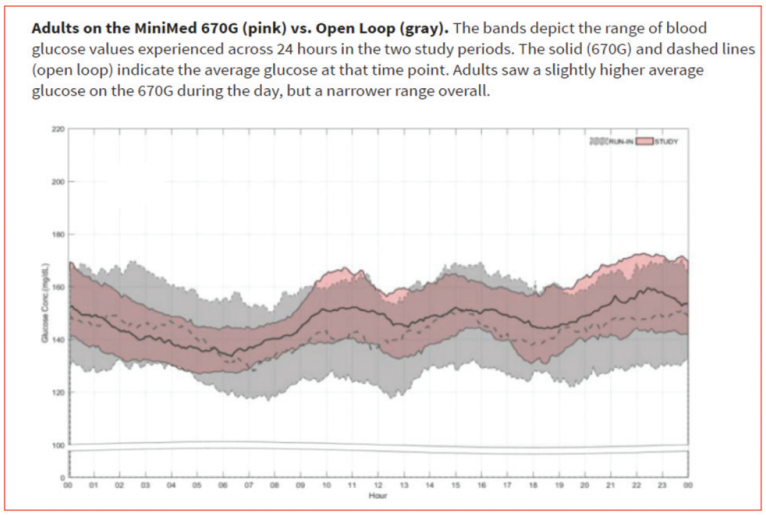

Figure 48. Adolescents and adults in the hybrid closed loop; downloaded CGMS data across 24 h.

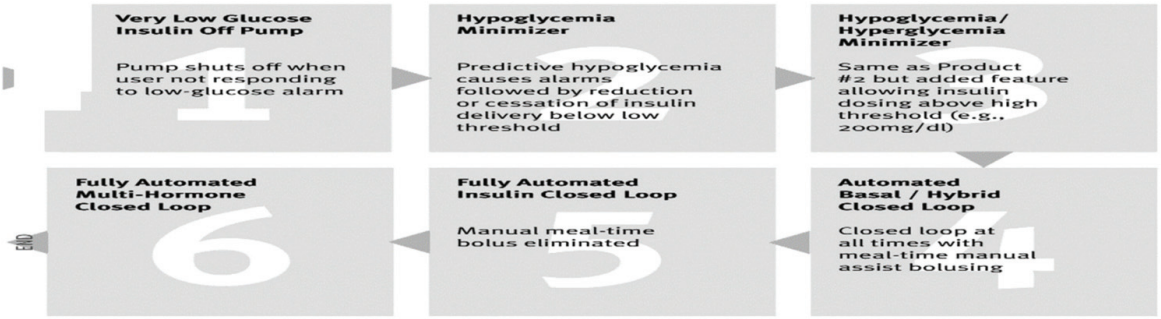

Figure 49. Kowalski JDRF road to closed-loop pump.

The JDRF has set its "Road to Fully Automated Closed-Loop Pumps", as presented by Kowalski [67] in Figure 50.

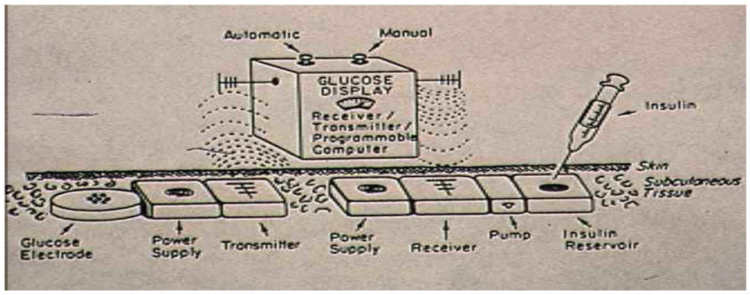

Figure 50. Dr. J. Stuart Soeldner's 1978 model of the future artificial mechanical pancreas.

We have moved through phases 1–3 and are now "officially" in phase 4 with the introduction of the newest hybrid closed-loop systems. We started back in the 1970s with proposed models of what the artificial mechanical pancreas, such as that shown in Figure 50 from Dr. Soeldner, the first "wearable" artificial pancreas that allowed blood glucose monitoring and insulin delivery. Figure 51 and a picture of the Ames Biostator as it was used in hospitals almost fifty years ago is shown in Figure 52. The first Ames Glucometer ®(the "brick") Figure 53 that brought bedside, in clinic and at home self blood glucose monitoring its early start.

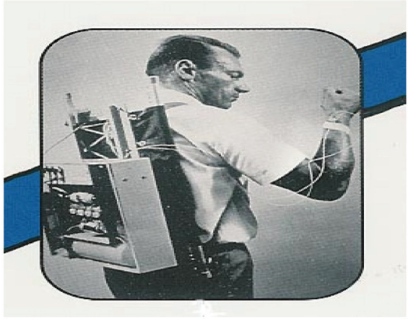

Figure 51. First wearable/portable artificial mechanical pancreas.

Figure 52. Ames Biostator: first "portable" intravenous monitor and insulin delivery system ~1974.

Figure 53. First Ames glucometer (the "brick").

The latest development over the past few years has also been the introduction of parents working with computers, opening up pumps, and advancing CGMS software to allow communication with each other, with their children and spouses, and also to talk to mobile phones in a more facilitated fashion. This has not happened with official government or industry sanction, and it has opened up some intriguing ownership, legal, and medial quandaries about who owns the software rights, computer maneuvering rights, and data when someone has "purchased" an insulin pump, blood glucose meter, or continuous glucose monitor. Nevertheless, the work has continued around the world, with the internet serving as a vehicle for communications, sharing, and improvement in software and hardware. Open Source, OpenAp, and NightScout (Figures 54 and 55) are some of the groups operating in this environment to bring improvements such as advanced waterproofing, better communication possibilities, better alarm notification, and improved quality of life for all utilizing these new developments in artificial intelligence, insulin pumps, monitoring, and access.

Figure 54. Night Scout poster for self-development of medical technology.

Figure 55. Open Source poster for future software and technology advances.

9. Pumps and CGMS

Pumps and CGMS are available. Communication between pumps, CGMS, computers, mobile phones, family members, and medical staff is also accessible, although expensive, and all of these items are improving rapidly. With an educated and motivated PWD and their family, an educated coordinated multidisciplinary health care team as well as reasonable and honest medical care insurance systems in addition to appropriate education and sophistication paying attention to the behavioral as well as medical aspects of a chronic disease like diabetes, the ability to adjust and adapt food, carb counting, and glycemic indexing allows for optimizing and analysis of these data using these new systems, allows the ability to add flexibility and to be reactive as well as proactive utilizing insulin, timing and activity to better mimic natural pancreatic function while at the same time minimizing hypoglycemia, decreasing persistent hyperglycemia, and improving time in range. [68] The knowledge that is more automatically generated and with added sensitivity and specificity can be added to these devices to improve quality of life and immediate short-term and long-term diabetes outcomes and hopefully to decrease short- and long-term complications of living with diabetes for all.

Evaluation of technology use in pediatric, adolescent, and young adult patients was also presented at the 2021 ISPAD annual meeting, as shown in Figure 56 [69].

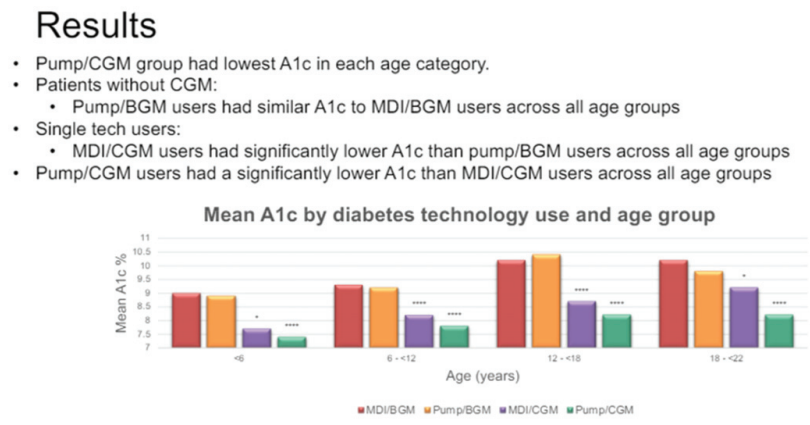

Figure 56. Pump and sensor technology results. Significantly different from the reference group(MDI/BGM) at a $p < 0.05$ *, $p < 0.0001$ ****.

10. Inhaled Insulins

Exubera® [70] was the first of the inhaled insulin to be tested and was approved in 2006 but then later withdrawn from the market shortly thereafter a year later due to poor sales and poor acceptance. After the "failure of Exubera [71]," Novo Nordisk and Eli Lilly/Alkermes discontinued further investigations of the AERx and AIR insulin systems, respectively, despite having had successful clinical trials proceeding. A more recent inhaled insulin (Figure 57), originally called Technosphere® and now called Afrezza® [72], was developed by Mankind and marketed by Sanofi-Aventis in 2014; it has two dose formulations with a significantly smaller inhaling device. Technosphere insulin inhalation powder [73] is composed of recombinant human insulin and fumaryl diketopiperazine (FDKP), an inert excipient. The FDKP does not facilitate drug absorption but functions only as a matrix to carry the insulin to the lung. Once the particles dissolve, the FDKP and the insulin are absorbed passively and independently of each other. The FKP is not metabolized and is excreted intact, primarily in the urine with a small amount in the feces. It is administered with a breath-powered, dry power inhaler in either three- or six-unit insulin equivalent doses; the inhaler itself has been described [74] by children, adolescents, and adults, including the elderly, as easy to use, small, and provides reproducible insulin delivery. The pharmacokinetic profile of the inhaled insulin delivered to the lungs appears to provide faster onset plus a shorter duration of action than either subcutaneous regular human insulin or aspart analog insulin [75]. As such, it can be administered either just before or just after a meal but must be used with basal insulin.

Figure 57. Inhaled insulin delivery devices.

Studies [76] have almost exclusively been done in type 1 (and type 2) adults (but some studies in type 2 adolescents) without much difference noted except for rather equivalent A1c results with control populations but earlier glycemic action [77] and short duration of action so that there was lower glycemic excursion noted and also less postprandial and nocturnal hypoglycemia because of the shorter tail effects (Figure 58) [78]. The most common side effect in all the clinical studies around the world has been immediate coughing after inhalation in about 7% of participants, which tended to disappear after seal weeks of inhaled insulin use. Short-term safety issues were addressed without significant concerns, although longer-duration use in those with asthma or chronic lung diseases as well as those with past and current smoking history remain potentially worrisome just as long-term pulmonary safety issues remain to be settled [79].

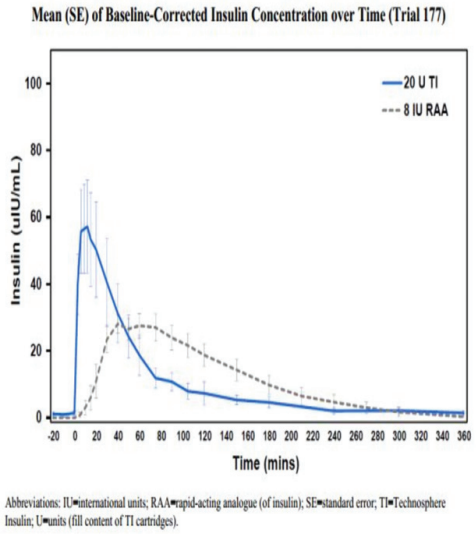

Figure 58. Inhaled insulin levels vs. rapid-acting injected analogs.

11. Super-Fast Insulin Analogs in Development

In an attempt to develop insulins where no waiting at all is required prior to food ingestion, analogs have been sought to achieve such results, as shown in Figure 59. Biodel has a product that originally was called VIAjet® and is now called Linjeta® that meets these criteria, and studies are ongoing for efficacy, side effects, and safety [80]. Linjeta involves zinc binding to decrease hexamer formation as well as citric acid added to prevent reaggregation from producing this "ultra-fast insulin analog". Novo Nordisk also has a similar product under development, Fiasp®, which has similarly faster initial uptake to counteract the initial food glucose rise and also faster removal from the circulation to help decrease the tail-effected hypoglycemia hours post-food [81]. Halozyme adds hyaluronic acid to the insulin preparation to promote the diffusion and thus speed up subcutaneous uptake to speed up the analog activity, while Insuline Corporation has developed a warming patch that increases site uptake by gently heating the injection area (Figure 60).

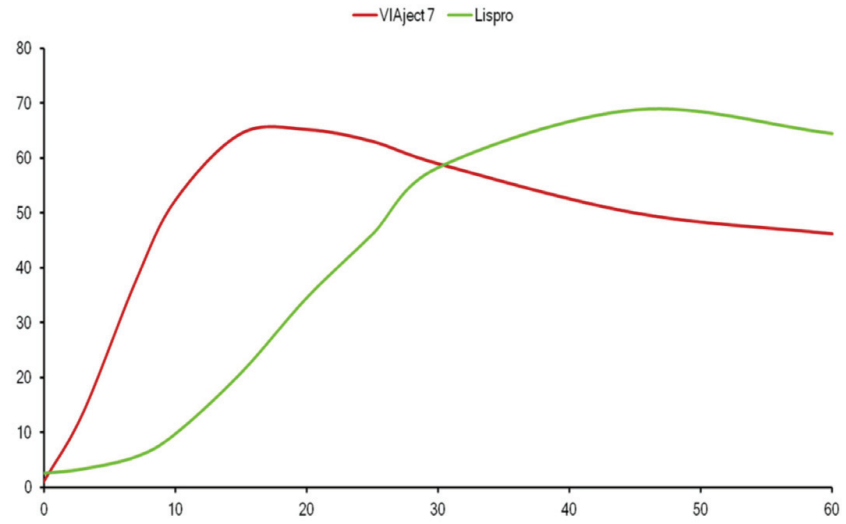

Figure 59. Super-fast insulin analogs.

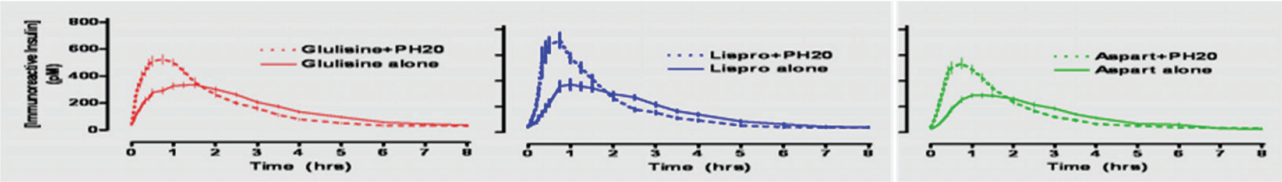

Figure 60. Comparisons of faster-acting insulins such as Fiasp® compared to glulisine, lispro, or aspart.

The rationale for these super-fast-acting approaches is similar to that proposed by the inhaled insulin: faster uptake (red curve), therefore better immediate post-food glycemic control. With a lower plus shorter tail effect, the duration of insulin activity is curtailed, and so there is potentially less hypoglycemia in the 3–6 h post-meal injection and, therefore, also nocturnally. With less food needed to balance the older insulins, presumably, there would be fewer calories required and less weight gain with insulin use in this fashion.

12. Ultra-Slow-Acting Insulins

The rationale for the "ultra-slow-acting insulins" is the same rationale as was proposed for the development of PZI, then CZI, then glargine and detemir insulins: smoother, longer duration, and perhaps needing fewer than daily injections to provide basal insulin coupled with less insulin exposure and therefore less hypoglycemia, particularly nocturnal hypoglycemia. They would be utilized in conjunction with prandial analogs designed to cover the immediate effects of food intake in a more physiologic fashion mimicking the normal pancreatic insulin delivery.

Novo Nordisk's degludec (Ideg or Tresiba®) has completed proof of concepts studies as well as initial safety and efficacy trials [82] with approval by some authorities already obtained in 2013 and with two pen dose formulations available (Figure 61). The half-life for degludec is about 25 h. Degludec consists of multiple hexamers with very slow release to monomers from the subcutaneous depot through adding a fatty acid side chain to the terminal aminoacid of the B chain of the human insulin molecule (Figure 62). The result [83] is insulin with twice the duration of activity of glargine or detemir and a flatter, smoother profile of effect as well—both desirable properties for basal insulin [84]. It can be given either in the morning or the evening and may not need a daily dose because of its long half-life in the circulation but would still require prandial insulins for meal and snack coverage. It is not yet approved for use in those under 18 years of age, but clinical studies are in progress to address safety and efficacy issues [85,86]. Postprandial as well as nocturnal hypoglycemia is the same or less than other products in testing to date [87].

Figure 61. Tresiba® pen.

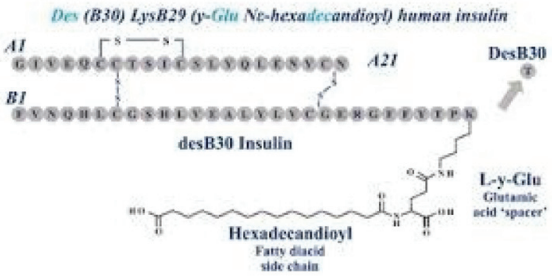

Figure 62. Degludec(Tresiba®) chemical schemata.

Lilly has developed a pegylated lispro insulin analog called peglispro (LY2605541) [88] with an even longer half-life of 48–72 h. Peglispro begins with the lispro analog that is then modified with a 28 KDa polyethylene glycol (Figure 63) to slow absorption as well as slow renal clearance. As with Ideg®, peglispro has a more consistent, flatter, and longer duration of activity and a decrease in overall hypoglycemia and especially a decrease in nocturnal hypoglycemia as a result. Clinical trial studies are ongoing [89,90].

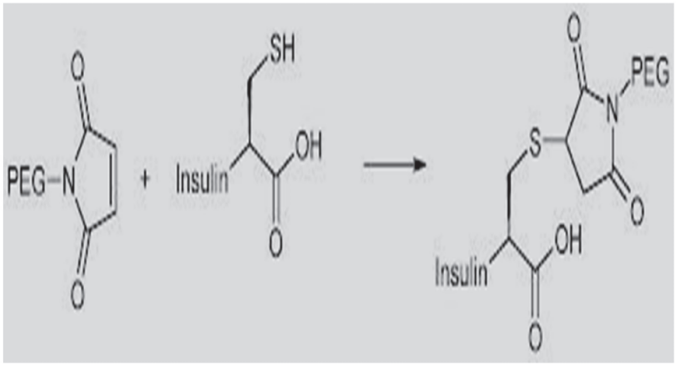

Figure 63. Peglispro chemical scheme.

Longer-lasting glargine analogs also have been studied and are coming to market with flatter curves of action and smaller or no peak effects with the hopes of decreasing hypoglycemia further and making basal insulin availability more standardized from day to day and therefore helping decrease glycemic variability.

Uniform multidisciplinary team care combined with more available monitoring, more adaptable food and snack choices, and more physiological insulin delivery using newer analogs, or insulin pumps combined with SBGM or automated or semi-automated more detailed BG testing have shown significant promise of overcoming the differences in different centers of specialty excellence around the world. The key improvement occurred when teams in both Sweden and the UK identified their discordant messages and actually worked very specifically to present a more unified approach for motivational teaching and training utilizing psychosocial approaches, learning theory, and group dynamics to form the basis of such programs—and produced significant improvements in A1c outcomes while reducing hypoglycemic episodes, glycosuria, postprandial hyperglycemia, etc. [91].

13. Insulin for Financially Distressed Parts of the World

Unfortunately, financial constraints continue to affect insulin treatment decisions, and there is persistent disparity in diabetes outcomes that reflect this as well as racial-ethnic disparities in management and outcomes [92–95]. This is true in the richest countries of the world where the costs of the new insulin analogs are significantly more than the costs of the older insulins and the balance between the clinical benefits, quality of life issues, long term complications risks, and these costs must continue to be weighed. In parts of the world where there are no reliable health care systems and where costs of the analogs are prohibitive, insulin must be donated to be reliably obtained; this is true for the older animal and semi-synthetic insulin products as well as the newer, most expensive analogs coming to market or being developed. There is a temptation to provide the least expensive premixed insulin products in those circumstances since this is "better than having no insulin at all". However, the restrictions of food access and affordability coupled with these premixed insulins increase the risks of significant hypoglycemic quite substantially. In parts of the world where there are few doctors, nurses, or dieticians and even fewer specialists in diabetes, the sophistication is often lacking for flexibility with any insulin. Coupled with the added prohibitive expense of blood glucose meters, batteries to run the meters, and the strips to test the BGs, information upon which to make treatment decisions is also often lacking, and such supplies need to be donated just as insulin needs to be

donated. Lack of updated information about strategies under these circumstances hampers use in a safe manner. For instance, insisting that everyone only use premised regular and NPH insulin pre-breakfast and pre-dinner by syringe and vial technique almost always produces severe episodes of nocturnal hypoglycemia at the peak of the background NPH. The only response often thought about is to decrease the insulin dosage and keep the blood glucose levels too high—thus avoiding severe hypoglycemia. Alternatively, when I travel to such countries to teach about new developments and different approaches, the audience of professionals as well as parents and children/adolescents is very often utterly surprised at the thought of giving just NPH at bedtime instead of at suppertime or of overlapping smaller doses of NPH, what we used to do before the current analog era of insulin options, in an effort to decrease and avoid the nocturnal insulin peaks and resultant hypoglycemia that can produce loss of consciousness and/or convulsions. Because the premixed insulins do not allow reasonable adjustment possibilities, this further adds to their non-safe efficacy in these circumstances. Quality of life is improved with analogs in poor countries just as it is in richer countries when the necessity of waiting 30 min instead of 15 min is added to the therapeutic milieu. More replicable and steady basal insulin needing only one instead of two injections each day would also improve quality of life if the expense of such new medications can be handled around the world. The ultimate goal is to have sufficient SMBG equipment to allow greater flexibility and adaptability to changes in food availability, paying particular attention to glycemic excursions and insulin delivery countering activity changes as well as intercurrent illness.

In Bangladesh, Sudan, Tanzania, Bolivia, Nigeria, China, Cambodia, India, and all around the world, these principles remain the same as they are in the richer nations with more developed health care systems. Programs such as Life for a Child (LFAC)® and Changing Diabetes in Children (CDIC)®, cosponsored by ISPAD, IDF and supported by donations of insulin, blood glucose testing equipment, and strips have all supported education, promotion of a multidisciplinary training program, professional education, and local government and academic initiatives to facilitate more availability of LFAC and CDIC with the hopes of saving children's lives, providing affordable or free insulin, and supporting the medical as well as psychosocial needs of young people with diabetes.

14. Summary

There is much excitement among diabetologists with the new and relatively rapid developments of bioengineered insulins aiming to improve absorption, glycemic coverage effects of foods eaten, and quality of life with fewer injections and better prandial coverage, thus decreasing hypoglycemic events in the process of improving control and lowering GHb levels closer to those of people without diabetes. Whether this is going to be accomplished with a fully artificial pancreas that makes dose decisions automatically, through the use of insulin pumps with the assistance of more reliable and easier to use continuous glucose monitoring systems coupled with the fastest-acting insulin analogs used in these pumps or with continued use of MDI regimens with prandial fats acting insulin analogs coupled with smoother and safer basal insulins is difficult to know for sure since all of these clinical research avenues continue to be explored around the world [96].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/diabetology3010010/s1, Figure S1. Lispro research studies documenting overnight glycemia. Figure S2. Lispro life-table analysis showing hypoglycemia comparison between regular insulin and lispro insulin. Figure S3. Episodes of mild hypoglycemia vs. A1c reduced on lispro vs. regular insulin.

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Review

Novel Insights into the Immunotherapy-Based Treatment Strategy for Autoimmune Type 1 Diabetes

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Abstract: Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing pancreatic β-cells by their own immune system, resulting in lifelong insulin deficiency. Continuous exogenous insulin replacement therapy is the current standard of care for T1D. Transplantation of primary pancreatic islets or the entire pancreas is a viable remedy for managing patients with autoimmune T1D. However, this strategy is not feasible due to several obstacles, including a scarcity of donors, islet cells, and poor vascular engraftment of islets post-transplantation, as well as the need for prolonged immune suppression. Innovative approaches must be developed to counteract pancreatic β-cell destruction and salvage endogenic insulin production, thereby regulating blood glucose levels. This review includes an overview of autoimmune T1D, immune cells involved in T1D pathophysiology, and immunotherapy-based strategies to treat and prevent autoimmune T1D. Recent immunotherapy progress toward targeting pancreatic islet-specific immune pathways tangled tolerance has fueled the advancement of therapies that may allow for the prevention or reversal of this autoimmune T1D while avoiding other adverse reactions associated with the previous attempt, which was mostly immunosuppressive. As a result, significant efforts are currently underway to improve the efficacy of immunotherapy-based approaches by leveraging the beneficial actions of immune cells, specifically effector $CD4^+$, $CD8^+$, and regulatory T cells. This review will provide an overview of currently available immune-based therapeutic options for T1D and will examine the growing evidence that supports the use of immune cell-based approaches to improve therapeutic outcomes in the prevention or reversal of autoimmune T1D.

Keywords: type 1 diabetes; autoimmune diseases; pancreatic β-cells; tolerance; regulatory T cells; immunotherapy

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1. Introduction

Autoimmune diseases, which are pathological conditions caused by abnormal immune responses to substances and tissues that are normally present or generated within their own body, affect nearly 23.5 million Americans, with nearly 80 percent of those affected being females. Around 100 or more autoimmune diseases have been reported, according to the American Autoimmune Related Diseases Association (AARDA). Many of them have similar symptoms, making them more difficult to diagnose and distinguish. Autoimmune diseases can affect almost any organ in the body, including the heart, muscles, eyes, brain, nerves, skin, joints, lungs, kidneys, glands, the digestive tract, and blood vessels. Although there are over 100 autoimmune diseases known to date, the most common autoimmune disorders are Type 1 Diabetes Mellitus (T1D), Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), Inflammatory Bowel Disease (IBD), Multiple Sclerosis (MS), Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy, Psoriasis, Graves' disease, and Hashimoto's disease [1,2].

As we all know, one of the immune system's most important functions is to protect the body by responding to attacking pathogens such as bacteria, parasites, or viruses by producing antibodies or sensitized lymphocytes to fight them. An immune response

against one's own body cannot be triggered or tolerated in healthy conditions. However, in some cases, the body's immune cells or tissue make a mistake and behave as if they are foreign because our immune system recognizes them as foreign entities and attacks their own body rather than protecting them. These irrational responses can result in a variety of autoimmune diseases. T1D, also known as Juvenile Diabetes, is a chronic autoimmune disease in which both T and B lymphocytes initiate a specific targeted immune response that leads to the destruction of insulin-producing pancreatic β-cells [3–5].

According to the Centers for Disease Control and Prevention, approximately 10% of the American population has diabetes, with type 1 diabetes accounting for 5–10% of those affected (T1D). The number of children diagnosed with various autoimmune diseases, in addition to autoimmune T1D, has increased. T1D is one of the most common childhood chronic diseases, affecting approximately 70,000 children each year. Worldwide, 111,1100 young people under the age of 20 have autoimmune T1D, and its prevalence in children is increasing by 3–5 percent per year, which is far too high. Pediatric T1D prevalence rates vary by region, but are typically lower in Asian countries and higher in the rest of the world [6,7]. Many autoimmune disorders, including T1D, are caused by flaws in the regulation of effector immune cell populations, which may be due to malfunctions in the immune-mediated suppression or immunological tolerance mechanisms.

T1D is a complex autoimmune disease characterized by genomic, epigenomic, and environmental factors that influence both adaptive and innate effector immune cell populations, culminating in pathological, chronic islet inflammation. The heterogeneity associated with human autoimmune T1D, as well as the nature of islet inflammation, reflects the individual's genotype and type of environmental insult. These factors influence which immune effectors are important in the pathophysiology of autoimmune T1D, the rate of disease progression, and the degree of pancreatic islet-specific β-cell dysregulation and/or death [8–11].

The emergence of autoimmune disorders resulted from the immune system's inability to control autoreactive cascading responses. The ability to reprogram the immune system to maintain homeostasis without ongoing treatment is the holy grail of autoimmune therapies such as T1D. The current treatment regimen for T1D patients is largely based on exogenous insulin injections or insulin pumps, which are lifesaving but not curative. While insulin replacement is beneficial for improving glycol-metabolic control, it is limited by its inherent inability to replicate endogenous insulin's biological functions; this puts T1D patients at risk for hypoglycemic episodes [12]. Due to the accelerated T1D disease, autoimmune T1D patients have a significantly reduced life expectancy. There are several ways to rebuild complete endocrine pancreatic function, but cadaveric whole pancreas or islets transplantation is only available to a small number of T1D patients. There was limited clinical success due to poor post-transplant engraftment of vascular cells, blood-mediated inflammatory response, hypoxia, hypoxia-reoxygenation injury, the alloimmune response against the graft, and persistent autoimmune attacks in T1D patients who received islets transplanted [13,14].

Several attempts have since been made to use emerging immunosuppressive approaches to provide immune protection to grafted islets or the entire pancreas. Traditional treatments for autoimmune T1D rely primarily on broad-spectrum immunosuppressive agents. They require much safer and more effective treatment due to the severe side effects of harsh immunosuppressive regimens.

Targeted and specific immunotherapies must be developed for the treatment of autoimmune T1D to re-establish tolerance mechanisms or eliminate pancreatic cell-specific immune responses generated by primary T and B lymphocytes, which can preserve the destructive β-cell mass for different functions and maintain insulin throughout life. Developing selective immunotherapies for autoimmune T1D will necessitate a thorough understanding of immunological responses to pancreatic cell-derived peptides, which serve as epitopes/antigens responsible for autoimmunity T1D, as well as how cognate T and B cells avoid tolerance checkpoints. This review discusses the general history of T1D, immune cells involved in the pathophysiology of autoimmune Type 1 diabetes, and immunotherapy-based treatment strategies for T1D.

2. Background of Autoimmune T1D

Autoimmune T1D occurs when our own immune system mistakenly destroys the insulin hormone-producing pancreatic β-cells in the Langerhans islets, schematically shown in Figure 1. T1D appears to have a genetic component and can be diagnosed in childhood and reported in later life, i.e., adulthood. Its causes are not completely understood or researched, and there is currently no likely treatment or cure. To survive, T1D patients must rely on injected or pumped insulin for the rest of their lives. T1D is found in both children and adults who exhibit symptoms such as frequent urination, dry mouth, increased thirst, itchy or dry skin, increased appetite, unexplained weight loss, and infections. Individuals with autoimmune T1D are typically diagnosed after exhibiting symptoms such as nausea, vomiting, extreme thirst, exhaustion, and/or malaise. As the body loses its ability to produce insulin, a hormone that allows the body to use the sugar found in everyday foods, known as glucose, as an energy source, patients with T1D must work closely with their endocrinologists to determine the insulin doses and lifestyle changes needed to manage their blood sugar levels. Autoimmune T1D patients are vulnerable to a variety of health issues, ranging from minor to severe; if you are not properly managed, your glucose levels will rise. Most T1D patients spend their lives managing it and have a problem with it, blood glucose levels are outside the clinically recommended beneficial range, which leads to potentially fatal hyperglycemia (high blood glucose) episodes and hypoglycemia (low blood glucose). Assume you do not maintain it and develop high blood sugar, which frequently leads to devastating health complications later in life, such as blindness, kidney failure, heart disease, and nerve damage, resulting in amputations.

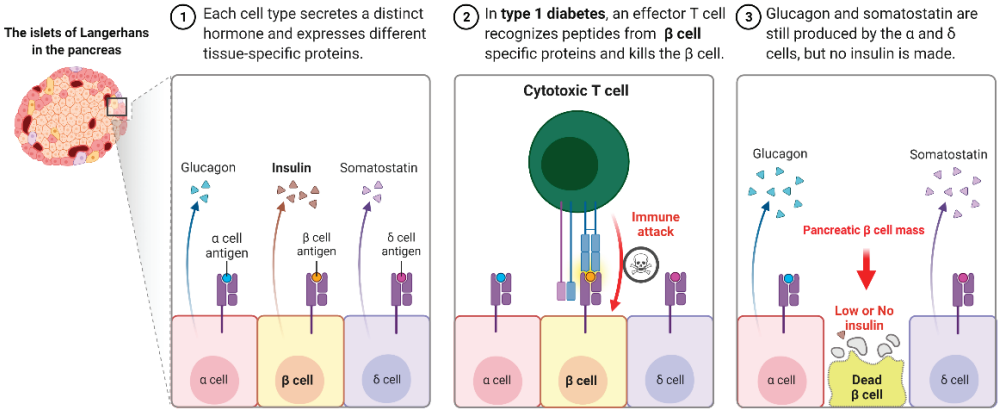

Figure 1. Type I Diabetes immune response.

Several factors are responsible for the causes of T1D; the scientific community does not distinguish between the exact causes of autoimmune T1D, but they do see some onset factors and triggers associated with the condition. There is frequently a family history of T1D, and the best diagnoses occur in people who have no family members with the disease. Having a family history of autoimmune T1D, on the other hand, puts people at an increased risk of developing the disease. Some scientists believe viral infections can cause or contribute to the onset of the disease. To clarify, developing a viral-based vaccine may be one method of preventing T1D. Some environmental factors may be linked to T1D, most likely because of changes in our environment, possibly via a few viral infections or other similar agents. Early exposure to those factors after birth may be linked to the development of autoimmune T1D. T1D is not caused by diet or lifestyle, but several studies point to genetics [15–18], ethnicity, age, and the likelihood of developing T1D; the presence of specific genes appears in many autoimmune T1D patients. As T1D does not discriminate, if someone in your family has had autoimmune T1D, you are more likely to develop the disease. Assume that people of all ethnic backgrounds have T1D, even though the prevalence increases in populations north of the equator. However, an individual can develop autoimmune T1D at any age, with the majority of cases being diagnosed in early

elementary school or as preteens. Hormonal changes, like those associated with growth spurts, can have an impact on the presentation and management of T1D.

2.1. Immune Cells Involved in the Pathophysiology of Autoimmune Type 1 Diabetes

Tolerance has two mechanisms: central and peripheral tolerance. Both contribute to the defense against autoimmunity (Figure 2). T1D autoimmune disease can be caused by critical insufficiencies or restrictions in peripheral (lymph nodes/circulation) and/or central tolerance mechanisms (bone marrow/thymus), presenting a therapeutic opportunity. In healthy people, our immune systems are perfectly balanced to distinguish which antigens are foreign and which are self-antigens. To control this process, our immune system has a tolerance mechanism that includes thymic (central) tolerance, which eliminates high-affinity auto-antigen-specific T lymphocytes and those that fail to recognize auto-antigens completely and excuses T lymphocytes that recognize auto-antigens with intermediate affinity. As the naive immune repertoire is positively selected on auto antigens, self-recognition is hard-wired in the immune system, blurring the distinction between unwanted autoimmunity and desired immunity. As recirculating lymphocytes are exposed to tissue antigens under non-inflammatory conditions, which result in a tolerant, anergic state, our immune system's peripheral tolerance system usually keeps potentially auto-specific lymphocytes in check. Nonetheless, in the presence of danger signals, such as infection and tissue damage, self-tolerance can be broken down, and autoimmune disease can progress. The immune pathogenesis of autoimmune T1D begins with a breakdown in self-tolerance, which results in the destruction of pancreatic β-cells by T lymphocytes (Figure 2).

Similarly, unwanted autoimmunity and desired host anti-pathogen specific immunity are inextricably linked, as effector immune responses that affect inflammatory tissue damage are analogous to those that mediate effective host defense. As a result, immunotherapeutic regimens that target the immune system's common pathways invariably have both desired and unintended consequences. Several strategies for eliminating auto-antigen-specific immune cells by breaking tolerance to self-antigens can result in autoimmunity, which has been thoroughly reviewed [19]. Recent findings emphasize the importance of inhibitory receptors (IRs), which trigger or regulate autoimmunity's peripheral tolerance. Later, deletion and blockade studies in animals and humans show a link to positive disease outcomes, highlighting the potential clinical benefits of enhancing IR signaling, specifically CTLA4, PD1, LAG3, TIM3, and TIGIT, to treat autoimmune diseases.

2.2. Immunotherapy-Based Approaches to Treating Autoimmune T1D

Drugs that target immune system elements provide relief for millions of people suffering from autoimmune diseases such as psoriasis and rheumatoid arthritis. Still, no single immunotherapy-based treatment for autoimmune T1D is currently approved. Few research organizations and pharmaceutical companies focus on multiple aspects of the immune system to develop an effective immune cell-based therapy to cure autoimmune T1D. Individuals with autoimmune T1D require lifetime medications in the form of regular insulin prescriptions to manage the condition, putting them at high risk of long-term complications. One day, the immunotherapy-based strategy will benefit T1D and become an insulin-free substitute to stop, prevent, and possibly cure this autoimmune T1D. They have focused on immune cell-based approaches for autoimmune T1D treatment since the 1980s, considering several possibilities such as the repair of unblanched immune tolerance, inhibition of diabetogenic T-cell or B-cell functionality, ex vivo regulatory T-cell (Treg) generation, repression of the innate arm of the immune system, inflammation, and HLA-matched islet transplantation rejection.

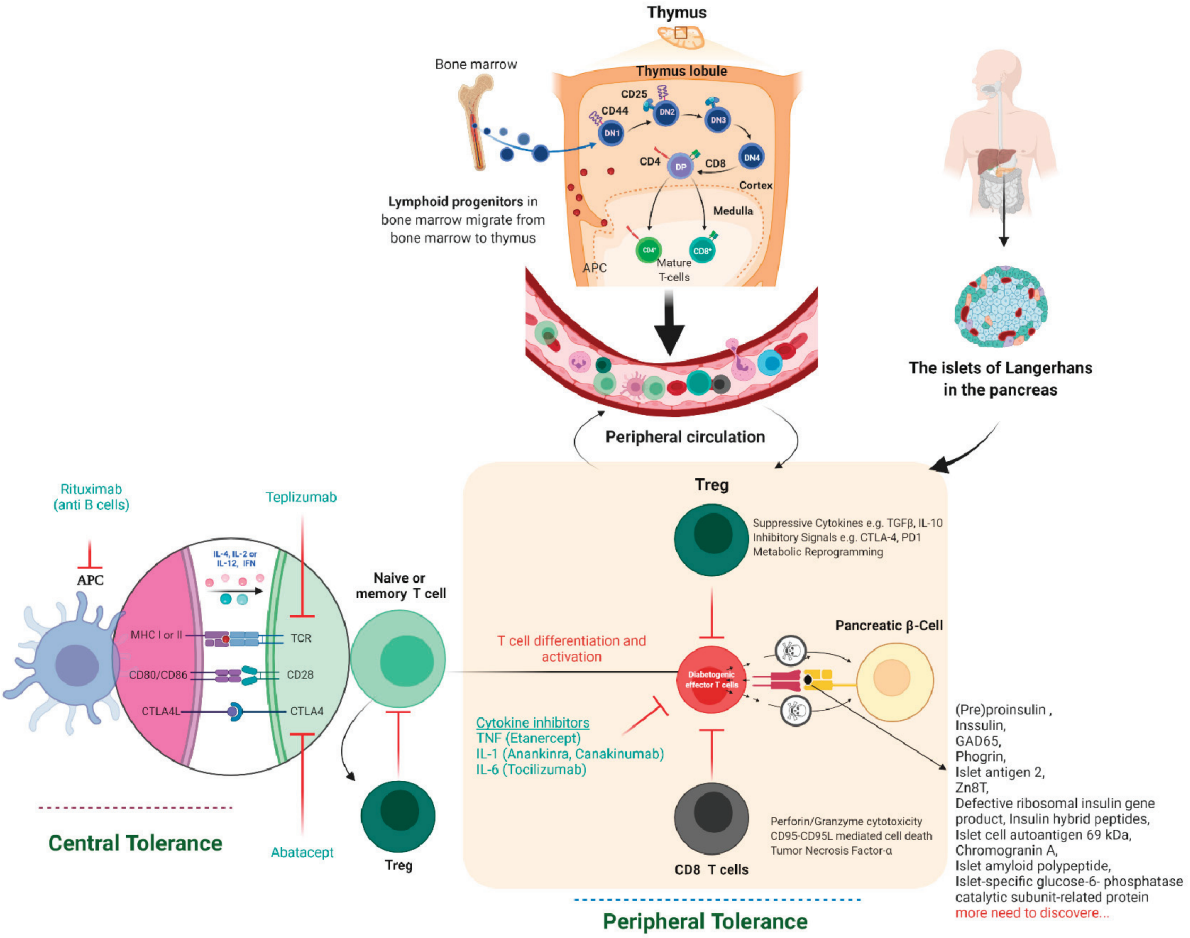

Figure 2. Immune tolerance, therapeutics, and pancreatic β-cell-specific antigen discovered till now in T1D. The lymphoid progenitor cells are initially generated in the host bone marrow by special hematopoietic stem cells. The T lymphocytes travel to the thymus, where the thymus-based central tolerance mechanisms train T cells to discriminate between self and non-self (adverse selection). CD4 regulatory T cells (Tregs) and diabetogenic T lymphocytes may identify self or pancreatic β-cell-specific antigens, but at different affinities, which might explain their destructive actions β-cell. Subsequently, live T lymphocytes arrive in the blood and lymph nodes' peripheral circulatory system and clash with their specific peptide-MHC/HLA complex. Specifically, in autoimmune T1D, these T lymphocytes are specific for pancreatic β-cell proteins such as insulin, GAD55, or so many others discovered to date. If these pancreatic β-cell-specific T lymphocytes and their respective antigen/epitopes are displayed by the MHC/HLA of antigen-presenting cells (DC, Macrophage, or B cells), T lymphocytes will become initiated in the lymph node, migrate to the pancreatic islets, and begin the demolition of β-cell in an antigen-specific manner. Tregs characterize the suppressive lymphocytes mainly responsible for peripheral immunological tolerance and try to inhibit these events. If the host body is unable to stop the autoimmune attack on pancreatic β-cells, insulin deficiency, hyperglycemia, and, eventually, autoimmune T1D will result. The majority of signaling events occur in the peripheral-local environment, in the lymph nodes and pancreas, and cannot be tracked using biomarkers.

2.3. There Are a Few Obstacles to Immunotherapy-Based Treatment for Autoimmune T1D Cure

First, thanks to the boon of recombinant technology, insulin has long been available, so there has not been a pressing need for the development of new T1D therapies. T1D patients must check their blood sugar levels regularly and calculate the amount of insulin to inject. Still, let us assume they can keep this up indefinitely. In that case, it is possible to live a fairly normal life. Second, the primary reason for the lack of approved immunotherapies

for T1D is that clinicians have been hesitant to refer patients with T1D to immunotherapy-based clinical trials. Due to endocrinologists' lack of understanding, new-generation immunotherapies have fewer unpleasant and potentially dangerous side effects than older immune-suppressing drugs or immunotherapy only for oncology, as it was initially explored or assumed. Third, there has been a lack of engagement from pharmaceutical companies and research organizations, and fourth, compared to a disease like RA, people living with T1D represent a relatively small business discussed [20–22].

The following section of this review provides an overview and understanding of previous immunotherapy-based trials, describes recent and ongoing combination immunotherapy trials, and speculates on future combination clinical interventions to preserve insulin-secreting pancreatic-cells in autoimmune T1D.

The first immunotherapy trials for T1D were conducted more than 35 years ago. No single study has demonstrated clinically significant benefits from therapy with an acceptable risk profile [23]. A French Cyclosporine Diabetes Study was the first clinical trial to test the immunological intervention in type 1 diabetes [24]. Cyclosporine A (CSA) disrupts TCR-mediated signal transduction, inhibiting T-cell activation and T helper IL-2 secretion [25]. Later, a few studies demonstrated a significant reduction in exogenous insulin requirement after one year of immune-suppressive drug CSA treatment [24,26]. After CSA removal, blood glucose control worsens, and autoantibody levels are return [27].

Furthermore, cyclosporin medication has been linked to renal and pancreatic β-cell toxicity [27]. Despite the lack of long-term effects and potential toxicity, these trials heralded a new clinical era centered on immunomodulatory strategies to delay or prevent T1D. Numerous interventions, including parenteral insulin administration, dietary exposures, broad-spectrum immunosuppressants, anti-inflammatory drugs, and T- or B-cell targeted immunosuppressants, have been tested to date. At the same time, a small number of clinical trials have revealed modest benefits. There are no clinically approved immunotherapies for autoimmune T1D now, but a few developments are listed in Table 1 or highlighted in Figure 2. Let us look at a few of these immunotherapies and how they might help T1D patients.

Table 1. Representative Immune-based therapeutic intervention study in autoimmune T1D and outcomes.

Therapeutic Agents	Study/Authors and Intervention	Outcome	Citations
T cell-based: • Anti-CD3 antibodies • Otelexizumab/teplizumab • prevent activation of T-cells, • deplete Teffs, and restore the Teffs/Tregs ratio	DEFEND-1, 2 (Otelexizumab)	There was no EBV in the therapy group, but there was no statistically significant difference in 2-h MMTT AUC C-peptide at 12 months.	[28]
	Protégé (Teplizumab)	At 1 year, there was no significant difference in HbA1c1 < 6.5 percent or insulin dose < 0.5 U/kg per day: At year 2, AUC C-peptide in the high dose group was considerably greater than in the placebo group.	[29,30]
	AbATE (Teplizumab)	The treatment group's baseline adjusted AUC C-peptide reduced at year 2 was considerably lower.	[31]
B cell-based: The monoclonal anti-CD20 antibody, which blocks the B cell function	Rituximab	HbA1c lowers as the rate of C peptide declines and insulin levels decrease.	[32,33]
Co-stimulation blockade	TrialNet CTLA4-Ig (abatacept); CTLA-4-IgG1 chimeric protein acts as a decoy receptor for CD80/86 and blocks CD28-CD80/86 induced co-stimulation of T-cells	Significantly higher stimulated C-peptide 2-h AUC in the treated group at the end of treatment and 1-year post-treatment	[34,35]
	TIDAL (alafacept); Alafacept: chimeric protein (2 LFA-3 molecule-IgG1) binds to CD2 and blocks T-cell-stimulation	Significantly higher stimulated AUC C-peptide in the treatment group compared to placebo; insulin use lower in the treatment group	[36]

Table 1. *Cont.*

Therapeutic Agents	Study/Authors and Intervention	Outcome	Citations
Cytokine-based: IL-2 agonist	Aldesleukin; IL-2 maintains Treg population and function	A dose-dependent elevation of Treg cells in the treatment group compared to placebo	[37]
TNF antagonism	Etanercept	HbA1c decreases while endogenous insulin production increases.	[38]
IL-1 receptor blockade	Anakinra	• No C peptide response • Lower insulin requirements compared with controls; lower insulin dose adjusted	[39,40]
IL-1beta antagonism	Canakinumab	There was no C peptide reaction	[39]
IL-1 receptor blockade IL-1beta antagonism	Anakinra/canakinumab	Immunomodulation/reverse relationship between inflammation and C peptide stimulation	[41]
Anti-IL-6 therapy	Tocilizumab in New-onset T1D (EXTEND)	Ongoing study	Clinical trial NCT02293837
Antigen-based therapy:	Antigen-specific therapies may involve direct targeting of pathogenic T cells and/or boosting Tregs for bystander suppression	Tregs were shown to be more prevalent in those who got a larger dose of oral insulin (62.5 mg)	[42]
Treg-based:	Expansion of autologous Treg cells	A subset of adoptively transferred Treg is still in circulation (25% of peak) at year 1, with no significant adverse effects. C-peptide preservation in those receiving a lower dose	[20]
DC-based:	In T1D individuals who get their autologous DCs exhibited limited output. In this study, autologous DCs were given by infused via abdominal intradermal injections each 2 weeks apart	The autologous DC-based therapy was very well tolerated; no important differences were seen in glycemia	[37]
Combination therapy	• Cyclophosphamide: immunosuppression • ATG and G-CSF: induction of Tregs	C-peptide significantly increased at 30 months follow up; increased side effects	[43]
		32% were insulin-free at 4 years, maintenance of C-peptide, but with increased side effects	[44]
		Mean AUC C-peptide at 12 months was significantly higher in the study group compared to the placebo group	Low-dose ATG + plus pegylated G-CSF [45]

AbATE = Autoimmunity Blocking Antibody for Tolerance in Recently Diagnosed T1Ds, ATG = anti-thymocyte globulin, AUC = area under curve, CTLA-4 = cytotoxic T-lymphocyte-associated antigen, DEFEND = Durable Response Therapy Evaluation for Early or T1D = Type 1 Diabetes, EBV = Epstein Barr virus, G-CSF = granulocyte colony-stimulating factor, HbA1c = glycated haemoglobin, IL-2 = interleukin-2, LFA-3 = leukocyte function antigen-3, MMTT = mixed meal tolerance test, Pre-POINT = Primary intervention with Oral Insulin for Prevention of T1D in infants at high genetic risk, TCR = T-cell receptor, Teffs = T effector cells, TIDAL = Type 1 Diabetes with Alefacept, Tregs = T regulatory cells, DC = dendritic cells.

Antibodies-based strategies: Suppression of effector or pancreatic β-cell attacking T-cells may aid in the protection of pancreatic β-cell destruction so that anti-T cell antibodies can be used. Based on the previous findings in the preclinical diabetic NOD mouse model, several clinical trials for T1D were conducted using a humanized monoclonal antibody against CD3 (anti-CD3, hOKT3 gamma1 (Ala-Ala), Teplizumab). Initially, two clinical trials lacked a placebo control group. They reported that endogenous C-peptide secretion was preserved while exogenous insulin requirements were reduced, with 5% of individuals achieving insulin independence for 48 weeks, which has been shown to preserve pancreatic β-cell function in people with T1D [46,47]. The most recent study demonstrates a delayed onset of T1D in high-risk individuals. According to a randomized, double-blind Phase

II clinical trial found that a single 14-day regimen of Teplizumab (anti-CD3) delayed the onset of autoimmune T1D by 24.4 months when compared to a placebo-treated group and that 29 percent of treated patients had HbA1c levels less than 7% and insulin dose requirements less than 0.5 U/kg per day [48]. This study highlights its potential as a preventative treatment for autoimmune T1D. The FDA has granted anti-CD3 Ab teplizumab an advanced treatment designation to expedite the process of determining whether there are additional results to support its approval [49]. However, the cytokine storm caused significant side effects in the first version of the anti-CD3 drug.

Chemokines and cytokines regulate immune system function and cascading. Generally, when cytokines interact with their receptors expressed on various immune cells, complex signaling events occur, leading to the activation of downstream markers such as transcriptional factors. Later, these factors promote the activation and differentiation of immune cells into specific lineages, for example, naive CD4 T lymphocytes can differentiate into various subsets of CD4+ T cells (Th1, Th2, Th17, Treg, TFH, Tf9, etc.). This subset differentiation is influenced by the cytokine environment, as we know that an imbalance between Treg and Th17 leads to autoimmunity [50].

The combination of cytokines such as TGFβ and IL-6 with growth factor IL-2 regulates naïve T lymphocyte function and its fate toward developing either the Treg or Th17 lymphocytes, ultimately affecting the overall autoimmunity [51]. Both cytokine TGF-β and IL-6 facilitated the Th17 pathway by activating transcription factors STAT3 and RORγt, which are associated with the destruction of pancreatic β-cells in T1D [52]. Whereas CD4 Treg lymphocytes play the opposite role, i.e., inhibit self-reactive cytotoxic T and Th17 lymphocytes, which initiate self-reactive cytotoxic T lymphocyte activation [53]. Self-reactive T lymphocytes are known to avoid clonal deletion or differentiation into the thymic Treg cell lineage early in life and enter the pancreatic lymph nodes [54]. To manipulate the cytokine axis to the equilibrated Treg/Th17 axis, cytokines, recombinant proteins, or antibodies are used. After antigen presentation, IL-2 triggers CD4 Treg lymphocytes; the dosage of recombinant IL-2 is the major one responsible for mimicking the Treg/Th17 pathway homeostasis [55,56].

Further possible antibodies for T1D therapy are anti-IL1 and anti-tumor necrosis factor-alpha (TNF-α), signaling molecules involved in inflammation and then destruction of pancreatic β-cells. Anti-TNF medications have previously been used to treat chronic pro-inflammatory autoimmune diseases, such as RA [57,58]. Despite the fact that etanercept (anti-TNFα) that binds soluble TNF was unable to prevent the development or progression of T1D [59]. Antibodies against TNF, Etanercept 24 weeks treatment, may benefit T1D since clinical studies have shown that this significant molecule decreases in HbA1c, and improves β-cell preservation compared to the placebo group [38].

There are increases in clinical trials that show a renewed interest in soluble cytokines, anti-cytokines recombinant monoclonal antibodies, or the corresponding cytokine receptors that are more often used [60]. The human monoclonal antibodies against TNF, such as adalimumab and etanercept, show the binding affinity towards the soluble TNFα than its TNFα receptors [61]. The TNFα binding with adalimumab (anti-TNFα] induces a conformational change that trimerizes TNFα receptors on Treg lymphocytes, which later causes their expansion [62]. The anti-IL17 recombinant monoclonal antibody Brodalumab, which suppresses the Th17 pathway, has been approved for a few autoimmune diseases [63]. While preclinical studies conducted in animal models suggest that the Th17 pathway is also associated with autoimmune T1D [64], clinical trials are necessary to reveal whether antibodies that suppress Th17 signaling have therapeutic effects on T1D. Based on a clinical study with an anti-IL21 drug that affects T lymphocyte activation. IL-21 is mainly secreted by CD4 helper T lymphocytes. IL-23 is another proinflammatory cytokine directed in immune intervention studies because shares the homology with p40 subunit with IL-12, another proinflammatory cytokine. Both IL-12 and IL-23 are involved in amplifying proinflammatory pathways and thus play critical roles in autoimmune processes. Ustekinumab is a mAb that deals with the homology/shared p40 subunit of IL-12 and IL-23, thereby

blocking subsequent signaling and differentiation of central immune pathways. It intensifies the cytotoxic activity of natural killer cells [65]. Later, there were two clinical trials conducted, the first one based on IL1 antagonists Canakinumab (anti-IL1 mAb) [41] and second, Anakinra (IL1R antagonist) [66], both unsuccessful in showing any statistically significant differences compared to placebo control group. This suggests that targeting single cytokine inhibition will likely not cure or provide a beneficial effect in the treatment of autoimmune T1D, due to the redundancy of pro-inflammatory cytokines associated in the onset and maintenance of the disease.

Suppressing B Cells: Despite the fact that autoimmune T1D is a T lymphocyte-mediated autoimmune disease, B lymphocytes also play a pathogenic role in antigen-presenting cells that modulate the pancreatic microenvironment [67]. Researchers linked B lymphocytes to the pathogenesis of autoimmune T1D via antigen presentation and T cell activation. CD20, a cell surface protein expressed on B lymphocytes, is required for B cell activation and proliferation and has emerged as a reliable therapeutic target with rituximab (anti-CD20). The TrialNet study group looked at how a short sequence of four infusions spread out over 30 days could delay the drop in C peptide by more than eight months in patients with newly diagnosed T1D. Nonetheless, the decline in C-peptide and β-cell function was essentially the same as the placebo control after two years [33].

Dendritic Cells based tolerogenic approach: The potent antigen-presenting and linker cells of innate and adaptive lymphocytes, i.e., dendritic cells (DCs) are generated from bone marrow's progenitor cells, which can maintain peripheral tolerance. The likelihood of creating tolerogenic DCs opens new therapeutic tactics in the prevention or remission of autoimmunity. DCs are the edge between innate and adaptive immune responses and are crucial for the initiation of antibody-humoral and cellular immunity [68], including T lymphocyte differentiation and activation [69]. They also support maintaining immunological tolerance by clearing apoptotic cells rapidly in the body. To govern if this DC can prevent autoimmune T1D by re-establishing peripheral immunological tolerance while using the primed DCs with dead cells from pancreatic β-cells, Marrin-Gallen et al. initially showed a lower disease incidence than the control group when NOD mice were injected with DCs primed with nitrilase-1 apoptotic bodies [70]. However, disappointing results were achieved in humans with T1D [71]. Later, a randomized, double-blind, phase I study was conducted with a few $n = 10$ subjects with T1D. These individuals were injected with ten million DCs into the abdomen intradermally once every 2 weeks for a total of four administrations and supervised for a year showed that a treatment regimen with autologous DCs, in a naive state or directed ex vivo to a tolerogenic immunosuppressive condition or state, is safe and well-tolerated. The DCs elevated the frequency of a potentially advantageous $B220^{+}CD11c^{-}$ B-cell population, at least in T1D autoimmunity [72]. However, several other human clinical studies resulted in disappointing results, which tells us that multiple agents contribute to failure compared to the success seen in the mice study. Similarly, this autoimmune T1D is associated with differential exposure to environmental factors [71].

Therapies that induce immune-suppressing T-cells: Pancreatic β-cells can be shielded from the autoimmune responses via the presence of Treg cells, which are immune-suppressive. Various strategies were investigated to expand the pancreas' Treg cells, including anti-CD3 mAb and IL-2 (a signaling molecule called interleukin-2).

Vaccines: In general, autoimmune diseases are characterized by the overproduction of specific cytokines, which primarily disrupt the immune system. As a result, many studies have focused on inhibiting excessive cytokine production to halt these autoimmune diseases. As a result, vaccine development is desirable in order to stimulate the immune system to neutralize the specific protein produced in excess. Tolerance can reverse pancreatic β-cell destruction if attacking T-cells expose a small number of specific proteins (or parts of proteins called peptides) from cells. C19-A2 proinsulin, a peptide, has been shown to regulate the immune response elicited by antigen-specific T lymphocytes.

Furthermore, this peptide was found to improve pancreatic β-cell function in T1D patients. In adults, a clinical trial using intramuscular DNA vaccinated with a proinsulin-

encoding plasmid (BHT-3021), which demonstrated overall safety, suggests that this strategy may have the potential to be used for treatment in the future. They discovered that antigen-specific $CD8^+$ T lymphocytes and preserved C-peptide levels were lower in the periphery while on this treatment. The primary immune cells found in the pancreas after infiltration include autoreactive B and T cells, natural killer (NK) cells, macrophages, and DCs [73]. The increased levels of IFN-I found in T1D patients' pancreatic islets and peripheral blood may contribute to disease progression; thus, blocking or downregulating this cytokine may aid in the prevention of autoimmune T1D pathogenesis [74]. Neovacs initiated IFNα-kinoid vaccine-based treatment approach for T1D.

Antigen-Specific Immunomodulatory Approaches: Several autoimmune diseases, antigen-specific therapeutic approaches have been studied [75], which modulate the inactivation of autoreactive T cells in T1D [76]. A list of antigen-specific immunomodulatory advances is shown in Figure 2 and Table 2.

Table 2. Autoantigen secreted by pancreatic β-cells in NOD mouse or T1D patients.

T1D Autoantigens	Tissue Distribution	Source (NOD Mouse or T1D Patients)	Effector CD4 and/or CD8 T Cells	Citations
(Pre) proinsulin	β cells, thymus	Mouse and human	CD4 and CD8	[77–80]
Insulin	Islet cells	Mouse and human	CD4 and CD8	[78,81–83]
A defective ribosomal insulin gene product	Islet cells	Human	CD8	[84]
Hybrid insulin peptides (HIPs)	Islet cells	Mouse and human	CD4	[85–88]
Glutamic acid decarboxylase (GAD65)	Islet cells, adrenal gland, CNS, neurons, testis, ovary	Mouse and human	CD4 and CD8	[89–92]
Zinc transporter 8 (ZnT8)	Pancreatic β cells	Mouse and human	CD4 and CD8	[93–98]
Tyrosine phosphatase like autoantigen or insulinoma antigen-2 (IA-2; ICA512, PTPRN)	Islets	Human	CD4 and CD8	[99–101]
IA-2β (Phogrin, PTPRN2)	Islets	Mouse and human	CD4	[102–105]
Islet cell autoantigen of 69 kDa (ICA69)	Pancreas, heart, and brain	Human	CD4	[106–110]
Chromogranin A	Neuroendocrine cells	Mouse and human	CD4 and CD8	[111–113]
Islet amyloid polypeptide (ppIAPP)	Islets	Mouse and human	CD4 and CD8	[114–117]
IGRP; islet-specific glucose-6-phosphatase catalytic subunit-related protein	Islets	Mouse and human	CD4 and CD8	[80,118–120]

Immune checkpoint-based therapy: Latest innovations focus on the considerable influence of inhibitory receptors (IRs) based therapy, facilitating peripheral tolerance in autoimmune diseases. The depletion or inhibition of specific blockade studies in animals, humans, and their association with inspiring disease outcomes highlight the prospective clinical benefits of enhancing IR signaling (agonism), specifically immune-checkpoint targets like CTLA4, PD1, TIM3, LAG3, and TIGIT to treat autoimmune diseases other than cancers [121]. A growing understanding of the significant role of IRs in cancer and recent developments in autoimmunity suggests that IR agonism may help prevent and manage autoimmune diseases, as reviewed in a review article by Stephanie Grebinoski and Dario AA Vignali [122].

Immune cell therapy: In autoimmune T1D, immune system cells mistakenly identify insulin hormone-producing pancreatic β-cells in pancreatic islets as foreign and dangerous; the primary goal of immune cell-based therapy is to disrupt these autoimmune signaling events. However, in the early stages of development, immune cell-based therapy is one of the most important ideas for developing a cure for autoimmune T1D. Replacing the missing insulin-producing β-cells may be able to restore average insulin production and cure T1D patients. However, initial attempts to transplant pancreatic cells have largely failed, owing to immune reactions. Another limitation is the scarcity of HLA-specific donors.

One promising approach is epitope-specific intervention, which can suppress the autoimmune response while avoiding the side effects of other therapies. Even though autoimmune T1D is polyclonal, with multiple T lymphocyte epitopes and pancreatic β-cell-specific autoantibodies, single epitope-specific medications can suppress the polyclonal immune response and reverse T1D in preclinical models [123]. The administration of specific immunomodulatory cells may aid in the reduction of the aggressive immune response against pancreatic β-cells. Tolerogenic DCs are known to suppress immune responses through a variety of mechanisms. Infusion of these cells, along with Treg cells, is being investigated as a potential strategy to prevent or reduce pancreatic β-cell destruction by the immune system. There are a few GMP-compliant procedures for isolating autologous CD4 Treg lymphocytes from peripheral blood [124]. The adaptive immune cell transfer of autologous CD4 Treg lymphocytes triggers a tolerogenic state [125], and few clinical trials are ongoing to determine whether it can efficaciously ameliorate autoimmunity. Few other new approaches develop Treg cells or cytotoxic T cells expressing specific receptors that enable the cells to target the pancreatic β-cells, increasing the therapy's effectiveness while reducing the cells' unwanted effects on other non-target regions of the body. In conclusion, a current roadblock to development in the cell therapy field is the lack of an efficient system to generate the "precise" auto or islet-Ag-specific cytotoxic CD4, CD8, or Treg lymphocytes that can be used for immune cell-based therapies in autoimmune diabetes or other diseases, as shown in Figure 3.

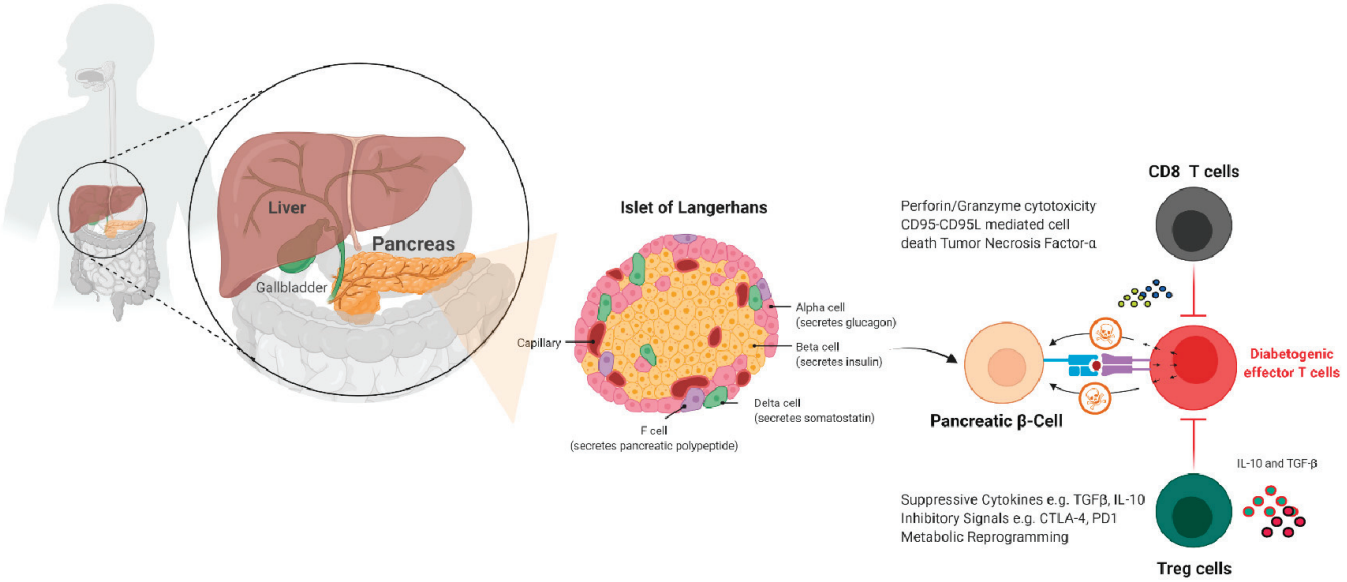

Figure 3. Pathogenesis of T1D and immunotherapy. This diagram depicts pancreatic islets and the various pancreatic cells found within them, such as delta, alpha, F, Acinus, and our target β-cell. Once a pancreatic β-cell is destroyed, its peptides are presented by immune cells, and the immune cell infiltrates the pancreas and destroys the remaining β-cells. We can protect these pancreatic-cells that secrete insulin by hiding or destroying diabetogenic effector T cells by generating β-cells that are epitope/antigen-specific cytotoxic CD4 or CD8 T cells or Treg cells that kill.

Tolerogenic autologous DC was generated in vivo and infused intradermally into a T1D patient. Later, in a clinical trial, they discovered that those patients tolerated the therapy well. Still, more research is needed to determine whether they can change the course of the disease [72]. Current exercise to find islet-specific T cell receptors and vital epitopes in autoimmune T1D, which may also allow for the generation of antigen-specific Treg or T lymphocytes in vitro using gene-editing tools [126].

Imcyse, a Belgian biotech company, develops imotopes, which generate pancreatic β-cell antigen-specific cytolytic memory $CD4^+$ lymphocytes while precisely eliminating antigen-presenting cells (APCs) presenting the same T-cell epitope through apoptosis. The pathogenic T lymphocytes activated by other epitopes on the same APC are also destroyed by the cytolytic memory $CD4^+$ lymphocytes (bystander effect). Imotope technology will now be able to precisely eliminate pathogenic auto-immune responses and cure autoimmune T1D. The lead molecule, IMCY-0098TM, is an imotope that has been developed to treat T1D and is currently in clinical trial 2b (NCT04524949). Imcyse's strategy, when used consistently, aids in the prevention and treatment of diseases for which there are no current therapeutic options, as well as the potential cure of patients without impairing immune defense.

Overcoming immunotherapy challenges: While various immunotherapies are being tested in clinical trials for the treatment of autoimmune T1D, several issues must be addressed. The factors involved in T1D differ among individuals with this condition, complicating treatment because a single type of treatment or therapy may not be sufficient for all patients. For example, not all T1D patients or individuals have islet cell inflammation. As a result, treatments aimed at reducing inflammation in islet cells may be ineffective in patients who do not have this type of inflammation. Given the individual differences in T1D characteristics, personalized medicine for individuals may be a highly beneficial approach in the coming year.

A new facet of T1D immunotherapy is achieving satisfactory efficacy. Triggering or targeting immune cells may result in a wide range of responses, reducing treatment efficacy and causing adverse effects. Physicians and researchers are attempting to address this issue through a variety of therapeutic approaches. Combining therapies that act through different mechanisms of action improves treatment efficacy. Varieties must be chosen in such a way that efficacy is improved, and side effects are minimized.

3. Conclusions and Future Perspectives

However, there is still a long way to go; new autoimmune diseases, particularly T1D immunotherapy, hold great promise for T1D patients. In the future, we may even be able to use single or multi-immunotherapy to prevent the condition before it manifests. This novel strategy is possible because we now have the technological capability to identify individuals at risk of T1D before significant damage to the pancreatic β-cells occurs. The past 30 years have taught us that immunotherapy has the potential to treat or preserve autoimmune T1D. However, we have yet to see a breakthrough because we have yet to crack the code in terms of an acceptable safety/efficacy balance for treating autoimmune T1D. Immune cell-based approaches to T1D treatment may offer the possibility of lifelong insulin administration in people with autoimmune T1D. Assume we used T1D patients in the early stages of the disease, when β-cell mass destruction is low or a significant percentage of β-cell mass remains. The in vivo expansion of pancreatic islet antigen-specific Treg therapy via adoptive transfer will be beneficial. In this case, attacking CD8 T lymphocytes and administering ex vivo prepared autologous tolerogenic DCs are promising immune cell-based therapies that may effectively preserve pancreatic β-cell mass and function. Since the discovery of insulin by Banting and Best in 1922, several recent developments have investigated autoimmune T1D management. In the near future, new skills will allow us to treat autoimmune diseases as well as prevent or reverse them.

There are still a few unanswered questions. Even though significant progress in our understanding of the immunobiology of autoimmune T1D allows for the development of novel therapeutics that may be beneficial and clinically safe for preserving autoimmune T1D.

Key questions that must be answered:

1. What factors/agents cause the initial destruction of pancreatic β-cells in T1D patients?
   - ○ Genetic or epigenetic modifications;
   - ○ Viral infection;
   - ○ Gut microbial flora;
   - ○ Environmental or diet and nutrition;
   - ○ Aging or developmental changes destroy β-cells.
2. Once pancreatic cells are destroyed, how do immune cells select their cognitive epitope; why do these epitopes express or present more? What factor or cells cause them?
3. Self-epitope/antigen enters circulation—how/why do CD4/CD8 T cells begin recognizing self-antigen, indicating that TCR rearrangement occurred previously with the corresponding epitope? What causes the immune tolerance system to fail to circulate these immune cells?
4. Once in the circulation, β-cells antigen/markers, specific T cells are generated; how do these T cells begin attacking remaining β-cell masses who give them command? Is there any expression of pancreas-specific signals or chemokine receptors on these cells? How they infiltrated the pancreatic islet to destroy-cells.
5. What causes Treg cells to become inactive?
6. How can we prevent an unwelcome immune attack on pancreatic β-cells? The best strategy is to hide and attack.

Hide: Modify pancreatic β-cell-specific antigen recognition/presentation to preserve β-cell mass. Attack: Attacking the rebel immune cells is another option; we can generate antigen-specific Treg or cytotoxic CD4/CD8 T cells to kill the rebel immune cells.

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Experiences of Diabetes Self-Management: A Focus Group Study among the Middle-Aged Population of Rural Pakistan with Type 2 Diabetes

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Abstract: Objectives: The middle-aged population from rural areas of Pakistan is disproportionately at risk of developing and mismanaging their diabetes. The purpose of this study was to explore the self-management experiences of two focus groups in the middle-aged population with type 2 diabetes mellitus living in rural Pakistan. Methods: The study design is based on the exploratory research using a qualitative approach. Purposive sampling was used to recruit patients with diabetes from the metabolic outpatient clinics of medical centers in rural areas of Pakistan. The data were collected for two focus groups consisting of 20 persons (10 men and 10 women) with type 2 diabetes mellitus, ranging in age from 40 to 65 years, who were receiving diabetic care at a local health facility. Focus group discussions with a sample size of 10 participants each were all recorded, transcribed, and analyzed. The data were evaluated thematically. Results: Participants described diabetes management as emotionally, physically, and socially taxing. The analysis of the data indicated three major themes: (1) diabetes as a challenging disease; (2) understanding diabetes and its challenges; (3) following diabetes self-management practices. Throughout the session, participants discussed the impact of diabetes on their daily life. This study provided new insights into the experiences of the middle-aged population of Pakistan regarding their self-management of diabetes. Conclusions: Healthcare professionals should become involved in diabetes self-management education as soon as feasible to alleviate patient worry and establish better patient-centered, culturally sensitive professional abilities. Along with monitoring patients' self-management, healthcare professionals should place a greater emphasis on patients' understanding of the disease and its challenges and associated complications. It is recommended to establish diabetes support groups to encourage patients to share their experiences of diabetes self-management.

Keywords: focus groups; self-management of type 2 diabetes; patients' experiences; healthcare professionals; middle-aged population

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1. Introduction

Diabetes mellitus is one of the most prevalent chronic diseases worldwide, creating a slew of health problems for the population [1]. According to the International Diabetes Federation (IDF), there are 463 million individuals worldwide who have diabetes [2], with type 2 diabetes being more prevalent in people aged 40 to 59 years [2,3].

The prevalence of type 2 diabetes in Pakistan is in the range of 7.6% (5.2 million populations) to 11% [3–5], and it is estimated that it will increase to around 15% (14 million

populations) by 2030. Pakistan ranks in the top 10 countries worldwide in terms of diabetes prevalence among people aged 20 to 79 years [2].

This population is at increased risk of type 2 diabetes due to a higher prevalence of overweight or obesity, physical inactivity, and unhealthy eating habits associated with a sedentary lifestyle [1,2]. Additionally, the Pakistani region has a population with numerous social and health inequities, which contribute to the region's high prevalence of diabetes and obesity [3–6].

Self-management is defined as a patient's active participation in his or her own treatment and well-being [7]. Self-management has been described by Corbin and Strauss [8] as having three main activities: (1) adherence to medication, which includes following the dietary recommendations; (2) behavioral management, which includes new lifestyle modification; and (3) emotional management, which includes coping with chronic disease-related feelings of frustration, fear, and despair. Because type 2 diabetes is a chronic disease involving just a few visits per year to health care providers, individuals must manage all of these components on their own for the rest of their lives.

The optimized self-management addresses all three main activities of the Corbin and Strauss framework [8] and encourages physicians and patients to work collaboratively to "identify problems, determine priorities, develop goals, build treatment plans, and resolve conflicts along the way" [9]. Previous research has demonstrated that successfully assisting patients with type 2 diabetes in self-management resulted in beneficial effect on their lifestyle and, eventually, helped in improving the health outcomes [10–12]. However, worldwide research indicates that the self-management support is still insufficient in many parts of the world [13].

Therefore, a thorough understanding of public health perspectives on self-management and how it is currently supported could aid in the creation of solutions that are more tailored to the requirements of patients, preferences, and skills. Appropriate self-management support is likely to improve health outcomes and care efficiency [13].

Self-management of diabetes is a critical activity for diabetes patients in their daily lives and is regarded as the cornerstone of diabetes care in the literature [14]. Numerous studies in the literature have emphasized the relevance of diabetes self-management and its association with increased diabetes awareness, responsible patient behavior toward their disease, and clinical success [15–17].

Diabetes mellitus has a negative impact on numerous facets on the daily life of a person. Therefore, structured individual care is generally connected with a better control of blood sugar [18]. Diabetic self-management education is the process by which individuals acquire the required knowledge and abilities for diabetes self-care [19]. Diabetes care on a daily basis (healthy diet, physical activity, blood sugar testing, and adherence to medication) is primarily the patient's responsibility; hence, people must be educated to carry out self-management activities properly. Substantial research work has been carried out to determine the numerous elements that influence self-management in people with diabetes [14,18,19].

The main objective of this research work was to explore the understanding and experiences of the focus groups about the self-management activities of Pakistan's middle-aged population with type 2 diabetes. Focus group discussion is frequently used as a qualitative approach to gain an in-depth understanding of social issues. Focus groups are about insight through guided group discussion, where participants share their thoughts, feelings, attitudes, and ideas on subjects. Exploring such rich experiences and gaining a better understanding of diabetes self-management activities promotes the development of more effective techniques for teaching diabetes self-management so that afflicted individuals may manage the condition effectively and coexist with it.

2. Methods

2.1. Study Design

The purpose of this qualitative study was to collect information regarding the self-management experiences of a middle-aged population with type 2 diabetes mellitus residing in rural Pakistan through focus group interviews [20]. For this study, two focus group interviews were conducted away from the medical clinics, in the main auditorium of Al-Rehman hospital in Abbottabad, Pakistan. The study design is based on the exploratory research using a qualitative approach. The purposive sampling was used to recruit patients with diabetes from the outpatient clinics of medical centers in rural areas of Pakistan.

2.2. Setting and Sample Size

Patients with diabetes were recruited through purposive sampling from metabolic outpatient clinics in rural medical centers in Abbottabad, Pakistan. The estimated population of Abbottabad city in 2010 was 1.1719 million [3,5]. The city is located in the north of Pakistan about 110 km from Islamabad (the capital city). Around 80% of the population lives in rural areas, there are 19 primary healthcare clinics in that area, and five of these clinics are associated with the hospital where this study was conducted [3,5].

The following criteria were used to determine inclusion: (a) a minimum age of 40 years, (b) a diagnosis of type 2 diabetes five years' prior, (c) a willingness to openly communicate their experiences and engage in conversation, and (d) willingness to be audio-recorded. The patients with coexisting liver, kidney, or thyroid disorder and under treatment at the time of the study were excluded. Patients with cognitive impairment, major psychiatric diagnosis, or surgery were also excluded.

A purposive sample of 20 participants was selected in this study. The purposive sampling is a suitable sampling method in qualitative studies to identify and select information-rich respondents to a phenomenon of interest, which was the self-management of diabetes mellitus in this study [20,21]. Therefore, this sampling approach was used to determine the sample size based on theoretical saturation, a point during data collection when new data does not bring additional insights into the research question or themes.

This small sample size is adequate enough to allow in depth exploration of the experiences of patients regarding the self-management of type 2 diabetes [20–22]. Carolan et al. [23] used a sample size of 22 participants to conduct the qualitative study for the focus groups. The aim of this study was to explore the experiences and concerns of individuals with type 2 diabetes mellitus, in a predominantly low socio-economic setting. Wu et al. [24] conducted a qualitative study with two focus groups, comprising a total of 23 participants, to collect data, and group discussions were held a total of four times in an education room. Mathew et al. [25] recruited 35 participants from a diabetes education center (DEC) in Toronto, Canada to conduct the qualitative study to explore men and women's diabetes self-management experiences among the focus groups.

2.3. Ethical Consideration

The "ethics committee of the University of New South Wales, Australia" (Ref. No.: HC16882) and Pakistan's "Ayub Medical Institution" approved the study. Participants were recruited as a result of responding to recruitment advertisements and leaflets. The information sheet was provided to all participants detailing the purpose of the research, potential risks and benefits, and concerns about confidentiality, in addition to the informed consent form. The researchers were straightforward in communicating the study's purpose and methods to the participants. At any point, a volunteer could withdraw from the research. Each participant signed an informed consent form.

2.4. Data Collection and Procedure

Focus groups were utilized in this study because they encourage discussion about the challenges of diabetes self-management in the middle-aged population of rural Pakistan [21]. In general, group dynamics generate valid ideas because discussions between

group members fosters diverse opinions and feelings [20]. The groups of males and females were separated for discussions as there are cultural limitations in the rural areas of Pakistan; in a male dominated society, women are unable to express their feelings openly [22].

Two sessions lasting approximately 50 min each were carried out. The discussion guide has been provided in Appendix A to initiate the discussions among the focus groups. This interview guide was also utilized by the authors who previously conducted the self-structured interviews for type 2 diabetes patients [22].

The authors explained the aims and objectives of this study to participants and they were informed that the discussions would be audio recorded and observations made to assure accurate data collection. The main author took part in the group activity, moderated the conversation, and prompted participants to share their experiences. The attendees were encouraged to share their stories and voice their concerns and ideas. Through engagement with others, they gained assistance and encouragement to express their opinions freely. The group activity ended once the participants no longer had thoughts or ideas to share.

2.5. Data Analysis

The data collected in this study were analyzed by thematic analysis because this method was developed to meet the needs of investigating the experiences, meaning, and reality of the participants [26]. Our thematic analysis involved initial independent coding by three academics. Thirty codes were identified in the preliminary analysis of the transcripts. Codes were then clustered and used to form nine sub-themes that integrated several of the originally identified codes and encompassed more general topics that were the focus of the transcripts. The detailed analysis led to the three major themes that illustrated the most significant and broad similarities or differences of diabetes self-management experiences of focus groups and barriers to self-management. These themes were further elaborated in the following section of results.

The following procedures were used to analyze the data: (1) all collected data, audio recordings, logs of observation, reflective conversations, and focus groups, were transcribed verbatim; (2) the transcripts were read repeatedly; (3) for the purpose of analysis, the sentences were shortened in the text; and (4) the discussions continued until all the authors agreed.

2.6. The Study's Reliability

The four dependability criteria were provided and applied to ensure the study's rigour [27,28]. The four criteria are as follows:

(1) True value: In order to enhance the true value of the analysis, the writers of this article examined data jointly, followed by the distribution of the article to six participants to review the article.
(2) Scope of Application: Confidentiality was assured for ideas voiced during the group sessions discussions by the participants to explore their experiences in a safe and trustworthy environment, resulting in extensive descriptions.
(3) Consistency: The researcher (the first author) led each group session and engaged personally in data collecting to avoid discrepancies in data collection procedures and the biases were avoided.
(4) Confirmability: The researcher checked and rechecked the data throughout the study to ensure the confirmability of the outcome of the data analysis.

3. Results

Participant Characteristics

Table 1 summarizes the participants' characteristics of this study (n = 20). Ten men and 10 women participated, ranging in age from 40 to 60 years (mean 55 years). Three participants had completed grade 9 education, four had completed school education, 10 completed education at the college level, and three had achieved a university degree. The majority of participants had married status and were employed. Men received diabetes

therapy for an average of 12 years, while women had treatment for an average of 10 years. The individuals' mean glycated hemoglobin (HbA1c) level between men and women was (8.2 and 8.5) percent respectively.

Table 1. Summary of participant demographics (n = 20).

Demographic	Men (n = 10)	Women (n = 10)	Total (n = 20)
Age (average, in years)	58	52	55
Marital Status			
Single/never married	4	2	6
Married	5	8	13
Separated/divorced	1	0	1
Widowed	0	0	0
Education			
Less than grade 9	2	1	3
Some/completed high school	2	2	4
Some/completed college or university	6	4	10
Graduate/professional degree	2	1	3
Employment			
Full/part-time, self-employed	8	7	15
Unemployed	1	3	4
Retired	1	0	1
Diabetes Duration (Mean years)	12	10	-
HbA1c level (Mean %)	8.2	8.5	-

Table 2 summarizes the analysis of the data resulting in three major themes: (1) diabetes as a challenging disease; (2) understanding diabetes and its challenges; (3) following diabetes self-management practices. These themes and sub-themes were discussed in detail in the context of the responses by the participants based on the detailed interview guide given in Appendix A for the two focus groups.

Table 2. Themes and sub-themes of self-management experiences of the middle-aged population in with type 2 diabetes in rural Pakistan.

Main Themes	Sub-Themes
Diabetes as a challenging disease	• Feelings of fatigue and fear • Blurred vision problem • Feeling hungry and thirsty
Understanding diabetes and its challenges	• Healthy diet and sugar control • Lack of physical activity • Emotions influencing Diabetes Self-management
Following diabetes self-management practices	• Diet challenges during social gatherings • Following physical activity schedule • Psychological burden with diabetes

Theme 1:

Diabetes Considered as "A Challenging Disease"

Patients with diabetes experience a range of discomfort, from disease diagnosis to the realization that the diabetes is irreversible. Throughout group discussions, individuals of varying ages and work position discussed their differing perspectives on the disease. The theme "diabetes considered as a challenging disease" brought three sub-themes for discussions, namely, the feelings of fatigue and fear, blurred vision, and feeling hungry and thirsty.

Some of the participants had co-morbidities and were concerned about future consequences. Due to the unseen nature of consequences, participants referred to diabetes as a challenging disease:

One patient noticed some apparent bodily changes and signs:

> "I am rather active at my workplace." However, if my physical power deteriorates significantly and I experience excessive exhaustion, I feel that my blood glucose level is gone up. . . " (Participant 14)

> "I have a new sensation, one of 'fatigue,' which may be related to my low blood glucose level. . . After being diagnosed with type 2 diabetes, I am compelled to monitor any physical changes that occur to me." (Participant 15)

> "I am living in terror of what may be impacted next. At the moment, I'm experiencing some nerve difficulties, which is really concerning. . . " (Participant 6)

Another concern that the middle-aged population confronts with diabetes is blurred vision.

> "I've noticed that I'm unable to see clearly but I used to see well, it also signals that I am suffering with some problem and may be my sugar level is elevated. . . " (Participant 5)

> "I have blurred vision and experience dizziness on occasion. . . When I am unable to see clearly, my heart rate increases. Occasionally, my sugar level drops so low that I am unable to see well." (Participant 2)

One patient mentioned that:

> "I have no idea when diabetes happened, I was feeling well, eating well but suddenly started feeling thirsty and hungry. . . " (Patient 10)

> "The most challenging part is to cope with the conditions due to changes in our daily routines particularly after the diagnosis of the disease—that is the time we need more support to understand the impact of the disease and our health condition." (Patient 1)

Theme 2:

Understanding Diabetes and Its Challenges

The theme "understanding diabetes and its challenges" in focus groups interactions and discussions included three emergent sub-themes: (1) healthy diet and sugar control; (2) lack of physical activity; (3) emotions influencing diabetes self-management. During the group discussions, the participants shared their diverse perspectives, personal experiences of understanding diabetes, and its issues.

Sub-Themes

1. Healthy Diet and Sugar Control

In Pakistani culture, the terms "eating" and "food" have historically meant more than simply satisfying one's "physical need". The participants in the focus groups utilized their own language and situations to convey and further improve their understanding of a variety of experiences.

> "I worked extremely hard for my career and was looking forward to enjoying delicious meals. I imagined that I might now have delicious meals daily and a great life. However, since being diagnosed with diabetes, I am unable to enjoy them at all." (Participant 5)

In group discussions and interactions, patients learned about the healthy food they need to eat as patients of diabetes. Though they are naturally not prohibited from eating these foods, they have to control what they eat.

The group members expressed a variety of viewpoints based on their personal experiences.

> "I understand that there is no problem in consuming delectable cuisine; it simply means that we must exercise control over how we eat nutritious food. . . " (Participant 8)

> "We can still enjoy delectable healthful foods, but adherence to a balanced diet is important in order to maintain blood sugar. . . " (Participant 7)

One of the female participants mentioned:

> "It is always difficult to cook the diabetic friendly food in a joint family set up. The food choices are very much dictated by the males living in the joint family." (Participant 9)

2. Lack of Physical Activity

Physical activity is crucial for diabetes patients to maintain a healthy blood glucose level. Patients discussed which exercises are appropriate for them and the effect of exercise on blood glucose control.

> "It is critical to engage in physical exercise on a regular basis, as this uses glucose and is critical for blood glucose control." (Participant 9)

Patients discussed during group talks which exercises are appropriate and easy to carry out on daily basis and its benefits to manage their diabetes.

> "I make it a point to workout at least three days a week. Every time, I chose to stroll for 30–40 min. . . " (Participant 8)

> "It is physically difficult for someone in their sixties to run or jump. The only form of exercise available to us is strolling. . . After meals, I'll take a half-hour stroll. . . " (Participant 15)

3. Diabetes Self-Management and Emotions

Controlling nutrition over an extended period of time causes patients with diabetes to experience varying degrees of psychological stress. They shared their experiences in group talks about psychological adjustment approaches for cultivating positive awareness in order to activate pleasant emotions and further control diabetes.

> "I know that eating cookies and cakes will increase my sugar level. However, I always eat cakes knowing that it brings high spikes to my blood sugar, I am emotionally influenced to continue doing it. . . " (Participant 7)

Theme 3:

Following Diabetes Self-Management Practices

The theme "following diabetes self-management practices" in focus group interactions and discussions included three emergent sub-themes: (1) dietary problems associated with social gatherings; (2) physical activity schedule; (3) psychological burden with diabetes. Following diabetes self-management practices is crucial for controlling the blood sugar and maintaining the healthy lifestyle. Diabetes self-management practices in day to day life involves overcoming diet challenges during social events, following a physical activity schedule, and coping with the psychological burden of diabetes. Therefore, patients with diabetes face great challenges in following up self-management practices.

Sub-Themes

1. Dietary Problems Associated with Social Gatherings

When confronted with restricted eating during special occasions, such as weddings and other events, patients frequently felt guilty if they did not restrict themselves and lost control. As a result, people with diabetes are constantly faced with the decision of whether to exercise self-control in such situations.

> "It is courteous to decline invitations in social gatherings with friends. If I abstain from something or eat less during social gatherings, my friends become unhappy. . . However, when I lose self-control, I experience guilt. . . " (Participant 12)

2. Adherence to a Physical Activity Schedule

The most challenging aspect for diabetic people is adhering to a physical activity regimen. While patients understand the importance of exercise, they frequently lack the ability to actually perform it.

> "I am aware that I must exercise... However, I am powerless to perform it. I am aware that no one wishes to suffer from chronic hyperglycemia, and that everyone wishes to maintain control of their physical health and illness status..." (Participant 10)

3. The Psychological Burden of Diabetes

Diabetes patients must modify their lifestyles, which includes adjusting their daily meals, making sure that they do physical activities regularly, consistently monitoring blood sugar levels, and adhere to their prescribed regimen. The long-term application of these self-care routines and acceptance of restrictive life limits impose a major psychological cost on the middle-aged diabetic population.

> "When I wake up in the morning, I ponder what should I eat and how much I consume... I need to get some workout. I am unable to take my medication till I have consumed food. I'm concerned that if I don't eat anything, my blood glucose will drop to low level... reduced activity may also result in poor glycemic control..." (Participant 12)

> "Whatever I do, I must keep my diabetes in mind. I must monitor and manage my diabetes on a daily basis... to come to terms with the fact that my diabetes disease is for the rest of my life. It's quite challenging and stressful..." (Participant 14)

4. Discussion

This study focused on the individual (with type 2 diabetes), not the disease. Instead of medical clinics or healthcare centers, researchers interviewed individuals at the lecturers' auditorium or other locations of their choice. This way, participants felt more at ease discussing diabetes and self-management. Participants expressed concern about medical experts criticizing their diabetes self-management style, as they focus on HbA1c values rather than patient type 2 diabetes issues.

Patients are responsible for taking care of their diabetes daily as patients of type 2 diabetes must make several decisions and perform difficult tasks to maintain sufficient glycemic control [29]. For middle-aged people, making this decision is more difficult as their fantasy of enjoying a variety of delicious foods during this stage of life after a lifetime of hard work can be shattered by diabetes [22].

Patients who were diagnosed within 5 to 6 years with stable, acceptable glycemic control may require less professional help if they receive it within a short duration after diagnosis. Previous research has identified that stable individuals have distinct support needs compared to episodic or progressing patients [29]. Unpredictable disease might reduce self-efficacy, making patients feel less capable of self-management and hence more in need of help [29,30].

The participants identified two instances of active self-management along their illness course. Patients reported inadequate self-management assistance, which is consistent with prior research [31]. They struggled to describe what is lacking, implying they are unsure about how the support can be improved. Patients of type 2 diabetes require self-management support [32].

Thus, a detailed grasp of patients experiences on self-management and how it is currently understood and supported may benefit in the development of solutions that are better customized to the needs, preferences, and expectations of individual patients. Appropriate self-management assistance is expected to improve health outcomes and efficiency of care [13].

4.1. Public Health Perspective

The focus of this study on non-medical issues is a major plus. This study looked at self-management from a public health perspective. A person-centered approach is important because patients are expected to manage type 2 diabetes on their own. Thus, the findings of this study may be used to design techniques that better support the self-management of

patients with type 2 diabetes. User-centered design develops methods from the standpoint of the user (patient) and these solutions may boost acceptability of interventions as they are reflecting the patients' requirements and expectations [19].

Our findings indicate two critical considerations for developing user-centered self-management support tools for people with type 2 diabetes. To begin, it is critical to provide support at the appropriate times, i.e., when patients require assistance due to changes in their daily routines or health. Our investigation discovered two such instances: immediately following diagnosis and when difficulties start (glycemic control deteriorates). In addition, previous research has demonstrated that as patients' health declines, their demand for self-management support increases [33].

4.2. Clinical Perspective

The qualitative study of two focus groups was carried out to gain a better understanding of the self-management experiences of rural areas of Pakistan's middle-aged population. The participants reported that diabetes is a silent disease with complications and presents numerous challenges to diabetes self-management. The effective diabetes treatment model is to ascertain the cause of a disease, accurately diagnose the disease, effectively treat the disease, and assist patients in recovering from the disease and regaining health [34].

However, patients who receive an accurate diagnosis of diabetes learn that the disease is incurable and must encounter a sequence of stressful events induced by the disease; this leads patients to interpret physical changes as warning indicators concerning their blood glucose levels [35]. Patients self-assessed their blood glucose levels and identified links between physical symptoms and blood glucose levels in this study. Patients of diverse ages and professional levels discussed how they might make sense of their current physical changes based on a variety of physical experiences during group interactions. Physical perception should be used to detect early symptoms, and detecting these warning signs prior to illness would assist diabetic patients in preventing disease onset [36].

Additionally, health care practitioners should consider ways to improve patients' self-observation of symptoms prior to beginning in order to further reduce the frequency of acute onset. Each individual's physical reaction to blood glucose levels is unique. If health care providers can assist patients in developing a connection with their bodies and empathize with the process of patient-centered disease empowerment, this will be meaningful development [37].

In rural Pakistan, meals are critical for the development of interpersonal ties. The traditional and cultural occasions and their associated family gatherings to celebrate these occasions put individuals with diabetes' food control to the test. According to relevant studies, the desire of individuals with diabetes to "not eat" at social events tends to isolate them from others [38]. We have found differences between the perception of self-management of type 2 diabetes between men and women in this study. The preparation of food is managed by women in Pakistani culture but the choice of food is dictated by males. Therefore, it is extremely difficult to prepare healthy food for the patients with diabetes.

As a result, when "unhealthy food" (not suitable for patients with diabetes) is served at gatherings, diet management becomes challenging. During the discussions, it was determined that diet control, namely limiting the desire to eat, was the most difficult component for the participants and these findings are in agreement with the findings by Huang et al. [39].

The cultural predisposition needs to be acknowledged and accepted as a necessary component of the diabetes care approach. Healthcare professionals should relax institutionalized health education constraints and integrate diet control into daily life of the patients. Physical Activity is the most cost-effective therapy for diabetes control. It has been shown to decrease insulin resistance and delay the onset of problems [40]. The majority of participants in this study were of the opinion that physical activity is a critical component of diabetes therapy; nonetheless, they reported difficulty maintaining an exercise routine.

However, the challenge for nurses is to incorporate physical activity into patients' everyday lives in order to further enhance the association between physical activity and disease, which is especially crucial for the middle-aged population. Lachance et al. [41] provided a compelling case that peer coaching and social support are beneficial for women's exercise self-management in communities. The current study proposes that diabetes care teams gradually increase the frequency of exercise management behaviors in the middle-aged diabetic population during a typical workday and social occasions.

Diabetes is a chronic condition that requires lifelong management. Diabetes patients must maintain strict control over their lives, which demands psychological and behavioral modifications as well as self-management [1]. Accepting life limits and following long-term self-care norms is very difficult for many patients, adding to the middle-aged population's psychological burden. These groups require more assistance with diabetes self-management, including physical, psychological, and interpersonal assistance. According to Cryer [38], elderly diabetes patients have higher illness-related discomfort and are more likely to experience it than other age groups of diabetes patients.

Individuals who are ill frequently endure mood fluctuations as the illness worsens. As a result, health care practitioners should place a greater emphasis on educating middle-aged patients about illness management rather than focusing exclusively on physiological treatment. The way a patient adjusts to an illness is greatly dependent on the physician–patient relationship, and a physician's attention to a patient's psychological difficulties can significantly improve the patient's diabetic care outcome [32]. It has been established that offering high-quality diabetes self-management education to patients improves their self-management [21].

Awareness of the complications from diabetes would help patients with diabetes to act with greater determination and motivation to carry out the self-management activities. The issue of awareness of complications is central because it is closely linked to the good medical practice of diabetes centers, particularly in rural areas [42]. The study carried out in Pakistan showed that people with diabetes have very low level of awareness about diabetes and its complications and management [43,44].

It is recommended that health care practitioners listen to their patients' self-management concerns and collaborate with them to develop strategies for making healthy lifestyle choices. Additionally, health-care teams should include clinical psychologists to assist patients in developing a more optimistic outlook on life, which will enable them to adjust to and coexist with the disease successfully.

5. Strength and Limitations

The main strength of this study is that it has provided culturally sensitive perspectives on the middle-aged population of rural areas of Pakistan with diabetes. The focus groups consisting of 10 women (group 1) and 10 men (group2) were divided and interviewed separately, provided excellent opportunity for women to express their point of view freely and without any reservations. This would have not been possible otherwise as women in rural areas of Pakistan are reluctant to express their point of view freely in front of male's participants. One of the limitations is related to selection bias: participants recruited had poor glycemic control; thus, they might have been more willing to take part in a clinical study in order to lower their HbA1c.

This study included outpatients at two clinics of the main hospital in Abbottabad's rural area; consequently, the study results may not accurately reflect the self-management requirements of all diabetes patients. Future studies should involve patients from a range of settings to ensure that our findings are generalizable. Second, the authors collected data on the diabetic self-management experiences of chosen focus groups at a single point in time; hence, the results may not accurately reflect an individual's perspective over time.

6. Relevance to Clinical Practice

This study's findings highlight a number of critical areas, and addressing these critical areas may result in an improvement in the experience and self-management outcomes of patients with type 2 diabetes. In the first instance, emotional assistance is clearly needed, as many participants described diabetes as a significant and ongoing emotional and psychological burden. Several participants reported a need for diabetes education and information as information searching may be challenging for persons who are underprivileged or have limited health literacy. Furthermore, family involvement in such groups is also vital for their own education about diabetes and for receiving information on how to best support a family member with type 2 diabetes [45].

7. Conclusions

This study highlighted the experiences of Pakistan's middle-aged population in rural areas regarding diabetes self-management. Healthcare professionals should be involved in diabetes self-management education as soon as feasible to alleviate patients' concern and aggravation about their glucose level and symptoms. Along with self-management, health practitioners should focus on the diabetes self-management activities of the patients. Our findings can assist healthcare professionals in developing better patient-centered, culturally sensitive therapeutic abilities. In addition, it is required to form diabetes support groups in rural areas of Pakistan to help patients of diabetes to share their self-management experiences. The majority of patients with diabetes in rural Pakistan are unaware of diabetes complications. Therefore, community based awareness programs should be launched in primary health care clinics and hospitals to decrease the morbidity and mortality associated with diabetes mellitus.

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Appendix A

Focus Group Interview/Discussion Guide

1. State your name, how long you've had diabetes for and why you decided to participate in the focus group.
   - What was going on in your life when you found out you had diabetes? (Describe your overall experience living with and managing diabetes over the past year)
   - Diet and nutrition
   - Physical activity
   - Taking medication
   - Managing stress
   - Care received
2. Overall, how well do you feel and think you are able to manage your diabetes?
3. What were your difficulties with having diabetes over the past year?
   - Diet: social gatherings, watching what you eat
   - Personal life
   - Treatment

- Fears/anxieties about having diabetes over the past year

4. What kinds of resources have been most helpful to you in managing your diabetes?
   - How do your family/friends help you?
   - Family physician
   - Books/Magazine/Media
5. Describe your experience during your visits to the Diabetes Clinic/Center.
   - Experience with the professionals and clerical staff
   - Experience with the programs (one-on-one visits included)
6. How were your difficulties addressed during your visits to the Center?
   - How were your fears/anxieties addressed during visits to the Center?
   - Draw on prior answers for difficulties and fears
7. How would you like to see improvement in Diabetes Center services?
   - Staff
   - Resources
   - Why didn't you come back to the Center?
   - What would keep you going back to the Center?
8. Overall, describe your experiences accessing healthcare in regards to your diabetes
   - Physician
   - Hospital
   - Specialists
9. Summarize the general themes of the focus group/ interview and ask:
   - Is this a good representation of what was said?
   - Does anyone else have anything to add?
   - Does it spark any ideas for anyone of you?

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Article

Is the FINDRISC Tool Useful in Screening Type 2 Diabetes and Metabolic Syndrome in an African Setting? Experience among Young Adults in Urban Tanzania

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Abstract: Background: Simple and less costly screening tools are needed to combat the rising non-communicable diseases epidemic. This study aimed to evaluate the utility of The Finnish Diabetes Risk Score (FINDRISC) as a screening tool for prediabetes, T2D, and metabolic syndrome (MetS) in a population of young adults in urban Mwanza, Tanzania. Methods: A cross-sectional community-based study was conducted among participants aged 18–35 years. The FINDRISC questionnaire was used to collect data and compute the FINDRISC scores for each participant. Socio-demographic, anthropometric, blood glucose, and lipid profiles data were collected accordingly. Results: A total of 259 participants were recruited into the study. The median age was 21 years (IQR 19–27), and more than half 60.2% (156) were females. In total, 32.8% (85) of the participants had at least a slightly elevated risk of developing T2D in 10 years' time. Compared to the Oral Glucose Tolerance Test (OGTT), FINDRISC had a sensitivity and specificity of 39.1% and 69.2%, respectively (aROC = 0.5). The FINDRISC score significantly correlated with MetS (p = 0.001). Conclusion: In this study, FINDRISC has shown low sensitivity and specificity in the screening of pre-diabetes/T2D. However, it has potential utility in the screening of MetS in a young-adult population.

Keywords: FINDRISC; prediabetes; diabetes; metabolic syndrome; young-adults

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1. Introduction

Type 2 diabetes mellitus (T2D) is a chronic disease that is characterized by a long pre-diabetic state before the development of a full-blown disease [1]. Prevalence of diabetes, diabetes-related deaths, and social-economic burden due to diabetes continue to rise globally [2]. The International Diabetes Federation (IDF) estimated a diabetes global prevalence of 8.8% in 2015, with a projection to increase to 10.4% in 2040 [2]. Further, the IDF estimated a 6.7% prevalence of Impaired Glucose Tolerance (IGF), 5 million deaths attributed to diabetes, and USD 673 billion global health expenditure in 2015 [2]. For individuals diagnosed early during pre-diabetes, it is possible to institute interventions that will halt the development of full-blown T2D [3]. Standard diagnostic tests, such as the Oral Glucose Tolerance Test (OGTT) and HbA1c, are expensive and difficult to scale up in a large population, especially in resource-limited settings, such as Tanzania [4]. Of late, cheaper and easy-to-use tools have been developed and tested in other countries and have shown to be cost-effective in the diagnosis of pre-diabetes and diabetes mellitus [5,6]. The Finnish Diabetes Risk Score (FINDRISC) is one such affordable and easy-to-use screening tool [7–10].

The FINDRISC questionnaire, originally used as a screening tool in the Finnish Diabetes Prevention Study, was found to be an effective tool in identifying individuals at a

high risk of developing T2D in 10 years' time [5]. Since then, the tool has been validated in several other studies and is used in adult populations for early diagnosis and prevention of overt T2D [7,9,11]. Several studies have been done to test the effectiveness of FINDRISC in screening forT2D and other chronic diseases, with promising results [11,12]. A validation study done in the Greek population, comprising adults less than 45 years to more than 64 years, showed FINDRISC to have high sensitivity as well as specificity in predicting unknown T2D [13]. Besides unknown diabetes, FINDRISC performed well in the cross-sectional detection of Impaired Fasting Glucose, Impaired Glucose Tolerance, as well as MetS [8,11].

Little is known of the utility of FINDRISC in African settings; to our understanding, no longitudinal study has been done in Africa to validate its prediction of the 10-year risk of developing diabetes. Nevertheless, few cross-sectional studies have been done in sub-Saharan Africa (SSA) and its utility has been documented [11,14]. In a recent study done among young adults in Nigeria, 9% of the participants were found to have moderate to high risk of developing diabetes in 10 years, although the predictive value for the current diabetes status was not reported [14]. Another study in Benin also found FINDRISC to be useful in screening for T2D [11]. Despite these findings, no study has described its utility in screening for current diabetes mellitus and MetS among young adult populations in sub-Saharan African settings, whose risk has been increasing [15].

Because of the challenges in accessing treatment for T2D and other non-communicable diseases in resource-limited settings in SSA, such as Tanzania, preventive measures are paramount [4]. For effective intervention, timely screening for diabetes is the key to diagnosing diabetes at the early stages and administering preventive measures before disease complications [16,17].

Using a community-based cross-sectional design, this study aimed to explore the utility of FINDRISC in predicting current diabetes mellitus and MetS among the young-adult population in an urban setting of Tanzania.

2. Materials and Methods

2.1. Study Design and Study Setting

This was a community-based cross-sectional study conducted between May and August 2018 in an urban setting of Mwanza city, Tanzania. The sample size was estimated using the Kish and Leslie formula [18], at a 95% confidence interval, 5% margin of error, and 80% sensitivity from a referred validation study [13]. The minimum required sample size was adjusted upwards to 252 participants to account for a 10% non-response rate. Using a multistage random sampling, three representative districts were randomly selected from 7 districts of the Mwanza region, and each district, two representing urban wards, and then four streets were randomly selected. Community leaders were utilized to announce to the public three days before review day, and all those who turned up at the survey center during the review day, met the inclusion criteria, and gave informed consent were randomly selected to participate in the study. A final total of 259 participants were enrolled, aged 18–35 years, who were not known to have the clinical diagnosis of diabetes at the time of enrollment.

2.2. Data Collection

2.2.1. Socio-Demographics and FINDRISC Characteristics

An investigator-administered structured questionnaire captured the socio-demographic data, including age, sex, education level, occupation, and employment status. The FINDRISC questionnaire was used to collect data on age group, body mass index (BMI) categories, waist circumference categories, physical activity status, vegetable eating behavior, history of hypertension and high blood glucose, as well as family history of diabetes mellitus, as indicated on the tool (details published elsewhere [19]). A total score was obtained by adding up scores for all parameters, and every participant had their FINDRISC scores recorded. Risk categories were categorized as per FINDRISC standard groups of

low risk (<7), slightly elevated (7–11), moderate (12–14), high (15–20), and very high risk (>20) [5,20].

2.2.2. Blood Pressure and Anthropometry

Clinical measurements included measurement of the systolic and diastolic blood pressure blood pressures taken three times at 5-min intervals using a calibrated digital sphygmomanometer (CH-432B, Citizen Systems Japan Co., Ltd., 6-1-12 Tanashi-cho, Nishi-Tokyo-Shi, Tokyo 188-8511, JAPAN). Mean arterial pressure (MAP) was calculated using the equation: MAP = DBP +1/3(SBP-DBP) [21]. Hypertension was defined as systolic blood pressure (SBP) $\geq$ 140 mmHg or diastolic blood pressure (DBP) $\geq$ 90 mmHg or a prior diagnosis of hypertension currently on anti-hypertensive therapy [22]. Weight, height, hip, and waist circumference were measured using a calibrated stadiometer and tape measure under WHO protocols [23]. The waist:hip ratio (WHR) was interpreted according to the WHO guidelines; in males, the ratio of $\geq$0.90 and females $\geq$0.84 were regarded as substantially increased [22]. BMI was calculated as weight (kg)/height^2 (m^2). Overweight was defined as a BMI of 25 kg/m^2–30 kg/m^2 and obesity as a BMI of more than 30 kg/m^2 [22]. MetS were defined based on the IDF criteria, which are the presence of central obesity plus any two of the following: raised triglycerides $\geq$ 1.7 mmol/L or history of specific treatment for this; reduced HDL cholesterol: <1.03 mmol/L in males, 1.29 mmol/L in females, or history of specific treatment; raised blood pressure $\geq$ 130 mmHg systolic and/or $\geq$ 85 mmHg diastolic or on antihypertensives; and raised FBG $\geq$ 7.0 mmol/L or previously diagnosed type 2 diabetes mellitus [24].

2.2.3. Oral Glucose Tolerance Test (OGTT)

Participants were contacted one day before the clinic visit and were instructed to come following overnight fasting. Upon arrival and before glucose testing, participants were asked if they had taken any food except water for at least 8 h before visiting the clinic to ascertain fasting. Those fasting were requested to provide venous blood for fasting blood glucose (FBG) measurement (ONCALL-PLUS device, ACON Laboratories, Inc., San Diego, CA, USA). Subsequently, participants were given 82.5g of dextrose monohydrate (equivalent to 75g of anhydrous glucose) diluted in 250 mls of drinking water to drink within 5 min for the Oral Glucose Tolerance Test (OGTT), and blood samples were collected after 2 h. FBG and 2-h postprandial glucose were recorded. Impaired Glucose Tolerance or pre-diabetes was defined as a fasting blood glucose of 5.8 mmol/L to 7.0 mmol/L or 2-h postprandial blood glucose levels of 7.8 mmol/L to 11 mmol/L. Diabetes mellitus was defined as fasting blood glucose of more than or equal to 7.1 mmol/L or 2-h postprandial blood glucose level of more than or equal to 11.1 [25].

2.2.4. Lipid Profile

A sample of fasting venous blood was collected for assessment of the lipid profiles at the clinic and was transferred in a cool box to Bugando Medical Center (BMC) hematology laboratory, and was stored at −20 °C before analysis. Under standard operating procedures, lipid profile tests were done using an ERBA XL Automated Chemistry Analyzer (Erba Lachema s.r.o, Brno, Czechia), where the fasting total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride were analyzed. Dyslipidemia was defined as the presence of either total cholesterol of more than 5.2 mmol/L, LDL of more than 3.3 mmol/L, triglycerides of more than 1.7 mmol/L, or HDL of less than 1.03 mmol/L in males or less than 1.29 mmol/L in females.

2.3. Statistical Analysis

Data were transferred from questionnaires to Microsoft Excel for cleaning and exported to STATA 13 (64-bit; StataCorp LLC 4905 Lakeway Drive College Station, TX 77845-4512, USA) for analysis. Continuous variables were summarized into frequency, means with standard deviations, or median with inter-quartile ranges based on distribution.

Categorical variables were presented as frequencies and proportions. The International Diabetes Federation (IDF) criteria were used to obtain the MetS traits and MetS of the study participants. Association between categorical variables was done using Pearson's correlation or Fisher's exact test where appropriate. Associations between the FINDRISC score and clinical as well as biochemical parameters that are not featured in the FINDRISC questionnaire were ascertained using linear regression. Two-side *p*-values of equal or less than 0.05 were considered statistically significant.

The predictive value of FINDRISC in detecting prediabetes and diabetes was evaluated using FINDRISC scores as a test and OGTT as the gold standard. FINDRISC scores of 7 and above (at least slightly elevated risk) were regarded as positive and those below 7 were regarded as negative. Participants with 2 h OGTT values of more than 7.8 mmol/L were regarded as having deranged blood glucose (inclusive of pre-diabetes and diabetes) while those with scores less than 7.8 mmol/L were regarded as not deranged (having normal blood glucose). From these values, the sensitivity, specificity, positive and negative predictive values, area under the receiver operator (ROC) curve, and their respective 95% confidence intervals were calculated.

3. Results

3.1. Background Characteristics of Study Participants

A total of 259 participants were enrolled in this study; the response rate of the recruited study participants was 100%. The median age was 21 (19–27) years and 60.2% (156) of the study participants were females. The majority of the study participants were university students 66.8% (173) (Table 1). The overall prevalence of hypertension, impaired glucose tolerance, diabetes, obesity, central obesity, dyslipidemia, and MetS were 35.1% (91), 15.5% (38), 7.8% (19), 8.1% (21), 14.7% (38), 44.4% (115), and 4.3%(11), respectively (Table 1). The Mean FINDRISC score was 5.2 $\pm$ 3.6, with a minimum score of 0 and a maximum score of 22. More than half of the participants had a low risk of developing diabetes mellitus in 10 years, while 32.8% of the participants had at least slightly elevated to a very high risk of T2D in 10 years (Table 2).

Table 1. Socio-demographic and clinical characteristics of the study participants.

Characteristics	Median (IQR)/*n* (%)
Number of subject enrolled, *N*	259
Age in years, Median (IQR)	21 (19–27)
Female Sex	156 (60.2)
Education level, *N* (%)	
None	1 (0.4)
Primary	33 (12.7)
Secondary	29 (11.2)
College and higher	196 (75.7)
Occupation, *n* (%)	
Employed	38 (14.7)
Not employed	9 (3.5)
Self employed	39 (15.0)
Students	173 (66.8)
Diabetes mellitus	19 (7.76)
Prediabetes	38 (15.51)
Hypertension	91 (35.1)
Obesity	21 (8.1)
Overweight	44 (17)
Dyslipidemia	115 (44.4)
Central obesity	38 (14.7)
Metabolic syndrome (MetS)	11 (4.3)

Table 2. Summary of FINDRISC.

Parameter			FINDRISC Points	*N* (%)
Age-groups (years)	<45		0	259 (100)
	45–54		2	-
	54–64		3	-
	>64		4	-
BMI categories (kg/m^2)	<25		0	192 (74.1)
	25–30		1	45 (17.4)
	>30		3	22 (8.5)
Waist circumference (cm)	Men	Women		
	<94	<80	0	162 (62.6)
	94–102	80–88	3	59 (22.8)
	>102	>88	4	38 (14.7)
Physically active?	Yes		0	65 (25.1)
	No		2	194 (74.9)
Eating vegetables daily	Yes		0	187 (72.2)
	No		1	72 (27.8)
Personal history of hypertension	No		0	247 (95.4)
	Yes		2	12 (4.6)
Personal history of hyperglycemia	No		0	249 (96.1)
	Yes		5	10 (3.9)
Family history of diabetes, *n* (%)	No		0	188 (72.5)
	Yes, first-degree relative		3	49 (18.9)
	Yes, second-degree relative		5	22 (8.6)
FINDRISC score	<7 (low risk)		0–6	174 (67.2)
	7–11 (slightly elevated risk)		7–11	74 (28.6)
	12–15 (moderate risk)		12–15	6 (2.3)
	15–20 (high risk)		15–20	4 (1.5)
	>20 (very high risk)		20–26	1 (0.4)

3.2. FINDRISC as a Predictor of Glucose Intolerance and Diabetes Mellitus

Comparing the FINDRISC score to the OGTT as a gold standard diagnostic test, the sensitivity and specificity of FINDRISC were 39.1% and 69.2% respectively, and the area under the ROC curve was (0.5, 95% CI: 0.47, 0.6), suggesting a weak ability of the FINDRISC to discriminate young adults with and without pre-diabetes or diabetes mellitus (Table 3).

Table 3. Diagnostic accuracy of the FINDRISC score for impaired blood glucose.

		95% Confidence Interval	
Prevalence	24.70%	19.60%	30.40%
Sensitivity	39.10%	27.10%	52.10%
Specificity	69.20%	62.20%	75.60%
ROC area	0.54	0.47	0.61
Positive likelihood ratio	1.27	0.88	1.84
Negative likelihood ratio	0.88	0.71	1.09
Odds ratio LR	1.44	0.81	2.59
Positive predictive value	29.40%	20.00%	40.30%
Negative predictive value	77.60%	70.70%	83.50%

FINDRISC scores were compared to the Oral Glucose Tolerance Test results. Participants with 7 points and above(slightly elevated risk to very high risk) were regarded as positive and below 7 points (low risk) as negative. OGTT levels less than 7.8 mmol/L were regarded as non-diseased while OGTT levels 7.8 mmol/L and above were considered diseased (with prediabetes and diabetes mellitus).

3.3. Diabetes and Metabolic Syndrome across the FINDRISC Categories

Proportions of Normal Glucose Tolerance, Isolated Fasting Blood Glucose, Impaired Glucose Tolerance, Diabetes Mellitus, MetS, and MetS traits across FINDRISC categories have been presented in (Table 4). Significant associations have been observed between

FINDRISC score with MetS, abdominal obesity, low High-Density Lipoprotein Cholesterol, and Fasting Blood Glucose.

Table 4. Diabetes and metabolic syndrome across the FINDRISC categories.

FINDRISC Score		0–6	7–11	12–14	15–20	20–26	Total (Row)	* *p*-Value
OGTT		174	74	6	4	1	259	
	NGT(202)	131 (69.3)	48 (25.4)	5 (2.7)	4 (2.1)	1 (0.5)	189	
	Isolated IFG	14 (66.7)	7 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	21	
	IGT	24 (60.0)	15 (37.5)	1 (2.5)	0 (0.0)	0 (0.0)	40	0.7
	DM	12 (60.0)	8 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	20	
Metabolic syndrome * (IDF)		2 (18.2)	5 (45.5)	0 (0.0)	3 (27.3)	1 (9.1)	11	0.001
MetS traits abnormality (IDF)	WC (Abd obesity)	18 (22.0)	53 (64.6)	6 (7.3)	4 (4.9)	1 (1.2)	82	0.001
	TG (high trig)	22 (73.3)	7 (23.3)	0 (0.0)	1 (3.3)	0 (0.0)	30	0.7
	HDL-C (Low HDL)	19 (57.6)	10 (30.3)	1 (3.0)	2 (6.1)	1 (3.0)	33	0.01
	BP (High)	61 (67.0)	24 (26.4)	2 (2.2)	3 (3.3)	1 (1.1)	91	0.3
	FPG (High)	4 (57.1)	2 (28.6)	0 (0.0)	0 (0.0)	1 (14.3)	7	0.001

Proportions of Normal Glucose Tolerance (NGT), Isolated Impaired Fasting Glucose (IFG), Impaired Glucose Tolerance (IGT), Diabetes Mellitus (DM), and MetS traits (Abdominal Obesity, triglycerides $\geq$ 1.7 mmol/L, HDL cholesterol: <1.03 mmol/L in males, 1.29 mmol/L in females, blood pressure $\geq$ 130mmHg systolic and/or $\geq$ 85 mmHg diastolic and Fasting Blood Glucose > 7.0 mmol/L) across FINDRISC categories. * Chi-Square.

3.4. FINDRISC as a Predictor of MetS and MetS Traits

Linear regression and analysis of variance were performed between FINDRISC and MetS, WHR, FBG, DBP, SPB, TG, and HDL. In univariable models, the FINDRISC score was significantly associated with Metabolic Criteria 2, 4, 5, and 6, and generally with MetS (Table 5). In the multivariable models, all response variables with *p*-value < 0.1 were adjusted for age and sex, and a significant association was observed between MetS2, MetS5, and MetS 6 as the outcome variable and the FINDRISC score as the predictor variable (Table 5).

Table 5. Linear regression on the predictive potential of the FINDRISC scores on MetS and IDF MetS traits as response variables.

MetS Criteria	Univariable		Multivariable	
	R^2	*p* Value	Adjusted R^2	*p* Value
MetS 1	0.00	0.96		
MetS 2	0.03	0.005	0.05	0.02
MetS 3	0.00	0.96		
MetS 4	0.04	0.001	0.04	0.07
MetS 5	0.14	0.001	0.13	0.001
MetS 6	0.05	0.001	0.04	0.002
MetS	0.13	0.001	0.13	0.001
WHR	0.12	0.001	0.36	0.001
FBG	0.02	0.05	0.02	0.2
DBP	0.04	0.002	0.14	0.06
SBP	0.00	0.8		
TG	0.00	0.3		
HDL	0.00	0.5		

MetS1:abdominal obesity, triglycerides $\geq$ 1.7 mmol/L, and high-density lipoprotein cholesterol < 1.03 mmol/L in males and < 1.29 mmol/L in females; MetS2: abdominal obesity, triglycerides $\geq$ 1.7 mmol/L, blood pressure $\geq$ 130 mmHg systolic and/or $\geq$ 85 mmHg diastolic; MetS3: abdominal obesity, triglycerides $\geq$ 1.7 mmol/L, FBG $\geq$ 7.0 mmol/L; MetS4: abdominal obesity, high-density lipoprotein cholesterol < 1.03 mmol/L in males and < 1.29 mmol/L in females, FBG $\geq$ 7.0 mmol/L; MetS5: abdominal obesity, high-density lipoprotein cholesterol < 1.03 mmol/L in males, blood pressure $\geq$ 130 mmHg systolic and/or $\geq$ 85 mmHg diastolic; MetS6: abdominal obesity, blood pressure $\geq$ 130 mmHg systolic and/or $\geq$ 85 mmHg diastolic, FBG $\geq$ 7.0 mmol/L; MetS: total, presence of any of the six criteria.

4. Discussions

T2D and other MetS-associated ailments are on the rise and account for significant morbidity and mortality worldwide, including among the young-adult populations. Developing cheap, effective, and easy-to-use screening tools for T2D is a cost-effective approach to control the disease and its complications, particularly in resource-limited settings where access to care is limited. Here, we report an alarmingly high prevalence of T2D, dyslipidemia, overweight, hypertension, and MetS in a young-adult population of sub-Saharan Africa and provide first-time data showing that, although the non-invasive FINDRISC tool was less effective at predicting current pre-diabetes and T2D, the tool significantly predicted MetS traits and MetS. The FINDRISC tool could therefore have utility in screening for MetS among a young adult population.

High levels of DM, hypertension, dyslipidemia, and central obesity have been observed in this study. Similar results have been observed recently in other studies with young adults under 40 years of age [26,27]. Nsanya et al. (2019) reported a 40%prevalence of high blood pressure in young adults and adolescents of Tanzania and Uganda [28], and a significantly higher prevalence of hypertension has been reported from studies done among school children and adolescents in Tanzania and other low-income countries [29,30]. Up to 36% of the young adults in India have been reported to have dyslipidemia from recent studies [31], and data from Tanzania shows a 12.96% prevalence of central obesity in the young-adult population (aged 18 to 30 years) [32]. However, the observed prevalence of diabetes mellitus (7.8%) is higher compared to the recently reported prevalence of diabetes in urban Tanzania (3.2–6.9% for the age group of 20–34 years) [2]. This value approaches the estimated prevalence of 9% projected for the year 2030 [33]. The trend in these diseases in the young population is alarming and indicates forthcoming danger in the absence of active and early interventions.

FINDRISC was developed to predict the 10-year risk for developing T2D [34]. Despite this use, the tool has been adopted for screening for T2D and MetS in different populations following several validation studies [12,13,35]. These studies were done in populations with mixed age groups and showed promising potential for the utility of FINDRISC as a screening tool, with over 80% sensitivity and specificity in screening for T2D and MetS [12,36]. Our study, however, showed lower sensitivity and specificity of FINDRISC as a screening tool for current T2D and this may be attributed to various reasons. Firstly, all participants were below 45 years of age, hence falling under one age category, making it difficult to discriminate a risk score based on age category. Secondly, the types of fruits and vegetables differ in this population compared to the Finnish population; hence, we need to customize the FINDRISC score to the African setting, which may increase its sensitivity. Thirdly, ascertaining family history of diabetes mellitus in a sub-Saharan African setting, as required in the current FINDRISC questionnaire, may not be realistic since most cases of diabetes mellitus remain undiagnosed due to poor health-seeking habits. Modifications of these aspects and validation of the modified tool may therefore increase the usefulness of FINDRISC as a tool for screening the current and future risk of T2D in the Tanzanian context [4,16,37].

Despite its limited utility in predicting current T2D, our study uncovered the potential utility of FINDRISC as a non-invasive screening tool for MetS. Particularly, our study showed significant associations between FINDRISC with the waist-to-hip ratio, diastolic blood pressure, mean arterial pressure, fasting blood glucose, and low-density lipoprotein levels. These findings are concordant with observations made in previous studies that validated FINDRISC as a screening tool to detect the occurrence of MetS [7]. In this study, FINDRISC was shown to significantly predict the current status of these parameters, opening up its potential to be used as a cheap and non-invasive screening tool for MetS in young adults.

Our study had several limitations. Firstly, the majority of study participants were college students, which limits the extension of study findings to the general population. Secondly, due to resource limitations, we were only able to perform OGTT but not HbA1C

for the diagnosis of prediabetes. Furthermore, given the cross-sectional nature of the study, we were only able to validate the utility of FINDRISC as a predictor of current pre-diabetes, T2D, and MetS; large community-based longitudinal studies are recommended to validate FINDRISC over a wider age range and more diverse subpopulations with the suggested modifications above, to be a predictive tool for future risk as well as a screening tool of T2D in sub-Saharan Africa.

5. Conclusions

Although limited in its ability to detect current pre-diabetes or T2D, the FINDRISC showed considerable potential for utility as a non-invasive screening tool for the MetS among young adults in sub-Saharan Africa. Further studies are needed to validate the utility of a modified FINDRISC tool as a predictor of the current and future risk of T2D and metabolic syndrome.

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Ultrasound Assessment of the Median Nerve Does Not Adequately Discriminate the Carpal Tunnel Syndrome among Patients Diagnosed with Diabetes

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Abstract: Background: Carpal tunnel syndrome is the most prevalent peripheral nerve entrapment condition of the upper limb. Among metabolic risk factors, diabetes is considered the most relevant. Although wrist ultrasound assessment of the median nerve has demonstrated a good correlation with the gold standard for the diagnosis of this syndrome, neurophysiological study, its usefulness in patients with diabetes is questionable because the compressive phenomenon is not the predominant one. Method: We conducted a retrospective study to compare the clinical and median nerve ultrasound features of patients with carpal tunnel syndrome previously diagnosed or not diagnosed with diabetes. Additionally, a linear multivariate regression analysis was performed to determine to what extent the cross-sectional area of the median nerve was dependent on the condition of diabetes by fixing other variables such as sex, age, or time of evolution. Results: We included 303 records of patients (mean age 44.3 ± 11.7 years old, 57.89% female, mean of time of evolution 13.6 ± 8.3 months) from 2012 to 2020. The cross-sectional area of the median nerve was 10.46 ± 1.44 mm^2 in non-diabetic patients and 8.92 ± 0.9 mm^2 in diabetic patients ($p < 0.001$). Additionally, diabetic patients had a shorter time of evolution (7.91 ± 8.28 months vs. 14.36 ± 0.526 months, $p < 0.001$). In the multivariate analysis, the resultant model (fixed R-square = 0.659, $p = 0.003$) included a constant of the following four variables: the evolution time (Beta coeff. = 0.108, $p < 0.001$ 95% CI 0.091 to 0.126, standardized coeff. = 0.611), the condition of diabetes (Beta coeff. = −0.623, $p < 0.001$ 95% CI −0.907 to −0.339, standardized coeff. = −0.152), the severity (Beta coeff. = 0.359, $p = 0.001$ 95% CI 0.147 to 0.571, standardized coeff. = 0.169), and the masculine sex (Beta coeff. = 0.309, $p = 0.003$, 95% CI 0.109 to 0.509, standardized coeff. = 0.103). Conclusions: Ultrasound assessment of the median nerve in patients with diabetes is not a useful tool to confirm whether carpal tunnel syndrome should be diagnosed or not diagnosed.

Keywords: carpal tunnel syndrome; ultrasound; power doppler signal; diabetes

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1. Introduction

Carpal tunnel syndrome (CTS) is the most prevalent peripheral nerve entrapment pathology. Its estimated annual incidence is 125 cases per 100,000 inhabitants [1]. It is 1.4 times more frequent in women than in men, and it is presumed that this increased risk is due to the higher prevalence of osteoarthritis in women [1–3]. However, other risk factors have been identified, such as diabetes and hypothyroidism [4]. Although CTS is pathophysiologically explained as a result of the continuous mechanical stress of the median nerve as it passes through the carpal tunnel, in the case of patients with diabetes, the origin of the symptoms is due to metabolic causes linked to peripheral neuropathy, rather than purely mechanical [5,6].

Recently, attempts have been made to evaluate the role of carpal ultrasound in the diagnosis of CTS [7–10]. In all cases, the initiative to validate ultrasound as a diagnostic

tool is justified by its greater availability and accessibility. However, since it is presumed that the biomechanical entrapment of the median nerve is not the main triggering factor of this disease in patients with diabetes, ultrasound evaluation in these patients would likely have a minor role.

The purpose of the present study is to determine the differences in the ultrasound examination of patients with CTS as a function of whether or not they were previously diagnosed with diabetes.

2. Method

A cross-sectional study of patients with an electrophysiological diagnosis of CTS based on available information from three databases was conducted between January and March 2021.

The records included patients diagnosed in three different centers in the Community of Madrid between 2012 and 2020. Only patients with at least one ultrasound examination of the carpus, including the cross-sectional area of the median nerve inside the carpal tunnel and detection of hyperemia using power Doppler (PD) signal, were included. Records of patients with thyroid disorders, osteoarthritis, amyloidosis, and connective tissue diseases were excluded. In addition, records corresponding to patients already treated due to CTS on the same hand were excluded.

All data were obtained from the corresponding electronic records. In the ultrasound examination, the cross-sectional area of the median nerve—measured in square millimeters—and the result of the detection of PD signal inside the carpal tunnel were extracted. Three different ultrasound devices were used (Logiq S9 General Electric®, Nemio XG Toshiba®, and MyLab 7 Esaote®); however, all the studies were performed by the same rheumatologist following the recommendations of Filippucci et al. for median nerve assessment [11]. Although it has been demonstrated that the place of measure along the carpal tunnel has no effect on the area of the median nerve [12], all measurements were performed at the level of the pisiform bone. In categorical, ordinal terms, disease severity was determined via neurophysiological evaluation and according to the definitions of the American Association of Electrodiagnostic Medicine [13]. The diagnosis of diabetes was only obtained directly from the medical chart when it was established at least 5 years ago.

Patients were grouped according to whether or not they were diagnosed with diabetes, and all other clinical, epidemiological, and ultrasound characteristics were compared. A multiple regression analysis was performed to correlate the median nerve area with the time of evolution and severity of CTS.

In order to determine clinical and epidemiological differences among CTS patients with or without a previous diagnosis of diabetes, we performed a bivariate analysis using chi-square and T-student tests (p-value significance fixed at 0.10). Additionally, to assess the relative weight of the prior diagnosis of diabetes among patients with CTS, a linear regression multivariate test was performed considering the section area of the median nerve as the dependent variable using the forward stepwise method of variable inclusion.

For purposes of multivariate analysis, female sex was categorized as 0 (male as 1), the severity of disease was categorized from 1 to 3 (mild to severe), dichotomic variables were categorized as present (1) and absent (0), and finally, treatment response was classified from 0 to 2 (none, partial and complete).

Our local scientific research ethics committee approved the conduct of the presen study.

3. Results

Three hundred and three records were included for analysis. Forty-seven patients (15.5%) had a diagnosis of diabetes. The mean age ± SD was 44.3 ± 11.7 years old. One hundred and seventy-five records (57.89%) corresponded to female patients. The distribution of severity of CTS according to the neurophysiological diagnosis study was as follows: 61 (20.1%) mild, 153 (50.5%) moderate, and 80 (29.4%) severe. The mean time of

evolution of CTS was 13.6 ± 8.3 months. Thirty-six patients (11.9%) had previously been diagnosed with CTS in the contralateral hand.

No significant differences in terms of age were detected in patients with or without diabetes; however, the cross-sectional area of the median nerve was 10.46 ± 1.44 mm^2 in non-diabetic patients and 8.92 ± 0.9 mm^2 in diabetic patients ($p < 0.001$). Intra tunnel power Doppler signal was detected in 12 non-diabetic patients (4.6%) and was not detected in non-diabetic patients. (Additionally, diabetic patients had a shorter time of evolution (7.91 ± 8.28 months vs. 14.36 ± 0.526 months, $p < 0.001$). Among diabetic patients, the antecedent of a previous contralateral CTS was present in 13 subjects (27.7%), while in non-diabetic patients, it was recorded in 23 (9.0%) ($p = 0.001$, OR 3.873; 95% CI 1.794 to 8.361). Table 1 shows the characteristics of the records included in the study, differentiating subjects according to their diabetic or non-diabetic patient condition.

Table 1. Demographic and clinical characteristics of the population of registries included in the study. *p*-value has been calculated for chi-square or Student's *t*-test as appropriate. CTS: carpal tunnel syndrome.

Variable	Patients with Diabetes N = 47	Non-Diabetic Patients N = 256	*p*-Value
Age (years ± standard deviation)	44.45 ± 11.25	44.2 ± 11.85	0.896
Female sex (%)	31 (65.9)	144 (56.3)	0.261
Time of evolution (months)	7.91 ± 5.67	14.36 ± 8.41	<0.001
Severity (%) Mild Moderate Severe	 18 (38.3) 28 (59.6) 1 (2.1)	 43 (16.8) 125 (48.8) 88 (34.4)	<0.001
Cross-sectional area of the median nerve (mm^2)	8.92 ± 0.90	10.46 ± 1.44	<0.001
Previous contralateral CTS diagnosis (%)	13 (27.7)	23 (9.0)	0.001
Historic response to conservative treatment (splint) (%) None Partial Complete	 37 (78.7) 9 (19.1) 1 (2.1)	 103 (40.2) 132 (51.6) 21 (8.2)	<0.001
Historic response to corticoids local administration (%) None Partial Complete	Patients treated = 46 35 (76.0) 6 (13.0) 5 (10.9)	Patients treated = 179 55 (30.7) 101 (56.4) 23 (12.8)	<0.001

In the bivariate analysis, Pearson correlation with the cross-sectional area of the median nerve was statistically significant with the time of evolution of the clinical manifestations (R = 0.782, $p < 0.001$). No other numerical variable showed a significant correlation with the cross-sectional area of the median nerve. Among categorical variables, besides the condition of diabetes, females had a smaller area than males (10.03 ± 1.57 vs. 10.48 ± 1.32 mm^2, $p = 0.007$).

In the linear regression multivariate analysis, the resultant model (fixed R-square = 0.659, $p = 0.003$ and ANOVA F-test = 147.231, $p < 0.001$) included a constant of 7.994 mm^2 and four variables: the evolution time, the condition and severity according to the neurophysiology study, and the masculine sex (Table 2). The predictive model including these four variables showed no significant differences with the real cross-sectional area of the median nerve (diff = −0.0041 ± 0.861, $p = 0.934$).

Table 2. Results of the linear multivariate regression analysis after a forward step-wise modeling. CTS: Carpal tunnel syndrome.

Variable	Beta Coefficient	95% Confidence Interval	*p*-Value	Standarized Coefficient
Evolution time	0.108	0.091 to 0.126	<0.001	0.611
Diagnosis of diabetes	0.623	−0.907 to −0.339	<0.001	−0.152
Severity of the CTS according to neurophysiology	0.359	0.147 to 0.571	0.001	0.169
Sex male	0.309	0.109 to 0.509	0.003	0.103

No significative differences in the cross-sectional area of the median nerve were detected when compared patients with and without the diagnosis of diabetes, according to their level of severity (data not shown).

4. Discussion

According to our results, ultrasound examination of the median nerve as it passes through the carpal tunnel is of scarce diagnostic value in diabetic patients since the classic reference of the increase in the cross-sectional area of the nerve does not seem to take place in these patients. In our series, we also identified a higher proportion of relapsing patients and a lower response to conservative treatment with splints and infiltrations.

Our study has certain limitations that we feel are appropriate to discuss. First, the purpose of the study is limited to determining to what extent the condition of diabetes influences the clinical characteristics of the disease and the fundamental diagnostic value of ultrasound. The detection of PD signal was not comparatively analyzed due to the absence of cases in the group of diabetic patients. Furthermore, because this was a retrospective study with data from three different ultrasound devices, PD signal detection may have been heterogeneous.

Another limitation to highlight is the accuracy of determining the magnitude of the dependent variable of the linear regression analysis. Two ultrasound devices yielded absolute values, while the third was sensitive to one-tenth of a square millimeter.

Finally, the neurophysiological studies, although they used the same classification pattern as a reference, were performed in three different centers and not by the sameprofessional.

The cross-sectional area of the median nerve was proposed for use as a diagnostic tool that is easy to obtain and correlates well with the results of neurophysiological studies [9,14–16]. Although its determination is simple and more accessible than the electromyogram, it has been suggested that its reliability could be related to specific anthropometric characteristics [17]. This would imply that the cut-off points of normality should be adjusted to body mass index [18,19] or carpal circumference [17].

In our study, diabetic status was a contributory variable in the nerve thickness prediction model, albeit in a negative sense. This can be interpreted to mean that the genesis of clinical CTS in diabetic patients is not due to mechanical nerve injury but to peripheral neuropathy [6]. It also implies that once the clinical manifestation has developed, patients are diagnosed earlier and, therefore, with a lesser degree of median nerve involvement. The causal relationship between diabetes and CTS is not due to the classic mechanical entrapment syndrome. The absence of cross-sectional changes in the median nerve favors a non-mechanical cause. Recent studies point to the development of nerve fiber fibrosis mediated by Transforming Growth Factor (TGF-β), Vascular Endothelial Growth Factor (VEGF) and certain interleukins [20,21].

The lack of therapeutic response to splints or infiltrations supports the idea that the cardinal lesion of CTS in diabetic patients is not necessarily a repetitive microtrauma. However, as it has been previously suggested, it can have a triggering relationship [21–23].

The asymmetric distribution of CTS severity between patients with and without a previous diagnosis of diabetes also suggests that the disease in people with diabetes tends to be milder. However, the degree of severity was also linked to the time of evolution [4]. The time to diagnosis of CTS was 50% shorter in diabetic patients than in non-diabetics.

Early diagnosis could be due to the follow-up that diabetic patients have and their greater degree of alertness around neurological symptoms.

5. Conclusions

In diabetic patients, the determination of the cross-sectional area of the median nerve via ultrasound should be used as a single discrimination tool for diagnostic purposes. This lack of diagnostic validity may be due to the earliness of the diagnosis, the lack of cardinal mechanical lesion, or the interaction of both considerations.

Thus, in diabetic patients, confirmation of the diagnosis of CTS, once a suspicious clinical picture has been established, should be made through neurophysiological studies.

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Informed Consent Statement: Patient consent was waived due to not patient was personaly recruited for purposes of this study. All the information used was obtained from electronic medical registries.

Data Availability Statement: The data set of the present study is available in Synapse™ repository (ID: syn26338284).

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

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Review

Urinary *N*-Acetyl-β-D-glucosaminidase (uNAG) as an Indicative Biomarker of Early Diabetic Nephropathy in Patients with Diabetes Mellitus (T1DM, T2DM): A Systematic Review and Meta-Analysis

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Abstract: Diabetic nephropathy (DN) is the main cause of chronic kidney disease in patients with type 1 (T1DM) and type 2 diabetes mellitus (T2DM). Renal tubular lysosomal enzyme activities like *N*-acetyl-β-D-glucosaminidase (NAG) have been shown to increase in patients developing DN. The aim of this systematic review and meta-analysis is to evaluate the diagnostic accuracy of NAG, as a preventional biomarker in the early stages of DN in patients with diabetes mellitus. Two impartial reviewers conducted a complete PubMed search until July 2021. A 2 × 2 contingency table was created for each trial and sensitivity and specificity were estimated using a bivariate random effects model. To pool data and estimate the area under the curve (AUC), the hierarchical summary ROC (hsROC) approach was utilized. Deek's test was used to estimate publication bias. The meta-analysis included 21 studies that evaluated 2783 patients with T1DM and T2DM, as well as 673 healthy individuals. The AUC of urinary NAG (uNAG) ranged from 0.69 (95% CI: 0.65–0.73) to 0.89 (95% CI: 0.86–0.92). According to the results, NAG in urine can be considered as a potential and effective biomarker for predicting DN in diabetic patients (T1DM, T2DM).

Keywords: *N*-acetyl-β-D-glucosaminidase (NAG); diabetic nephropathy; chronic kidney disease (CKD); meta-analysis; systematic review

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1. Introduction

Diabetic nephropathy (DN), is a metabolic disease and one of the most frequent microvascular complications of type 1 (T1DM) and type 2 (T2DM) diabetes mellitus [1]. The prolonged exposure of the body to high blood glucose levels (hyperglycemia) due to diabetes, affects proper functioning of the kidneys by damaging specific units responsible for removing waste products from the body and filtering essential substances to pass into the bloodstream [1]. DN is independently associated with cardiovascular risk in diabetic patients, especially in patients with T2DM [2]. Therefore, early detection and treatment is of major importance, as it can prevent critical complications of the disorder. DN is the leading cause of chronic kidney disease (CKD) and its diagnosis is based on the current level of albuminuria leading in three stages [3]: DN normoalbuminuria, microalbuminuria, and macroalbuminuria. More specifically, the confirmation of the disease is based on the persistent albuminuria in early morning urine samples, due to glomerular hyperfiltration [4,5]. Healthy individuals excrete small amount of albumin on a daily basis which does not exceed 30 mg/g. Albumin-to-creatinine ratio (ACR) is the gold standard method to detect elevated protein excretion in urine samples of diabetic patients. The onset stage of DN is defined by moderately increased albuminuria, known as microalbuminuria and it is diagnosed by the detection of a significant amount of albumin in the urine, which ranges from 30–300 mg/24 h [5]. The progression of the disorder refers to a gradual decline in

GFR and is characterized by severely abnormal increased levels of albuminuria, known as macroalbuminuria (proteinuria). It is increasingly appreciated that both glomerular and tubular interstitial damage have an essential role in the pathophysiology and development of DN [6].

However, according to recent studies, in 30% of diabetic population diagnosed with microalbuminuria, the course of the disease has shifted to a new clinical picture, defined by normal albuminuria and GFR after 10 years of follow up [7–9]. Even though microalbuminuria measurement is the gold standard method to predict and monitor the progression of DN, significant efforts have been made to investigate and validate alternative biomarkers for the diagnosis of DN, allowing the early identification of diabetic renal lesions [10]. Multiple biomarkers have been reported and classified due to their ability of detecting specific disorders [11]. Studies have shown promising preliminary results, suggesting that increased levels of the potential biomarkers are associated with the presence of renal damage in patients with T1DM and T2DM. According to studies, some of the effective biomarkers of glomerular injury are: adiponectin [12,13], transferrin [14], and ceruloplasmin [15]. Studies have also shown potential biomarkers which reflect tubular injuries, these include kidney injury molecule-1 KIM-1, a1- and b2-microglobulin [16–18], liver-type fatty acid binding protein L-FABP [17], and neutrophil gelatinase-associated lipocalin (NGAL). Recent meta-analysis suggests that NGAL is a potential valuable biomarker for early prediction of DN in diabetic patients [19].

In addition, *N*-acetyl-β-D-glucosaminidase (NAG) is a lysosomal enzyme found in proximal renal tubular cells and its significant concentrations during tubulointerstitial damage, are related with renal dysfunction [20]. In previous studies, increased urinary levels of NAG concentrations were present in diabetic patients diagnosed with normoalbuminuria rather than the control group. In addition, urinary NAG (uNAG) has been shown to increase progressively along with the DN stages, indicating that it might be an early predictive biomarker for DN [21,22]. Thus, the aim of this study is to conduct a systematic review and meta-analysis to evaluate the diagnostic accuracy of uNAG, as a preventional biomarker in the early stages of DN in patients with diabetic mellitus.

2. Materials and Methods

2.1. Search Strategy and Study Selection

This systematic review and meta-analysis was conducted according to the standard PRISMA (Preferred Reporting Item for Systematic Reviews and Meta-analyses) guidelines [23]. A literature search was performed on the PubMed database by two independent reviewers until the end of July 2021, using a clearly formulated query of terms and keywords ("urinary biomarker" OR "N-Acetyl-beta-D-Glucosamine" OR "GLcNAc" OR "N-AcetylGlucosamine" OR "urinary NAG" OR "serum NAG" OR "urinary lysosomal enzyme") AND ("diabetes" OR "diabetic nephropathy" OR "diabetic kidney disease"). To eliminate local literature bias, the study search was comprehensive and did not include language limitations. In addition, further search was conducted in other electronic engines, such as Google Scholar, the duplicates records were removed. The study selection was based on specific predefined criteria and each reason for inclusion or exclusion was recorded. All of the studies were included after reviewing the abstracts and full text of each article.

2.2. Inclusion and Exclusion Criteria

Studies chosen for meta-analysis were based on specific inclusion criteria. The rationale for the criteria of the study selection was predefined and clearly stated. The meta-analysis included studies, in which uNAG was determined in healthy individuals and in patients with diabetes mellitus. Diabetic patients were divided in the three following categories: patients with normoalbuminuria (UACR < 30 mg/g), patients with microalbuminuria (UACR = 30–300 mg/g), and patients with macroalbuminuria (UACR > 300 mg/g). Studies eligible for the meta-analysis also included the degree of DN determined by the

estimation of the UACR using a 24-h urine sample, or a random morning urine sample, according to the American Diabetes Association [23].

2.3. Data Extraction

NAG concentration in urine samples and uNAG concentration normalized to the urinary creatinine (uNAG/Cr), were extracted from each study. Furthermore, data synthesis included the extraction of the first author's name, study location, year of publication, age and sex of the participant groups, type of diabetes and clinical characteristics of each study group. Finally, a 2 × 2 contingency table was constructed using the absolute data of true positive (TP), false positive (FP), true negative (TN), and false negative (FN) for comparing and combining the effects of different research.

2.4. Quality Assessment of Safety Studies

The study selection was performed guided by the mentioned criteria, followed by an in-depth quality assessment, using the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS–2) [24]. The QUADAS tool consists of 14 questions of four different key domains. The risk of bias and the applicability of the studies were examined for each domain. The following judgements were used to complete the signaling questions: low, high, or unclear risk. For the quality analysis, the Review Manager Software (RevMan 5.4) was used.

2.5. Meta-Analysis

A random effects meta-analysis was performed in order to synthesize quantitative information from related studies. The approach method of the bivariate meta-analysis involves estimating sensitivity (logit-Se) and specificity (logit-Sp) by van Houwelingen [25,26].

According to the available raw data, the following statistical calculations were used for estimating the mean and *SD*. Therefore, in the studies where the 95% confidence interval (*CI*) was specified, *SE* was determined according to the following equation recommended by Cochrane Handbook [22]:

$$SE = (upperlimit - lowerlimit)/3.92 \quad (1)$$

In the studies where median (*M*) and inter-quartile range (*IQR*) were provided, for calculating mean and *SD* we followed the Cochrane Handbook [27]. The median was utilized as a mean estimator, while the *SD* was determined as follows:

$$SD = \frac{IQR}{1.35} \quad (2)$$

In the studies where median (*M*) and range were provided, we used the principles specified by Hozo and co-workers [28]. The following equation was used for sample sizes $n < 25$:

$$\overline{x} = \frac{\min + 2M + max}{4} \quad (3)$$

In the studies where the sample size was $n > 25$, median was chosen as the appropriate value over the mean value. *SD* for sample sizes $n < 15$ was calculated by the following equation:

$$SD^2 = \frac{1}{12}\left(\frac{(min + 2M + max)^2}{4} + (max - min)^2\right) \quad (4)$$

while, for $n > 25$ was calculated by the equation:

$$SD = \frac{R}{4} \quad (5)$$

The three following groups of diabetic patients were considered in the meta-analysis: patients with normoalbuminuria, patients with microalbuminuria, and patients with

normo/microalbuminuria. Normoalbuminuria was defined using as status variables healthy individuals (controls) vs. normoalbuminuric patient group whereas for the prediction of microalbuminuria, normoalbuminuric vs. microalbuminuric patients, patient groups were used as status variables.

In order to assess the diagnostic performance of uNAG and uNAG/Cr in early diagnosis of DN, the hierarchical summary ROC curve (hsROC) was constructed using sensitivity, specificity, and parameters of the bivariate normal distribution. For each study, the absolute number of TPs, FPs, FNs, and TNs were computed by altering the threshold values (log cutoff) calculated by the raw extracted data from the articles, such as the mean and standard deviation (*SD*) of uNAG and uNAG/Cr, assuming a normal distribution. The Youden index at its maximum value, represents the ideal discrimination limit, which is calculated as Y = sensitivity + specificity − 1 [29].

The interpretation of the curve was based on the following principals proposed by Swets [30]: Low ($0.5 \geq$ AUC ≤ 0.7), moderate ($0.7 \geq$ AUC ≤ 0.9), and high ($0.9 \geq$ AUC ≤ 1.0) accuracy. The between-study heterogeneity was estimated by using the Cochran Q-test and I^2 statistic and was presented as a forest plot [31]. For the publication bias were used calculation methods according to Deek's et al. [32]. The current study's data synthesis and statistical analysis were carried out using Stata software v.13 (College Station, TX, USA: StataCorp LLC).

3. Results

3.1. Included Studies and Trial Characteristics

Literature search from the databases yielded 353 citations, of which 3 were duplicates and discarded, resulting in 350 unique citations. Following the first review of the titles and abstracts, 283 articles were excluded for not meeting the inclusion criteria. Further, following a detailed examination of the 69 full-text articles, 48 articles were removed due to misclassification of DN or the unclear statement of the methods used in the study. Consequently, a total of 21 studies were found eligible and were included in the meta-analysis. The PRISMA flow of the review process is shown in Figure 1. These studies consisted of 2783 patients and 673 healthy in total. Table 1 represents the population's characteristics extracted from each study. Precisely, studies included 1196 patients with T1DM, 1587 patients with T2DM, and 673 healthy individuals. The group of patients with T1DM involved 644 patients (53.8%) with normoalbuminuria, 477 patients with microalbuminuria (39.8%), and 75 patients (6.3%) with macroalbuminuria. The group of patients with T2DM involved 760 patients (47.8%) with normoalbuminuria, 663 patients (41.7%) with microalbuminuria, and 164 patients (10.3%) with macroalbuminuria. Patients with macroalbuminuria were not included in the meta-analysis due to the insufficient number of studies. Furthermore, 50.5% of the patient group, were male with a mean age of 53.4 years old. In the healthy group 50.1% were male with a mean age of 51.3 years old. The clinical features collected from the studies are expressed by the weighted average and the concentrated standard deviation and are presented in supplementary Table S1.

Table 1. Detailed characteristics of the included studies in the meta-analysis for controls and diabetic patients.

	Controls			Diabetic Patients												
				Normoalbuminuria			Microalbuminuria			Macroalbuminuria						
Country	Sample Size (*n*)	Sex (%.Male/Female)	Age (Mean)	Sample Size (*n*)	Sex (%.Male/Female)	Age (Mean)	Sample Size (*n*)	Sex (%.Male/Female)	Age (Mean)	Sample Size (*n*)	Sex (%.Male/Female)	Age (Mean)	NAG Type	Determination Method of NAG	Data	Reference
Ghana	65	44.6/55.4	51.2	39	–		26	–	–	–	–	–	uNAG	Spectophotometric *	Mean, *SD*	[33]
Ghana	65	44.6/55.4	54	39	–		26	–	–	–	–	–	uNAG/Cr	Spectophotometric	Mean, *SD*	[33]
Iran	25	60/40	55.2	24	62.5/37.5	58.2	8	62.5/37.5	53.1	–	–	–	uNAG/Cr	Immunoturbidimetry	Mean, *SD*	[34]
Egypt	40	40/60	15.1	48	–	14.6	11	–	16.8	–	–	–	uNAG/Cr	Colorimetricanalysis **	Mean, *SD*	[35]
Poland	42	28.5/71.5	56	14	–	–	89	–	–	27	–	–	uNAG/Cr	Spectophotometric	Median *IQR*	[36]
India	48	–	45.6	94	–	–	102	–	–	–	–	–	uNAG/Cr	ELISA **	Mean, *SD*	[37]
Egypt	20	60/40	51	20	50/50	51.3	25	44/56	52.9	25	48/42	51.7	uNAG	Spectophotometric	Median *IQR*	[38]
Poland	32	37.5/62.5	61.9	29	38/62	63.4	32	34.3/65.7	63.4	29	34.5/65.5	62.4	uNAG	ELISA	Mean, *SD*	[39]
USA	38	50/50	43	363	44/56	39	296	61/39	41	–	–	–	uNAG	Spectophotometric	Mean, *SD*	[40]
Egypt	10	60/40	47.3	10	80/20	51.36	20	50/50	48.6	20	40/60	52.8	uNAG	ELISA	Mean, *SD*	[41]
Japan	57	59.6/40.4	44.5	90	–	47.5	–	–	–	–	–	–	uNAG/Cr	RIA	Mean, *SD*	[42]
India	48	–	45.3	94	–	–	102	–	–	–	–	–	uNAG/Cr	Spectophotometric	Mean, *SD*	[37]
Japan	–	–	–	20	45/55	57.1	17	35.2/64.8	62.7	–	–	–	uNAG/Cr	–	Median *IQR*	[43]
Spain	32	46.8/53.2	60	25	52/48	60	60	48.3/51.7	59	75	48/52	64	uNAG	Colorimetric analysis	Median *IQR*	[44]
Japan	20	55/45	57	19	84.2/15.8	62	7.8	18/82	72.2	19	56.2/43.8	60	uNAG	Colorimetric analysis	Median *IQR*	[45]
UK	20	50/50	45	20	–	–	20	–	–	–	–	–	uNAG	EIA	Mean, *SD*	[46]
UK	15	–	48	12	58.3/41.7	48	12	41.7/58.3	48	12	50/50	48	uNAG	EIA	Mean, *SD*	[47]
China	28	46.4/53.6	48.3	61	–	–	24	–	–	16	–	–	uNAG	Colorimetric analysis	Median *IQR*	[48]
Italy	31	32.2/67.8	61.1	43	37.1/62.9	64.2	–	–	–	–	–	–	uNAG	Colorimetric analysis	Median *IQR*	[49]
China	42	54.8/45.2	54.3	144	57.6/42.4	54.3	94	55.3/44.7	55.49	49	57.1/42.9	59.2	uNAG	Immunonephelometric	Median *IQR*	[50]
Skopje	30	66.6/33.4	33	170	56.4/43.6	50	115	56.5/43.5	57.3	–	–	–	uNAG	–	Mean, *SD*	[51]
Egypt	30	50/50	51	26	39/61	51	30	53/47	57	30	53/47	56	uNAG	ELISA	Mean, *SD*	[52]

* NAG absorbance for spectophotometric analysis: OD = 400–405 nm ** NAG absorbance for ELISA/colorimetric analysis: OD = 400 nm.

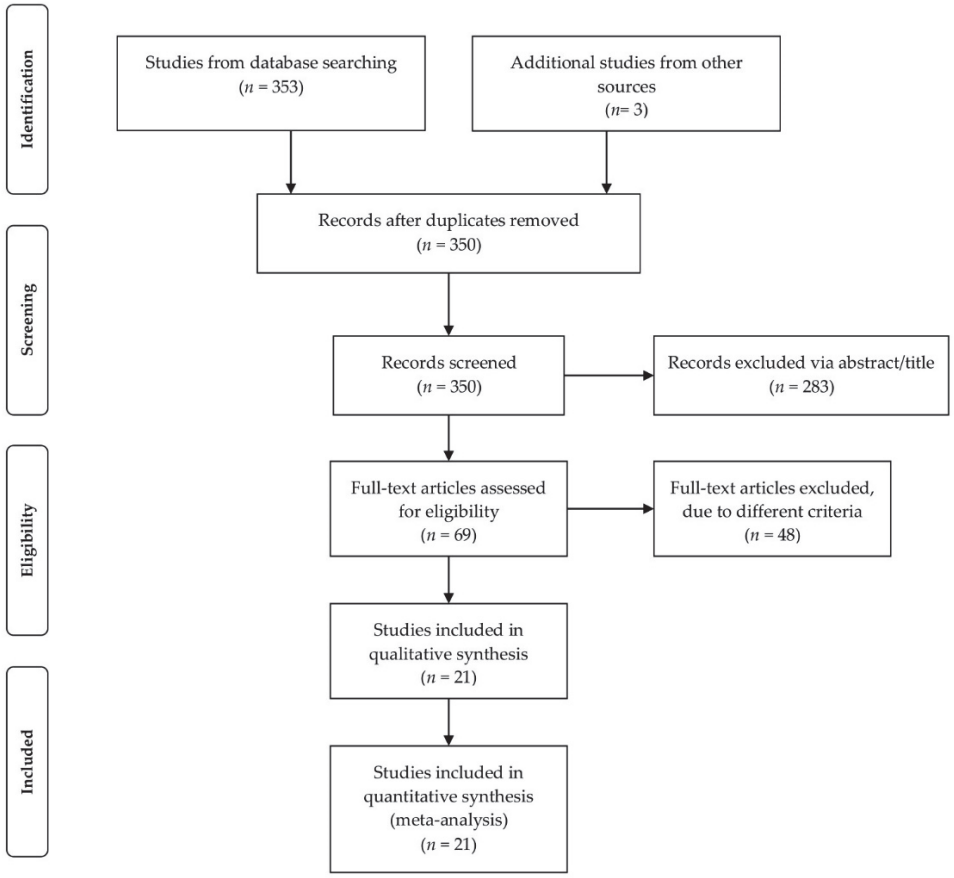


Figure 1. PRISMA flow diagram for literature search and study selection.

3.2. Quality Assessment of the Included Studies

The outcome of the comprehensive quality assessment of the 21 included studies are shown in the Figure 2. The unclear risk of bias in some studies on patient selection was present due to lack of information about the characteristics of the patient group with microalbuminuria and macroalbuminuria, such as the total number of patients, age, and gender. There was a concern regarding the applicability of the index test due to the different processing of the uNAG sample.

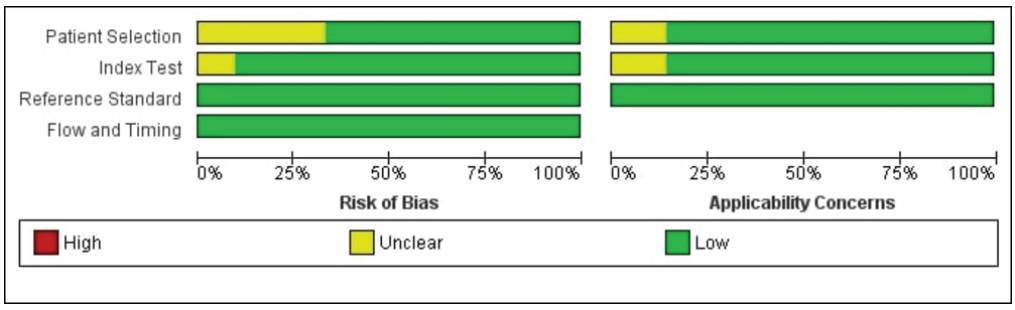
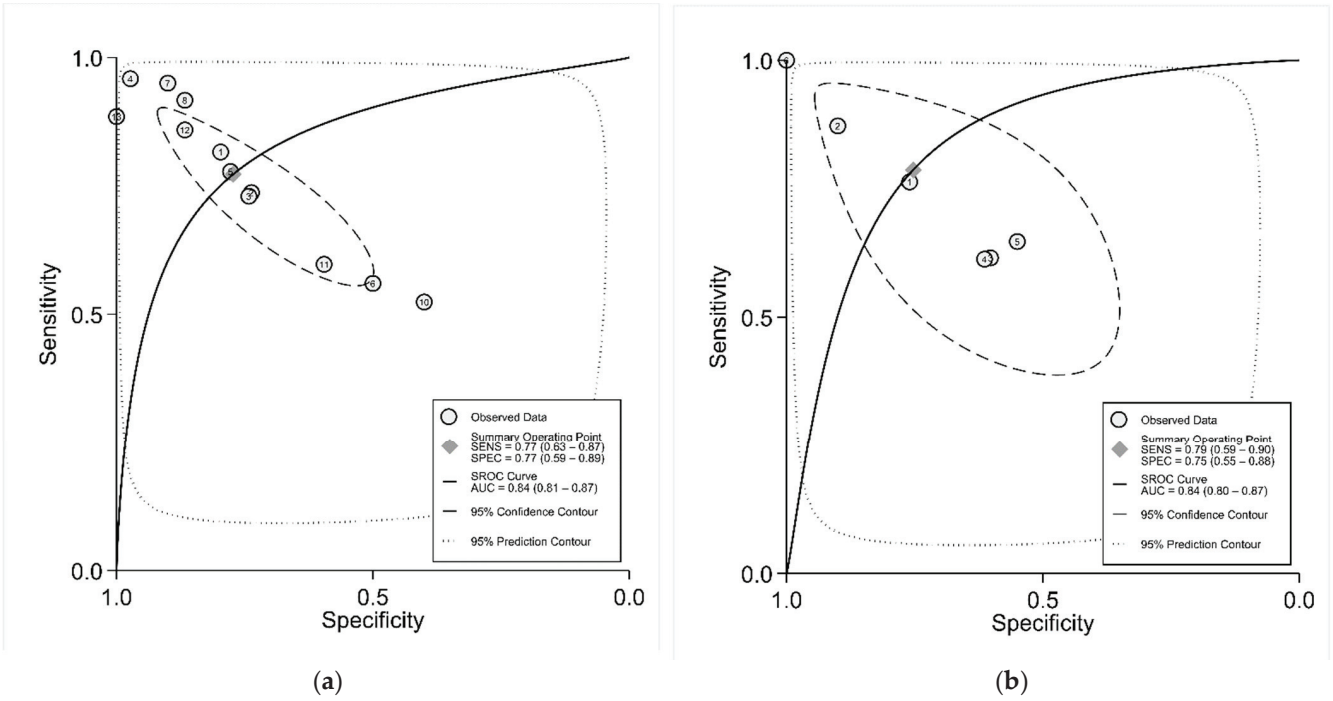


Figure 2. The Quality Assessment of Diagnostic Accuracy Studies–QUADAS. The figure represents the risk of bias and the applicability concern for the included studies. Each risk of bias is illustrated as percentage (%).

3.3. Diagnostic Accuracy and Summary ROC Curve

Overall, pooled sensitivity and specificity in diabetic patients, ranged from 0.65 (95% CI: 0.38–0.85) to 0.84 (95% CI: 0.77–0.89) and 0.65 (95% CI: 0.41–0.83) to 0.88 (95% CI: 0.67–0.97) respectively. An hsROC curve was created for each category (uNAG, uNAG/Cr)

and the AUC was calculated, along with the 95% CI. The diagnostic accuracy of uNAG estimated by AUC for predicting DN in diabetic patients, for all groups ranged from 0.69 (95% CI: 0.65–0.73) to 0.89 (95% CI: 0.86–0.92) (Figure 3 and supplementary Figures S1–S2). These results show moderate to excellent diagnostic accuracy of uNAG and uNAG/Cr. In addition, the best predictive performance was shown by uNAG and uNAG/Cr to discriminate between diabetic patients with normo-microalbuminuria and the healthy group with an AUC = 0.89 (95% CI: 0.86–0.92) (Figure 3). In the meta-analysis were used the following values, which are presented in Table 2: TP, FN, FP, and TN, paired sensitivity and specificity, along with the corresponding 95% CI and the cutoff values for each individual study. Table 3 provides the diagnostic and prognostic values for uNAG and uNAG/Cr.



(**a**) (**b**)

Figure 3. The hierarchical summary Receiver Operating Characteristic (hsROC) curve of uNAG (**a**) and uNAG/Cr (**b**) to differentiate controls (healthy individuals) from normoalbuminuric diabetic patients.

Table 2. Contingency table for uNGAL and uNGAL/Cr in diabetic patients, along with paired sensitivity and specificity of individual studies.

uNAG: Controls vs. Patients with Normoalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP ***	**FN ***	**TN ***	**FP ***	**Sensitivity (95%.CI)**	**Specificity (95% CI)**
27594733	Anane H.A.	Ghana	2016	2	11.15	31	7	51	13	0.80 (0.63–0.90)	0.79 (0.68–0.88)
23966807	Heba S. Assal	Egypt	2013	2	8.25	14	5	14	5	0.72 (0.48–0.90)	0.72 (0.48–0.90)
25519006	Zurawska Plaksej E.	Poland	2014	2	156.5	27	10	23	8	0.73 (0.56–0.86)	0.71 (0.53–0.86)
20980978	Vaidya S. V.	USA	2011	1	1.15	347	15	36	1	0.95 (0.92–0.97)	0.96 (0.86–0.99)
25717442	Gehan S.	Egypt	2015	2	1	7	2	7	2	0.70 (0.34–0.93)	0.70 (0.34–0.93)
16935891	Navarro J.F.	Spain	2006	1	1	14	11	16	16	0.56 (0.34–0.76)	0.50 (0.32–0.68)
17910281	Kalansoopiya A.	UK	2007	2	1	19	1	18	2	0.95 (0.75–0.99)	0.90 (0.68–0.98)
18236735	Kalansoopiya A.	UK	2007	2	1	11	1	13	2	0.91 (0.61–0.99)	0.86 (0.60–0.98)
21779943	Fu W.	China	2011	2	1	11	49	3	24	0.18 (0.09–0.30)	0.11 (0.02–0.29)
26904288	Muro P.D.	Italy	2015	2	1	22	20	16	24	0.52 (0.36–0.68)	0.40 (0.24–0.56)
31218128	Zhang D.	China	2019	2	1	86	58	25	17	0.59 (0.51–0.67)	0.40 (0.25–0.56)
-	Nikolov G.	Skopje	2013	2	1	146	24	26	4	0.85 (0.79–0.90)	0.86 (0.69–0.96)
32601635	Shrouq F.A.H.	Egypt	2020	2	1	23	3	30	0	0.88 (0.69–0.97)	1.00 (0.88–1.00)
uNAG/Cr: Controls vs. Patients with Normoalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP**	**FN**	**TN**	**FP**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
27594733	Anane H.A.	Ghana	2016	2	9.2	22	17	38	27	0.56 (0.39–0.72)	0.58 (0.45–0.70)
23105632	Ambade V.	India	2006	1.2	6.5	68	26	32	16	0.72 (0.62–0.81)	0.66 (0.51–0.79)
15016173	Salem M. A. K.	Egypt	2002	1	4.6	38	10	31	9	0.79 (0.65–0.89)	0.77 (0.61–0.89)
2881186	Shimojo N.	Japan	1987	1	2.3	99	1	56	1	1.00 (0.95–1.00)	1.00 (0.95–1.00)
23105632	Ambade V.	India	2003	1	6.2	65	29	33	15	0.68 (0.53–0.81)	0.69 (0.58–0.78)
16641878	Piwowar A.	Poland	2006	2	0.3	9	17	27	35	0.34 (0.17–0.55)	0.43 (0.31–0.56)
18022929	Karakani A. M.	Iran	2007	1	3.6	23	1	24	1	1.00 (0.85–1.00)	1.00 (0.85–1.00)
uNAG: Patients with Normoalbuminuria vs. Patients with Microalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP**	**FN**	**TN**	**FP**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
27594733	Anane H.A.	Ghana	2016	2	12.9	2	1	1	1	0.53 (0.37–0.69)	0.52 (0.33–0.73)
23966807	Heba S. Assal	Egypt	2013	2	13.8	1	3	2	4	0.76 (0.50–0.91)	0.91 (0.73–0.99)
25519006	Zurawska Plaksej E.	Poland	2014	2	193.5	2	1	2	1	0.54 (0.38–0.71)	0.48 (0.29–0.65)
16966829	Fujita H.	Japan	2006	2	20	18	0	19	0	1.00 (0.81–1.00)	1.00 (0.82–1.00)
25717442	Gehan S.	Egypt	2015	2	1.2	6	3	12	7	0.62 (0.26–0.87)	0.60 (0.36–0.80)
16935891	Navarro J.F.	Spain	2006	1	4	34	26	14	11	0.56 (0.43–0.69)	0.56 (0.34–0.75)
21779943	Fu W.	China	2011	2	12.7	16	8	41	20	0.66 (0.44–0.84)	0.67 (0.54–0.78)
20980978	Vaidya S.V.	USA	2011	1	2.5	2	6	2	4	0.82 (0.78–0.86)	0.84 (0.79–0.88)

Table 2. *Cont.*

uNAG/Cr: Patients with Normoalbuminuria vs. Patients with Microalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP**	**FN**	**TN**	**FP**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
27594733	Anane H.A.	Ghana	2016	2	15	29	9	19	6	0.76 (0.60–0.88)	0.75 (0.56–0.91)
15016173	Salem M. A. K.	Egypt	2002	1	9.8	41	6	9	1	0.85 (0.72–0.93)	0.87 (0.58–0.99)
16641878	Piwowar A.	Poland	2006	2	1.1	8	5	53	35	0.57 (0.28–0.82)	0.60 (0.49–0.70)
23105632	Ambade V.	India	2003	1	9.6	57	36	62	39	0.61 (0.51–0.71)	0.60 (0.50–0.70)
16373913	Narita T.	Japan	2005	2	3	11	6	11	9	0.67 (0.38–0.85)	0.55 (0.31–0.76)
18022929	Karakani A. M.	Iran	2007	1	6.2	23	0	7	0	1.00 (0.85–1.00)	1.00 (0.85–1.00)
uNAG: Controls vs. Patients with Normo-Microalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP**	**FN**	**TN**	**FP**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
23966807	Heba S. Assal	Egypt	2013	2	10	38	7	19	1	0.84 (0.70–0.93)	0.93 (0.75–0.99)
25519006	Zurawska Plaksej E.	Poland	2014	2	160	53	17	23	9	0.75 (0.63–0.85)	0.72 (0.53–0.86)
20980978	Vaidya S. V.	USA	2011	1	1.3	597	62	38	0	0.90 (0.88–0.92)	1.00 (0.90–1.00)
25717442	Gehan S.	Egypt	2015	2	1	21	9	8	2	0.70 (0.50–0.85)	0.79 (0.44–0.97)
uNAG /Cr: Controls vs. Patients with Normo-Microalbuminuria											
PubMed ID	**Author Name**	**Country**	**Year**	**Type of Diabetes**	**Cut-Off**	**TP**	**FN**	**TN**	**FP**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
15016173	Salem M. A. K.	Egypt	2002	1	5.2	53	6	33	7	0.89 (0.79–0.96)	0.82 (0.67–0.92)
16641878	Piwowar A.	Poland	2006	2	0.5	55	48	17	25	0.53 (0.43–0.63)	0.40 (0.25–0.56)
18022929	Karakani A. M.	Iran	2007	1	4	32	0	25	0	1.00 (0.89–1.00)	1.00 (0.89–1.00)
23105632	Ambade V.	India	2003	1	6.5	142	54	34	14	0.72 (0.65–0.78)	0.70 (0.55–0.83)
27594733	Anane H.A.	Ghana	2016	2	11	45	20	44	21	0.69 (0.56–0.80)	0.68 (0.54–0.78)

* TP: True Positive, * FN: False Negative, * TN: True Negative, * FP: False Positive.

Table 3. Pooled diagnostic and prognostic accuracy of uNAG in T1DM and T2DM patients.

Number of Studies	Sensitivity (95% CI)	I^2 (%)	Specificity (95% CI)	I^2 (%)	PLR (95%CI)	NLR (95% CI)	DOR (95% CI)	AUC (95% CI)	*p*-Value
uNAG: Controls vs. patients with normoalbuminuria									
13	0.77 (0.63–0.87)	64.65 (37.83–91.46)	0.77 (0.59–0.89)	58.22 (25.48–90.96)	3.4 (1.5–7.6)	0.29 (0.14–0.06)	12 (3–52)	0.84 (0.81–0.87)	0.89
uNAG/Cr: Controls vs. patients with normoalbuminuria									
7	0.82 (0.56–0.94)	93.22 (89.64–96.80)	0.79 (0.57–0.92)	93.95 (90.87–97.04)	3.9 (1.4–11.1)	0.23 (0.07–0.79)	17 (2–159)	0.87 (0.84–0.90)	0.63
uNAG: Patients with normoalbuminuria vs. patients with microalbuminuria									
8	0.65 (0.38–0.85)	64.65 (37.83–91.46)	0.65 (0.41–0.83)	58.22 (25.48–90.96)	1.8 (0.7–4.8)	0.54 (0.20–1.49)	3 (0–24)	0.69 (0.65–0.73)	0.66
uNAG/Cr: Patients with normoalbuminuria vs. patients with microalbuminuria									
6	0.79 (0.59–0.90)	82.49 (69.37–95.61)	0.75 (0.55–0.88)	85.76 (75.66–95.87)	3.2 (1.4–7.4)	0.28 (0.11–0.70)	11 (2–61)	0.84 (0.80–0.87)	0.13
uNAG: Controls vs. patients with normo-microalbuminuria									
4	0.83 (0.73–0.89)	87.99 (78.95–97.04)	0.92 (0.66–0.99)	74.65 (51.70–97.59)	10.8 (1.9–61.9)	0.19 (0.11–0.33)	58 (6–540)	0.90 (0.88–0.93)	0.49
uNAG/Cr: Controls vs. patients with normo-microalbuminuria									
5	0.84 (0.56–0.95)	96.43 (94.53–98.32)	0.81 (0.48–0.95)	93.13 (88.69–97.56]	4.4 (1–19]	0.20 (0.05–0.85)	22 (1–388)	0.89 (0.86–0.92)	0.08

3.4. Subgroup Analysis and Publication Bias

The forest plot graphical presentation had shown notable heterogeneity in sensitivity and specificity in all sets examined. The degree of heterogeneity in sensitivity and specificity for all groups ranged from 64.65% to 96.43% and from 58.22% to 93.95%, correspondingly. The between study heterogeneity for the prediction of normoalbuminuria and microalbuminuria in diabetic patients is presented in supplementary Figures S3–S5. The Deek's funnel plot estimated the evaluation of publication bias, in which *p*-value for all the groups, ranged from 0.08 to 0.89 and is showed in supplementary Figures S6–S8. Potential bias was present in the studies with data involving uNAG, specifically for distinguishing controls and normoalbuminuric or microalbuminuric diabetic patients.

4. Discussion

Diabetic nephropathy is considered to be the main cause of end-stage renal disease and a critical complication in patients with T1DM and T2DM. Therefore, the prediction of the disease at an early stage of its development is considered of the highest value. The diagnosis of DN is based on microalbuminuria estimation [5]. However, according to recent studies, diabetic patients diagnosed with microalbuminuria, have shifted back to normoalbuminuria and high GFR [7–9]. In addition, pathogenesis of DN evolves an interaction between metabolic and hemodynamic factors which cause glomerular and tubular interstitial injury. Therefore, researchers have been questioning and reevaluating the diagnostic value of the gold standard method, proposing an alternative approach, using potential tubular biomarkers such as NAG, for the early prediction of DN or other glomerular and markers of oxidative stress or inflammation. The attempt of this systematic review and meta-analysis was to evaluate the diagnostic accuracy of uNAG and provide comprehensive information for the accuracy of uNAG, as a preventional biomarker in the early stages of DN in patients with T1DM and T2DM.

Anane et al. [33] in their research study showed that the values of uNAG and uNAG/Cr ratio from patients' urine samples, had an increasing rate as the values of albuminuria increased and the rate of glomerular infiltration (eGFR) decreased, in patients with T2DM compared to the control group. Sheira's et al. [41] study showed statistically significant increase of uNAG and uNAG/Cr ratio and decrease of the estimated GFR in patients with microalbuminuria compared to patients with normoalbuminuria and in all patient groups compared to control group. Moreover, a parallel increase of the urinary excretion of NAG with the deterioration of DN has been observed, which indicates the severity of kidney damage and disease progression [40,41]. Kim et al. in their study showed that the levels of urinary NAG had a moderate positive correlation with the levels of urinary ACR in T2DM and that increased levels in urinary NAG may be associated with glycemic parameters reflecting glucose fluctuation [53].

The main result of our meta-analysis is the high value of AUC for uNAG and uNAG/Cr in distinguishing the control group from normo-microalbuminuric diabetic patients. This finding shows that uNAG/Cr can be considered a potential, good biomarker to predict early diabetic nephropathy in patients with diabetes mellitus. Moreover, according to the guidelines by Swets, the diagnostic accuracy values of uNAG and uNAG/Cr showed moderate accuracy in the other settings as well. These findings support the hypothesis for renal tubule damage even in the initial stage stages of DN, before the presence of pathological amounts of albuminuria, indicating a promising diagnostic accuracy of the biomarker. It is worth mentioning that uNAG/Cr seemed to present higher capability than uNAG in the diagnosis of DN, as designated by the comparison of the corresponding AUC values for each group.

According to the forest plot, there was significant presence of heterogeneity between studies. Possible causes of heterogeneity were mainly due to (a) the design of the studies which indicated different methodology of estimating creatine's concentration, (b) the different choice of urine collection for estimating ACR (spot urine or 24 h urine collection), and (c) the disparate race and ethnicity of the study population. In addition, a significant

decrease in heterogeneity was observed among the category of diabetic patients with normoalbuminuria and diabetic patients with microalbuminuria. The low presence of heterogeneity was caused due to the common procedure followed in each study.

Furthermore, according to Deek's charts, publication bias was absent in the majority of studies. Publication bias was evident in studies that performed the discrimination between controls and normo-microalbuminuric diabetic patients using uNAG/Cr. This bias may be introduced due to unclear methodology used for estimating the values of microalbuminuria in some of the studies. As evidenced by the QUADAS quality assessment, the vague risk of bias in some studies on patient selection was present due to insufficient information about the diabetic patients' characteristics with normoalbuminuria and microalbuminuria.

In conclusion, the final results of this systematic review and meta-analysis indicate that uNAG is a promising biomarker (raw and creatinine-normalized) for early and valid prediction of diabetic nephropathy in patients with T1DM and T2DM. Meta-analysis findings indicate that uNAG/Cr has higher efficiency in all patient groups. In particular, higher accuracy was observed in identifying the presence of DN in normo-microalbuminuric patients with T1DM and T2DM.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/diabetology2040025/s1, Table S1. Clinical characteristics of the included studies in the meta-analysis for controls (healthy individuals) and diabetic patients with normoalbuminuria and microalbuminuria. Figures S1–S2. The hierarchical summary Receiver Operating Characteristic (hsROC) curve of uNAG and uNAG/Cr to discriminate normo-, micro-, normo/microalbuminuric diabetic patients. Figures S3–S5. Forest plot for sensitivity and specificity of uNAG and uNAG/Cr to distinguish normo-, micro-, normo/microalbuminuric diabetic patients. Figures S6–S8. Deek's funnel plot for the evaluation of publication bias of uNAG and uNAG/Cr.

Author Contributions: P.G.B. and G.V.K. conceived the study. A.R.D. and G.V.K. participated in data collection. A.R.D. performed the analysis. G.V.K. and P.G.B. participated in data collection and in the interpretation of the results. All authors participated in drafting the manuscript. All authors have read and agreed to the published version of the manuscript.

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Perspective

Deciphering the Neurosensory Olfactory Pathway and Associated Neo-Immunometabolic Vulnerabilities Implicated in COVID-Associated Mucormycosis (CAM) and COVID-19 in a Diabetes Backdrop—A Novel Perspective

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Abstract: Recent Mucorales-mediated outbreaks of infections and an association of fungal infection with COVID-19 cases, as observed for COVID-19-associated mucormycosis (CAM), have posed new challenges for the management of patients in critical care units. Diabetes and hyperglycemia are integrally linked to the severity of COVID-19, and uncontrolled diabetes mellitus and COVID-19 have recently been (independently or in combination) associated with the emergence of aggressive mucormycosis due to attendant defects in innate immune recognition pathways. Therefore, the identification of novel global cellular stressors upregulated during diabetes to understand the contribution of diabetes-associated metabolic vulnerabilities can help build a Metabolic-Stress-Associated Interactome (MSAI). This interactome can help reshape the metabolic inflammation (meta-inflammation) underlying the clinical manifestations of COVID-19 to facilitate the rational design of effective therapies for COVID-19 and CAM. Accordingly, an important area of research in COVID-19 therapeutics is engaged with identifying diabetes-associated pan-cellular stressors to understand their role in immune deregulation during COVID-19 and CAM, including investigating the distant trans-neurovascular–endocrine axis's role in coordinating cellular-stress recognition, transmission, compensation, and decompensation during inter-organ regulation of metabolic homeostasis in diabetes. We reviewed clinico-pathological and laboratory data to propose potential diabetes-linked novel neo-vulnerabilities that can reshape the olfactory mucosal immune landscape during airway infections such as COVID-19 and CAM.

Keywords: COVID-19; SARS-CoV-2; ACE2; TMPRSS2; mucormycosis; COVID-associated mucormycosis (CAM); mucins; Diabetic Keto Acidosis (DKA); Metabolic-Stress-Associated Interactome (MSAI); olfactory neurovascular niche; serine proteases; ferroptosis; redox-iron stress

1. Introduction

1.1. Current Taxonomy of Mucorales and Congeners

The Mucoromycota phylum comprises the Mucormycotina, Glomeromycotina, and Mortierellomycotina sub-phyla that fall into the Mucorales, Umbelopsidales, and Endogo-

nales orders, respectively. Mucorales comprise a group of versatile and ecologically highly diverse environmental molds. They are considered to be the cosmopolitan saprobes with a variety of unique features—they are omnipresent in human habitation because they thrive in the same environmental conditions as mankind. Generally, they are closely associated with the organic substrates, and the spores may be released into the soil or remained suspended in air. The medically important order Mucorales comprises 55 genera and 261 species. With the advent of molecular sequencing tools such as the barcoding of Mucorales using internal transcribed spacer (ITS) region and phylogenetic analyses, new species are continuously being identified and added to the list [1–4].

Recently, the identification of *Cunninghamella (C.) arunalokei* sp. nov. (as seen in an infection in an immunocompetent host) potentially expanded the list of medically important Mucorales to >38 etiological agents causing a life-threatening condition coiled as mucormycosis [2]. Hoffman et al. assigned various genera of Mucorales into family and species by clinical relevance. The opportunistic Mucorales that cause mucormycosis are usually thermotolerant and may include opportunistic genera and species such as *Actinomucor*, *Apophysomyces*, *Cokeromyces*, *Cunninghamella*, *Lichtheimia*, *Mucor, Rhizomucor*, *Rhizopus (R.), Saksenaea*, *Syncephalastrum*, and *Thamnostylum.* Two species of the genera *Rhizopus-R. arrhizus* (syn. *R. oryaze*) and *R. microsporus* cause 70% of worldwide mucormycosis and predominant COVID-19-associated mucormycosis (CAM). The three most virulent Mucorales primarily associated with diabetes mellitus (DM) are *C. bertholletiae* (77%), *Rhizopus* spp. (57%), and *Mucor* spp. (41%) [1,3,4]. Furthermore, the prevalence of rare species such as *R. homothallicus, Saksenaea vasiformis, Apophysomyces variabilis, Thamostylum lucknowense, Mucor irregularis*, and (most recently) *C. arunlalokei* nov. sp. in the Indian subcontinent suggests the unique ecological niche and epidemiological significance of mucormycosis and its implications and burden on public health [5–7].

Uncontrolled DM is the major comorbid risk factor along with a high spore burden for acquiring mucormycosis. There has been an increase in the trend of DM prevalence worldwide, and the situation in India is alarmingly high, as nearly 8% of adults aged $\leq$20 years have DM [8]. Inarguably, infectious diseases alone attribute to over 12% of mortality in DM cases worldwide [3]. Secondly, 7–14% of COVID-19 patients have DM as their predominant comorbidity, and COVID-19-associated acute or stress-induced hyperglycemia cases comprise half of the hospitalized patients in the ongoing pandemic [3,9–13]. Ergo, COVID-19 itself leads to new-onset DM and precipitates into diabetic keto acidosis (DKA) due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus and the inappropriate use of systemic corticosteroids (CS) [6,12,14]. Furthermore, CAM, a complication in individuals with severe or critical COVID-19 as a virus-induced endothelial dysfunction, has proven detrimental with worsening outcome [7,15,16]. The emergence of CAM in India is multifactorial and involves the immediate environment, as an abundance of mucoralean spores—indoors, outdoor, and in the soil—can be acquired by air inhalation, ingestion, or traumatic inoculation. Host factors include uncontrolled DM, inappropriate SC therapy, iron overload, iatrogenic factors, and COVID-19 virus involving endothelial dysfunction and immune dysregulation [7]. Similar to Coxsackie, Influenzae, and SARS-CoV-1 viruses, SARS-CoV-2 can also induce an acute diabetes state mediated by the high-level expression of angiotensin-converting enzyme 2 (ACE2) receptors in the pancreatic islet cells, which results in the widespread destruction of beta cells in the pancreas, the diminution of insulin production, an enhanced secretion of cortisol, and resultant stress exacerbating the progression of insulin resistance. Furthermore, in type 2 DM and DKA, along with a chronic inflammatory state, there is a reduction in the master regulatory cytokine interleukin-10 (IL-10) synthesis by lymphocytes and macrophages in place of increased glycosylation, which also hampers the extravasation and chemotactic abilities of phagocytic cells on the path to overall dysregulated and dysfunctional immune responses. All these events fail to arrest spore germination and resultant morbid culmination of the pathogenesis of CAM [17,18].

1.2. COVID-Associated Mucormycosis (CAM)

The estimated global cases of mucormycosis per year pre-COVID era were 10,000. Startlingly, during the second wave of the COVID-19 pandemic, mediated by the delta variant, the averages rose 2–3 times, with more than 47,000 CAM cases being reported alone from the Indian subcontinent in just three months, i.e., May to July 2021, and continuing to wreak havoc and straining healthcare systems to the breaking point [2,6,7,10,11]. Furthermore, the prevalence of cases per million inhabitants of mucormycosis was 14.0 per year, which was highest global average, and Asia stands in the highest prevalence zone, with 29.9 cases per million inhabitants per year [19]. Mucormycosis usually develops in 10–14 days after hospitalization according to Muthu et al., who systematically reviewed the cases first reported during COVID-19 from India and compared them with the rest of the world [6]. They categorized cases into early CAM (wherein mucormycosis is diagnosed simultaneously with COVID-19) and late CAM at proportions of 25% and 36%, respectively; in other parts of the world, late CAM develops from seven days to 3 months following COVID-19 infection, with a mean average of 19.5 days. Similarly, the predominant presentation of CAM was rhino-orbital-cerebral-mucormycosis (ROCM), reaching 89% in India and 64% globally [20]. A study post-June 2021 from India revealed that the case fatality rate reported from India versus the rest of the global averages was comparatively lower, i.e., 36.5% vs. 61.9%, respectively. This may have been due to a diagnostic dilemma in picking pulmonary mucormycosis (PM) and probably due to the predominance of ROCM in the Indian context, wherein healthcare is overburdened with radical surgeries, orbital exenteration, the extensive dissection of the sinuses, and critical shortages of antifungal drugs [6,11]. First, the phenomenon was unique to India, but soon several countries of various continents started reporting a similar surge in CAM cases including Pakistan, Iran, Egypt, Brazil, USA, Mexico, Chile, Honduras, Paraguay, Uruguay, and European countries. The list is increasing in the wake of yet another wave of COVID-19 due to the delta and newly emerged omicron variants [21–23]. The analysis of CAM in 18 countries by Hoenigl et al. revealed that 53% of the cases were from India. A multi-center study from the first wave of COVID-19 concluded that CAM predominately presented as ROCM followed by the PM type and was caused by three important comorbid factors, diabetes, inappropriate SC (more than 6 mg of dexamethasone use for more than recommended duration), and SARS-CoV-2 itself [24,25]. The estimated prevalence of CAM in India at epidemic proportions during the pandemic is unprecedented and nearly 70 times that of the global average [26]. This uncanny upsurge was termed by many mycologists and infectious disease experts as a "tsunami of black fungus in COVID stricken India." This maiden mayhem of CAM in India raised alarm bells, warranted swift federal government interventions with rapid response groups, and led to the naming of mucormycosis as the national "notifiable disease" [27]. This initiative further developed a comprehensive framework by strengthening the national registry of mucormycosis cases, monitoring systems, early recommendations for diagnosis, treatment (in places with shortages of antifungals), and management strategies. Under the aegis of the Fungal Infection Study Forum (FISF), a systematic registry of cases was initiated under the name of "Fung-I-Reg" in India and stood as a pivotal forum in recommending the guidelines on limiting and managing CAM. Furthermore, in harmony with the International Society of Human and Animal Mycology (ISHAM), European Confederation of Medical Mycology (ECMM), and Mycoses Study Group (MSG), FISF proposed global guidelines for the diagnosis and management of mucormycosis in low and middle-income countries (LMIC) such as India [12,28].

1.3. Mucormycosis-Associated Diabetes (MAD)

Hyperglycemia may be induced by SC. During the COVID-19 pandemic, SC were indiscriminately used to circumvent oxygen desaturation, especially in the second wave [6,7]. Firstly, SCs act as a substrate of oxidative stress metabolism through lipolysis and proteolysis from hepatocytes, resulting in a hyperglycemic state, insulin resistance, and continuous ketogenesis [17]. Secondly, this results in inefficient cellular functions such as neutrophil migration,

ingestion, and phagolysosome fusion, thereby suppressing the microbicidal activity of activated macrophages, the antagonism of macrophage maturation and differentiation, and the depression of inflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-alpha (TNF-α), and other mediators of macrophages; virtually all phagocytic and respiratory burst mechanisms are dampened, which has broadened the scope for the establishment of mucormycosis [7,17]. Recent studies in the immunopathogenesis of mucormycosis-associated with diabetes mellitus (MAD) have documented a well-accepted hypothesis that emphasizes the pivotal role of macrophages and their subsets, i.e., M1 and M2 and their pattern recognizing receptors (PRRs) or Toll-like-receptors (TLRs), as the initiators of the efficient killing of fungal cells by phagocytosis. Among the two subsets of macrophages, those of M2 are anti-inflammatory and release IL-10, which is involved in the immune response to fungal cells. Conversely, in patients with MAD, hyperglycemia triggers stress response in the endoplasmic reticulum, the overexpression of the glucose-regulated protein-78 (GRP78 protein), increase in reactive oxygen species (ROS), and free fatty acids (FFA), and a plethora of pro-inflammatory cytokines (TNF-α and IL-1β) and chemokines from the liver, muscle, and hypertrophic cells of the adipose tissues, resulting in a persistent inflammatory state. This ensures the massive tissue recruitment of the activated M1 subset of macrophages and resultant infiltration, along with the dampening of the M2 macrophage regulatory response [17].

Furthermore, *Rhizopus* spp. thrives by relying on iron siderophores, high serum glucose levels, and an insulin resistance state as its energy source [3,7,17]. Incontrovertibly, DKA is a double-edged sword that Mucorales easily exploit by affecting glutathione restoration, and an increased glucose metabolism results in increased glycosylation end-products and ROS, which accumulate in organs and tissues, thus further increasing susceptibility to *Rhizopus* infections [3]. Alternately, these patients require hemodialysis with deferoxamine chelation therapy to correct the DKA, which in turn makes them vulnerable to mucormycosis due to augmented levels of serum-free iron readily available for Mucorales to flourish. Furthermore, COVID-19-associated hyperferritinemia may act as a lucrative source iron for thriving and invading by Mucorales. Lastly, the intensive use of antimicrobial therapy creates a favorable nidus, amplifying a conductive microenvironment for the progression of an invasive fungal infections such as mucormycosis [3,7,17].

1.4. Immuno-Pathobiology of CAM and the Interface between COVID-19 and Mucor

In the above-described subset of diabetic patients, a potentiated inflammatory state is caused by the continuous and persistent recruitment of local immune cells such as macrophages and neutrophils, which in turn recruit a variety of inflammatory cytokines [17]. This phenomenon, which paradoxically switches yet another robust inflammatory phenotype when SARS-CoV-2 is positively flagged in these subsets of patients, predisposes patients to a variety of secondary infections including mucormycosis, such as COVID-19-associated pulmonary aspergillosis (CAPA), disseminated fusariosis, and invasive candidiasis. The first was extremely devastating during the second wave of COVID-19 in the Indian subcontinent and resulted in a dual-disease term called CAM. Much of the immunopathogenesis of Mucorales in DM patients comes from the prototype species *Rhizopus* spp. [29].

Secondary infections in these subsets of patients caused by Mucorales are mediated via the hyper-secretion of pro-inflammatory cytokines by immune reactive cells that open up divergent pathways leading to a vicious cycle of CAM and the widespread insult of the host tissues. Moreover, a plethora of interventions in the management of COVID-19 have further potentiated serum ferritin and free iron levels, drastically restricting the efficiency of macrophage immunity. Evidently, through siderophores, Mucorales efficiently acquire free iron from patients with DKA, as higher serum levels of free iron result in acidosis and poor binding to proteins. Secondly, in a setting of uremia wherein desferrioxamine chelation may act as a source (similarly to a COVID-19-induced hyperglycemia (indiscriminate use

of CS) and chronic inflammatory state), Mucorales facilitate the pivotal mechanism in the immunopathogenesis of CAM [6,7,29].

Two host cell receptors—the ACE2 receptors (which are expressed in abundance in host endothelium) and glucose-regulated protein 78 (GRP78) (in addition to its role in MAD) as a co-receptor by recognizing the spike protein (S) of SARS-CoV-2 and efficiently internalizing the virus into the host cells [30]—are also involved in endothelial cell barrier disruption, inflammation, coagulation, endotheliatis, and apoptosis. In severe cases of COVID-19, abnormally elevated levels of D-dimer, fibrinogen, and Willebrand factor (VWF) and profound increases in inflammatory cytokines such as IL-1, Il-6, IL-8, and TNF-alpha are indications of multi-organ dysfunction due to widespread venous thrombosis, systemic vasculitis, and vascular coagulopathy [29]. Rayner et al. found that the suppression of GRP78 expression resulted in the inhibition of SARS-CoV-2 replication [31]. The pre-existing hyper immune-reactiveness, endothelial damage, and multi-organ dysfunction in ongoing COVID-19 are important risks for the emergence of complications owing to angioinvasive Mucorales [7,29,30]

Recently, Franco et al. reviewed host–pathogen and molecular interactions in the pathogenesis of *Rhizopus* spp. in DM patients. Mucormycosis has six main clinical presentations, of which ROCM is the most predominant [3]. Though the occurrence of ROCM was preponderantly seen in immunocompromised patients in the pre-COVID-19 era, CAM has since created a similar niche by mimicking immunocompromised (IC) status in the present pandemic situation [32]. Augmenting this scenario, there have been multiple reports of mucormycosis in immunocompetent hosts, presenting as chronic ROCM, skull base invasion, and renal mucormycosis caused by these seemingly thought opportunistic invaders [33,34]. Regardless of presentation, the angioinvasiveness, dissemination to the contiguous tissues resulting in necrotic eschars and thrombosis along with mucoralean spores in IC hosts with notably high mortality rates [3]. Coincidentally, the angioinvasive process and thrombotic microangiopathy in the disease spectrum are the mimickers of both COVID-19 and mucormycosis pathogenesis. Furthermore, the striking mortality of mucormycosis in DM patients is as high as 77% due to infections by *Cunninghamella bertholletiae*, followed by 57% due to *Rhizopus* spp. and 41% due to *Mucor* spp., which indicates the need for the efficient management of these rapidly progressive conditions [35].

The spread pathways of Mucorales in ROCM were described by Hosseini and Bhorgei in 2005 [36]. In the inhalation mode, the pterygopalatine fossa serves as the main reservoir for mucormycosis, and the spores and hyphae lodge in the nasal passages and contiguous sinuses. GRPs are a group of immunoglobulin-binding proteins (BiPs) that include GRP78 (a molecular chaperone belonging to the heat shock protein 70 (HSP70) family located in the lumen of the endoplasmic reticulum) and its isoform GRP78va (located in the cytosol), which have pivotal roles in folding, assembly, and quality control of proteins and misfolded protein degradation. Furthermore, they are expressed in diverse types of inflammatory, cancerous, viral, and invasive fungal spores including the Mucorales [37,38]. Hyperglycemia-induced stress in *Rhizopus* spp. infection in DM patients results in the translocation of GRP78 onto the cell surface (cs) as csGRP78 and the overexpression of receptors for signaling, also leading to anti-csGRP78 autoantibodies. With the expression of fibronectin and type I collagen, fibrosis is a consequential event of the sequential interactions of csGRP78 with β1 integrin and the activation of the focal adhesion kinase (FAK) and downstream protein kinase Akt (van). Interestingly, a similar mechanism of receptor signaling and entry of the pathogen into the host cell was proposed for Dengue, Ebola, Coxsackie, and SARS-CoV2 viruses, along with *Rhizopus* spp. [39,40].

Receptor interaction studies of nasal and alveolar epithelia with homologous spore coating proteins (coat protein homolog (CotH) present on Mucorales such as *Rhizopus delemar* and *Rhizopus* spp. have revealed that csGRP78 and integrin-β1 are only overexpressed in the nasal epithelia of DM patients. This complex microenvironment, holding high glucose, iron, and DKA levels, promotes superficial tissue level invasion by favoring the interactions between CotH (specifically, CotH3) on *Mucorales* with that of the abundant

csGRP78 and integrin-β1 on the nasal epithelium. Furthermore, this expands the virulence of *Rhizopus* spp. via coating with laminin and collagen IV potentiating the invasion, endothelial damage, and continuous expression of GRP78 in the contiguous tissues planes and endothelium, eventually establishing the clinical entity ROCM in DM [36–39].

Beguilingly, Mucorales have adapted to the novel acquisition of virulence factors by harboring endosymbiotic nosocomial Gram-negative bacteria including various species of *Burkholderia* and *Ralstonia (R.) pickettii*. The former releases potent toxic metabolites called rhizoxins by endosymbiosis, and the latter plays a role in the intracellular survival of macrophages and triggers a profuse pro-inflammatory cytokine release [41,42]. Intriguingly, *R. pickettii* endosymbiosis with *Rhizopus microspores* has complicated several nosocomial outbreaks of Mucorales due to their propensity to contaminate sterile water, saline medical solutions, and hospital disinfectants [40,42].

1.5. The Complex Interplay of Various Factors: Mucosal Proteases and Iron Redox Stress

Human environments, whether community settings, indoor or outdoor areas, or air-conditioned or non-air-conditioned hospital areas, allow mucoralean spores to become airborne and inhaled by immunocompetent hosts without any apparent clinical disease. However, in an event of a source with a high hyphal burden, the polymorphoneutrophils (PMNs) response is overwhelmed, thus surpassing the primary barriers of immunity. The usual modes of transmission are inhalation, ingestion, or direct inoculation. The size of sporangiospores may also contribute to their role in establishing human disease, as larger *R. arrhizus* spores tend to settle in the upper respiratory tract and the smaller *Cunninghamella* or *R. microsporus* spores reach the lower respiratory tract; the latter is highly virulent and leads to rapid disease [6,43,44]. Furthermore, molecular and epidemiological studies to delineating the mechanisms of acquisition of hypervirulence, Mucorales genus-specific differences in interactions with the host cells (GRP78 expression and CotH3 interactions), the role of SARS-CoV-2 in potentiating diseases leading to CAM, other co-infections, and nosocomial outbreaks in increased CAM cases in aggravating seemingly opportunistic Mucorales infection are currently underway [6,43,45].

2. Potential Immuno-Metabolic Vulnerabilities That Can Prefigure COVID-19 and CAM-Synergistic Action of Diabetes-Associated Proteolytic and Metabolic Stress

2.1. Expanding the MSAI-Proteolytic Stress as a New Player in COVID-19 Arena

The potential diabetes-associated cellular-stress pathways emerging from multi-faceted high throughput omics approach are facilitating expansions on MSAI and the development of the understanding of the pathogenesis of beta-cell dysfunction in COVID-19, which is incompletely understood even though integrated stress response (ISR) is integral to the development of diabetes. The impairment of insulin-producing pancreatic beta-cells is a key contributing factor to the diabetic pathogenesis by SARS-CoV-2. However, whether beta-cells are damaged by the coronavirus through a direct action is less clearly understood. The pancreatic cells are governed by intricate inter-organ network regulation involving soluble humoral factors from diverse organs that crosstalk with their neuro-vascular conduits. Therefore, linking the cause of pancreatic beta-cell damage in subjects with COVID-19 to the direct infection of SARS-CoV-2 or alternate indirect effects including distant organ damage, heralding inflammatory loops, and yet-unexplored cellular stressor(s) remains challenging. However, the potential of direct infection with SARS-CoV-2 and the subsequent cell fate reprograming of pancreatic cells has been recently demonstrated [46–48]. Notably, heterogenous host pancreatic (endocrine, exocrine, and acinar) cellular-stress responses caused by SARS-CoV-2 infections have displayed the transdifferentiation of insulin-producing beta cells into glucagon-producing alpha and trypsin-producing acinar cells in the pancreas, which has been linked to an ISR pathway. Remarkably, a trans-ISR inhibitor treatment was found to increase insulin expression and reduce glucagon, trypsin, and cell stress-associated genes in SARS-CoV-2-infected human islets [47], indicating a possible role of co-secreted pancreatic trypsin/proteolytic stress in reshaping diabetes-associated ISR.

2.2. Enhanced SARS-CoV-2 Transmissibility and Role of Olfactory Mucosal Proteases

Viral proteases are reported to have potential ancillary functions extending beyond the polyprotein/spike clipping that are linked to host immune evasion [49]. However, the biological functions (outside fusogenic potential) of cellular host proteases activated during COVID-19 remain largely unexplored. Importantly, the TMPRSS2 and trypsin serine proteases have been identified as important molecular players in the underlying pathology and emergence of coronaviruses, respectively. In particular, the trypsin treatment (proteolytic processing) of viral spike proteins has been identified as a potential species barrier for the emergence of zoonotic coronaviruses, hence posing a potential threat for future outbreaks driven by the cross-species transmission of coronaviruses [50–54]. It was proposed that additional small intestine proteases such as trypsin could enhance SARS-CoV-2′s viral infection and pathogenesis capability by triggering more robust cell fusion [55]. The increased transmissibility of SARS-CoV-2 compared to SARS-CoV is linked to the occurrence of a unique furin-cleavable polybasic motif (RRAR) at the S1/S2 boundary, as the cleavage results in a C-terminally exposed RRAR peptide that is capable of binding to neuropilin-1 (NRP1), which has been demonstrated to be an important host factor receptor for SARS-CoV-2 by facilitating entry and infectivity [50,56,57]. The novel core region with the SPRRAR (^{S}680-^{V}687) polybasic insert of SARS-CoV-2 harbors positively charged arginine at 683 and hydrophobic alanine at 684, which makes the site susceptible to promiscuous binding/cleavage by not only serine proteases such as furin or furin-like PCs but also mono- and dibasic amino acid targeting serine proteases such as matriptase, human airway trypsin, TMPRSS2, and kallikrein. Importantly, a plethora of serine proteases (SPs) such as furin, TMPRSS2, furin-like PCs, and trypsin-like proteases in the nasal microenvironment have facilitated the heightened transmissibility of emerging SARS-CoV-2 variants that have enhanced susceptibility to SP-cleavable polybasic amino acids; for instance, a mutation of non-polar proline at 681 to more positively charged arginine in delta variant (P681R) in spike protein resulted in enhanced transmissibility due to more cleavability at S1/S2 [58,59], and a mutation of proline to histidine (P681H) in omicron variant's spike protein [60] was recently reported and affirmed the trend of enhanced proteolytic susceptibility and concomitant high transmissibility. The nasal serine proteases are therefore integrally linked to the increased infectivity of SARS-CoV-2 due to indispensable prerequisite of proteolytic-priming of spike protein by diverse nasal SPs. It is a reasonable speculation that emerging variants of concern will display increased infectivity and transmissibility due to the subsequent shedding of biologically functional cleaved fragments that tend to retain the potential to not only bind olfactory cells (e.g., RRAR binding to NPR-1) but are also secreted into the mucous and exported as respiratory droplets to transmit infection, as has been previously reported [57].

2.3. Neuro-Vascular Olfactory Mucosal Niche in Diabetes and SARS-CoV-2 Pathogenesis

The neurological symptoms of COVID-19 and long COVID including anosmia, headache, encephalitis, and neurovasculopathy are becoming increasingly recognized (reported in up to 85% of ICU patients); however, neuroinvasion/neurotropism or the presence of SARS-CoV-2 in olfactory neurons and the brain remains unproven [61–64]. The transport of viruses through the neuro-vascular niche of olfactory mucosa all the way to CNS is debatable, but damage to neuro-vascular immune units of the brain comprising neural-crest-derived vascular pericytes and neuroglial astrocytes that participate in neuro-inflammation is more accepted [61,65]. The most well-documented step in the pathophysiology of SARS-CoV-2 is the primary route of infection, the olfactory mucosa, wherein the nasal-ciliated cells and non-neuronal sustentacular cells are the primary targets of the virus replication during the early stages of COVID-19 [62,66].

Interestingly, though anosmia and olfactory dysfunction are hallmark and consistent neurological symptoms of COVID-19, mechanistic insights of how neurosensory perception is altered and the involvement of the olfactory-nerve-mediated transport of viruses to the CNS are unresolved [67–70]. Despite the abundant expression of viral entry proteases

(ACE2 and TMPRSS2) in human and mouse olfactory systems and indications of sustentacular cells as prime targets of SARS-CoV-2 [71–73], viral replication in the non-neuronal olfactory epithelia of patient samples could be demonstrated only recently [62] due to the technical challenges of accessing the olfactory epithelium (OE) during active infection.

It is noteworthy that the major target sustentacular cells (SNCs) are glia-like in functionality. Given the paucity of literature on human SNCs, comparisons to animal models are challenging; nevertheless, in a rat model, SNCs were proposed to be glucose-transporting cells from the blood across the apical mucus to fuel olfactory sensory neuron (OSN) cilia [74,75]. Additionally, SNCs have been implicated in sheathing OSNs [76,77], which suggests a highly supportive role of SNCs in OSNs survival and maintenance, and any dysregulation in these supporting cells could lead to olfactory transduction pathway defects such as anosmia. Furthermore, the existence of a closely apposed olfactory neurovascular unit (not much explored) could also underlie neuro-inflammatory events resulting in massive immune dysregulation in the upper respiratory pathway during COVID-19 and paving the path for the aggressive presentation of mucormycosis. Investigating the odorant receptor repertoire is yet another potential area to inform on any association with inflammatory networks, but it is challenging to map receptor expression in COVID-19-active subjects during the acute phase of the disease marked by anosmia; nevertheless, the olfactory epithelia of deceased patients did not show any differences [62]. The strong correlation of diabetes to COVID-19 and CAM/ROCM infection suggests potential "pro-viral/pro-fungal" cellular host factors in OE, which could be linked to glucose homeostasis deregulation and immune derangement in the olfactory mucosae of diabetic patients, hence explaining the predisposition of upper airway infections in diabetic subjects. This premise may be supported by a recent study that reported the identification of a *UGT2A1/UGT2A2* single locus gene encoding for UDP glucuronosyltransferase enzyme in COVID-19 patients with anosmia [78], thereby reemphasizing the involvement of a glucose stress-associated anomalous glycosylation process that is integrally linked to disturbances in mucosal homeostasis and inflammation [79]. The possible role of glycosylation in altering the olfactory mucosal immune landscape is discussed in a later section. We first discuss the association and role of proteases in inflammatory pathways triggered by protease-activated signaling cascades in infectious inflammatory diseases.

2.4. Proteases as Signal Transducers—A Role beyond Spike Clipping

Olfactory infection and dysfunction occur early on during COVID-19, overlapping with severe invasive fungal co-infections including ROCM in primarily hyperglycemic patients with compromised immune status, and CAM was recently characterized by an unprecedented upsurge in fungal cases during a delta variant outbreak in India. Given the high proteolytic priming requirement and increased infectivity of the delta variant (as discussed above), it is reasonable to propose that a protease-rich neuro-immune unit of olfactory mucosa could play a crucial role in the rapid spread and systemic dissemination of fungal infections via neuro-inflammatory responses mediated by dysregulated olfactory transduction pathways, thereby compromising the anti-fungal immunity in diabetic patients with COVID-19 during ROCM infection. Nevertheless, any strong correlation between host protease-mediated immune dysregulation has not been reported for COVID-19 so far. However, the majority of the serine proteases in OE have been reported to have cognate signaling receptors, and many such protease-receptor signaling pathways have been established for other microbial diseases.

Emerging paradigms suggest an increased trend towards "trypsin-reliance" for zoonotic coronavirus emergence that might predict the cross-species transmission of coronaviruses possibly employing alternative route(s) such as the digestive tract/gut, which has been proposed to be a potential site for future coronavirus emergence events in humans, and alternative but unidentified ACE2-independent host–cell receptors [53,54,80,81] that continue to be reported with broader host–receptor repertoire linked to an enhanced infectivity and transmissibility of SARS-CoV-2 [58]. The ACE2-independent or partially dependent entry

of SARS-CoV-2 is becoming increasingly recognized by studies supporting the hypothesis that host cells infected by SARS-CoV-2 morph into virus-permissive cells that preset low ACE2 expression [82], indicating lower ACE2 thresholds for successful infection in some tissues or the presence of alternative receptors for viral entry. SARS-CoV-2-infected trachea transcriptomic signatures were found to parallel hematopoietic lineage progenitor cells, indicating a possibility of hematopoiesis induction by SARS-CoV-2 [83]. These paradigms are suggestive of potential mechanisms that might be active at the trypsin (protease)–host interface, which may extend beyond the layer of "viral-spike processing" and could be linked to "host–receptor alterations", as previously proposed [54].

Recent findings from diverse groups support the "co-emergence/-existence of SARS-CoV-2 infection and cell fate reprogramming events", indicating extended SARS-CoV-2-associated reprogramming trajectories including possible hematopoiesis, [83] preferred enterocyte progenitor (low ACE2) permissive replication [82], and transit-amplifying cells or intestinal stem cells supporting SARS-CoV-2 infections [84]. Furthermore, recent studies on SARS-CoV-2 infected olfactory mucosa reported that the infected nasal epithelial cells were subsequently regenerated by basal stem or other niche cells [62,66], which has been also reported for the regeneration of trachea, intrapulmonary airways, and alveoli in COVID-19 subjects [85]. The incorrectly timed growth factor responsiveness has been linked to disease severity [86], and tissue reparative growth factor signatures have been linked to recovery from moderate COVID-19 [87], thus indicating orchestrated spatio-temporal wound healing to be an important component in determining the extent and severity of COVID-19.

The regulation of the protease/antiprotease balance during pathologic insults (marked by the up- or downregulation of serine protease/SERPIN) is very complex and underlies tissue integrity and inflammation via various growth-factor-linked signaling pathways. Type II transmembrane serine proteases such as matriptase and kallikreins are known to activate protease-activated receptor-2 (PAR-2) and be regulated by SERPIN SPINT1/HAI-1 [88]. Recent studies have implicated coagulation proteases such as TF, FVIIa, and FXa in the activation of signaling receptor PAR-2, which is linked to pro-viral responses via TLR3 [89–97]. The authors of recent study proposed that targeting FXa and thrombin by using specific anticoagulant drugs can inhibit PARs, which may have beneficial role in human inflammatory diseases [97]. PAR-2 signaling can also be triggered by trypsin, tryptase, neutrophil elastase, GPI-anchored serine protease (PRSS8) prostatin, membrane-anchored hepsin, and TMPRSS2. PAR-2 targeting can have beneficial effects in the context of infectious pathology; notably, PAR-2-mediated NETosis was recently linked to pathogen benefits [98].

2.5. In Vitro Disease Modelling to Recapitulate COVID-19 and Diabetic Pathways

Given the potential of invoking inflammatory signaling cascades by serine proteases, as encouraged by our previous findings [99–102], we speculated that protease–antiprotease disbalance could induce concomitant metabolic reprogramming events driving viral-competent neuro-immunomodulatory pathways. We recently tested a potential hypothesis regarding whether proteolytic stress (proteolytic activity associated with COVID-19-activated TMPRSS2, furin, complement coagulation serine proteases, DPP4, HAT, neutrophil elastase, etc.) can upregulate metabolic stress and intensify metabolic reprogramming, thus resulting in altered neuro-immune responses in monocytic cells. Neural-crest-originating astrocytes and pericytes are becoming increasingly appreciated responder cells underlying neurovascular abnormalities in COVID-19 pathobiology. We explored whether developmental epithelial–mesenchymal transition (EMT) events initiated by active proteases during neural-crest generation could be recapitulated by providing exogenous proteolytic exposure to pathology competent cells in vitro, resulting in the upregulation of developmental multipotent neural-crest-like cells with ectomesodermal potential capable of upregulating neurogenesis/gliogenesis (neurons and astrocyte) and

vasculogenesis (microvascular pericyte), crucial cellular processes underlying neuroinflammatory diseases [Sharma et al., unpublished data].

3. Methods

3.1. In Vitro Virus-Free Model-Establishment and Characterization of a Novel Proteolytically Tunable Plasma Based Cellular Stress Model for COVID-19 Modeling

A novel in vitro virus-free model of COVID-19 was designed to investigate the effect of serine protease trypsin as a potential cellular stressor on a disease-competent monocytic THP-1 cell line that could result in COVID-19-associated pathways upon transcriptomic profiling. The cellular model was a slight modification of our previous findings [102], as we incorporated healthy plasma in the current model in addition to the use of serum-free media and the testing of escalating doses of trypsin. Briefly, monocytic THP-1 cells were cultured on a combination of serum-free media and plasma (control, C); THP-1 cells cultured on control/C supplemented with low trypsin (30 μM) were named low proteolytic stress (LPS), and THP-1 cells cultured under control/C supplemented with high trypsin (100 μM) were named high proteolytic stress (HPS). Finally, we evaluated the growth factor responsiveness of THP-1 cells in the presence of 100 μM of trypsin. Briefly, adult retinal pigment epithelial (ARPE-19) cells were cultured in 100 μM of trypsin in serum-free media at a density of 1 million cells/mL for 72 h. The condition media were collected and used to treat THP-1 cells for a further 72 h. The ARPE-19-conditioned media well-characterized by us and is marked by the secretion of biologically active bFGF (basic fibroblast growth factor) and interleukin IL-1ß, as determined with multiplex assays [99–101]. The ARPE-19-conditioned media therefore contained bFGF, IL-1ß and active trypsin (as qualitatively determined by gelatin zymography—data not shown), but absolute concentrations of trypsin were not determined with a commercial trypsin substrate. We named this group HPS-GFC (high proteolytic stress growth factor cytokine). The THP-1 cells cultured under trypsin conditions were incubated for 72 h at 37 °C and 5% CO_2. Each experiment was conducted in triplicate.

3.2. Transcriptomic Profiling

RNA sequencing was outsourced to Redcliffe Life Tech, Noida, India, which performed transcriptomics using an NGS Illumina platform with a 150 read length and paired end sequence layout. Quality control was carried out using the fastp tool (0.20.1) to provide clean data for downstream analysis. NGS sequencing reads were mapped with the Hisat2 (2.1.0) alignment tool. For abundance estimation, the data input was subjected to the Counts (2.0.1) tool in either the Sequence Alignment Map or Binary Alignment Map format. Differential gene expression and visualization were carried out using the DeSeq2 (1.8.3) tool. Proteolytic stress was tested to assess the upregulation of COVID-19-relevant inflammatory pathways using unbiased transcriptomic profiling.

Gene Set Enrichment Analysis ridge plots were used for significant KEGG terms. Ridge plots are density plots of the frequency of log2 fold-change values per gene within each enriched KEGG group, which helps to interpret the up- or downregulation of that KEGG category. Here, plots were created in clusterProfiler using KEGG orthologue annotations and log2 fold changes per gene calculated by DESeq2 during differential expression analysis. *X*-axis is the log2 fold change in expression for genes present in each plotted KEGG category, with positive values indicating upregulated expression in replicates and negative values indicating downregulated expression in replicates. Peaks are colored by corrected *p*-value, as shown by the legend, and corrected *p*- and q-values are shown per KEGG category.

4. Results

Interestingly, the monocytic cells upregulated COVID-19-associated pathological pathways upon escalating proteolytic stress while concomitantly upregulating metabolic stress, suggesting the importance of ensuing proteolytic stress underlying COVID-19 pathology. The combined role of metabolic and proteolytic stress resulted in the upregulation of

COVID-19 pathways that co-segregated with complement–coagulation, olfactory transduction, steroid biosynthesis, iron-ion binding, ferroptosis, and maturity-onset diabetes (Figures 1–5).

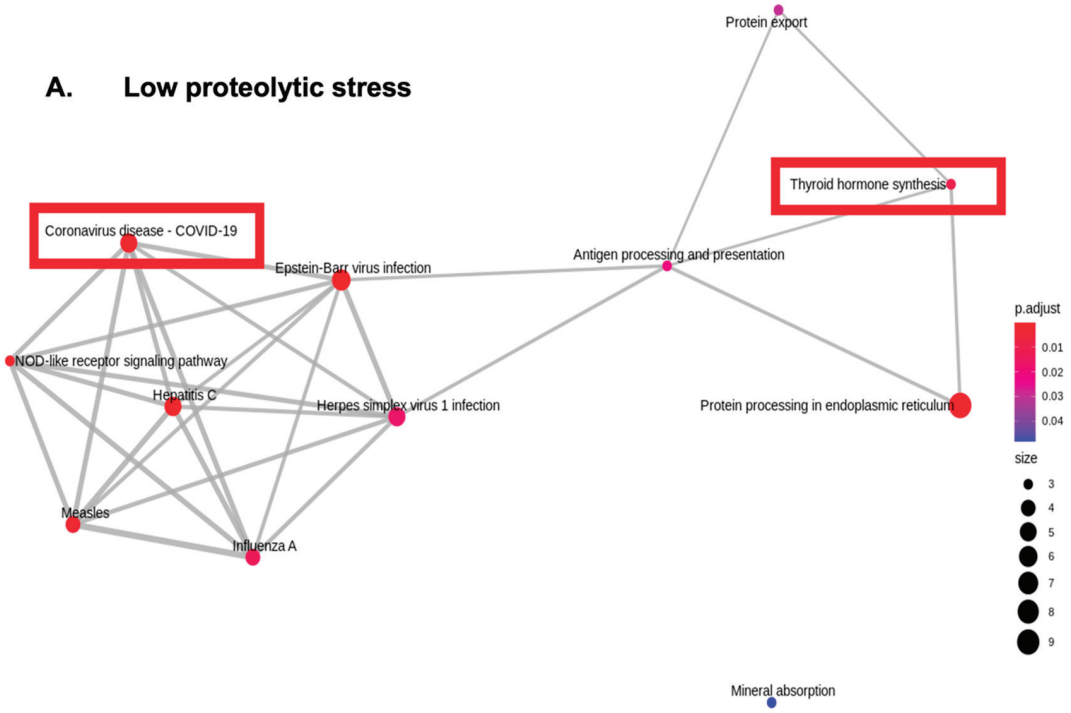

Figure 1. *Cont*.

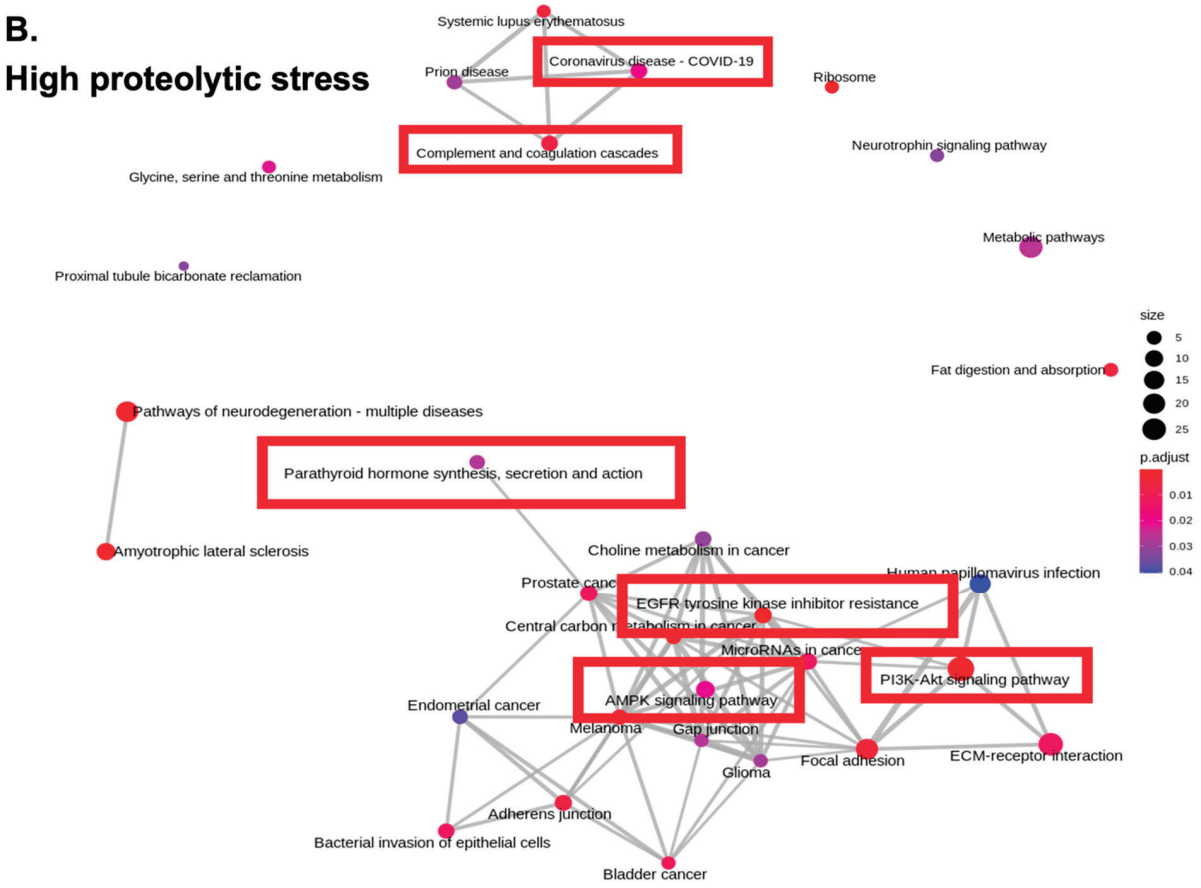

Figure 1. GO plots of enrichment analysis Panel (**A**): COVID-19 pathways are upregulated following proteolytic stress. Transcriptomic profiling data of monocytic THP-1 cells treated with prototypic serine protease trypsin (proteolytic stress) at a low concentration are represented as low proteolytic stress (LPS) compared to control/C in Panel (**A**). GO plots of enrichment analysis Panel (**B**): Those treated with trypsin at escalating concentrations are represented as high proteolytic stress (HPS) compared to control/C in Panel (**B**). The LPS pathway network indicates upregulation of antigen processing and presentation, thyroid hormone synthesis, and COVID-19 pathway upregulation. The interferon response pathway genes were also found to be overexpressed under LPS conditions (data not shown), suggestive of a mild-COVID-19-like disease phenotype. The HPS condition was characterized by upregulation of COVID-19 pathway along with complement–coagulation, metabolic, neurodegenerative, and several bacterial and viral infectious pathways, thus indicating a hyper inflammatory or severe COVID-19-like disease pathway in vitro. HPS was found to result in concomitant upregulation of metabolic stress, which may have implications in pathophysiology of COVID-19 as a plethora of trypsin-like serine proteases including TMPRSS2, furin, DPP4, complement–coagulation serine proteases, neutrophil elastase, and trypsin are upregulated during the inception and course of the disease.

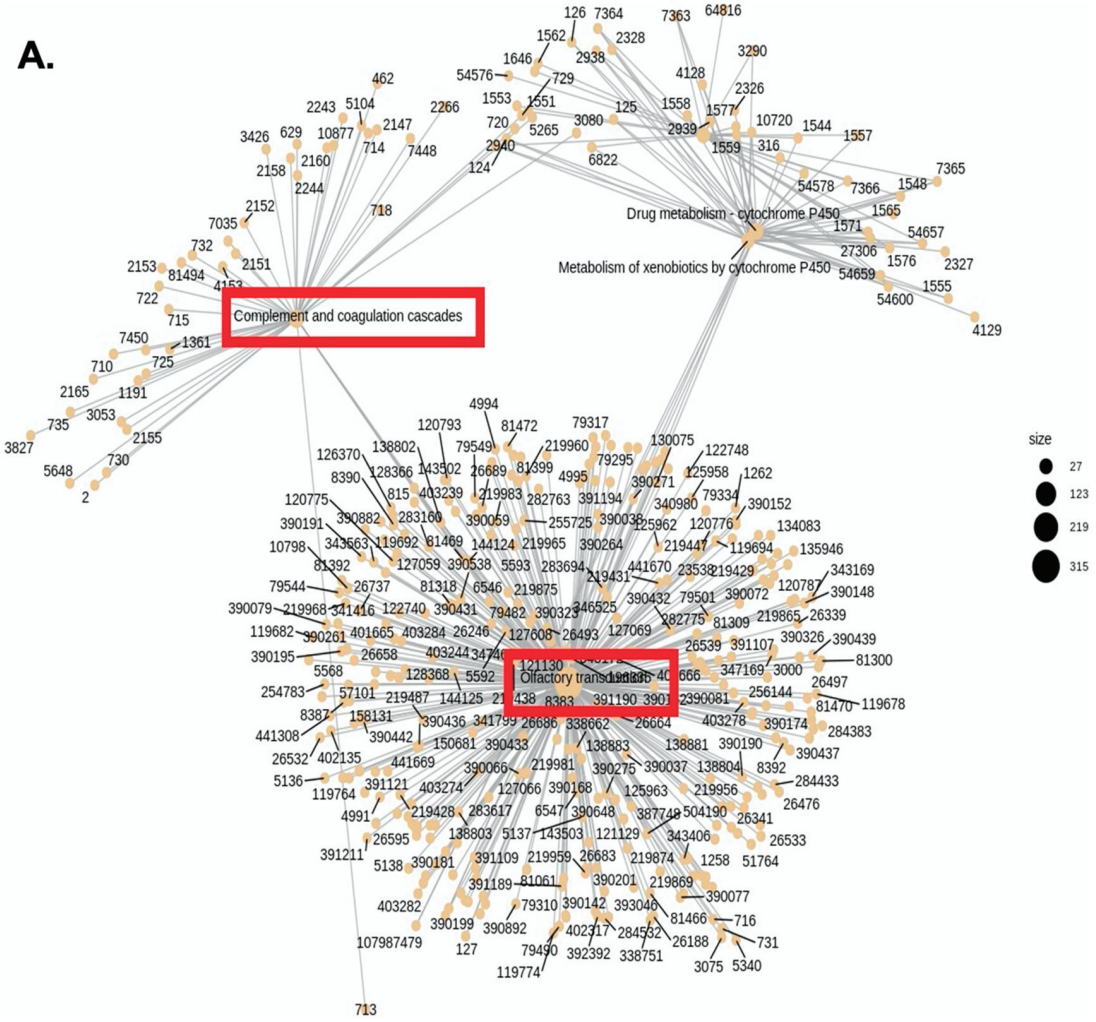

Figure 2. *Cont*.

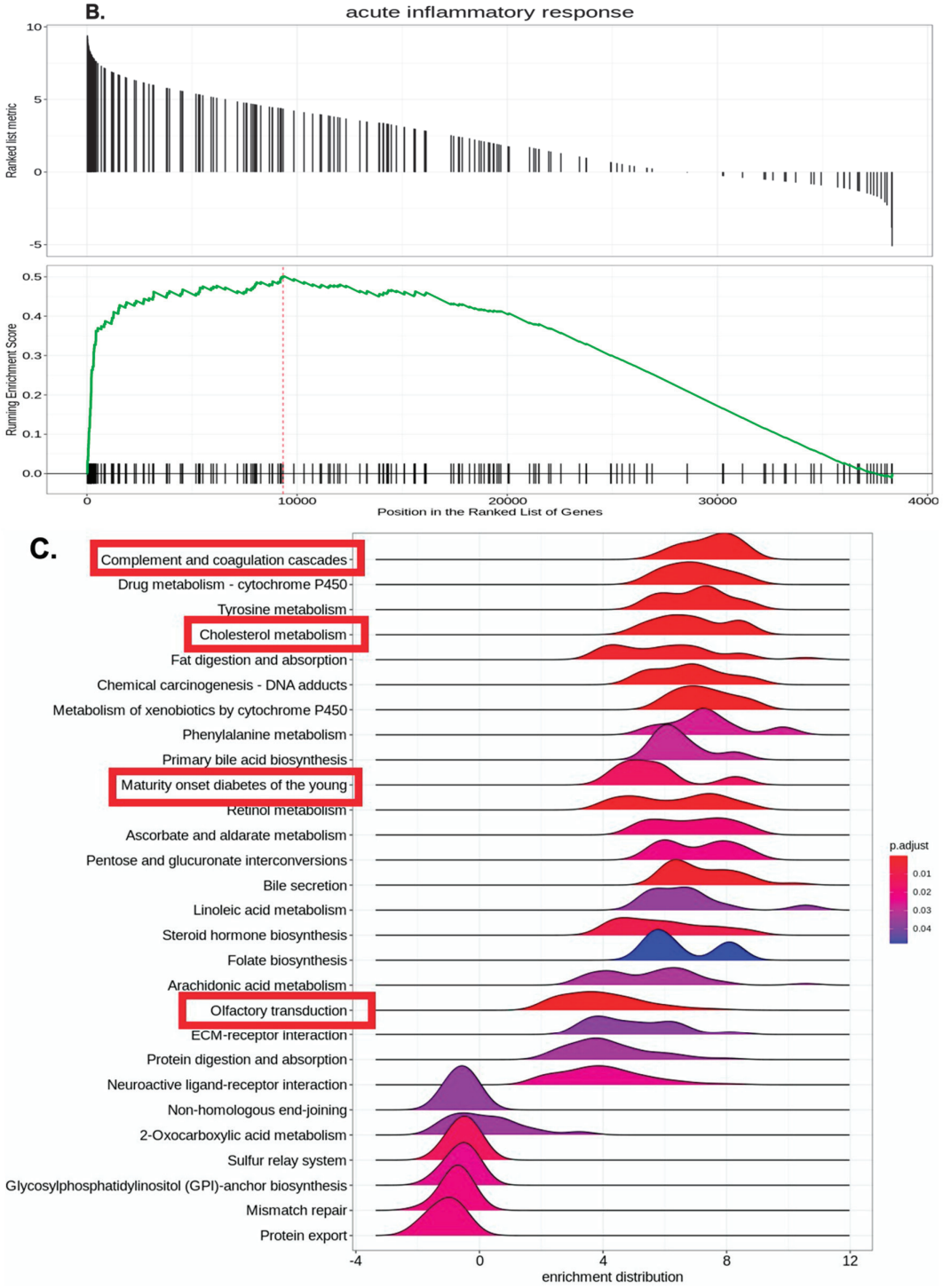

Figure 2. Category Net plot: HPS treatment compared to control/C is marked by the upregulation of neurosensory olfactory transduction and complement coagulation pathways Panel (**A**); GSEA (Gene Set Enrichment Analysis) plot: acute inflammation Panel (**B**); and ridge plot for GO enrichment pathways: metabolic and maturity-onset diabetes pathways Panel (**C**). HPS was found to result in concomitant upregulation of metabolic stress, which may have implications in pathophysiology of COVID-19 because trypsin-like serine protease TMPRSS2 is upregulated in olfactory epithelium following SARS-CoV-2. This can induce metabolic stress, olfactory dysregulation (anosmia), and hyperinflammation.

Figure 3. *Cont.*

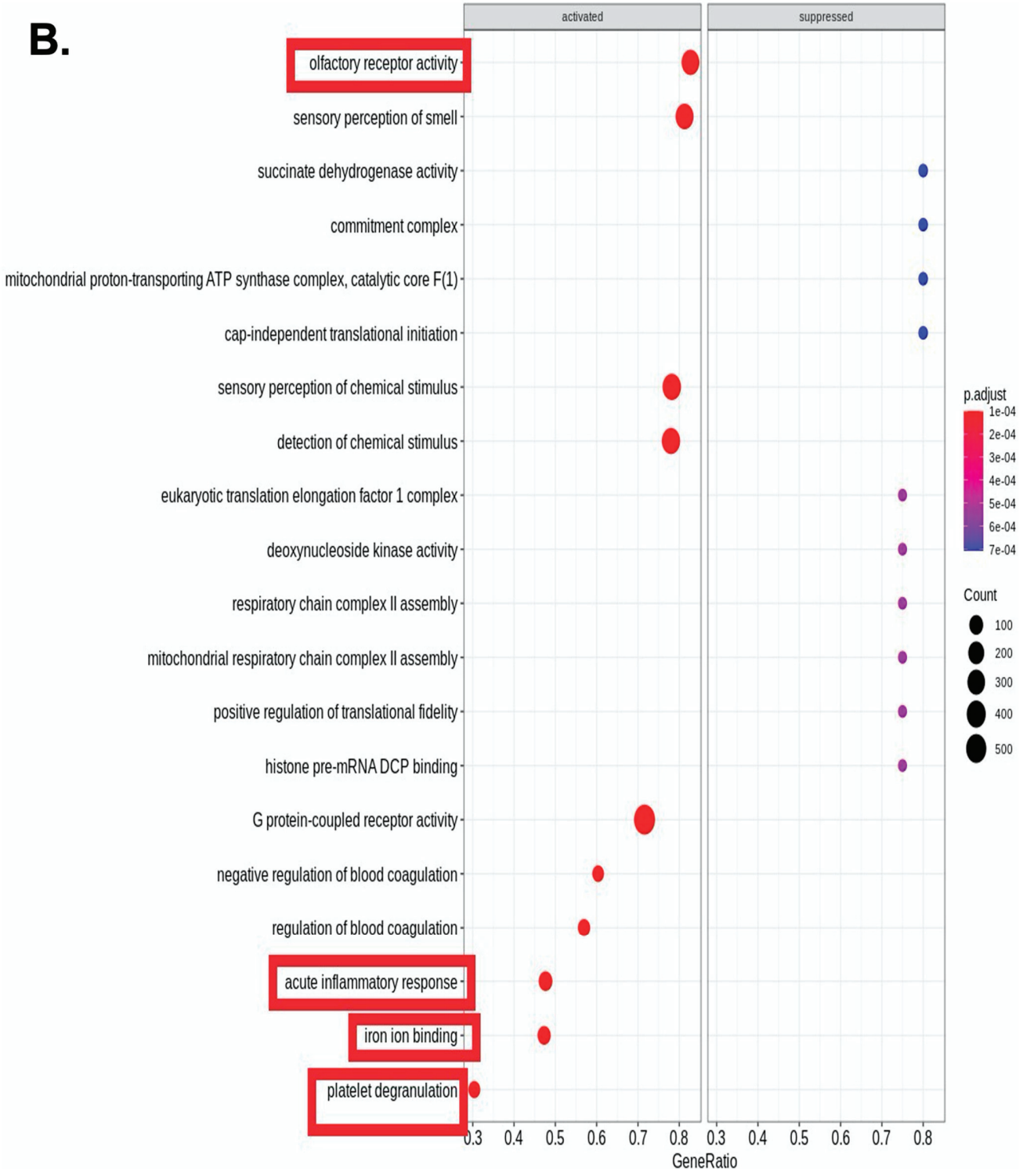

Figure 3. GO plot of enrichment analysis: HPS treatment compared to control/C is marked by upregulation of iron-ion binding Panel (**A**); dot plot for GO enrichment pathways: deregulation of neurosensory, acute inflammatory, and metabolic (diabetes) pathways Panel (**B**). This may have implications in pathophysiology of COVID-19-associated new onset diabetes and COVID-19-associated mucormycosis (CAM) because iron is an important nutrient for mucor growth and virulence.

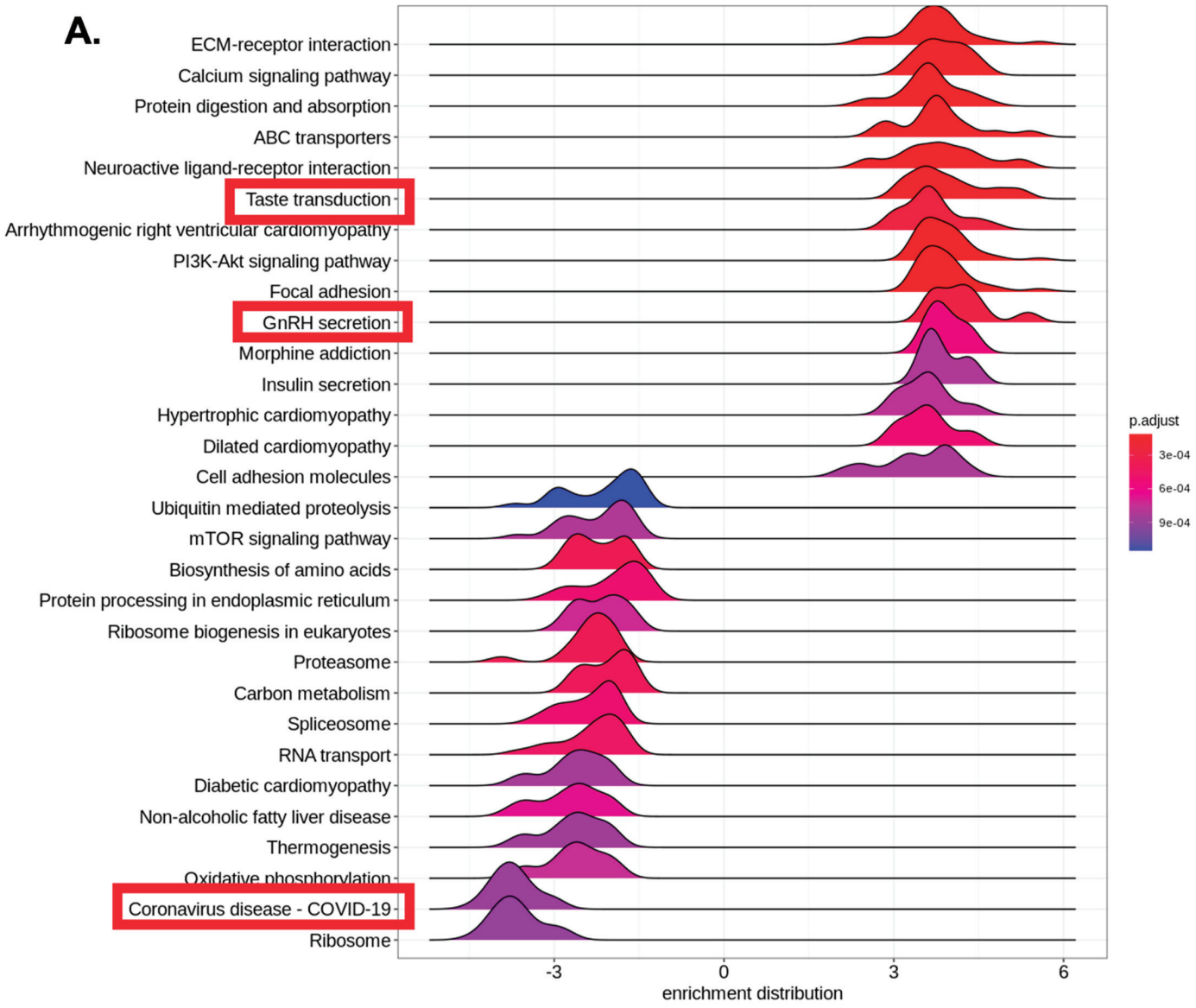

Figure 4. *Cont*.

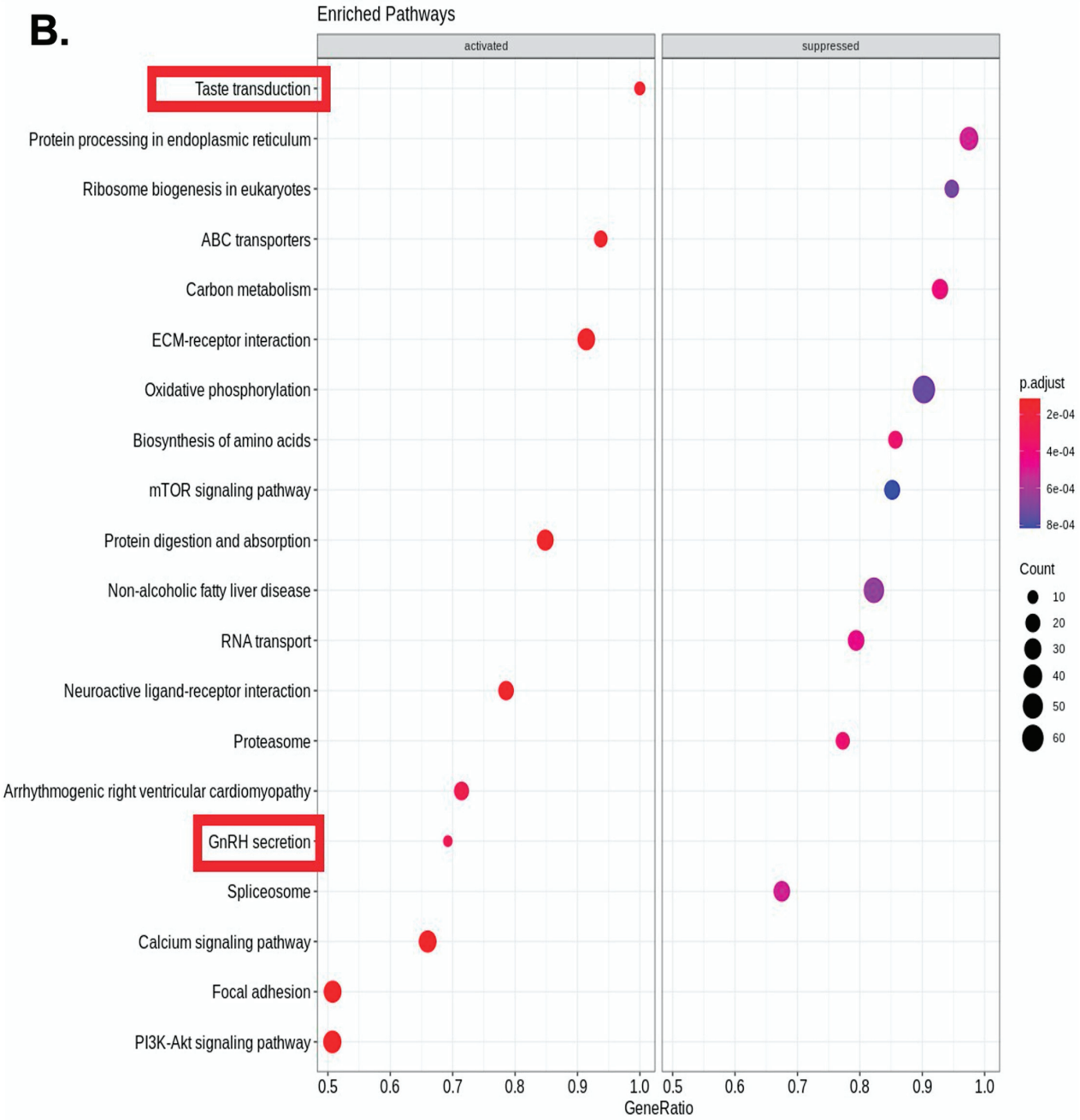

Figure 4. Ridge plot for GO enrichment pathways Panel (**A**): HPSGFC treatment is a unique condition that combines high proteolytic stress and growth factor levels and cytokine effects (for details, refer to the methodology section). Dot plot for GO enrichment pathways Panel (**B**): HPSGFC treatment compared to control/C is marked by the upregulation of neuroactive ligand receptor interaction pathways: the neurosensory (olfaction and taste), neuroendocrine (GnRH), and metabolic rebalancing (insulin secretion) pathways Panels (**A**) and (**B**): This treatment group resulted in downregulation of the COVID-19 pathway and associated carbon metabolism, spliceosome, oxidative phosphorylation, and RNA transport pathways. This has implications for COVID-19 and CAM because selective growth factor and cytokine responsiveness can help control COVID-19 severity, rescue metabolic health, and ameliorate neurosensory regeneration. The reversal of diabetes (insulin secretion) was linked to the downregulation of the COVID-19 pathway in our in vitro disease modelling. A regulated IL-1 beta/inflammasome activation pathway can have beneficial effects in controlling COVID-19.

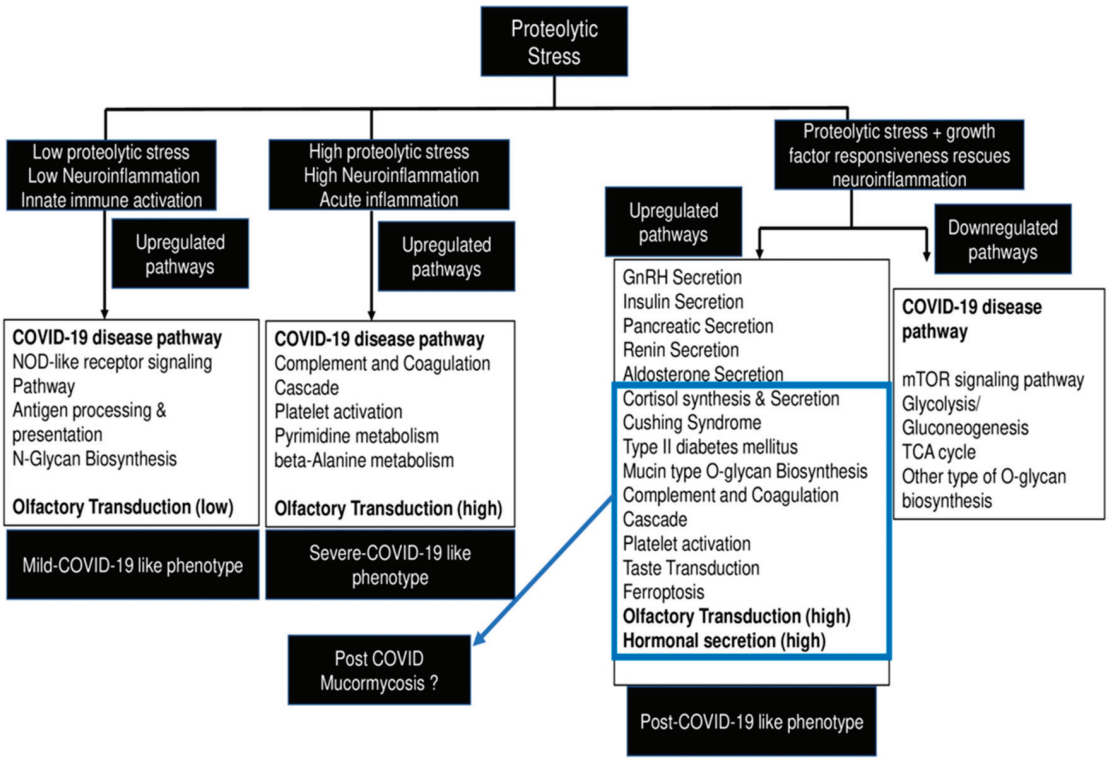

Figure 5. A working model of the development and course of COVID-19 based on our in vitro data. Transcriptomic profiling data of monocytic THP-1 cells treated with prototypic serine protease trypsin (proteolytic stress) at a low and escalating concentrations are represented as low proteolytic stress (LPS) and high proteolytic stress (HPS) compared to control/C, respectively. HPSGFC condition is depicted in the extreme right under the heading "proteolytic stress + growth factor responsiveness". The addition of neuroregenerative growth factor to the high proteolytic environment is speculated to ameliorate COVID-19, but the upregulation of cortisol secretion can result in development of T2DM and post-COVID mucormycosis, even in the presence of the sufficient secretion of insulin. Olfactory transduction can be linked to both COVID-19 development and downregulation depending on the presence of growth factor responsiveness. We present the differentially regulated significant biological pathways under various treatment groups, data collected with the extended GO biological pathway enrichment analysis tool (data not shown). We use the term "neuroinflammation" to link co-expression of olfactory transduction and coagulation–complement pathways, and we hypothesize that neurosensory olfactory transduction can keep immune responses under check and limit hyperinflammation in presence of soluble growth factors and cytokines, as in HPSGFC conditions. Endocrine and neuroendocrine reshaping is also associated with growth factor and cytokine responsiveness. Therefore, olfactory dysfunction or anosmia alone may not be strong predictors of the course of COVID-19. Additional parameters such as growth factor assessment, proteolytic activity and neuroendocrine peptides in the plasma and nasopharyngeal swabs could help improve stratifications of disease severity.

Furthermore, comparisons of diverse treatments allowed us to classify groups that displayed the following: (a) the COVID-19 pathway along with the co-expression of upregulated interferon response, oligoadenylate synthetase, double-stranded RNA binding, and innate immune recognition pathways and a downregulated olfactory transduction pathway, thus suggesting a mild-COVID-19-like pathological response; (b) COVID-19 pathway co-segregating with upregulated complement coagulation, acute inflammatory response, and steroid biosynthesis pathways, thus suggesting a severe COVID-like pathological response; and a unique group (c) a downregulated COVID-19 pathway co-segregated with upregulated olfactory transduction, an RNA interference pathway, and a TCR receptor pathway. We hypothesize a potential (novel) adaptive immune reshaping linked to

olfactory transduction through a neuropeptide-signaling pathway that could reshape inflammatory responses by inducing antigen-specific T-cell responses by involving the RNA interference/RISC pathways of gene silencing and regulation. We are in the process of establishing the significance of these pathways in diabetic patients with COVID-19 and CAM [Sharma et al., unpublished work]. We earlier proposed pathogen-invoked proteolytic activation as a novel cellular stress-inducing process that could facilitate viral-competent immunomodulation through reprograming RNA metabolism and homeostasis in a process involving the protease-induced transcriptomic/epi-transcriptomic reshaping (PITTR) of host cells to counter cellular stress [102].

The pre-symptomatic or acute phase of COVID-19 marked by high viral loads/ replication in the nasal epithelium makes it a potential target to inhibit the intensification of infection by limiting spread of the infection. Therefore, the prophylactic/therapeutic targeting of the olfactory epithelium by employing intranasal sprays is envisioned as a potential approach to prevent COVID-19 severity. The use of intranasal drug formulations are highly encouraged in COVID-19 and CAM treatment due to initially higher viral loads in the nasal microenvironment and their direct detrimental impact on infection inception and clinical course. Recently developed imidazole compounds have been reported to enhance anti-viral interferon responses upon intranasal administration in animal settings [103]. Similarly, recent phase-II clinical trials from Canada and the UK, showed the efficacy of nitric oxide nasal spray (NONS) in reducing SARS-CoV-2 viral loads for treatment of COVID-19 infections [104,105]. We envision strong anti-diabetic and antimicrobial effects of the combination of anti-metabolic and anti-proteolytic (MPI) inhibitors. Metabolic stress/hyperglycemia intensifies systemic SARS-CoV-2 and nasal/olfactory ROCM spread. We expect a prophylactic use of the MPI intranasal formulations in diabetes patients that could exhibit beneficial effects in regulating ferroptosis, promoting iron homeostasis, and facilitating the management of COVID-19 and CAM due to its anti-diabetic, anti-inflammatory, anti-oxidant, and anti-chelating properties.

5. Discussion

5.1. Hexosamine Biosynthetic Pathway of Glycosylation and Metabolic Stress Calibration in Olfactory Mucosa

Metabolic rewiring is a common mode of adapting to cellular stress to restore homeostasis due to its plasticity in cellular metabolism. As evidenced in uncontrolled hyperglycemia, persistent metabolic alterations can result in the irreversible resetting of the metabolic machinery, thus resulting in the desynchronization of the functional outputs. The primary bioenergetically tuned metabolic pathways include AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), the hexosamine biosynthetic pathway (HBP), and iron handling. Major cellular calibrants of energy stress include the innate immune myeloid lineage monocyte/macrophages and neutrophils that respond to metabolic stress with remarkable resilience by adjusting to distinct metabolic states by displaying phenotypic, transcriptomic, functional, and spatial heterogeneity, thereby diversifying the inflammatory immune responses. Accordingly, burgeoning metabolic diabetes disorders are linked to myeloid cell dysfunction, which has been recognized as important feature in SARS-CoV-2 infection. Diabetes and hyperglycemia have been integrally linked to the severity of COVID-19, and uncontrolled diabetes mellitus and COVID-19 have recently been independently or in combination associated with the emergence of aggressive mucormycosis [16,19,106–116]. The induction of diabetes in SARS-CoV-2 infectious settings has also been demonstrated [47,48,117], suggesting an important role of metabolic resetting during COVID-19 disease course.

Myeloid lineage macrophages are central to anti-fungal/anti-Mucorales immunity. Therefore, "long COVID" and "post-infectious hyperinflammatory diseases" marked by metabolic and myeloid-immune deregulation are outcomes of COVID-19 that offer secondary fungal infections at the forefront due to ensuing immune deregulation after COVID. A consistent observation during the current unprecedented mucormycosis surge is under-

lying diabetes, which is also a strong comorbidity for COVID-19 severity, thus suggesting a potential role of metabolic sensors in the regulation of immune responses during infectious diseases. However, the mechanistic insights behind the correlative T2DM-associated deregulation of epithelial–endothelial barrier integrity and mucosal immunity remain relatively underexplored [20,118–130].

Importantly, infection-triggered (SARS-CoV-2/Mucorales) inflammasome-mediated pyroptotic cell death has been intricately linked to release of DAMPs and extracellular ATP, monocyte/macrophage activation, epithelial and endothelial cell damage, and vascular leakage, all resulting in inflammatory immune cell infiltration into the nasal/respiratory mucosa. The proinflammatory cytokines consequently lead to mucosal mucin reshaping and the activation of thromboinflammation. What remains underappreciated is the fact that the pathogenic microbes (SARS-CoV-2/Mucorales) have to traverse the bulky polyanionic–mucus barrier (20 μm) and heparan sulfate proteoglycans (HSPGs) (50 nm), embedded in the cell membranes to reach and destroy the nasal/airway epithelial cells. The mucins (terminally charged sialic acid and hydrophobic fucose) and polyanionic HSPGs offer potential structural frameworks for mediating interactions with primary binding motifs in the infecting microbes (e.g., cationic RBD groove of SARS-CoV-2 and fungal lectins attach to mucins and HSPGs to facilitate binding and entry to the host cells). Following binding, the microbes can be inactivated/cleared-off by mucociliary clearance or be subjected to infection spread, depending on the cellular/extra cellular metabolic milieu. The anti-pathogenic and rheologically flexible properties of mucus come at the cost of the high energy consumed in the process of mucous hydration in the airway system, which is highly dependent on the pathologically overexpressed metabolites (alarmin/DAMPs) including extracellular ATP and UDP-sugars derived from the HBP pathway (glucose/galactose) [131–135]. Therefore, it is reasonable to hypothesize that diabetes-associated metabolic pathways participate in reshaping mucin biology and innate immunity in the upper airways, thus making them susceptible to infection and the spread of microbes.

It could be speculated, that T2DM-responsive bioenergetically tuned HBP deregulation alters the "metabolic glycan-code input"; as opposed to the primary metabolic sensing by myeloid "cellular sensors" such as macrophages/monocytes. This metabolic perturbation (altered glycosylation) underlying diabetes is first read by the innate immune "structural sensors", such as airway epithelial mucins and endothelial glycocalyx that are heavily glycosylated macromolecules. Epithelial mucins and endothelial glycocalyx are arranged as multiscale hierarchal structured fluids in nasal and respiratory epithelia, therefore acting as the frontline structural barricades of the mucosal innate immune system. The altered glycosylation patterns in nasal/respiratory mucosa and endothelial glycocalyx compromise epithelial–endothelial barrier integrity and offer easy pathogenic access to the underlying epithelium/endothelium, thus resulting in enhanced spread, enhanced vascular permeability, and thromboinflammation. Concomitantly, there is heightened purinergic signal transduction via the HBP intermediate UDP-sugar (ligand)-mediated activation of metabotropic purinoreceptor P2Y14 (expressed on adipocytes, myeloid immune cells, hematopoietic stem cells, kidneys, lungs, and intestines). The P2Y14 receptor was recently implicated as a strong target for neutrophilia attenuation in severe COVID-19 [132]. However, no data on COVID-19 or mucormycosis patients are available to date. P2Y14 expression is linked to glucose and lipid homeostasis [136], as well as the amplification of allergen-induced hyperinflammatory airway eosinophilia [135]. The HBP pathway has also been recently linked to ferroptosis sensitivity [137], thereby indicating a potential druggable "diabetes–HBP–mucin–iron axis" in COVID-19 and mucormycosis infections. Diabetes-associated, HBP-mediated altered glycosylation patterns in epithelial mucins and the role of the HBP-derived, UDP-sugar-activated P2Y14 purinoreceptor in myeloid immune deregulation remain subjects that have yet to be explored.

5.2. Diabetes and Metabolic Iron Redox-Stress- Macrophages as Ferrostats

It is important to note that the immune cells that act as primary metabolic sensors with high plasticity in metabolic switching (glycolytic vs. oxidative phosphorylation) are myeloid lineage macrophages, which are also central to anti-fungal/anti-Mucorales immunity. The longitudinal profiling of respiratory and systemic immune revealed myeloid cell-driven inflammatory events in COVID-19 [114]. The differential activation potential of NLRP3 inflammasome among myeloid cells is suggested to be a biomarker for the course of COVID-19 [115]. SARS-CoV-2 was shown to engage inflammasome and induced pyroptosis in monocytic cells [116]. Therefore, the metabolic reprograming of macrophages during hyperglycemia potentially alters the gene expression and regulation of immune pathways important for innate immunity and subsequent macrophage-sculpted adaptive immunity. Histone acetylation was recently reported to be upregulated in macrophages following concerted increases in glycolytic flux and ATP-citrate lyase activity [109]. Monocytopenia and morphological monocytic defects were found to be associated with hyperinflammation in COVID-19 in T2D patients [110], and hyperglycemia was shown to drive proinflammatory M1 polarization in macrophages via the TLR4–IRF5 pathway [111]. The defective macrophages thus fail to efficiently reshape the adaptive immune responses during inflammatory diseases such as COVID-19.

The enhancement of $CD8^{+}$T cell effector functions (shaping extra-follicular adaptive immune responses) and the identification of repertoire of neutralizing antibodies to offer protective immunity post-infection are intensive areas under research to prevent infection, re-infection, or superinfections. Persistent alterations of iron homeostasis/hyperferritinemia in COVID-19 are also associated with immune deregulation [138–162]. Importantly, glycans/glycosylation is becoming increasingly relevant in shaping T cell effector functions and immunological synapses [163].

Macrophages are well-appreciated for their role in systemic iron homeostasis, in which they sequester iron during pathogenic infections and release iron for erythropoiesis. Importantly, immunometabolic resetting in macrophages is increasingly becoming linked to their handling of systemic iron recycling by regulating the unidirectional flux of non-transferrin-bound iron (NBTI/non-Tf-Fe^{3+}) and erythrophagocytosis driven by CD163, CD91, and CD47 receptors. Therefore, iron overload can be highly toxic and needs to be strictly governed to maintain iron homeostasis [164,165]. Macrophages have also been highly implicated in metabolic disorders such as T2DM and neurodegenerative diseases, as iron-dependent lipid peroxidation can cause oxidative damage in these diseases via Fenton chemistry. The crosstalk between iron and immune cells (wherein iron trafficking is regulated by cytokines and acute phase proteins) and the iron-dependent cell fate determination of macrophages and lymphocytes during inflammation underscore the requirement for the tight control of iron metabolism and homeostasis [166–173].

5.3. Iron Metabolism and Homeostasis—Can Ferroptosis Be the Game Changer?

The authors of a recent case-control study from our health care center in India on COVID-19 patients with and without mucormycosis compared the baseline iron parameters including iron, ferritin, total iron-binding capacity, and percentage transferrin saturation. However, these iron indices did not reveal any significant differences between CAM survivors and non-survivors [174], indicating that much needs to be understood regarding the complexity of iron biology during infectious pathologies such as CAM. Iron or iron-containing complexes, such as heme and iron–sulfur (Fe–S) clusters are integral to several biological processes such as oxygen transport (hemoglobin), oxygen storage (myoglobin), bioenergetic pathway intermediates (cytochrome-c), metabolic pathway intermediates (amino acid oxidases, fatty acid desaturases, and lipoxygenases), cellular detoxification (cytochrome P450), and cellular defense (nitric oxide synthase, NADPH oxidase, and myeloperoxidase). Notably, the cellular homeostatic machinery is highly reliant upon the cellular redox status, which is determined by the total cellular output of the ROS and antioxidant system. Iron availability is central in maintaining the cellular redox state

because it is available in two highly interconvertible forms: reduced Fe^{2+} (soluble) and oxidized Fe^{3+} (insoluble). Iron cofactors such as heme, iron–sulfur clusters (Fe–S), and iron-oxo systems participate in a wide range of biological processes in electron transfer reactions and are central to metabolic pathways, mitochondrial function, cell cycle, and more [175–181]. The use of ferritin-bound iron is a cells' protective mechanism of storing iron in macrophages and hepatic cells. Ferritin-bound iron release is a tightly regulated process known as ferritinophagy that involves the nuclear receptor coactivator 4 (NCOA4)-mediated transfer of ferritin to autolysosomes, as well as degradation and iron release for the biosynthetic pathways [182].

The overt production of ROS via chemically and reactive Fe^{2+} ions is detrimental to cellular membranes because it oxidizes membrane phospholipids and results in membrane rupture and release of DAMPs, which trigger sterile inflammation in iron-dependent regulated cell death known as ferroptosis. Ferroptosis comprises a rapidly evolving research field with enormous therapeutic potential, as it is becoming increasingly linked to inflammatory diseases, infectious diseases, and inflammation-associated immunosuppression. Ferroptosis can be induced by extrinsic (the inhibition of membrane cystine/glutamate antiporter system XC^-) or intrinsic pathways (the inhibition of anti-oxidant glutathione peroxidase/GPX4). Though the terminal effectors of ferroptosis are not clearly understood, it is a form of iron-dependent, peroxide-mediated regulated cell death (RCD) that is distinct from other RCD modes including apoptosis, necroptosis, and pyroptosis because it is independent of the activities of the caspases (apoptosis), MLKL (necroptosis), and gasdermin D (pyroptosis) molecular effectors [183–196].

Ferroptosis has been increasingly linked to the regulation of inflammatory networks in innate immune cells such as neutrophils and macrophages that exhibit graded polarities and differential sensitivities to ferroptosis [197], with pro-inflammatory M1 macrophages marked by the secretion of high iNOS, cytokines, and lipid mediators displaying ferroptotic-resistant phenotypes, as opposed to pro-ferroptotic propensity of pro-resolving alternatively activated M2 macrophages that secrete anti-inflammatory molecules. The intracellular redox regulation driven by metabolic iron, thiols, and lipid peroxides is emerging as a new mode of cellular reprograming under the term "redox-stress" that may have potential implications in the resetting of pro-inflammatory pathways driven by the ferroptotic mode of cell death, which is marked by the loss/rupture of the oxidation sensitive phospholipid plasma membranes followed by the release of intracellular DAMPs, resulting in the propagation of sterile inflammation. This is in striking contrast to the anti-inflammatory apoptotic mode of cell death that is marked by the preservation of cell membrane integrity and the containment of intracellular contents that are cleared by hydrolytic digestion and phagocytic engulfment by macrophages [198,199]. Iron-regulatory processes tend to have cell-specific mechanisms [200,201]. Iron-overload-triggered ferroptosis can be inhibited by iron chelators such as deferoxamine, deferiprone, and ciclopirox that decrease intracellular iron levels [202]. Ferroptosis has become increasingly linked to bacterial and yeast infections [203–205], but further detailed investigations are warranted in many contexts before targeting ferroptosis as a feasible strategy in infectious pathologies. Nevertheless, emerging opportunities for anti-ferroptotic agents in neurological disease settings are already in progress.

5.4. Pro-Ferroptotic Labile Iron Pool (LIP) and RNA-Binding Proteins (RBPs) in Regulation of Ferroptosis and Diabetes

The reactive iron-free radicals in the LIP can oxidatively damage the plasma membrane unsaturated lipids (PUFAs) [175,206]; therefore, the LIP is intrinsically under tight control by the action of iron-buffering compounds such as iron–glutathione (Fe–GSH) complexes (cytosolic GSH occurring at 2–10 mM and complexing Ferrous ions occurring at 1:1 stoichiometry) [207] and iron chaperones. Fe–GSH complexes are reported to be chaperoned by the poly(rC)-binding protein (PCBP) family that dictates intracellular iron distribution and transport [208]. PCBPs (1/2) chaperone iron (at low micromolar affinity) to and from

iron transporters (DMT1, ferroportin 1), iron storage pools of ferritin, and iron-containing prolyl hydroxylase (PHD), deoxyhypusine hydroxylase (DOHH), heme-oxygenase (HO-1), and BolA2. PCBPs are RNA-binding proteins (RBPs) with intrinsically disordered regions (IDRs) composed of hnRNP-K-homology (KH) domains that preferably bind to Fe–GSH and facilitate iron transport to client proteins. As the affinities of Fe–GSH complexes and PCBP1 coordination lie in sub-micromolar ranges, it has been suggested that the majority of the cytosolic LIP exists in the PCBP1–Fe–GSH format [199,207]. Therefore, RBPs such as PCBPs intracellularly regulate the chemical reactivity and trafficking of pro-ferroptotic LIP. Ferritin sequesters iron in large amounts as inert ferric oxyhydroxides that can be mobilized by the cells in a process of ferritin degradation via lysosomes or ferritinophagy [209,210] (which is marked by the binding of ferritin to its cognate autophagic cargo receptor NCOA4), and the consequently liberated iron is transported back to the cytosol or mitochondria (erythroblasts) to begin heme synthesis; ferritinophagy and ferroptosis are accordingly implicated in the management of metabolic diseases [211]. PCBP1- and NCOA4-driven ferritinophagy could mobilize large pools of stored iron that may have pro-ferroptotic roles via the delivery of PCBP1-mediated iron to client ferroptotic proteins. PCBP1 chaperones can deliver ferrous ions to mono- or di-iron centers such as HIF-1α (pro-inflammatory macrophages are marked by mitochondrial ROS production and HIF-1α stabilization [212]), the regulating PHD2 enzyme (mono iron center), and the DOHH monooxygenase enzyme (di-iron center), which participates in the hydroxylation of the modified amino acid hypusine that is exclusively present in the eIF5a translational initiation factor. Deoxyhypusine synthase (DHPS)-driven eIF5A hypusination was recently shown to be a feature of proinflammatory macrophages, and *DHPS* deletion in macrophages resulted in improved insulin sensitivity and glucose homeostasis. Furthermore, hypusination is being increasingly implicated in metabolic diseases and microbial infections [213,214], hypusine polyamine precursors have been reported in intestinal epithelial renewal and M2 macrophage polarization [215], and dietary spermidine was recently shown to boost eIF5A hypusination and protect mitochondrial dysfunction during brain aging [216]. The polyamine pathway intermediate spermidine was recently reported to induce anti-inflammatory pathways following release from apoptotic cells [217]; it has protective effects in metabolic diseases and has emerged as a well-tolerable caloric restriction mimetic and provider of nitric oxide and arginine bioavailability [218]. Interestingly, the SAT1 (spermidine N1 acetyltransferase) gene was recently reported as a metabolic transcription target for p53 and a pro-ferroptotic molecule resulting in lipid peroxidation upon ROS stress [219]. Targeting the polyamine metabolic pathway was reported as a promising approach to control viral infections including SAR-CoV-2 [220]. SAT1 is a catabolic enzyme in the polyamine pathway that leads to the conversion of anti-inflammatory spermidine to N1 acetylspermidine. We recently observed the upregulation and downregulation of N1 acetylspermidine in COVID-19 and CAM plasma samples, respectively, via unbiased metabolomic profiling (unpublished data). Additionally, SAT1 and other ferroptotic pathway genes were differentially regulated in our in vitro culture model that recapitulated the disease modeling of COVID-19 pathways (Figure 6), thereby warranting detailed investigation to understand the role of metabolic ferroptotic and polyamine pathway intermediates in infectious diseases such as COVID-19 and CAM. Amino acid metabolism modifications, such as hypusination, require further investigations to understand their role in regulating infectious and metabolic diseases.

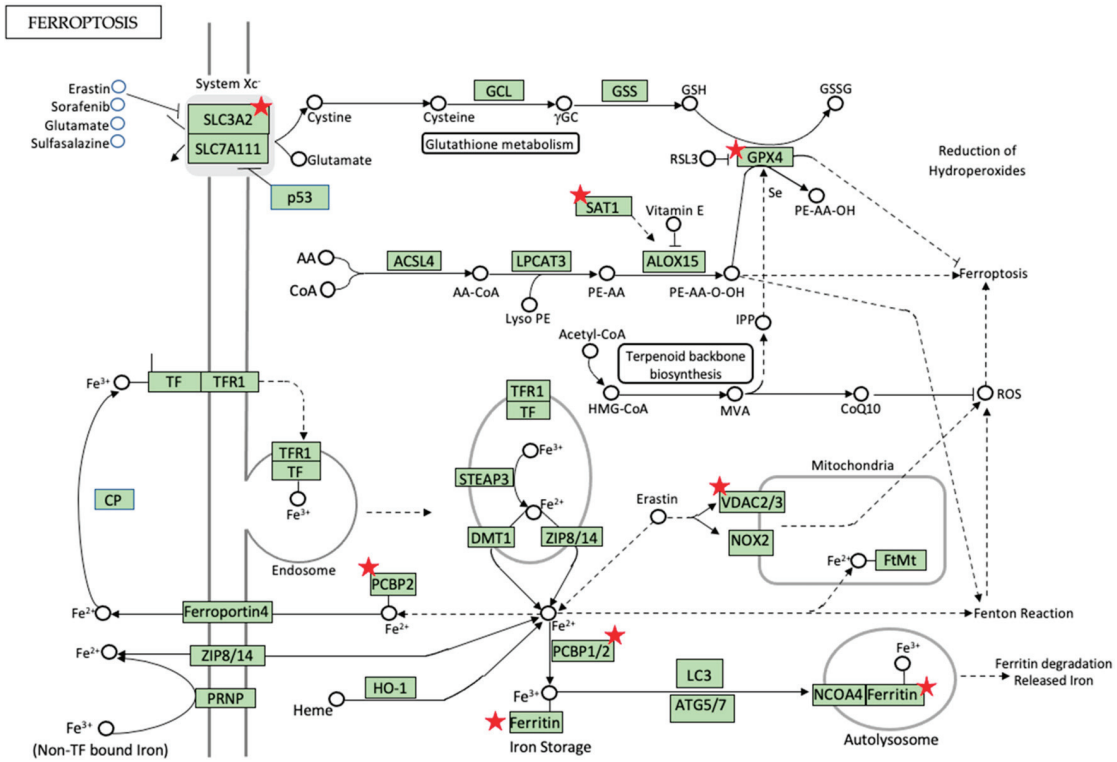

Figure 6. Proteolytic stress coupled to growth factor responsiveness is marked by downregulation of ferroptotic pathway genes (highlighted in red), indicating that resistance to ferroptosis could have a beneficial effect in controlling diabetes-associated COVID-19 and CAM. Iron overload is associated with peroxidation of unsaturated fatty acids in plasma membrane, resulting in ferroptotic cell death, inflammation, production of DAMPs, and propagation of inflammatory pathways. Therefore, downregulation of pro-ferroptotic genes could be a strategy to handle iron-overload-mediated inflammatory deregulation in diabetes-associated COVID-19 severity and prevent the spread of CAM. The KEGG pathway was published with slight modifications with permission from Kanehisa laboratory.

The authors of merging studies are trying to leverage post-transcriptional control of immune responses that is regulated by RBPs [130] such as tristetraprolin, Regnase-1, Roquin, and RNA methylases, which coordinate the inflammatory networks in immune cells and associated niches by modulating the mRNA pool of immune pathway genes [221]. Interestingly, PCBP1 RBP was recently implicated as an intracellular checkpoint for shaping T-cell responses by promoting T effector immune response as opposed to T regulatory functions [222]. Diabetic endotheliopathy (possibly driven by the HBP deregulation of endothelial glycocalyx) also posttranscriptionally alters endothelial gene expression via RBPs that are becoming increasingly linked to posttranscriptional immune regulation; RBPs generated during mRNA processing are emerging as frontiers in the regulation of pancreatic beta cell function [223–225].

We propose a posttranscriptional gene silencing (RNAi) regulation of TCR genes in COVID-19 pathology, which may intersect with tRNA biology pathway enzymes such as tRNA aminoacyl synthetases that are emerging as important players of immuno-metabolic dysregulation and linked to the intergenerational inheritance of metabolic traits in diabetes mellitus [226–228].

6. Routes of Infection and Current Perspectives in Clinical Presentation and Diagnosis

There is temporal variability, i.e., seasonal, etiological, geographical, and ecological, in the epidemiology of the transmission of Mucorales of human importance. Frequent hospital outbreaks of cutaneous mucormycosis involving contaminated surgical dressing, adhesive bandages, and tongue depressors have been reported. Furthermore, high spore counts in hospital environments (especially in the ongoing expansion and construction of premises) and contaminated air-conditioners together account for nearly 9% of nosocomial mucormycosis [229]. Regarding CAM, much speculation has been focused on ventilators, catheters, air humidifier bottles, industrial oxygen cylinders, steam inhalation practices, and the extensive usage of masks, which warrant systematic aero-mycological and surface mycological investigations. Furthermore, the Centers for Disease Control (CDC), Atlanta, GA, USA, published a systematic tool that aids in performing environmental assessments when investigating healthcare-associated disease outbreaks due to fungi including Mucorales [6,20,230].

The first step in the diagnosis of CAM is the knowledge of warning symptoms and signs, as well as a high index of clinical suspicion, that includes the identification of DM with or without DKA, the use of SC therapy; the use of immunosuppressants such as tocilizumab; identification of immunodeficiency; iatrogenic suppression using mechanical ventilation; and the identification of hemato–oncological patients, bone-marrow/solid organ transplantation recipients, iron overload, and obviously COVID-19 in the present context. The extent of damage in ROCM or PM needs a multidisciplinary investigation and management with swift decisions from a team of experts, e.g., those in the otorhinolaryngology, ophthalmology, pulmonology, radiology and imaging, internal medicine, infectious disease, neurology, neurosurgery, anesthesiology, maxillofacial surgery, mycology/microbiology, pathology, and clinical pharmacy fields [28,231,232].

In radiological studies using magnetic resonance imaging (MRI) of the paranasal sinuses, contrast-enhanced MRI has been used to identify the infection of vital tissue and assess cerebral invasion in ROCM, diagnostic nasal endoscopy, and color Doppler ultrasonography studies. The computed tomography (CT) of the thorax and the plain and high-resolution (HR) CT for PM have also been used [231,232]. Clinical and radiological findings have overlapped with COVID-19, so the diagnosis of disseminated and pulmonary mucormycosis is challenging. The reverse halo in peripheral lung locations that is suggestive of pulmonary mucormycosis in IC patient(s) and serves as a useful indicator for pre-emptive antifungal therapy is not an indicator for COVID-19, as it can be a commonly overlapping finding. Recently, EORTC and MSG updated the criteria for proven invasive fungal disease (IFD) [231], with recommendations for senso stricto among patients with cancer, transplant recipients, and other severely IC hosts. Even though the diagnosis of IFD via radiological findings remains the gold standard, helping in early detection and swift course of actions, it lacks sensitivity. The most significant pulmonary radiological features of IFD in non-cancer patients and among the trio of invasive pulmonary aspergillosis (IPA), mucormycosis, and COVID-19 overlap with "atypical" non-nodular pattern, consolidation, and ground-glass opacities [231].

The laboratory diagnosis and confirmation of mucormycosis is easy if the biopsy samples are available, as in the case of ROCM, whereas the diagnosis of disseminated or pulmonary mucormycosis is difficult due to the nonavailability of biopsy specimens due to various limitations. The microscopic examination of the tissues using KOH mount and Calcofluor white aids the rapid confirmation of diagnosis. Under microscopy, the Mucorales hyphae classically appears hyaline, broad (2–8 μm diameter), ribbon-like (often folded), pauci-septate, or aseptate with a right-angled branching. Unlike other molds, Mucorales grow faster within 24–48 h and appear cottony. However, the sensitivity of cultures is not more than 80%. The species-level identification of cultures can be made with conventional phenotypic, morphological, and physiological features such as growth rate and incubation temperatures on various media, but it needs expertise in mycological techniques and identification. The method of choice for the species identification of Mucorales is therefore

ITS sequencing, which is mostly restricted to reference laboratories. Even though ITS barcoding has been a preferred molecular marker for the identification of various Mucorales species, it has a few limitations such as the failure to differentiate *R. microsporus* and *R. azygosporus* because both belong to the same species [233].

Therefore, attempts to directly diagnose mucormycosis from clinical specimens have been made by standardizing the Mucorales PCR using conventional, nested, RFLP, and real-time PCR (qPCR) formats on blood samples and other matrices such as bronchoalveolar lavage (BAL) or tissue specimens, and several in-house assays have shown good sensitivity. The commercially available multiplex, pan-Mucorales real-time (qRCR) kit-MicroGenius assay by PathoNostics readily detects several Mucorales species including *Rhizopus, Lichtheimia, Rhizomucor,* and *Cunninghamella* in sera and BAL, thus showing promising rapid diagnostic utility in present clinical setups. In this era of rapid diagnosis, lower turnaround-time (TAT), and laboratory quality initiatives, several technologies such as matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF) have increasingly gained momentum and will start influencing the clinical microbiology laboratories in the future. The negative results of the serological markers such as (1→3)-β-D-glucan (BDG) and galactomannan (GM) (used in other IFD) can aid the diagnosis of invasive mucormycosis (IM) during strong suspicion of IFD. The evaluation of these surrogate markers in proven IM using an enzyme-linked immunospot (ELISpot) assay (leveraging Mucorales-specific T-cells with a derived diagnostic cut-off) has been reported, and elevated IL-10 and IL-4 levels in symptomatic patients and elevated Mucorales-specific T-cells with a higher IFN-γ levels in non-symptomatic patients were observed. These results indicate the assay's promising utility in hematological patients and warrant rigorous investigation into its potential as a surrogate marker in IM and other patient populations [234].

Clinical Perspective of Rhino-Orbital Mucormycosis and Patient Management

Mucormycosis, primarily caused by Mucorales in immunocompromised or immune-competent hosts with diabetes/trauma, is a critical scenario for every clinician dealing with this condition. Rhino-orbital mucormycosis is the most common form of the disease; its fulminant nature, the extensive angio-invasion-induced ischemic necrosis-related inflammation of tissues, delays in suspicion in primary care, and lack of effective medical antifungal therapy have worsened diagnosis challenges.

Rhino-orbito-cerebral mucormycosis patients present with a history of facial heaviness, fever, purulent nasal discharge, headache, nasal congestion, and sinus pain. The disease can progress and present with the destruction of the turbinates and the nasal septum, paleness, blackening in the nasal and palatal mucosa, and ulcer formation in the palate with exposed underlying osteomyelitic bone. Peri-orbital swelling decreases vision, proptosis, and ophthalmoplegia with or without diplopia in patients of orbital involvement, and it alters neurological function and consciousness in cases of cerebral involvement, thus indicating ominous disease aggressiveness. Diagnosis primarily rests on microbiological analysis with KOH wet mounts of scrapping or tissue samples taken from pale, necrotic, and inflamed areas. Radiological imaging with CT/MRI and histological analysis of tissue samples aid diagnosis.

A diagnosis or high clinical suspicion of diagnosis leads to management that primarily involves the following steps:

- Continued treatment of the primary immune deficiency condition.
- Correction of biochemical parameters and management of associated diabetic status.
- Aggressive debridement of the necrotic tissues of the rhino-orbital-facial region with the aim to clear all necrotic tissue and osteomyelitic bones to the maximum extent until the tissues bleed, with caution used in case of cerebral involvement to not to debride brain tissue.
- Post-debridement wound and cavity local care and adjuvant medical management with amphotericin B and/or posaconazole and isavucanazole.

- Continuation of cavity care after the completion of therapy with the regular clearance of crusts and saline irrigations for 3–6 months after treatment.
- Prosthetic rehabilitation and/or reconstruction of the defect.

The morbidities and functional compromises associated with disease and management lead to significant losses of productivity and hinder the quality of life of patients, especially during the current COVID-19 pandemic, in which instances of mucormycosis have increased to overwhelming levels due to underlying immune dysregulation by COVID-19 and associated steroid therapies. Hence, high clinical suspicion and early referrals are key to early management and the preservation of the quality of life in cases of rhino-orbital mucormycosis.

7. Future Perspective—A Working Hypothesis to Explain Development of COVID-19 and CAM in the Backdrop of Diabetes

Serine proteases can have cell-autonomous functions (besides SARS-CoV-2 activation) in olfactory microenvironment, in which they can activate and signal through cognate GPCR family of protease-activated receptors (PARs) that participate in transducing downstream inflammatory and calcium signaling pathways, resulting in the regulation of ion channels and transporters. In diabetic patients, endotheliopathy in the vascular olfactory niche can further add to the PAR-transducing protease repertoire by offering pre-activated TF-FVIIa and FXa serine proteases that could have Janus faces during inflammatory propagation. TF-FVIIa and FXa can activate PAR signaling concomitant to the complement coagulation pathway intensification that is central player in COVID-19 pathogenesis [235], and the resultant inflammatory loops can have pro-fibrogenic functions as the associated plasmin and uPAR–uPA signaling modulators are known to have growth factor-binding, TGF-beta-activating, integrin-signaling, and ECM-remodeling features. We report that protease stress is associated with concomitant metabolic deregulation and the differential expression of olfactory transduction pathway genes including the olfactory/odorant receptors (ORs). Importantly, the majority of the olfactory receptors are orphan receptors, and deorphanization is as a major challenge. Many fatty acid and lipid metabolites are potential activating and inhibitory ligands of ORs, and emerging studies have suggesting a broader expression pattern of ORs beyond the olfactory epithelium, including adipocytes, airways, kidneys, livers, lungs, and adipocytes. Extra-nasal ORs have been reported to perform seminal functions including blood pressure regulation via the renin–angiotensin–aldosterone pathway in kidneys and the hypoxia-sensing ORs in the glomus cells of the carotid body. Hypoxia metabolite lactate is a partial agonist of carotid body ORs. Hepatic ORs regulate hepatic lipid accumulation via the regulation of GLP-1, resulting in glucose and insulin tolerance. Airway smooth muscle cells express ORs that regulate bronchoconstriction through calcium signaling via the cAMP pathway. However, ligand-dependent contractility effects that are linked to inflammatory pathways of IL-8 and GMCSF have also been reported. The adipocytic effects of ORs are mediated by hepatic fatty acid oxidation and brown adipose tissue thermogenesis. Epicardial adipose tissue and diabetes mellitus patient plasma contain medium chain fatty acids that can activate cardiac muscle cells and regulate ionotropic effects [236,237]. The appreciable overlap of ORs expression pattern with affected organs in COVID-19 and the OR regulation of RAAS and hypoxia functions led us to propose their potential contribution in disease pathology and their use as therapeutic targets for diabetes, COVID-19 pathology, and CAM.

Importantly, neural-crest-originating astrocytes and pericytes are becoming increasingly linked to underlying neurovascular abnormalities in COVID-19 pathobiology. We also observed the upregulation of neural crest pathways in protease-treated monocytic cells. It is a possibility that developmental epithelial–mesenchymal transition (EMT) events initiated by active proteases during embryonic neural-crest induction could be recapitulated by providing exogenous proteolytic exposure to pathology competent monocytic cells in vitro, resulting in the upregulation of developmental multipotent neural-crest-like stem cells with ectomesodermal potential that capable of upregulating neurogenesis/gliogenesis

(neurons and astrocyte) and vasculogenesis (microvascular pericyte), crucial cellular processes underlying neuroinflammatory diseases [Sharma et al., unpublished data]. The OR-expressing sustentacular cells in olfactory epithelia are glia-like, and the neuroendocrine cells have also been reported to be neural-crest-derived; their participation in the lung neuroinflammation in context of ARDS has been proposed with the pharmacological neuromodulation of the vagus nerve as a potential therapeutic approach to treat COVID-19 [238]. The hypothalamus–pituitary–adrenal gland axis (HPA), along with the vagal reflex and the possible involvement of the carotid body, has been proposed for the neural control of inflammation wherein the deregulated vagal anti-inflammatory reflex is central to inflammation and metabolic diseases including type 2 diabetes. The HPA axis under stress releases cortisol-releasing hormones, which induce the secretion of anti-inflammatory glucocorticoids from adrenal glands. Vagal anti-inflammatory reflex sense inflammation and the activation of efferent vagus nerve fibers results in dampened cytokine production following the binding of neurotransmitter acetylcholine to its receptor nicotinic α7nAChR on immune cells such as macrophages. Furthermore, the efferent sympathetic output to adrenal gland medulla chromaffin (neuroendocrine) cells triggers epinephrine release to activate specific receptors in immune cells to resolve inflammation [239]. Carotid body chemoreceptors have been proposed as afferent arms of the anti-inflammatory reflex and are becoming increasingly linked to inflammatory diseases such as sepsis and intermittent hypoxia [240]. The glomus cells of the carotid body that act as hypoxia sensors bear olfactory receptors and are neural-crest-derived, so we hypothesize carotid body chemosensors as central players in driving neuroinflammatory events in COVID-19/post-COVID neurological sequelae including anosmia.

Notably, small molecule neurotransmitters and neuromodulators generally belong to the class of amino acid metabolites including glutamate, sulfur-containing cysteine and methionine, proline, GABA, lysine, arginine, glycine, serine, alanine, aromatic amino acids, and branched-chain amino acids. These amino acid neurotransmitters recycle between neurons and astrocytes and conduct nerve impulse transmission through ionotropic channels, and a few of these amino acids participate in generating mitochondrial oxidative and nitrosative stress. The aromatic amino acid tyrosine is the precursor for the dopamine, norepinephrine, and epinephrine neurotransmitters that regulate neuroinflammation and are implicated in many neurocognitive diseases [241]. We hypothesize that the oxidative and nitrosative stress in macrophages due to metabolic deregulation during diabetes and COVID-19 can alter iron homeostasis and therefore lead to the lipid peroxidation and ferroptosis that compromise macrophage functionality and anti-Mucorales prowess. Vascular macrophages have been shown to upregulate ORs that bind to lipid metabolites and activate inflammasome and IL-1 production [242]. The parallel deregulation of the olfactory transduction pathway via metabolic-stress-triggered lipid and amino acid metabolites (proposed as potential ligands for ORs) and the activation of PARs due to serine proteases can drive the co-deregulation of olfactory transduction and complement coagulation pathways in macrophages (vascular immune) and olfactory epithelium (neurosensory) cells causing anosmia and neuroinflammation. The subsequent generation of DAMPs, as reported during inflammasome activation, can be passed onto the astrocytes and pericytes of the brain, which results in neurocognitive and neurodegenerative features. The post-viral effects of COVID-19 in the olfactory system and their neurological connections were described recently [69]. The pre-symptomatic or acute phase of COVID-19 is marked by high viral loads/replication in the nasal epithelium, which makes it a potential target to inhibit the intensification of infection by limiting the spread of the infection. Therefore, the prophylactic/therapeutic targeting of the olfactory epithelium by employing intranasal sprays could be a potential approach to prevent diabetes-associated COVID-19 and CAM (Figure 7).

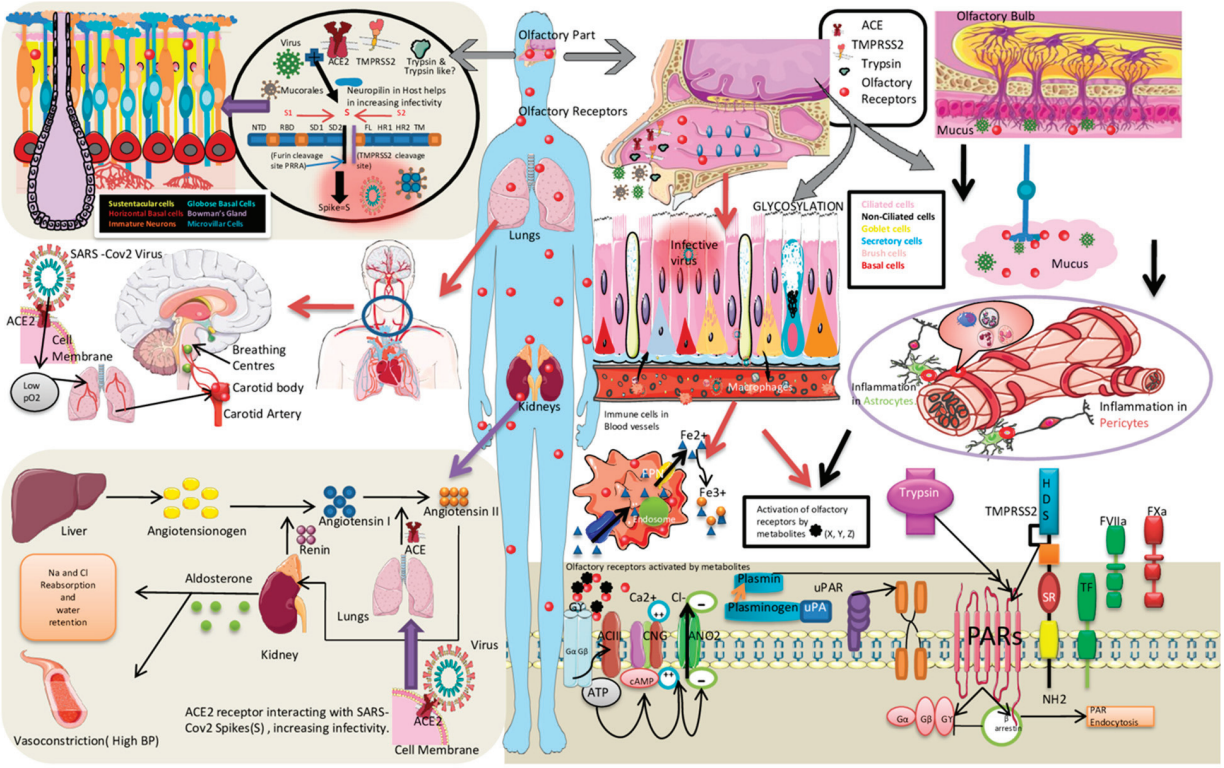

Figure 7. A working hypothesis to explain the development of COVID-19 and CAM in the context of diabetes. We acknowledge Servier Medical Art Available online: https://smart.servier.com (accessed on 2 January 2022) [243] for providing the various cartoon components that comprise this illustration. We acknowledge SARS-CoV-2, ACE2, and TMPRSS2 receptor image credits as adapted from an image by Davian Ho for the Innovative Genomics Institute." https://innovativegenomics.org/free-covid-19-illustrations/ (accessed on 2 January 2022) [244].

8. Conclusions

The mechanistic insights of chemosensory/neurosensory deregulation in the olfactory epithelium and the attendant immune landscape reshaping during COVID-19 infection remain largely unexplored due to infection control concerns in disease-active patients. We leveraged the proteolytic predominance of olfactory microenvironment during acute phase of the disease to develop a novel virus-free cellular model to identify a potential host-associated serine protease-interactome that displays COVID-19-like disease signatures including maturity-onset diabetes, complement–coagulation, and olfactory transduction. Our data support the role of olfactory dysfunction in predicting neurological sequelae in CNS and suggest a new paradigm that could involve olfactory transduction-mediated neuroinflammation in the respiratory tract and peripheral tissues with resident macrophages expressing neuropeptide receptors. We also gathered appreciable patient data from COVID-19 and CAM plasma metabolomics and proteomics that suggest the beneficial effects of rescuing olfactory epithelium health with the potential repurposing of anti-metabolic drugs such as 2DG (2-deoxy-D-glucose) and nutraceutical azoles with anti-inflammatory and anti-oxidative effects. Our preliminary data demonstrated the anti-fungal activity of 2DG and L-carnosine (growth inhibition assay) against clinically relevant Mucorales species, a novel anti-fungal action (unpublished data). Both drugs have proven anti-diabetic and potential in-silico anti-COVID-19 effects, so our next work is directed towards the development of novel intranasal anti-diabetic sprays for controlling infectious airway diseases such as COVID-19 and CAM (Sharma et al., unpublished work).

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Abbreviations

ACE2	Angiotensin-Converting Enzyme 2
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
BAL	Bronchoalveolar Lavage
BDG	β-D-Glucan
BiPs	Binding Proteins
CAM	COVID-19-Associated Mucormycosis
CAPA	COVID-19-Associated Pulmonary Aspergillosis
CDC	Centers for Disease Control
CotH	Coat Protein Homolog
COVID-19	Coronavirus Disease 2019
CS	Cell Surface
CT	Computed Tomography
DAMPs	Damage-Associated Molecular Pattern
2DG	2-Deoxy-D-Glucose
DHPS	Deoxyhypusine Synthase
DKA	Diabetic keto acidosis
DM	Diabetes Mellitus
DMT1	Divalent Metal Ion Transporter 1
DOHH	Deoxyhypusine Hydroxylase
DPP4	Dipeptidyl-peptidase 4
eIF5a	Eukaryotic Translation Initiation Factor 5 a
ELISpot	Enzyme-Linked Immunospot
EMT	Epithelial–Mesenchymal Transition
EORTC	European Organization for Research and Treatment of Cancer
FAK	Focal Adhesion Kinase
Fe–GSH	Iron Glutathione
Fe–S	Iron Sulfur
FFA	Free Fatty Acids
FISF	Fungal Infection Study Forum
GM	Galactomannan

GnRH	Gonadotrophin-Releasing Hormone
GPI	Glycosylphosphatidylinositol
GRP78	Glucose-regulated proteins 78
HBP	Hexosamine Biosynthetic Pathway
HPS	High Proteolytic Stress
HR	High-Resolution
HSP70	Heat Shock Protein 70
HSPGs	Heparan Sulfate Proteoglycans
IC	Immunocompromised
IDRs	Intrinsically Disordered Regions
IFD	Invasive Fungal Disease
IFN-γ	Interferon Gamma
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-1β	Interleukin 1 Beta
Il-6	Interleukin 6
IL-8	Interleukin 8
IL4	Interleukin 4
IM	Invasive Mucormycosis
IPA	Invasive Pulmonary Aspergillosis
ISHAM	International Society of Human and Animal Mycology
ISR	Integrated Stress Response
ITS Sequencing	Internal Transcribed Spacer
ITS	Internal Transcribed Spacer
KOH Mount	Potassium Hydroxide Mount
LIP	Labile Iron Pool
LMIC	Low- and Middle-Income Countries
LPS	Low Proteolytic Stress
MAD	Mucormycosis-Associated diabetes
MALDI–TOF	Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry
MLKL	Mixed Lineage Kinase Linked Domain
MPI	Anti-Metabolic and Anti-Proteolytic Inhibitors
MRI	Magnetic Resonance Imaging
MSAI	Metabolic-Stress-associated Interactome
MSG	European Confederation of Medical Mycology (ECMM), and Mycoses Study Group
MTOR	Mammalian Target of Rapamycin
NBTI	Non Transferrin Bound Iron
NCOA4	Nuclear Receptor Coactivator 4
NONS	Nitric Oxide Nasal Spray
NRP1	Neuropilin-1
OE	Olfactory Epithelium
OSNs	Olfactory Sensory Neuron
PAR-2	Protease-Activated Receptor-2
PCBP	Poly(rC) Binding Protein
PHD	Prolyl Hydroxylase
PITTR	Protease-Induced Transcriptomic/Epi-Transcriptomic Reshaping
PM	Pulmonary Mucormycosis
PMNs	Polymorphoneutrophils
PRRs	Pattern Recognizing Receptors
PRSS8	Serine Protease-8
PUFAs	Plasma Membrane Unsaturated Lipids
qPCR	Quantitative Polymerase Chain Reaction
RBP	Receptor Binding Protein
RBPs	RNA-Binding Proteins
RCD	Regulated Cell Death
RFLP	Restriction Fragment Length Polymorphism

RNAi	RNAInterference
ROCM	Rhino-Orbital-Cerebral-Mucormycosis
ROS	Reactive Oxygen Species
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SAT1	Spermidine N1 Acetyltransferase
SC	Systemic Corticosteroids
SNCs	Sustentacular Cells
SPs	Serine Proteases
T2DM	Type 2 Diabetes Mellitus
TAT	Turnaround-Time
TCR	T-Cell Receptor
TLR4–IRF5	Toll-Like Receptor 4–Activated Interferon Regulatory Factor 5
TLRs	Toll-Like-Receptors
TMPRSS2	Transmembrane Serine Protease 2
TNF α	Tumor Necrosis Factor α
UDP	Uridine Diphosphate
VWF	Willebrand Factor

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