SKELETAL MUSCLE MITOCHONDRIAL BIOENERGETICS IN TYPE 1 DIABETES

EXAMINATION OF MITOCHONDRIAL BIOENERGETICS IN SKELETAL MUSCLE BIOPSIES FROM ADULTS WITH TYPE 1 DIABETES

By

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LAY ABSTRACT

Type 1 diabetes (T1D) is a complex disease that still has no known cure. Current treatment focuses on managing blood sugar levels with exogenous insulin injections and frequent blood sugar checks. However, over time, people with T1D still develop serious complications that inevitably impact their quality of life and lifespan. A potential adjuvant therapy to prevent complications in T1D is improving the health of skeletal muscle through exercise given its role in stabilizing blood sugar/lipid levels and whole-body insulin sensitivity. However, this area continues to be severely understudied in the T1D population. Thus, this thesis examined skeletal muscle metabolic 'health' from adults with T1D who do not have major diabetes complications and manage their blood glucose moderately-well. Through a series of novel experiments, we found that young and middle- to older-aged adults with T1D have alterations in the metabolic engines of their muscles, and depending on biological sex, the alterations manifest as either heightened or degraded cellular function. These findings are the first to provide a comprehensive cellular investigation of the impact of T1D on the metabolic health of skeletal muscle in people with T1D and provide the foundation for future research examining skeletal muscle as an essential and early adjuvant therapy in this population.

ABSTRACT

The overall objective of this thesis was to examine mitochondrial bioenergetics in muscle biopsies from humans with type 1 diabetes (T1D) to gain a deeper understanding of the cellular mechanism(s) underlying changes to skeletal muscle health reported in T1D, a phenotype we have referred to as 'diabetic myopathy'. It was hypothesized that humans with T1D, compared to their matched counterparts without diabetes (control), would demonstrate significant deficiencies in muscle mitochondrial function and ultrastructure/content as determined by the gold-standard *in vitro* methodology: high-resolution respirometry and transmission electron microscopy, respectively. It was further hypothesized that sex differences would not exist in mitochondrial function with T1D, and mitochondrial deficiencies would be more dramatic at an earlier age with T1D. Adults with uncomplicated T1D and strictly matched controls (age, sex, BMI, self-reported physical activity levels) were recruited from surrounding university-dwelling communities. Site-specific deficiencies in mitochondrial respiration, H_2O_2 emission, and calcium retention capacity were found in young, physically active adults with T1D despite normal mitochondrial content. Further experiments revealed that muscle mitochondrial respiration in women and men differentially adapt to the T1D environment where men with T1D have lower complex II but higher complex I respiration compared to women with T1D, while women (irrespective of T1D) have lower ADP sensitivity. Women with T1D also demonstrated lower H_2O_2 emission compared to men with T1D. In contrast, despite a lower mitochondrial content in middle- to older-aged

adults with T1D, mitochondrial respiration (normalized to content) was either normal or increased in adults with T1D compared to control, with observable differences between sexes. Overall, this research has demonstrated that despite being recreationally to physically active, adults with uncomplicated T1D with moderately well-managed glycemia demonstrate alterations in skeletal muscle mitochondrial function and ultrastructure, including differences between sexes.

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PREFACE

This thesis is a “sandwich” style thesis prepared in the format outlined in the School of Graduate Studies’ Guide for the Preparation of Theses. Chapter 1 is the general introduction which includes the electronic version of the published reprint of two review papers. The second review paper in this chapter consists of only two pages from the published reprint, with the remainder of the reprint presented in Chapter 4, which sets the context for Chapter 5. Chapters 2 and 3 include original research papers published and presented as the electronic version of the published reprint. Chapter 5 is an electronic version of a manuscript in print. Chapter 6 is the general discussion and conclusions. The candidate is the first author on all the papers. The declaration of academic achievement is found in the preface of each chapter including author contributions and the citation. Copyright permission is indicated at the beginning of each chapter and found in Appendix A. All chapters have been reproduced with the permission of each co-author.

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LIST OF ALL ABBREVIATIONS AND SYMBOLS

[H+]	Hydrogen ion concentration
^{31}P-MRS	Phosphorus-31 magnetic resonance spectroscopy
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BMI	Body mass index
Cr	Creatine
CRC	Calcium retention capacity
CVD	Cardiovascular disease
ETC	Electron transport chain
G6P	Glucose-6-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HbA1c	Hemoglobin A1c
HK	Hexokinase
LDH	Lactate dehydrogenase
mG3PDH	Mitochondrial glycerol-3-phosphate dehydrogenase
mPTP	Mitochondrial permeability transition pore
OXPHOS	Oxidative phosphorylation
PCr	Phosphocreatine
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
ROS	Reactive oxygen species
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
U	Units
ULK1	Unc-1 like autophagy activating kinase 1

CHAPTER 1 — GENERAL INTRODUCTION

PREFACE

Type 1 diabetes (T1D) is a complex, autoimmune-mediated metabolic disease characterized by hyperglycaemia and insulin deficiency. Millions of people worldwide are afflicted with T1D (Xu et al., 2018), and the prevalence has consistently risen over the last decade (You & Henneberg, 2016). Insulin therapy continues to be the primary treatment option for people with T1D, and while advances in technology (e.g., insulin pumps and continuous glucose monitors) have facilitated the clinical management of this disease, the unfortunate reality is that diabetes complications (e.g., cardiomyopathy, neuropathy, nephropathy, retinopathy) still develop and remain the major cause of morbidity and premature mortality in this population (Mameli et al., 2015). Thus, it is clear that insulin therapy is not a cure, and if we are to improve the healthy lifespan of people living with T1D, greater research efforts are urgently needed towards unravelling the mechanisms underlying the development of diabetes complications in multiple organs and tissues[a].

This introductory chapter contains a published review paper in its entire form and an excerpt from another published review paper. Together, they serve as the literature review of this thesis. The review paper introduces diabetic myopathy, current available evidence of diabetic myopathy in T1D humans, and proposes mitochondrial dysfunction as an underlying cellular mechanism. The excerpt provides a brief overview of mitochondrial physiology and succinctly describes the impact of mitochondrial dysfunction on skeletal muscle health in T1D humans.

[a]paragraph is an excerpt from a published reviewer paper as first author. Full article available in Appendix B.

1.1 Diabetic Myopathy: current understanding of this novel neuromuscular disorder

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1.1 PREFACE

Author contributions:

CMFM drafted the review article outline, wrote the initial manuscript draft, designed the figures, and worked in refining the final draft of the manuscript based on coauthor feedback. CGRP edited the manuscript and worked in refining the final draft of the manuscript. TJH contributed to the drafting of the review article outline, edited the manuscript and worked in refining the final draft of the manuscript.

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REVIEW

Diabetic Myopathy: current molecular understanding of this novel neuromuscular disorder

Cynthia M.F. Monaco[a], *Christopher G.R. Perry*[b], *and Thomas J. Hawke*[a]

Purpose of review
Here we summarize the evidence from human studies of the impairments to the structural, functional, and metabolic capacities in skeletal muscle in those with type 1 diabetes (T1D) – a condition known as diabetic myopathy. Given the importance of skeletal muscle for blood lipid and glucose management, the development and progression of diabetic myopathy would not only lead to increased insulin resistance, but also impact the ability to mitigate dysglycemic/dyslipidemic burdens.

Recent findings
Despite the importance of skeletal muscle in whole-body metabolic control, studies investigating diabetic myopathy are startling limited. Recent findings have demonstrated that those with T1D exhibit decreased force production, increased fatigability, loss of muscle stem cells, and a greater reliance on glycolytic metabolism, as a result of reduced mitochondrial capacity.

Summary
We propose a mechanistic model for the development of diabetic myopathy based on the human findings to date. This model suggests that repeated insulin injections in those with T1D leads to recurrent periods of intracellular hyperglycemia in myofibers. Resultant reductions in mitochondrial function lead to greater reliance on glycolytic metabolism and a concomitant shift in fiber type composition. Studies defining the scope and magnitude of diabetic myopathy and testing the veracity of this model are urgently needed in order to develop appropriate therapeutic strategies to maximize muscle health in those with T1D.

Keywords
complications, mechanisms, mitochondria, skeletal muscle, type 1 diabetes

INTRODUCTION

Type 1 diabetes mellitus (T1D) results from the autoimmune-mediated destruction of the pancreatic β cells, rendering a person hypoinsulinemic and hyperglycemic. Although insulin therapy is an essential treatment for managing T1D, it is not a cure and it does not prevent the development of devastating complications that invariably shorten the lifespan by as much as 15 years [1]. T1D complications include: nephropathy, retinopathy, neuropathy, and cardiovascular diseases (CVD) such as myocardial infarction and stroke. T1D is associated with a 10-fold increase risk of CVD [2,3] and is the most common cause of death in T1D [4]. However, a largely unstudied complication of T1D is the impact of this chronic disease on skeletal muscle. We have termed this complication diabetic myopathy and it is characterized by reductions in skeletal muscle structural, functional, and metabolic capacities. As discussed in detail later, the evidence supports myopathy as a diabetic complication that develops independent of neuropathy, but is worsened with the development and progression of neuropathy. In this review, we will define the importance of skeletal muscle in managing T1D, followed by how this chronic disease affects skeletal muscle health and conclude by offering a mechanism underlying the development and progression of this critical, yet often undiagnosed complication.

DIABETIC MYOPATHY: A COMPLICATION OF CLINICAL SIGNIFICANCE

Normally, pancreatic β cells release insulin into the portal circulation, allowing the liver to be exposed

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KEY POINTS

- By-passing the liver with peripheral insulin injections forces skeletal muscle to be the primary tissue mediating insulin's action. Functional and metabolic (mitochondrial) dysfunction in skeletal muscle could expedite T1D complications through reduced capacity for physical activity, impaired substrate oxidation, and reduced insulin sensitivity.
- Recent data from human studies highlights significant functional impairments (loss of force production, increased fatigability, loss of muscle stem cells) and mitochondrial (metabolic) dysfunction that would impact the ability of muscles to mitigate dysglycemia and dyslipidemia.
- The evidence to date supports denoting diabetic myopathy as a novel neuromuscular disorder and a diabetic complication of clinical significance. The links between skeletal muscle health and overall well-being are now well-established. Defining the scope of myopathy in those with T1D will uncover mechanistic insights into the adaptive capacities of T1D muscles and lead to evidence-based therapeutic/exercise strategies to improve the functional and metabolic capacities of skeletal muscle in T1D.

to three to five times more insulin than the periphery [5,6]. In the non-T1D state, insulinizing the liver disproportionately to muscle allows the liver to play the prominent role in glucose homeostasis and allows muscle and liver to share in storing a glucose load [6,7]. However, in T1D, subcutaneous insulin injections bypass this canonical 'liver-first' model [5,7]. Further impacting hepatic function is the fact that peripherally administered insulin must be done at levels consistent with the systemic circulation (as compared to portal circulation) to prevent hypoglycemia [8,9▪]. Thus, peripheral injections shift the emphasis of insulin's action to the peripheral tissues, of which skeletal muscle is by far the largest and most metabolically active. Insulin resistance and recurrent dysglycemia and dyslipidemia are considered the primary contributors to the development of most of the clinically relevant diabetic complications [10–12]. Impairments to muscle health, in particular it is metabolic capacities, would make muscle less capable of handling the large fluctuations in glycemia and lipidemia that characterize T1D and invariably, insulin resistance develops. Consistent with this idea, a recent comprehensive metabolomic profiling of T1D and matched nondiabetics identified widespread metabolomic abnormalities in T1D with poor glycemic control [13▪▪]. Interestingly, many of these perturbations identified in those with poor glycemic control were unchanged in those with chronically good glycemic control [13▪▪].

Strong evidence now links skeletal muscle health with overall health and well-being. In fact, measures of muscle function (grip strength, gait speed, chair rise test) are used clinically and are strong prognostic indicators of mortality risk in the elderly and diseased states [14–16]. The positive relationships between these measures and survival exist independently of other variables (e.g. smoking, sex), highlighting the importance of a healthy muscle mass to overall health. Therefore, the development of diabetic myopathy would not only lead to weakness and increased fatigability, but also, potential impairments to muscle's metabolic capacities would negatively impact insulin sensitivity, as well as the ability to manage glycemic and lipidemic loads after each meal – a situation that would exacerbate the development and progression of other diabetic complications.

CHANGES TO SKELETAL MUSCLE IN DIABETIC MYOPATHY

Derangements in skeletal muscle's physical and metabolic capacity with T1D are commonly observed in humans (see [17] for review). In the following sections we have summarized our current knowledge on diabetic myopathy with a focus on structural, functional, and metabolic changes.

T1D and alterations in muscle structure

Abnormalities in the morphology of skeletal muscle from T1D individuals were first brought to light in the late 1960s and early 1970s. These studies noted changes in myofibrillar size, glycogen content, and mitochondria in long-standing patients with T1D [18–20]. However, it was not until 1977 that this was investigated in more depth. Reske-Nielsen *et al.* [21] analyzed the ultrastructure of striated muscle from 29 patients with T1D with varying durations of T1D: recent onset (less than 1 week to 28 weeks), short-term (17 months to 5 years), and long-term (7–32 years). In the short-term group, without evidence of peripheral neuropathy, these authors observed a reduction in myofiber and myofibril diameter as a result of myofilament loss, and displaced A- and I- bands and undulating Z-lines (Fig. 1). In the long-term group, where all patients exhibited peripheral neuropathy, these alterations were more pronounced and were also accompanied by large areas of granular material, small mitochondria, and degenerated nuclei in place of the destroyed sarcomeres. Distinguishing the direct contributions of T1D on skeletal muscle from the indirect effects

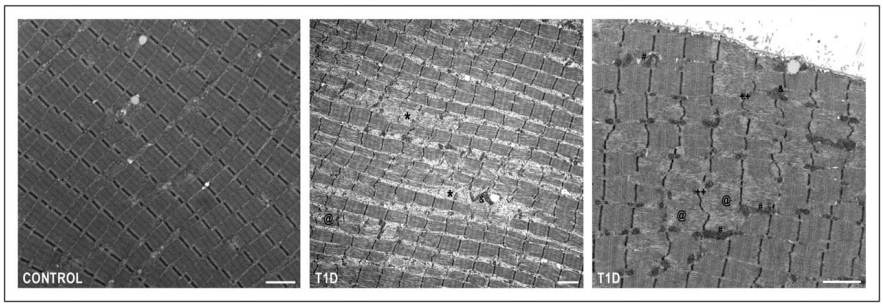

FIGURE 1. Electron microscopy images of human skeletal muscles with and without type 1 Diabetes. The left panel illustrates the vastus lateralis muscle of a 23-year-old female without T1D (control). The middle and right panels illustrate the ultrastructure of the vastus lateralis muscle of a 19-year-old T1D male with no history of neuropathy. Consistent with previous work [21], we observe loss of myofibrillar material, replaced with nonmyogenic structures (*), sarcomeric destruction (@), displaced I- and A- bands, and undulating Z-lines (++). There is also evidence of abnormally shaped mitochondria (&) and mitochondria exhibiting disassociation with sarcomeres (#). Scale bar in each image represents 2 μm. These images are unpublished images belonging to authors C.M.F.M., C.G.R.P., and T.J.H.

on muscle ultrastructure resulting from diabetic neuropathy is an important, but as of yet, unexamined question. However, the fact that these observations occurred in all groups regardless of the presence of diabetic neuropathy, highlights the sensitivity of skeletal muscle to the T1D environment.

Losses in muscle mass (atrophy) have been previously reported in those with T1D [22–24]. In addition to the contributions of protein turnover, which have been demonstrated to be unbalanced in T1D [25,26], skeletal muscle relies on its muscle stem (satellite) cell population to maintain a healthy myonuclear content and repair from injury [27]. Only one study has investigated muscle stem (satellite) cells in T1D humans. D'souza *et al.* [28▪▪] found that young adults with T1D (18–22 years old who reported no other diabetic complications) exhibited significant reductions in muscle satellite cell content. Using the Akita, T1D, mouse model to decipher the underlying mechanisms for the satellite cell loss, the authors found a persistent activation of the Notch signaling pathway in satellite cells that attenuated the ability to exit quiescence and initiate proliferation. Thus, not only is there less of this critical muscle stem cell population, but the cells present appear less effective in initiating repair in the diabetic state. Clearly, future studies in this area are needed as evidence from rodent models suggests that muscles in those with T1D are also more prone to damage [28▪▪,29]; findings that may help explain the weakness and decreased time to exhaustion in humans with T1D [17].

Type 1 diabetes and reductions in muscle function

The reductions in muscle function, including decreased strength and increased fatigability, often observed in adolescents and young adults with T1D [30–33] contribute to the increased risk of physical disability and sarcopenia invariably observed in older adults with the disease [23,34,35,36▪]. Although the mechanism for such an association has been reported to be a late complication of diabetic peripheral neuropathy [34,35], recent studies have reported that muscle strength impairments actually develop earlier in the course of diabetes – prior to, and independent of – neuropathy. Specifically, Orlando *et al.* [37▪▪] found that T1D adults (HbA_{1c} ~7.1%), without evidence of diabetic neuropathy, display lower maximal isometric force production and increased muscle fatigability compared to age-matched healthy non-T1D controls. This group also studied a cohort of patients with T1D with diabetic neuropathy and found they exhibited even lower force production and increased fatigability. In those with type 2 diabetes (T2D), a similar relationship between disease status and muscle function has also been established with reductions in muscle quality strongly associated with longer T2D duration and glycemic control [38–40]. In these studies, lower leg strength in those with T2D was reduced and the presence of peripheral neuropathy produced only a small, additional effect [40–42].

Taken together, the evidence indicates that impairments in muscular function in those with

T1D develops independent of, but is aggravated by, diabetic neuropathy. This conclusion is consistent with the work of others [42,43] who have reported muscle weakness in T1D and T2D is only partly related to motor nerve impairment, and is dependent on contraction velocity – a finding which may help shed light on inconsistencies in the literature. Certainly, impairments in muscle function developing independent of neuropathy would be more consistent with the ultrastructural changes observed by Reske-Nielsen *et al.* [21] in the short-term and mid-term cohorts.

Type 1 diabetes and impaired muscle metabolism

The alterations in muscle morphology and function are largely consistent with negative changes in skeletal muscle metabolic capacities that have been reported. Early studies of young T1D adults by some of the great researchers in the field of skeletal muscle metabolism (e.g. Costill, Saltin, Graham) have reported alterations in skeletal muscle metabolic enzymes, including increased lactate dehydrogenase activity [44,45] and lower hexokinase and succinate dehydrogenase activity [44–46], suggesting a greater reliance on anaerobic metabolism for energy production. The reliance on anaerobic metabolism is consistent with the greater size and/or number of glycolytic fibers which have been more recently reported [47]. A challenge in extrapolating these early findings to persons with T1D today is the huge disparity in insulin therapies. For example, patients with T1D in the Costill study [46] took insulin (Lente) once per day (avg. = 48 U/day; range = 20–58 U/day) whereas exercise capacity testing in the study by Saltin *et al.* [44] were done on patients with T1D who had not been administered insulin for 24 h and had an average resting blood glucose of 18.3 mM. Contrast that with the variety of short-acting, intermediate-acting, and long-acting insulin analogs currently available, and the use of pump-based therapies, and it becomes easier to appreciate the need to re-evaluate these previous studies to determine whether their findings remain true in the face of more aggressive insulin therapies.

Some recent work, however, does support these previous findings of a shift in the metabolic profile in the muscle of those with T1D despite improved insulin therapies. Cree-Green *et al.* [48▪▪], using ^{31}P-MRS to measure the rate constant of phosphocreatine (PCr) re-synthesis (infers *in vivo* mitochondrial function/oxidative capacity), found a greater reliance on glycolytic metabolism and a delayed skeletal muscle mitochondrial ADP recovery following contraction in youth with T1D (HbA1c ~8.2%). These findings were consistent with earlier work by Crowther *et al.* [49] who found that well-controlled T1D men (HbA1c < 7.0%, no symptoms of neuropathy) also had a slower rate of PCr re-synthesis following a 30 s exercise bout, in addition to glycolytic flux beginning earlier during exercise and reaching a higher peak rate compared to the non-T1D patients. This suggests that despite being on intensive insulin therapy and having more well-controlled blood glucose, muscle in those with T1D becomes more reliant on glycolytic metabolism, and this may be due, in part, to an attenuation in mitochondrial function. Another study [50] observed a greater plasma lactate and $[H^+]$, and muscle lactate immediately following intense exercise despite no differences in maximal activities of key metabolic enzymes (hexokinase, citrate synthase, and pyruvate dehydrogenase) at rest, in young adults with T1D (HbA1c ~8.6%, neuropathy exclusion criteria) compared to controls. It is worth noting however that differences in pretraining VO_{2max} values (reported only as absolute values) may have indicated greater baseline fitness in the T1D group.

CURRENT MECHANISTIC THEORIES UNDERLYING DIABETIC COMPLICATIONS DEVELOPMENT

Despite myopathy increasingly becoming recognized as significant complication of T1D, the studies investigating this neuromuscular disorder are limited. Thus, our fundamental understanding of the mechanisms underlying the development and progression of diabetic myopathy remain relatively naïve.

Given the findings from human T1D studies previously described, below we proffer a novel mechanism for discussion to guide the design of future investigations into the study of diabetic myopathy (schematized in Fig. 2). The theory of hyperglycemia-mediated myopathy proposed here is an extension of the independent theories put forth by Brownlee (reviewed in [51]) and Sharma (reviewed in [52▪▪]) to explain the development of cellular damage in cell types unable to regulate intracellular glucose concentrations. Although skeletal muscle is capable of regulating its own intracellular glucose concentration in the non-diseased state, the repeated instances of peripheral hyperinsulinemia in those with T1D (resulting from recurrent bolus insulin injections) causes substantially higher rates of glucose flux into muscle, consequently leading to repeated instances of intracellular hyperglycemia (i.e. glucose 'overload'; [50]).

The hyperglycemic intracellular environment creates a 'substrate overload' situation for the

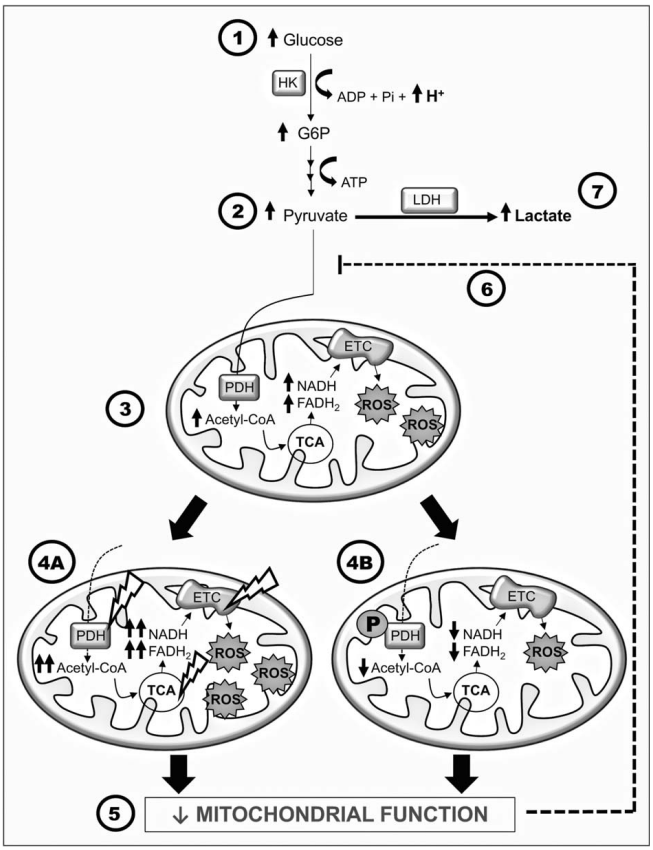

FIGURE 2. Proposed mechanism underlying the development of diabetic myopathy in individuals with type 1 diabetes. Subcutaneous bolus insulin injections expose skeletal muscle to recurrent hyperinsulinemia, leading to increased glucose flux into the muscle and ultimately glucose 'overload' (1). Intracellular hyperglycemia results in an increased glycolytic flux and an abundance of pyruvate (2). In the non-T1D state (3), pyruvate is transported into the mitochondria and undergoes oxidation to acetyl-CoA via pyruvate dehydrogenase (PDH) which then enters the tricarboxylic acid (TCA) cycle resulting in the production of electrons donors NADH and FADH2. These electron donors drive the electron transport chain (ETC) which culminates in the production of ATP. In the T1D state, the intracellular hyperglycemia is proposed to result in reduced mitochondrial function in one of two ways. First, substrate overload would result in significantly elevated reactive oxygen species (ROS) production, leading to oxidative damage of mitochondrial proteins and reduced ETC activity (mitochondrial respiration; 4A). Alternatively, elevated substrate flux through the mitochondria would result in phosphorylation (inhibition) of PDH activity and a concomitant decrease in ETC activity (mitochondrial respiration) by reducing substrate entry into the mitochondria (4B). The intracellular hyperglycemia-mediated decrease in mitochondrial function (5) (through negative feedback on PDH and other rate limiting pathways) prevents further substrate entry into the mitochondria (6) and instead shifts pyruvate toward lactate production (7), placing a greater reliance on glycolytic rather than oxidative metabolism for energy production. Whether the changes in metabolic profile occurring within the muscle of those with T1D directly results in structural and functional deficits described is unclear at this time. Based on data from other disease models (e.g. mitochondrial myopathies, aging), we would suggest that while the structural and functional impairments of muscle are likely multifactorial in origin, mitochondrial dysfunction, either directly or indirectly, is a primary driver behind many of the changes occurring. ATP, Adenosine 5′-triphosphate; HK, hexokinase; G6P, glucose-6-phosphate; LDH, lactate dehydrogenase.

myofibers. According to Sharma's theory, intracellular hyperglycemia (or nutrient stress) would lead to an eventual decrease in mitochondrial superoxide production through inhibition (i.e. phosphorylation) of pyruvate dehydrogenase (PDH). The inhibition of PDH would thus reduce pyruvate entry into the tricarboxylic acid (TCA) cycle and thereby reduce mitochondrial electron transport chain (ETC) activity. As Sharma points out, although reduced mitochondrial ETC activity may be a positive short-term adaptation, long-term reductions in mitochondrial oxidative phosphorylation activity would not only result in an increased flux through lactate dehydrogenase but, would also be associated with the release of oxidants from nonmitochondrial sources and release of proinflammatory and profibrotic cytokines.

Conversely, Brownlee's unified theory implicates hyperglycemia-mediated overproduction of mitochondrial superoxide as the precipitating factor in diabetic cellular damage. Brownlee further postulates that the common link to the cellular damaging cascades is a decrease in the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH). This initiating step would increase glycolytic intermediates upstream of GAPDH and lead to the formation of advanced glycation endproducts, increased hexosamine pathway activity, protein kinase C activation, and an increased polyol pathway flux.

MECHANISM UNDERLYING THE DEVELOPMENT AND PROGRESSION OF DIABETIC MYOPATHY

Although there is evidence for a decrease in PDH content/activity in the skeletal muscle of T1D rodents [53,54,55▪▪], the evidence in humans is less equivocal [50,56]. Similarly, changes in muscle expression/activity of GAPDH are generally not reported in the mouse or human T1D literature. As well, an increased generation of mitochondrial ROS within skeletal muscle of T1D rodent models has been observed [57] but, to the best of our knowledge, no similar studies have been undertaken in humans. Although the theories proposed by Brownlee and Sharma to explain the development of diabetic complications (in the kidney, vasculature, and nervous system) are very helpful and provide clear starting points for therapeutic consideration, they admittedly represent a reductionist view of a very complex process. Large-scale proteomic studies have calculated no less than 20,000 skeletal muscle redox-sensitive proteins (i.e. capable of oscillating between a reduced and oxidized state as a redox couple). The emergent picture reveals hundreds of dynamic redox control elements that could support redox signaling and integration of metabolic functions [58,59]. Thus, it is likely that many metabolic enzymes are dysregulated in humans with T1D, and a more comprehensive interrogation of the metabolic (and myofibrillar) pathways and ROS sources are needed if we are to fully appreciate the etiology of diabetic myopathy. Consistent with this, K.S. Nair's group [55▪▪] used a proteomic approach to demonstrate that insulin-treated diabetic rodents downregulate mitochondrial proteins of the ETC, TCA cycle, and β-oxidation while upregulating proteins involved in glycolysis and glycogen breakdown. Unexpectedly, despite decreases in β-oxidation proteins, increases in muscle proteins involved in uptake of fatty acids were observed.

Based on the evidence presented above, a model for the development and progression of T1D myopathy emerges whereby recurrent administration of subcutaneous insulin results in transient bouts of hyperinsulinemia that leads to intramuscular hyperglycemia. These short-term, repeated increases in glycemic levels within the myofiber lead to increased glycolytic flux shuttled towards lactate production rather than oxidative metabolism due to reductions in mitochondrial respiration. Whether the reductions in skeletal muscle mitochondrial respiration observed in humans with T1D are through phosphorylation of PDH or mediated through nonenzymatic glycation/ROS-induced protein damage is currently unknown. While not elaborated in detail within this brief review, it is also worth considering that a mismatch of β-oxidation and fatty acid uptake (observed in insulin-treated T1D rodents) could lead to an accumulation of incomplete fatty acid oxidation products and a resultant increase in oxidative stress and insulin resistance [55▪▪]. Whether the changes in metabolic profile occurring within the muscle of those with T1D results in the structural and functional deficits previously described is unclear at this time. We would suggest that although the structural and functional impairments of muscle are likely multifactorial in origin, mitochondrial dysfunction either directly or indirectly, is a primary driver behind many of the changes occurring.

CONCLUSION

The evidence presented in this review clearly supports myopathy, a hereunto little recognized complication of T1D, as a novel neuromuscular disorder of clinical significance. With peripheral injections shifting the emphasis of insulin's action toward skeletal muscle, the importance of muscle health to overall well-being in T1D cannot be understated.

Thus, it is surprising that the information available on the impact of T1D on skeletal muscle health is so limited. Future studies are clearly needed to not only reveal the scope and magnitude of myopathy, but also to definitively demonstrate the underlying mechanisms for this complication. These studies will pave the way for the discovery of novel adjuvant therapies and/or appropriate exercise strategies to maximize mitochondrial function in those with T1D.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ■ of special interest
- ■■ of outstanding interest

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1.2 Brief Overview of Mitochondrial Physiology

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Showing pages 102 to 103.

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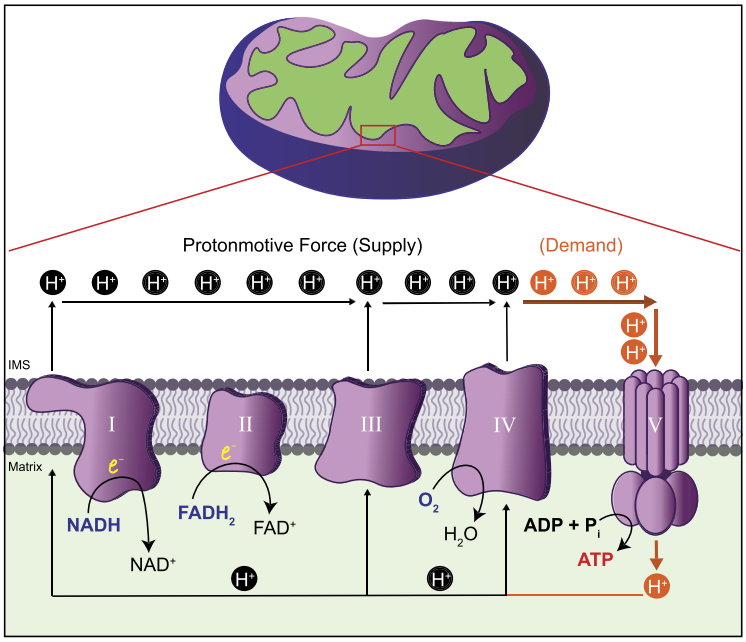

Figure 2. Schematic representation of mitochondrial oxidative phosphorylation. The electron carriers NADH and $FADH_2$, derived from the metabolism of carbohydrates and lipids, donate electrons (e-) to complex I and complex II, respectively. The electrons are then passed down in a series of redox reactions until they reach the final electron acceptor, oxygen (O_2), producing water (H_2O). Free energy is released during the transfer of electrons, which is captured and used by complexes I, III, and IV to pump protons (H^+) from the matrix into the IMS against an electrochemical gradient to create the protonmotive force (the "energy supply"). The protonmotive force, or membrane potential, in turn can drive the production of ATP from ADP (the "energy demand") and inorganic phosphate (P_i) by driving protons back into the matrix through complex V.

Brief Overview of Mitochondrial Physiology

Mitochondrial respiration

Mitochondria produce ATP via the mechanism of oxidative phosphorylation (also referred to as mitochondrial respiration). As depicted in Figure 2, a series of specific proteins (complexes I through V) located in the inner mitochondrial membrane, termed the electron transport chain (ETC), catalyze the oxidation of respiratory substrates (electron carriers) NADH and $FADH_2$, generated during the metabolism of carbohydrates (glycolysis) and lipids (B-oxidation). Electrons are then transferred into the ETC and, through a series of redox reactions, are passed down from complex I or complex II all the way to complex IV until they reach the final electron acceptor, oxygen. Free energy is released during these chemical reactions, which is captured and used to "pump" protons across the mitochondrial inner membrane and into the intermembrane space (IMS) against an electrochemical gradient. This creates a proton-motive force, also known as the mitochondrial membrane potential ($\Delta\Psi$), that in turn drives protons back into the matrix through the ATP synthase (complex V), rotating a part of the enzyme that drives the phosphorylation of ADP into ATP.

Mitochondrial ROS production

Approximately 2% of the total oxygen consumed during "normal" oxidative phosphorylation results in the production of ROS due to natural electron leakage from the ETC. These leaked electrons are highly unstable and react quickly with nearby oxygen to produce oxygen free radicals, such as the superoxide radical ($O_2^{\bullet}$-) and hydroxyl radical ($^{\bullet}OH$), as well as nonradicals, such as hydrogen peroxide (H_2O_2). The mitochondrial matrix and cytoplasm are equipped with enzymatic (*e.g.*, glutathione and catalase) and nonenzymatic (*e.g.*, vitamin E) antioxidant defenses to counterbalance these oxidants. At low physiological levels, ROS acts as an important signaling molecule for various processes, including muscle contraction, cell proliferation, and cell adaptation to exercise training. However, when ROS levels exceed antioxidant capacity (as may occur with excess substrate delivery, dysfunctional mitochondria, or reduced antioxidant defences), it becomes pathological and causes oxidative stress.

Mitochondrial Ca^{2+} uptake

Mitochondria also contain a Ca^{2+} uniporter that allows Ca^{2+} flux into the matrix, a process that is essential for ATP production and for regulating muscle contractile function and programmed

cell death. The influx of Ca^{2+} into the matrix not only contributes to shaping of the sarcoplasmic Ca^{2+} transients but also simultaneously stimulates mitochondrial energy production by activating select Ca^{2+}-sensitive mitochondrial dehydrogenases (56). This ensures that adequate energy is provided to support muscle contraction. Moreover, mitochondria can aid in the buffering of cytoplasmic Ca^{2+} by chelating excess levels of this ion with inorganic phosphate in its matrix. However, Ca^{2+} overload or the combination of excess Ca^{2+} and damaged mitochondria can induce opening of the mPTP. mPTP opening collapses the $\Delta\Psi$ and leads to decreased ATP production, disruption of ionic homeostasis, and swelling. As the mitochondrial swells, the cristae of the inner mitochondrial membrane begin to unfold and the increased pressure on the outer mitochondrial membrane causes it to rupture and releases proapoptotic factors that activate caspase cascades in the cytosol to initiate cellular fragmentation and ultimately, cell death.

Mitochondrial Dysfunction and the Impact on Skeletal Muscle Health

At this point in time, the term mitochondrial dysfunction is ill-defined owing to the multiplicity of mitochondrial functions in a cell, as described previously, as well as the fact that it remains unclear as to whether altered mitochondrial function reflects a physiological adaptation, a pathological maladaptation, or simply a pathological phenomenon. For the purpose of this review, mitochondrial dysfunction will entail any abnormality in the key physiological roles (*e.g.*, respiration, ROS, Ca^{2+} uptake) of a mitochondrion (*i.e.*, intrinsic mitochondrial function).

Depending on the metabolic state of the cell, mitochondria regulate skeletal muscle mass, function, and metabolism via either the activation of anabolic or catabolic signaling pathways, some of which feed-forward to myonuclei to either upregulate or downregulate the expression of genes important for muscle protein synthesis/degradation, muscle contraction, and substrate oxidation (reviewed in (57)). For example, excess ROS production, caused by either 1) nutrient excess, 2) a combination of nutrient excess and reduced mitochondrial respiration, 3) damaged mitochondria, 4) depleted antioxidant defenses, or 5) a combination of these factors, can induce the expression of atrogenes via activation of the JNK/FoxO signaling pathway as well as the activation of endoplasmic reticulum stress, which in turn suppress protein synthesis and promote protein degradation, and hence, muscle atrophy (57). Excess mitochondrial ROS also can lead to opening of the mPTP and thus increase apoptotic potential. Opening of the mPTP also can be triggered by impairments in the ability of the mitochondria to retain excess levels of Ca^{2+} due to failure of cytosolic Ca^{2+} homeostasis and ionic disturbances. Irrespective of the cause, the resultant increase in apoptosis invariably promotes muscle atrophy. Increased mitochondrial ROS also can lead to nuclear and mtDNA mutations/deletions, protein damage (including ETC enzymes), and lipid peroxidation — in other words, oxidative stress — all of which can directly and indirectly impact muscle mass, function, and metabolism. For instance, damage to enzymes of the ETC caused by ROS can lead to even greater ROS production/damage, creating a vicious cycle that perpetuates mitochondrial dysfunction and apoptotic cell death. Mitochondrial dysfunction, specifically impaired mitochondrial respiration, can lead to the accumulation of incompletely oxidized substrates and toxic metabolites, which have been implicated in impaired muscle contraction (57). Furthermore, impaired mitochondrial respiration not only drives mitochondrial ROS production but also increases the AMP/ATP ratio, which in turn leads to the activation of the energy sensor molecule AMP-activated protein kinase (AMPK). Increased AMPK activity inhibits anabolic pathways, including muscle protein synthesis by inhibiting mTOR and directly phosphorylating FoxO3 (see Fig. 1).

1.3 Objectives and Hypotheses

The overall objective of this thesis was to examine mitochondrial bioenergetics in muscle biopsies from humans with T1D to gain a deeper understanding of the mechanism(s) underlying the evidence of diabetic myopathy in people with T1D. It was hypothesized, *a priori,* that humans with T1D, compared to their matched counterparts without diabetes (control), would demonstrate significant deficiencies in muscle mitochondrial function and morphology/content as determined by the gold-standard *in vitro* methodology: high-resolution respirometry and transmission electron microscopy (TEM), respectively.

1.3.1 Study 1 (CHAPTER 2)

The objective of this study was to systematically examine skeletal muscle mitochondrial bioenergetics, specifically respiration, ROS emission (mROS), and Ca^{2+} retention capacity (mCRC), as well as mitochondrial content/ultrastructure in *Vastus lateralis* biopsies from otherwise, healthy and physically active young adults (females and males) with T1D.

It was hypothesized that compared to control participants: 1) all mitochondrial respiratory assays as well as mCRC would be decreased, whereas mROS increased, in T1D participants. 2) Mitochondrial ultrastructure would be abnormal in T1D participants. 3) Mitochondrial content would be decreased in T1D participants.

1.3.2 Study 2 (CHAPTER 3)

The objective of this study was to build from Study 1 and determine whether sex differences in muscle mitochondrial bioenergetics – respiration, mROS, mCRC – and content exists in otherwise healthy and physically active young women and men with T1D.

It was hypothesized that compared to men with T1D: 1) Mitochondrial respiration and mCRC would be decreased, and mROS increased in women with T1D. 2) Mitochondrial content would be decreased in women with T1D compared to men with T1D.

1.3.3 Study 3 (CHAPTER 5)

The objective of this study was to systematically assess mitochondrial respiratory function and content/ultrastructure in *Vastus lateralis* biopsies in otherwise healthy, recreationally active adults (females and males) with T1D that are beyond young adulthood.

It was hypothesized that compared to control participants: 1) All mitochondrial respiratory assays would be decreased in participants with T1D. 2) Mitochondrial content would be decreased in T1D participants. 3) All mitochondrial measures would negatively correlate with age, disease duration, and glycemia.

CHAPTER 2 — Altered mitochondrial bioenergetics and ultrastructure in the skeletal muscle of young adults with type 1 diabetes

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PREFACE

Author contributions:

CMFM, MCH, MAT, TJH and CGRP designed the experiments. **CMFM**, TJH and CGRP wrote the manuscript. **CMFM**, MCH, SVR, NEV, CL and CM performed experiments. **CMFM**, MCH, SVR, NEV, CL, FAR, CM, MAT, MPK, RL, TJH and CGRP analyzed and interpreted data. All authors edited the manuscript.

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ARTICLE

Altered mitochondrial bioenergetics and ultrastructure in the skeletal muscle of young adults with type 1 diabetes

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Abstract

Aims/hypothesis A comprehensive assessment of skeletal muscle ultrastructure and mitochondrial bioenergetics has not been undertaken in individuals with type 1 diabetes. This study aimed to systematically assess skeletal muscle mitochondrial phenotype in young adults with type 1 diabetes.

Methods Physically active, young adults (men and women) with type 1 diabetes (HbA_{1c} 63.0 ± 16.0 mmol/mol [7.9% ± 1.5%]) and without type 1 diabetes (control), matched for sex, age, BMI and level of physical activity, were recruited (n = 12/group) to undergo vastus lateralis muscle microbiopsies. Mitochondrial respiration (high-resolution respirometry), site-specific mitochondrial H_2O_2 emission and Ca^{2+} retention capacity (CRC) (spectrofluorometry) were assessed using permeabilised myofibre bundles. Electron microscopy and tomography were used to quantify mitochondrial content and investigate muscle ultrastructure. Skeletal muscle microvasculature was assessed by immunofluorescence.

Results Mitochondrial oxidative capacity was significantly lower in participants with type 1 diabetes vs the control group, specifically at Complex II of the electron transport chain, without differences in mitochondrial content between groups. Muscles of those with type 1 diabetes also exhibited increased mitochondrial H_2O_2 emission at Complex III and decreased CRC relative to control individuals. Electron tomography revealed an increase in the size and number of autophagic remnants in the muscles of participants with type 1 diabetes. Despite this, levels of the autophagic regulatory protein, phosphorylated AMP-activated protein kinase (p-AMPKα^{Thr172}), and its downstream targets, phosphorylated Unc-51 like autophagy activating kinase 1 (p-ULK1^{Ser555}) and p62, was similar between groups. In addition, no differences in muscle capillary density or platelet aggregation were observed between the groups.

Conclusions/interpretation Alterations in mitochondrial ultrastructure and bioenergetics are evident within the skeletal muscle of active young adults with type 1 diabetes. It is yet to be elucidated whether more rigorous exercise may help to prevent skeletal muscle metabolic deficiencies in both active and inactive individuals with type 1 diabetes.

Cynthia M. F. Monaco and Meghan C. Hughes are joint first authors. Thomas J. Hawke and Christopher G. R. Perry are joint senior authors.

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Research in context

What is already known about this subject?

- Previous research using ^{31}P-magnetic resonance spectroscopy (MRS) has reported a reduction in ATP synthesis rates in the skeletal muscle of those with type 1 diabetes; a finding that suggests mitochondrial impairment
- Skeletal muscle, by virtue of its mass, is the largest site for postprandial glucose disposal and is indispensable for glycaemic control
- Skeletal muscle mitochondrial impairments have been implicated in a number of pathophysiological states, including insulin resistance, type 2 diabetes, ageing and cachexia

What is the key question?

- Do skeletal muscle metabolic and ultrastructural abnormalities exist in physically active young adults with moderately controlled type 1 diabetes?

What are the new findings?

- Type 1 diabetes causes site-specific alterations in mitochondrial bioenergetics, including increases in H_2O_2 emission and decreases in mitochondrial respiration, as well as decreased mitochondrial Ca^{2+} retention capacity (CRC)
- Increases in autophagic debris were noted in the skeletal muscle of those with type 1 diabetes, without increases in the autophagic regulatory protein phosphorylated AMP-activated protein kinase (p-AMPKα^{Thr172}) and downstream autophagic proteins phosphorylated Unc-51 like autophagy activating kinase 1 (p-ULK1^{Ser555}) and p62
- Individuals with type 1 diabetes did not have reduced mitochondrial content or skeletal muscle capillary density, suggesting differences in mitochondrial bioenergetics and CRC are the result of intrinsic alterations

How might this impact on clinical practice in the foreseeable future?

- These findings indicate that recreational physical activity may be insufficient to prevent skeletal muscle metabolic deficiencies in type 1 diabetes. Further studies are required to elucidate whether more rigorous exercise may help to prevent skeletal muscle metabolic and ultrastructural abnormalities

Keywords Calcium tolerance · Capillary density · Mitochondria · Myopathy · Reactive oxygen species · Skeletal muscle · Type 1 diabetes

Abbreviations

AMPK	AMP-activated protein kinase
CRC	Ca^{2+} retention capacity
mG3PDH	Mitochondrial glycerol-3-phosphate dehydrogenase
mPTP	Mitochondrial permeability transition pore
MRS	Magnetic resonance spectroscopy
OXPHOS	Oxidative phosphorylation cocktail
PDC	Pyruvate dehydrogenase complex
ROS	Reactive oxygen species
TEM	Transmission electron microscopy
ULK1	Unc-51 like autophagy activating kinase 1

Introduction

Skeletal muscle accounts for almost half of our body weight and, as such, is responsible for up to 75% of insulin-stimulated glucose disposal after a meal [1, 2]. In fact, blood glucose uptake into muscle and its subsequent conversion to glycogen is a major determinant of insulin sensitivity [3, 4]. Given the importance of skeletal muscle for glucose management, as well as its vital role in insulin action, impairments in skeletal muscle health in people with type 1 diabetes diminishes their ability to mitigate dysglycaemic burdens, promotes the development and progression of complications [5, 6] and, ultimately, expedites the accelerated physical disability that characterises diabetes [7]. However, our understanding of the impact of this chronic disease on the health/quality of human skeletal muscle is extremely limited.

Mitochondria play a fundamental role in energy metabolism and ATP production throughout the body. Based on their role in fuel oxidation, mitochondria can also generate considerable amounts of reactive oxygen species (ROS). It remains controversial whether impaired skeletal muscle mitochondrial respiratory function contributes to insulin resistance and dysglycaemia in obesity and type 2 diabetes [8]. However, a consistent observation has been increased mitochondrial ROS in obese/type 2 diabetes relative to lean control individuals

[8]. In addition, using ^{31}P-magnetic resonance spectroscopy (MRS), people with type 1 diabetes have been shown to exhibit slowed post-exercise ATP resynthesis [9–11]; the underlying mechanism(s) for this impairment is currently unknown and would require a direct and comprehensive exploration of site-specific mitochondrial respiratory function. Moreover, the effect of type 1 diabetes on skeletal muscle ultrastructure, last reported in 1977 [12], has not been re-examined since the introduction of more aggressive insulin therapies (e.g. basal and fast-acting insulins, insulin analogues and pump therapy), and its effect on muscle mitochondrial ROS production remains to be elucidated.

Here, we hypothesised that otherwise healthy, young adults with type 1 diabetes would display site-specific decrements in skeletal muscle mitochondrial respiration, increased ROS production and altered muscle ultrastructure relative to non-diabetic controls.

Methods

Participants

Twelve untrained participants with type 1 diabetes were recruited and closely matched with twelve participants without diabetes (control group) for age, sex, BMI and level of physical activity. Demographics (Table 1) and participant number was determined by power calculations from our previous studies in human muscle [13, 14]. All participants with type 1 diabetes used insulin (insulin pump or multiple daily injections) and reported no complications. Prior to giving informed consent, all participants were given oral and written information about the experimental procedures. All procedures were approved by the Research Ethics Board at York University (REB number e2013-032) and conformed to the Declaration of Helsinki.

Study design

Participants reported to York University in the early morning or early afternoon and were instructed to consume a standardised meal 1.5–2 h prior to their visit. Participants with type 1 diabetes were also instructed to continue their habitual use of insulin. Upon arrival, body mass and height measurements were taken to determine BMI, and a blood sample was obtained using venepuncture, for HbA_{1c} analysis. Skeletal muscle samples were then obtained from the vastus lateralis muscle by a microbiopsy percutaneous needle, as described previously [13], and used for mitochondrial bioenergetic analyses, transmission electron microscopy (TEM), histological analysis and western blotting, as described below.

Mitochondrial bioenergetics

All mitochondrial bioenergetic experiments were performed in vitro using permeabilised muscle fibres prepared from fresh muscle samples that were immediately placed in ice-cold BIOPS containing (in mmol/l): 50 MES, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 imidazole, 0.5 dithiothreitol (DTT), 20 taurine, 5.77 ATP, 15 PCr and 6.56 $MgCl_2 \cdot 6H_2O$ (pH 7.1), as previously described in detail [13].

Mitochondrial respiration Using the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria), high-resolution measurements of mitochondrial oxygen consumption were conducted in permeabilised myofibres placed in 2 ml of respiration medium MiR05 [15], containing 0.5 mmol/l EGTA, 10 mmol/l KH_2PO_4, 3 mmol/l $MgCl_2 \cdot 6H_2O$, 60 mmol/l K-lactobionate, 20 mmol/l Hepes, 20 mmol/l taurine, 110 mmol/l sucrose and 1 mg/ml fatty acid-free BSA (pH 7.1), at 37°C. Experiments were conducted at an initial oxygen concentration of 350–375 μmol/l with constant stirring at 750 rev/min. Respiration medium was supplemented with 20 mmol/l creatine (C0780;

Table 1 Demographics of participants

Characteristic	Control ($n = 12$)	Type 1 diabetes ($n = 12$)
Sex (male/female)	5/7	5/7
Age (years)	26 ± 2	26 ± 4
Weight (kg)	65.4 ± 12.1	73.0 ± 13.4
Height (m)	1.70 ± 0.07	1.69 ± 0.13
BMI (kg/m^2)	22.5 ± 2.8	25.4 ± 3.7*
HbA_{1c} (mmol/mol)	33.0 ± 2.2	63.0 ± 16.0***
HbA_{1c} (%)	5.2 ± 0.2	7.9 ± 1.5***
Diabetes duration (years)	–	15.2 ± 7.9
Diabetes onset (years of age)	–	11.5 ± 7.3
Physical activity (min/week)[a]	233.1 ± 152.6	201.0 ± 168.6

Data are means ± SD

[a] Based on a 7 day activity recall record of moderate-to-vigorous activity levels

*$p < 0.05$, ***$p < 0.001$ vs control participants; unpaired, two-tail Student's t test

Sigma, St Louis, MO, USA) to enhance mitochondrial phosphate shuttling [16]. ADP-stimulated respiratory kinetics were determined through ADP (A5285; Sigma) titrations in the presence of 5 mmol/l blebbistatin (CAY13013; Cayman Chemical, Ann Arbor, MI, USA), in order to prevent spontaneous contraction of fibres [13, 17, 18], 5 mmol/l pyruvate (P2256; Sigma) and 2 mmol/l malate (M1000; Sigma). Complex I kinetics were determined through standard pyruvate and glutamate (GLU303; BioShop, Burlington, ON, Canada) titrations in the presence of 5 mmol/l blebbistatin, 5 mmol/l ADP and 2 mmol/l malate, while Complex II kinetics were determined through standard succinate (S2378; Sigma) titrations in the presence of 5 mmol/l blebbistatin, 5 mmol/l ADP and 10 μmol/l rotenone (R8875; Sigma; to prevent superoxide generation at Complex I). During each titration, Cytochrome *c* (192-10; Lee Biosolutions, Maryland Heights, MO, USA) was added last to determine intactness of the outer mitochondrial membrane. Any samples that exceeded a 10% increase in respiration after Cytochrome *c* addition were excluded [19].

Mitochondrial H_2O_2 emission Mitochondrial H_2O_2 emission was measured fluorometrically (QuantaMaster 40; HORIBA Scientific, Edison, NJ, USA) at 37°C in buffer Z [18], containing 105 mmol/l K-MES, 30 mmol/l KCl, 10 mmol/l KH_2PO_4, 5 mmol/l $MgCl_2{\cdot}6\,H_2O$, 1 mmol/l EGTA, 5 mg/ml BSA (pH 7.1), supplemented with 20 mmol/l creatine, 10 μmol/l Amplex UltraRed reagent (A36006; Life Technologies, Carlsbad, CA, USA), 0.5 U/ml horseradish peroxidase (P8375; Sigma) and 40 U/ml Cu/Zn superoxide dismutase (SOD1; S9697; Sigma), as described previously [13]. Various sites were assessed by titrating the following substrates [20, 21]: (1) 2.5 μmol/l antimycin (A8674; Sigma) for assessment of Complex III; (2) 10 mmol/l pyruvate and 2 mmol/l malate, to assess Complex I via generation of NADH; (3) 10 mmol/l succinate, to assess Complex I (via reverse flow) via generation of $FADH_2$; (4) 10 mmol/l pyruvate and 0.5 μmol/l rotenone, to assess pyruvate dehydrogenase complex (PDC); rotenone blocks NADH entry into Complex I; and (5) 20 mmol/l glycerol-3-phosphate, for the assessment of mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH; G7886; Sigma). In addition, fibres used for Complex I (pyruvate + malate), PDC and mG3PDH measurements were treated with 35 μmol/l 1-chloro-2,4-dinitrobenzene (237329; Sigma) during permeabilisation to deplete glutathione [22].

Mitochondrial Ca^{2+} retention capacity Mitochondrial Ca^{2+} retention capacity (CRC) was measured in duplicate, fluorometrically (QuantaMaster 40; HORIBA Scientific), at 37°C in buffer Y, containing 250 mmol/l sucrose, 10 mmol/l Tris-HCl, 20 mmol/l Tris base, 10 mmol/l KH_2PO_4, 2 mmol/l $MgCl_2{\cdot}6H_2O$ and 0.5 mg/ml BSA, with 1 μmol/l Calcium Green-5N (C3737; Life Technologies), as described previously [23]. Briefly, Ca^{2+} uptake was initiated with 8 nmol pulses of Ca^{2+} ($CaCl_2$) and then subsequent pulses of 4 nmol Ca^{2+} were titrated until opening of the mitochondrial permeability transition pore (mPTP) was observed [23].

TEM

Fresh muscle was immediately fixed in 2% (vol./vol.) glutaraldehyde (111-30-8; Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 mol/l sodium cacodylate buffer pH 7.4 (Canemco, Lakefield, QC, Canada) and processed as described previously [24]. To quantify mitochondria, representative micrographs from eight unique fibres (containing a portion of the subsarcolemmal region adjacent to the nucleus, with most of the image containing the intermyofibrillar area) were acquired at ×15,000 magnification. Blinded quantification of mitochondrial size (mean area, μm^2), distribution (number per μm^2) and density ($\mu m^2 \times$ number per $\mu m^2 \times 100$) was achieved using Nikon Imaging Software (NIS)-Elements AR (v 4.6; Nikon, Melville, NY, USA) by manually outlining mitochondria and converting to actual size using a calibration grid [25].

Electron tomography

Samples were oriented and trimmed to allow longitudinal sectioning of fibres, which were visually localised. Ultra-microtomy (Leica UC7; Leica Microsystems, Wetzlar, Germany) was performed with a 35° diamond knife (Diatome, Nidau, Switzerland), and 65 nm thick sections were mounted onto copper slot grids (Plano, Wetzlar, Germany). Electron micrographs for tomography tilt series were acquired on a JEM-2200FS electron microscope (JEOL, Akishima, Tokyo, Japan) (200 kV) equipped with a TemCam-F416 camera (TVIPS, Munich, Germany) and using a 25 electron volt (eV) energy filter slit. Under-focus was adjusted to 4000 nm. Micrographs were acquired two-fold binned for tomography. The resulting pixel size was 2.4 nm. Tilt series was collected over a total angular tilt range from −70° to +70°, at 2° increments. Image series was aligned by patch tracking mode using the IMOD software package (v 4.7; http://bio3d.colorado.edu/imod) [26]. A single reconstructed volume was computed from each tilt series by radially weighted back projection. A 3D density distribution (tomogram) was obtained. A virtual section with a 24 nm thickness was generated with the slicer tool in IMOD. Major organelles were coloured utilising Adobe Photoshop CC 2015 by a blinded researcher.

Histology and immunofluorescence analysis

Biopsied muscle was immediately embedded in Tissue Tek optimum cutting temperature (O.C.T.) compound (4583; VWR, Radnor, PA, USA), frozen in liquid nitrogen-cooled isopentane and stored at −80°C until analysis. Cryosections (−20°C) that were 8 μm thick were transferred onto static-free microscope slides (CA48311-703; VWR), allowed to air dry and fixed with ice-cold 4% (wt/vol.) paraformaldehyde. Sections were then incubated in blocking solution (5% [vol./vol.] normal goat serum in 1X PBS; S-1000; Vector Laboratories, Burlingame, CA, USA) for 30 min and then co-stained with CD31 (platelet endothelial cell adhesion molecule-1 [PECAM-1]) overnight. An antibody against CD41 was added the following day and incubated for 1 h. The appropriate secondary antibodies were then applied for 1 h: Alexa Fluor 594 and Alexa Fluor 488. Nuclei were counterstained with DAPI. CD31 was used as a marker of capillary content and CD41 to measure platelet aggregation. The antibodies were validated by manufacturers for immunofluorescence analyses (see electronic supplementary material [ESM] Table 1 for antibody details). H&E stains were used for the determination of muscle fibre area in order to calculate capillary:fibre ratio, as previously described [14]. Images were captured with a Nikon 90 Eclipse microscope (Nikon) and analysed using NIS-Elements AR software (v 4.6; Nikon) at original magnification of ×40.

Western blotting

A portion of muscle was quickly snap-frozen and stored at −80°C until analysis. Frozen muscle (~10–30 mg) was homogenised with a tapered teflon pestle in ice-cold buffer containing 40 mmol/l Hepes, 120 mmol/l NaCl, 1 mmol/l EDTA, 10 mmol/l $NAHP_2O_7{\cdot}10H_2O$ pyrophosphate, 10 mmol/l β-glycerophosphate, 10 mmol/l NaF and 0.3% CHAPS detergent (pH 7.1, adjusted using KOH). Protein concentrations were determined using a BCA assay (Life Technologies) and then loaded equally (50 or 100 μg), using Ponceau S (P7170; Sigma) as a loading control. Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, blocked for 1 h and incubated overnight at 4°C using commercially available antibodies validated by manufacturers for western blotting: human total oxidative phosphorylation cocktail (OXPHOS; to detect individual complexes of the electron transport chain [I, II, III, IV, V]), phosphorylated (Thr172) and total AMP-activated protein kinase (AMPKα), phosphorylated (Ser555) and total Unc-51-like autophagy activating kinase 1 (ULK1), sequestosome (SQSTM1; herein referred to as p62) and vinculin. After overnight incubation, membranes were washed in tris-buffered saline, 0.1% (vol./vol.) Tween 20 (TBS-T) and incubated for 1 h at room temperature with anti-mouse infrared fluorescent secondary antibody (IRDye 800CW-conjugated) for OXPHOS, and horseradish peroxidase conjugated secondary antibody for all other proteins (see ESM Table 1 for antibody details). Immunoreactive proteins were detected by infrared imaging (Odyssey CLx; LI-COR Biosciences, Lincoln, NE, USA) or chemiluminescence (Gel Logic 6000 Pro Imager; Carestream, Rochester, NY, USA) and quantified by densitometry using Image J (v 1.51t; http://imagej.nih.gov/ij/).

Statistical analysis

All results are expressed as mean ± SEM except for participant characteristics, which are expressed as mean ± SD. An unpaired two-tailed Student's *t* test was performed for all experiments except for CRC and mitochondrial respiration kinetics where an unpaired one-tailed *t* test and a two-way ANOVA were used, respectively. Significance was established at $p \leq 0.05$.

Results

Skeletal muscle mitochondrial bioenergetics

Control of oxidative phosphorylation by ADP ADP is a primary regulator of mitochondrial bioenergetics and signals the net cellular metabolic demand to mitochondria in times of energetic stress. Therefore, we first determined if mitochondria were sensitive to low, physiologically relevant concentrations of ADP, as well as maximal concentrations (oxidative capacity) in type 1 diabetes. We found that both sensitivity to low ADP levels and maximal capacity for respiration were significantly lower ($p < 0.05$, main effect) in participants with type 1 diabetes compared with control participants (Fig. 1a).

Substrate-specific impairments We then determined if mitochondrial sensitivity to specific substrates was different in the muscle of those with type 1 diabetes vs control individuals. No detectable change in Complex I-supported mitochondrial respiration was observed, as evidenced by the lack of a significant difference in the sensitivity or capacity of the mitochondria to oxidise pyruvate (an index of glucose oxidation) (Fig. 1b). This suggests that mitochondrial oxidation of pyruvate from glucose is not impaired in type 1 diabetes, and neither is Complex I oxidation of NADH from pyruvate dehydrogenase. There were also no detectable changes in glutamate-supported respiration (NADH oxidation) (Fig. 1c), which further suggests there is no deficiency in Complex I activity in type 1 diabetes. In contrast, Complex II-supported respiration by succinate ($FADH_2$ synthesis) was significantly lower ($p < 0.001$, main effect) at both low (sensitivity) and maximal (oxidative capacity) succinate concentrations in participants with type 1 diabetes, suggesting a reduction in

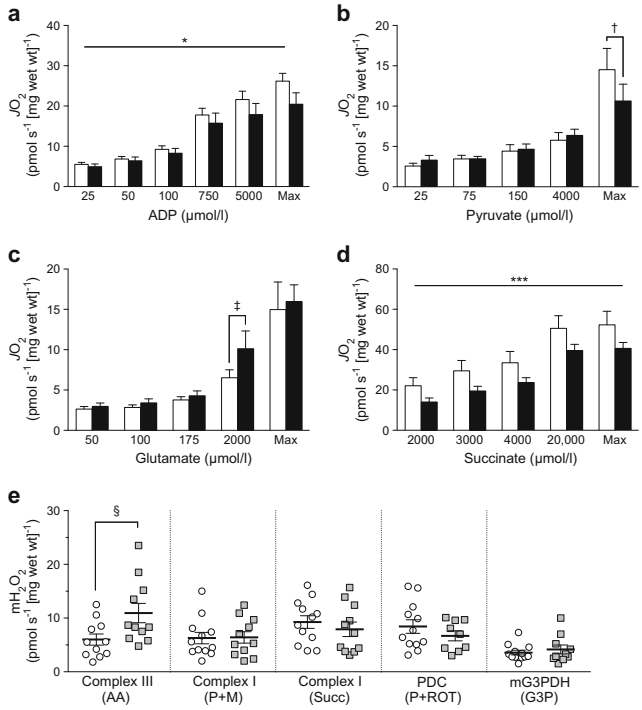

Fig. 1 Skeletal muscle mitochondrial bioenergetics in young adults with and without type 1 diabetes. Rates of mitochondrial oxygen consumption (JO_2) were measured in permeabilised muscle fibres in the presence of carbohydrate-derived substrates. (**a**) Submaximal and maximal (oxidative capacity) ADP-stimulated respiration in the presence of pyruvate and malate. Concentrations of ADP required to reach maximum varied between 6 and 18 mmol/l (both groups, $n = 12$). (**b**) Pyruvate-supported respiration (Complex I sensitivity and oxidative capacity) in the presence of 5 mmol/l ADP. Concentration of pyruvate required to reach maximum varied between 6 and 38 mmol/l (control group, $n = 11$; type 1 diabetes group, $n = 9$). (**c**) Glutamate-supported respiration (Complex I sensitivity and oxidative capacity) in the presence of 5 mmol/l ADP. Concentration of glutamate required to reach maximum varied between 4 and 20 mmol/l (control group, $n = 12$; type 1 diabetes group, $n = 8$). (**d**) Succinate-supported respiration (Complex II sensitivity and oxidative capacity) in the presence of 5 mmol/l ADP and rotenone. Concentration of succinate required to reach maximum varied between 22 and 26 mmol/l (both groups, $n = 11$). (**e**) Rates of mitochondrial H_2O_2 emission (mH_2O_2) derived from Complex III (antimycin A [AA]), Complex I forward electron flow (pyruvate + malate [P + M]) and reverse electron flow (succinate [Succ]), PDC (pyruvate + rotenone [P + ROT]) and mG3PDH (glycerol-3-phosphate [G3P]). White bars, control; black bars, type 1 diabetes; white circles, control; grey squares, type 1 diabetes. $^{*}p < 0.05$, $^{***}p < 0.001$, main effect of type 1 diabetes; $^{\dagger}p = 0.278$; $^{\ddagger}p = 0.117$; $^{\S}p < 0.05$. Max, maximum; Wt, weight

Complex II activity in type 1 diabetes compared with control individuals (Fig. 1d).

Mitochondrial H_2O_2 emission Next, we measured mitochondrial H_2O_2 emission potential. In the participants with type 1 diabetes, Complex III-supported mitochondrial H_2O_2 emission was significantly elevated ($p < 0.05$) compared with control participants but was not statistically different at Complex I (reverse [succinate] and forward electron flow [pyruvate + malate]), PDC and mG3PDH (Fig. 1e).

Skeletal muscle mitochondrial content

Considering that a reduction in mitochondrial respiration can be caused by lower mitochondrial content, we next examined the spatial organisation and abundance of mitochondria, as well as the expression of individual electron transport chain proteins. Using TEM, no differences in mean mitochondrial size, number of mitochondria per muscle area or mitochondrial area density in either the subsarcolemmal or intermyofibrillar regions of the muscle

were observed (Fig. 2a). Similarly, the levels of individual proteins were not significantly different between type 1 diabetes and control participants (Fig. 2b, c), further supporting no difference in total muscle mitochondrial content between the groups. Likewise, there were no differences in p-AMPKα^{Thr172} (relative to total AMPKα; Fig. 3d), a known regulator of mitochondrial biogenesis [27]. Collectively, this data indicates that the lower respiration per mg of muscle was not due to reductions in mitochondrial content, suggesting that impairments intrinsic to the mitochondria themselves might exist in people with type 1 diabetes. However, no differences were observed in ADP-stimulated respiration, Complex I (pyruvate)- and Complex II (succinate)-supported respiration when normalised to OXPHOS protein content (ESM Fig. 1).

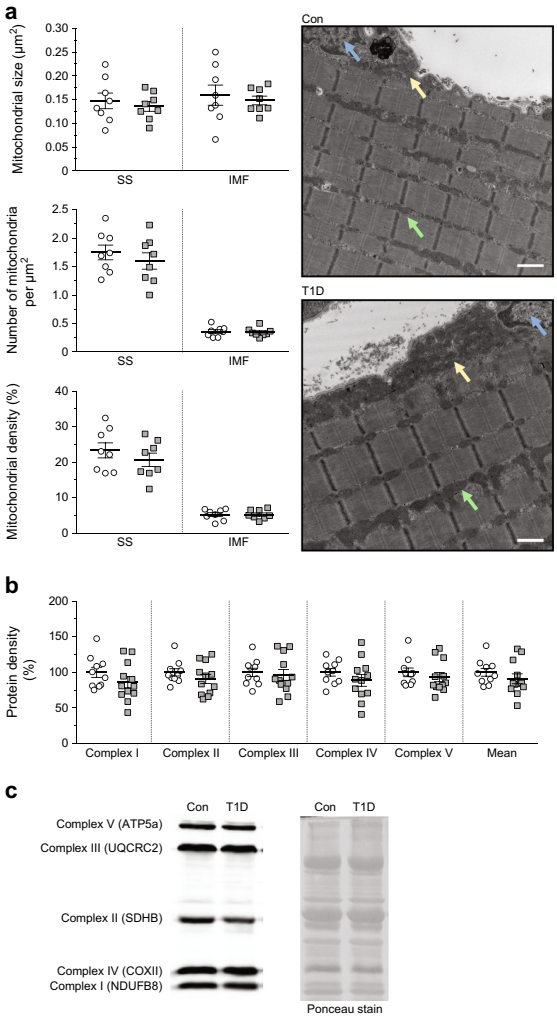

Fig. 2 Skeletal muscle mitochondrial content. (**a**) Transmission electron micrographs were acquired and analysed for mitochondrial size (mean area, μm^2), distribution (number per μm^2) and density (μm^2 × number per μm^2 × 100) within the subsarcolemmal area (SS) and intermyofibrillar area (IMF) of the muscle. Blue arrow, nucleus; yellow arrow, SS mitochondria; green arrow, IMF mitochondria. Scale bar, 2 μm. (**b**) Protein levels of the individual complexes of the electron transport chain (I, II, III, IV, V; as measured by the OXPHOS antibody cocktail) as well as mean expression of all complexes combined. (**c**) Representative immunoblot of Complex I (~18 kDa), Complex II (~29 kDa), Complex III (~48 kDa), Complex IV (~22 kDa) and Complex V (~54 kDa) and Ponceau stain (loading control). White circles, control; grey squares, type 1 diabetes. ATP5a, ATP synthase subunit alpha 5; Con, control; COXII, cytochrome oxidase subunit II; NDUFB8, NADH: ubiquinone oxidoreductase subunit B8; SDHB, succinate dehydrogenase [ubiquinone] iron-sulfur subunit B; T1D, type 1 diabetes; UQCRC2, ubiquinol-cytochrome c reductase core protein 2

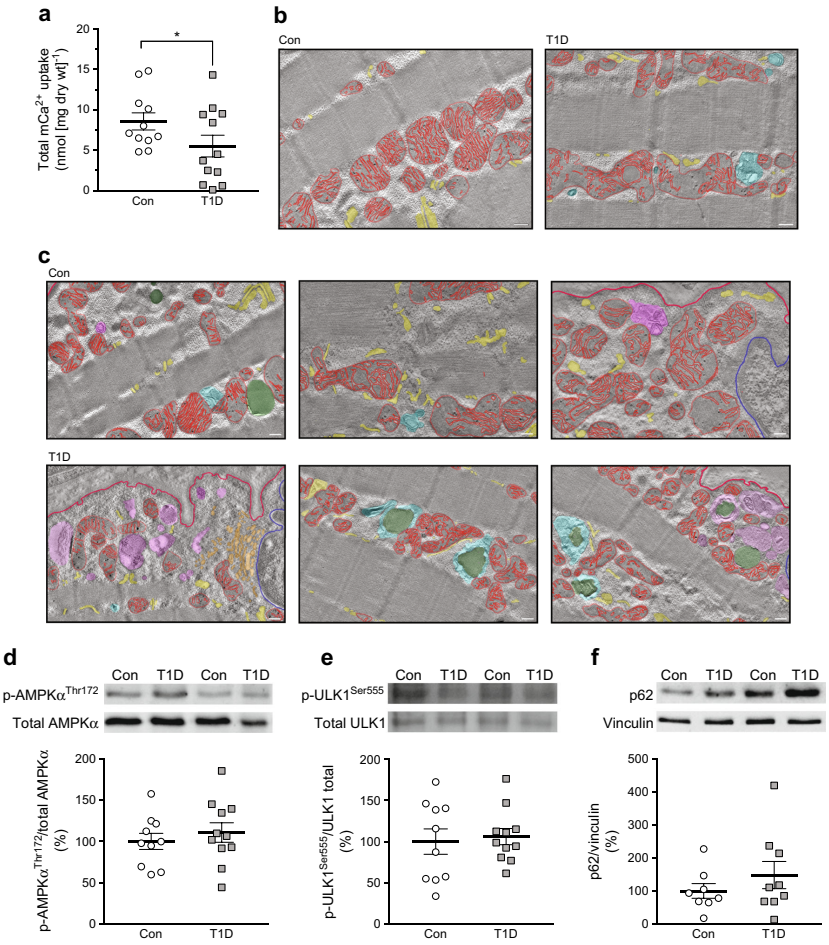

Fig. 3 Mitochondrial CRC, ultrastructural analysis and measurement of autophagic regulatory proteins. (**a**) Total mitochondrial Ca^{2+} (mCa^{2+}) uptake (a.k.a. CRC) was measured in permeabilised muscle fibres by sequential titrations of Ca^{2+} pulses until opening of the mPTP was observed. (**b**) Representative electron tomography (3D) images of irregular organisation of mitochondrial cristae (highlighted in red) in type 1 diabetes compared with control individuals. Cyan highlighting, intermyofibrillar autophagic remnants; yellow highlighting, triads/sarcoplasmic reticulum. Scale bar, 200 nm. (**c**) Representative electron tomography (3D) images of increased presence of autophagic remnants in both subsarcolemmal (highlighted in purple) and intermyofibrillar (highlighted in cyan) regions of the muscle, which were also frequently associated with intramyocellular lipid droplets (highlighted in green) in those with type 1 diabetes vs control individuals. Red highlighting, mitochondrial cristae; magenta outline, subsarcolemma; blue outline, nucleus; yellow highlighting, triads/sarcoplasmic reticulum; orange highlighting, Golgi apparatus. Scale bar, 200 nm. (**d**) Quantification of p-AMPKα^{Thr172} normalised to total AMPKα with representative blot (p-AMPKα^{Thr172} and total AMPKα, ~62 kDa). (**e**) Quantification of p-ULK1^{Ser555} normalised to total ULK1 with representative blot (p-ULK1^{Ser555} and total ULK1, ~150 kDa). (**f**) Quantification of p62 normalised to loading control vinculin, with representative blot (p62, ~62 kDa; vinculin, ~124 kDa). White circles, control; grey squares, type 1 diabetes. *$p < 0.05$. Con, control; T1D, type 1 diabetes

Instead, a main effect ($p < 0.05$) for higher Complex I (glutamate)-supported respiration was observed in those with type 1 diabetes vs control participants when normalised to OXPHOS (ESM Fig. 1c). However, we take caution in this observation given the heterogeneity in both respiration and OXPHOS protein expression

between individuals, which may warrant larger sample sizes for such ratiometric comparisons.

Skeletal muscle mitochondrial Ca^{2+} tolerance and electron tomography

We next tested Ca^{2+} handling within the mitochondria. Opening of the mPTP has been linked to swelling and energetic dysfunction [28]. We hypothesised that reductions in Ca^{2+} handling within the mitochondria (i.e. reduced CRC) would lead to an increased sensitisation of the mPTP and, ultimately, attenuations in mitochondrial respiratory function. As hypothesised, mitochondrial CRC was significantly lower ($p = 0.046$) in skeletal muscle from participants with type 1 diabetes relative to control participants (Fig. 3a).

We next used electron tomography to assess the skeletal muscle ultrastructure. This powerful technique allows for the 3D study of cellular architecture at the nanoscale level. Consistent with our observed attenuations in mitochondrial respiration, electron tomography revealed irregularities in the organisation of cristae in the participants with type 1 diabetes that were uncommon in control participants (Fig. 3b; mitochondria and cristae highlighted in red). Furthermore, consistent with the reductions in mitochondrial Ca^{2+} handling previously noted, the muscles from participants with type 1 diabetes also exhibited an increase in size and number of autophagic remnants compared with the control group, and this was visible within both the subsarcolemmal and intermyofibrillar areas (Fig. 3c; highlighted in purple and cyan, respectively). A close association of autophagic remnants to intramyocellular lipid droplets was also frequently observed in individuals with type 1 diabetes (Fig. 3c; lipid droplets highlighted in green). Despite these observations, no differences in the autophagic regulatory protein p-AMPKα^{Thr172} (as noted previously) or its downstream targets p-ULK1^{Ser555} or p62 were observed between groups (Fig. 3d–f).

Skeletal muscle microvasculature

To ascertain whether changes in mitochondrial bioenergetics were the result of microvasculature loss and concomitant hypoxia, capillary density was quantified by staining frozen muscle sections with an antibody against CD31. No significant difference between groups was observed, regardless of whether the data was collated as per cent density or ratio of total number of capillaries to myofibre area (Fig. 4a). It was also worth considering that, while capillary density may not be diminished, the capillaries present may be obstructed given the reported increases in prothrombotic factors, such as plasminogen activator inhibitor 1 (PAI-1), in type 1 diabetes [29]. We speculated that there may be greater prevalence of thrombi within the capillaries of individuals with type 1 diabetes, consequently resulting in a decreased regional blood flow. We assessed platelet aggregation by co-staining frozen muscle sections for CD31 and CD41. No significant difference between groups was demonstrated in the number of $CD41^+$ areas overlaying $CD31^+$ cells (Fig. 4b) indicating that in this physically active, young adult cohort with type 1 diabetes, impairments in skeletal muscle microvasculature did not mediate the observed alterations in mitochondrial bioenergetics and ultrastructure. Moreover, this finding also suggests that deterioration in skeletal muscle quality may precede the eventual vascular complications that arise in type 1 diabetes.

Discussion

The findings of this work highlight mitochondrial and autophagic differences within the muscles of young adults with type

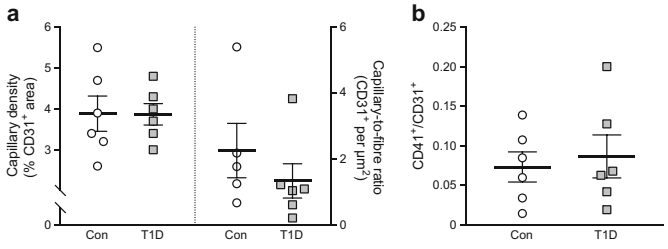

Fig. 4 Skeletal muscle capillary density and analysis of platelet aggregation. (**a**) Capillary density expressed as the percentage of areas positively stained for platelet endothelial cell adhesion molecule ($CD31^+$) relative to total muscle area analysed and as ratio of total number of $CD31^+$ to myofibre area. (**b**) Positively stained areas of platelet aggregation ($CD41^+$) within capillaries ($CD31^+$) measured as the ratio of $CD41^+$ to $CD31^+$. White circles, control; grey squares, type 1 diabetes. Con, control; T1D, type 1 diabetes

1 diabetes with moderate glycaemic control and who exceed recommended activity levels (>150 min of moderate-to-vigorous intensity activity per week) according to the American and Canadian diabetes associations [30, 31]. Specifically, the work described herein demonstrates, for the first time, site-specific alterations within the mitochondria of young adults with type 1 diabetes compared with (matched) control participants without diabetes. These alterations in mitochondrial bioenergetics occur in the absence of a loss of mitochondrial content or changes in skeletal muscle microvasculature (as assessed by capillary density and platelet aggregation). Furthermore, TEM investigations revealed that type 1 diabetes negatively affects skeletal muscle ultrastructure, as evidenced by disorganised mitochondrial cristae and an increased presence of autophagic remnants. These early alterations precede the well-characterised complications (e.g. cardiovascular disease, polyneuropathy) of type 1 diabetes and occur in large, proximal muscle groups (in contrast to distal limb muscles, which are more readily affected by polyneuropathy), suggesting that the early alterations seen in this study are unlikely to be owing to neuropathy but, instead, intrinsic alterations in muscle, and that this skeletal muscle metabolic dysregulation is a primary outcome of this chronic disease that may contribute to dysglycaemia.

Skeletal muscle is not only the primary organ system responsible for physical capacity but is also a critical metabolic organ since it is: (1) the major site of fatty acid catabolism [32]; (2) a principal mediator of whole-body glucose homeostasis [2]; and (3) a major determinant of whole-body insulin sensitivity [2, 3]. Thus, impairments in skeletal muscle quality, particularly in conditions of metabolic disease (e.g. diabetes mellitus), could have profound long-term consequences by increasing the incidence of primary contributors to the development of diabetic complications, such as recurrent dysglycaemia, dyslipidaemia and insulin resistance [33–35]. Consistent with the importance of skeletal muscle in overall health, if one considers other chronic diseases (e.g. heart disease and cancer), loss of muscle mass, strength and metabolic function (referred to as cachexia) are commonly observed, and this decline in muscle health is an important determinant in overall survival [36, 37].

While changes in skeletal muscle health of rodent models of type 1 diabetes has been more intensely studied (previously referred to as 'diabetic myopathy' [38, 39]), the extent to which this is happening in human skeletal muscle of those with type 1 diabetes has yet to be fully elucidated [40]. In the clinical diabetes literature, skeletal muscle mitochondrial ATP synthesis is one of the most widely investigated variables, often assessed indirectly in vivo by monitoring the resonance of phosphate in ATP using ^{31}P-MRS. Previous studies using ^{31}P-MRS have frequently, but not always [41], reported a ~20–25% reduction in mitochondrial ATP synthesis rates in the skeletal muscle of those with type 1 diabetes [9–11]. While ^{31}P-MRS has the obvious advantage of capturing ATP kinetics under intact physiological conditions, a drawback of this approach is the lack of mechanistic understanding underlying the changes in mitochondrial function that may be observed.

Consistent with the work of Cree-Green et al [9] and others [10, 11], we observed a ~20% reduction in mitochondrial oxidative capacity in the muscles of those with type 1 diabetes (Fig. 1a, Max), suggesting that our in vitro (permeabilised muscle fibre method) assessment is consistent with ^{31}P-MRS studies. What was unanswered from the aforementioned ^{31}P-MRS studies, however, was whether the mitochondria are reduced in content and/or impaired, or whether nutrient delivery is the cause of the resulting poor mitochondrial function in those with type 1 diabetes. Here we provide the mechanistic insight to answer these questions.

First, regarding whether the deficits observed in previous ^{31}P-MRS studies were directly related to differences in mitochondrial content or impairments of mitochondrial quality, we now offer invaluable insight using ultrastructural analysis. Using TEM, we observed no difference in the amount, distribution and size of mitochondria within the skeletal muscle of those with type 1 diabetes compared with control individuals. Within the mitochondria, we observed no differences in the protein expression of the individual electron transport chain protein subunits using western blot analysis (total OXPHOS cocktail), further suggesting that mitochondrial content is not reduced in young, physically active adults with type 1 diabetes vs control individuals. In contrast, we did observe structural abnormalities in mitochondria using electron tomography; disorganised cristae and more abundant autophagic structures were present in the muscles of individuals with type 1 diabetes compared with control participants. In line with this, we also observed a significant reduction in mitochondria viability in permeabilised myofibres of those with type 1 diabetes, as assessed by CRC [23].

Second, these findings suggest that nutrient delivery in the muscles of young adults with type 1 diabetes is not likely impaired. Histological/immunofluorescent examination of skeletal muscle biopsies revealed that capillary density within the vastus lateralis muscle was not different between those with type 1 diabetes and those without. Furthermore, as increases in prothrombotic factors have been reported in those with type 1 diabetes [29], we investigated whether the capillaries present were occluded by platelet aggregates (thrombi) and found that they were not. While we do not see visible evidence for impaired blood flow in the present study, we cannot rule out the possibility of a functional impairment resulting in reduced blood flow and delivery of oxygen and nutrients within the muscles of those with type 1 diabetes [42].

Collectively, these novel results provide evidence of direct alterations in skeletal muscle mitochondria in those with type 1 diabetes. The underlying mechanism(s) for these mitochondrial alterations in type 1 diabetes may be revealed by the

mitochondrial bioenergetics investigations related to mitochondrial H_2O_2 emission in this study. To the best of our knowledge, mitochondrial-derived ROS production has never been examined in skeletal muscle of humans with type 1 diabetes. We observed a significant increase in mitochondrial H_2O_2 emission potential at the level of Complex III, with no significant differences owing to Complex I, intermembrane (mG3PDH) or matrix (PDC) proteins. Work using isolated mitochondria [43, 44], has demonstrated that Complex III significantly contributes to ROS production in response to fatty acid oxidation. While we did not assess this in the current study, future work should be undertaken to discern differences in the propensity for ROS production at Complex III in response to glucose and lipid substrate availability in those with type 1 diabetes. The tissue requirements for the comprehensive assessment of mitochondrial phenotype prohibited additional assessment of major mitochondrial antioxidant enzymes (e.g. glutathione peroxidase, thioredoxin reductase and superoxide dismutase) and markers of oxidative stress in the present study. Nonetheless, it has been suggested that Complex III-generated ROS serves as a signalling molecule, particularly in the adaptive response to hypoxia (for review see [45]). This latter point may be physiologically important when considering the peripheral vascular disease that is known to develop in older adults with type 1 diabetes.

Perspectives

This investigation revealed that young, active people with type 1 diabetes possess mitochondrial and muscle ultrastructural abnormalities that precede reductions in capillary content. While we have demonstrated site-specific alterations in mitochondrial bioenergetics, further investigation is required to identify the precise signalling pathway leading to these changes. Though our observations of increased autophagic remnants in muscles of those with type 1 diabetes were not associated with increases in autophagic markers (p-ULK1^{Ser555} and p62), it is possible that these signals had already been transmitted and pathways initiated. Alternatively, recent work [46] has suggested that p62 activity is redox sensitive and may promote autophagy in the absence of changes in content. Clearly, future work in this area will help further elucidate this important observation.

It seems likely that the relationship between skeletal muscle abnormalities and dysglycaemia in type 1 diabetes are reciprocal, particularly given previous suggestions that excessive glucose provision to mitochondria elevates ROS production [47, 48]. Another possible mechanism may relate to peripheral insulin administration. Specifically, in people without type 1 diabetes, large rises in blood glucose levels following a glucose load are attenuated by delivering insulin to the liver first via the portal circulation. However, in those with type 1 diabetes, peripheral insulin administration bypasses the canonical 'liver-first' model and results in greater elevations in circulating blood glucose [49]. The peripheral insulin administration shifts a greater burden of the glycaemic load to skeletal muscle, forcing it to accept a greater glycaemic load then it otherwise would [1, 2], thereby increasing glycaemic stress akin to that previously described [50] for cell types that could not regulate cellular glucose entry. An important avenue for future research is to investigate this altered relationship between blood glucose levels, peripheral insulin injections and the muscle abnormalities reported in the type 1 diabetes population. Indeed, this relationship might be considered in the context of potential cellular abnormalities in other relevant tissues, such as liver and adipose tissue.

A major clinical concern in this study is that the mitochondrial/metabolic alterations observed were in young adults with type 1 diabetes, who had self-reported moderate-to-vigorous activity levels above the American and Canadian diabetes associations' recommendations. In addition, these changes occurred in large, proximal muscle groups without a detectable loss of capillary density. Clearly, larger cohort studies are needed to assess the impact of type 1 diabetes in those who are sedentary, as well as those who meet or exceed physical activity recommendations, to determine whether an optimal level of physical activity exists that is sufficient to restore/overcome the metabolic abnormalities that characterise the muscle of those with type 1 diabetes.

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Data availability All relevant data are included in the article.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement CMFM, MCH, MAT, TJH and CGRP designed the experiments. CMFM, TJH and CGRP wrote the manuscript. CMFM, MCH, SVR, NEV, CL and CM performed experiments. CMFM, MCH, SVR, NEV, CL, FAR, CM, MAT, MPK, RL, TJH and CGRP analysed and interpreted data. All authors edited the manuscript. All authors provided final approval of the version to be published. All people

designated as authors qualify for authorship, and all those who qualify for authorship are listed. TJH and CGRP are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of data and the accuracy of the data analysis.

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CHAPTER 3 — Sexual dimorphism in human skeletal muscle mitochondrial bioenergetics in response to type 1 diabetes

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PREFACE

Author contributions:

CMFM, RL, CGRP, and TJH conceived and designed research; CMFM, CAB, MCH, SVR, and CGRP performed experiments; **CMFM**, CAB, CGRP, and TJH. analyzed data; **CMFM**, MCH, CGRP and TJH interpreted results of experiments; **CMFM** prepared figures; **CMFM** and TJH drafted manuscript; **CMFM**, CAB, MCH, SVR, RL, CGRP, and TJH edited and revised manuscript; **CMFM**, CAB, MCH, SVR, RL, CGRP, and TJH approved final version of manuscript.

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RAPID REPORT | *Mitochondria Dysfunction in Aging and Metabolic Diseases*

Sexual dimorphism in human skeletal muscle mitochondrial bioenergetics in response to type 1 diabetes

Cynthia M. F. Monaco,[1] Catherine A. Bellissimo,[2] Meghan C. Hughes,[2] Sofhia V. Ramos,[2] Robert Laham,[2] Christopher G. R. Perry,[2] and Thomas J. Hawke[1]

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Monaco CMF, Bellissimo CA, Hughes MC, Ramos SV, Laham R, Perry CGR, Hawke TJ. Sexual dimorphism in human skeletal muscle mitochondrial bioenergetics in response to type 1 diabetes. *Am J Physiol Endocrinol Metab* 318: E44–E51, 2020. First published December 3, 2019; doi:10.1152/ajpendo.00411.2019.—Sexual dimorphism in mitochondrial respiratory function has been reported in young women and men without diabetes, which may have important implications for exercise. The purpose of this study was to determine if sexual dimorphism exists in skeletal muscle mitochondrial bioenergetics in people with type 1 diabetes (T1D). A resting muscle microbiopsy was obtained from women and men with T1D (n = 10/8, respectively) and without T1D (control; n = 8/7, respectively). High-resolution respirometry and spectrofluorometry were used to measure mitochondrial respiratory function, hydrogen peroxide (mH_2O_2) emission and calcium retention capacity (mCRC) in permeabilized myofiber bundles. The impact of T1D on mitochondrial bioenergetics between sexes was interrogated by comparing the change between women and men with T1D relative to the average values of their respective sex-matched controls (i.e., delta). These aforementioned analyses revealed that men with T1D have increased skeletal muscle mitochondrial complex I sensitivity but reduced complex II sensitivity and capacity in comparison to women with T1D. mH_2O_2 emission was lower in women compared with men with T1D at the level of complex I (succinate driven), whereas mCRC and mitochondrial protein content remained similar between sexes. In conclusion, women and men with T1D exhibit differential responses in skeletal muscle mitochondrial bioenergetics. Although larger cohort studies are certainly required, these early findings nonetheless highlight the importance of considering sex as a variable in the care and treatment of people with T1D (e.g., benefits of different exercise prescriptions).

bioenergetics; female; mitochondria; sex differences; type 1 diabetes

INTRODUCTION

Sexual dimorphism in the development of type 1 diabetes (T1D) complications exists and evidence indicates a greater negative impact in women. Women with T1D have a twofold increased risk of developing and dying from cardiovascular disease as well as a near 40% greater excess risk for all-cause mortality compared with men with T1D (2, 5, 6). In addition, it is known that women with T1D typically have greater struggles with insulin management (5), have worse glycemic control (8), and suffer from increased adiposity (13) and a greater degree of insulin resistance (9) compared with men with T1D. This is striking considering the clinically reported cardioprotective effects of estrogen in the female sex as well as the beneficial effects of estrogen on insulin action and metabolism (3). Thus, with accumulating evidence of sex differences in diabetes complications development, it is becoming evermore evident that a sex-specific approach to diabetes research and disease care/management is necessary.

Although the mechanisms for the metabolic effects of female sex hormones (e.g., estrogen) are still being unraveled, there is a growing appreciation that these hormones play a role in regulating insulin signaling and action, substrate oxidation, and mitochondrial bioenergetics (3, 15). With respect to estrogen, recent findings in healthy, recreationally active women and men suggest that sex may influence mitochondrial respiratory function in skeletal muscle (1, 19, 12). This is of particular interest in the context of T1D, considering the recent findings from our group (11) and others (4) of site-specific alterations in mitochondrial functions in the skeletal muscle of young adults with T1D and considering the fact that skeletal muscle is our largest metabolic organ, by mass, thus making it a major determinant of whole body insulin sensitivity and a critical player for glycemic control. Whether there is a differential response in skeletal muscle mitochondrial bioenergetics between women and men with T1D is not currently known. Therefore, to address this gap in knowledge, we systematically assessed mitochondrial bioenergetics and content in skeletal muscle biopsies from women and men with and without T1D.

RESEARCH DESIGN AND METHODS

Participants

Eighteen volunteers with T1D (women n = 10/men n = 8) and fifteen volunteers without T1D (controls; women n = 8/men n = 7) were recruited and closely matched for age, sex, and body mass index (Table 1). A subset of these volunteers (T1D, women n = 7/men n = 5; controls, women n = 7/men n = 5) was derived from our previously published cohort (11). Menstrual cycle phase was not controlled for in this study. All procedures were approved by the Research Ethics Board at York University (REB# e2013–032) and conformed to the Declaration of Helsinki.

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Table 1. *Participant demographics*

	Control		Type 1 Diabetes (T1D)	
	Women (n = 8)	Men (n = 7)	Women (n = 10)	Men (n = 8)
Age, yr	26 ± 2	25 ± 3	26 ± 5	25 ± 4
Height, m	1.65 ± 0.06	1.76 ± 0.02†††	1.63 ± 0.08	1.79 ± 0.11**
Weight, kg	57.9 ± 9.7	76.5 ± 4.8†††	67.3 ± 15.2	81.6 ± 9.5*
BMI, kg/m^2	21.1 ± 2.4	24.7 ± 1.7††	25.2 ± 4.5	25.4 ± 2.2
HbA_{1c}, %			8.1 ± 1.5	6.9 ± 0.8*
Disease duration, yr			13 ± 10	12 ± 5
Disease onset, yr			12 ± 7	13 ± 7
Physical activity, min/wk	321 ± 197	246 ± 150	240 ± 162	180 ± 100

Data are expressed as mean ± SD. BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}. Physical activity was based on a 7-day activity recall record of moderate-to-vigorous activity levels. No difference was found between sexes in either the control group ($P = 0.459$) or T1D group ($P = 0.430$) using an unpaired, two-tail Student's t test. ††$P < 0.01$, †††$P < 0.001$ vs control women; unpaired, two-tail Student's t test; *$P < 0.05$, **$P < 0.01$ vs T1D women; unpaired, two-tail Student's t test.

Study Design

The study design has been described in detail previously (11). Briefly, participants arrived at the laboratory at 8:30 AM or 2:30 PM and were instructed to consume a standardized meal 1.5–2 h before their visit and refrain from any form of exercise for at least 24 h. Prior to giving informed consent, all participants were given oral and written information about the experimental procedures. Body mass and height were recorded to determine body mass index, and blood was collected using venepuncture for hemoglobin A_{1c} analysis. Skeletal muscle samples were then obtained from the vastus lateralis muscle by a micropunch percutaneous needle under local anesthetic and utilized for mitochondrial bioenergetic and Western blot analyses, as described below.

Mitochondrial Bioenergetic Analyses

Freshly obtained muscle samples were placed in ice-cold BIOPS and immediately used to prepare permeabilized muscle fibers for the assessment of in vitro mitochondrial oxygen consumption, mitochondrial H_2O_2 emission (mH_2O_2) and mitochondrial Ca^{2+} retention capacity (mCRC) as previously described in detail (11). Briefly, respiration assays were undertaken in MiR05 respiration medium supplemented with 20 mM creatine using the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria), and mH_2O_2 assays were measured spectrofluorometrically (QuantaMaster 40, HORIBA Scientific, Edison, NJ) and undertaken in buffer Z supplemented with 10 μM Amplex UltraRed reagent (#A36006, Life Technologies, Carlsbad, CA), 20 mM creatine, and 40 U/ml Cu/Zn superoxide dismutase (#S9697, Sigma Aldrich, St. Louis, MO). Fiber bundles used for complex I (pyruvate + malate)-, pyruvate dehydrogenase complex-, and mitochondrial glycerol-3-phosphate dehydrogenase-derived mH_2O_2 were also treated with 35 μM 1-chloro-2,4-dinitrobenzene (#237329, Sigma) during permeabilization. mCRC assays were measured spectrofluorometrically and performed in duplicate in buffer Y supplemented with 1 μM Calcium Green-5N (C3737; Life Technologies).

Western Blot Analyses

Frozen muscle samples were homogenized in 10 μL/mg muscle of ice-cold lysis buffer [in mM: 40 HEPES, 120 NaCl, 1 EDTA, 10 $NaHP_2O_7 \cdot 10H_2O$ pyrophosphate, 10 β-glycerophosphate, 10 NaF and 0.3% CHAPS detergent (pH 7.1) supplemented with 1 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, and ¼ tablet of SIGMAFAST protease inhibitor (SLBR7552V, Sigma)]. Homogenized samples were agitated for 1 h at 4°C followed by centrifuging for 10 min. The supernatant was aliquoted, analyzed for total protein using a bicinchoninic acid assay (Thermo Scientific, Waltham, MA), and diluted in 4× SDS loading buffer [62.5 mM Tris(HCl) pH 6.8, 2% SDS, 10% glycerol, 1 mM of 0.2 M EDTA, 100 mM DTT and 0.25% bromophenol blue]. Total proteins (50 μg equally loaded per lane) were separated by SDS-PAGE, transferred to a PVDF membrane, blocked for 1 h [Tris-buffered saline (TBS), 5% non-fat dry milk (wt/vl), 0.1% Tween 20 (TBS-T)] and incubated overnight at 4°C with the commercially available antibody human total oxidative phosphorylation cocktail (OXPHOS; 1:208 in block; cat. no. ab110411, Abcam, Cambridge, MA). Following this, the membrane was washed, incubated with secondary antibody mouse IgG conjugated to horseradish peroxidase (1:10,000 in TBS-T, CST#7076, Cell Signaling, Danvers, MA) for 1 h, and visualized by chemiluminescence (Gel Logic 6000 Pro Imager, Carestream, Rochester, NY). Bands were quantified by densitometry using Image J. Membranes were incubated with Ponceau S (cat. no. P7170, Sigma) for loading control.

Statistics

All data were analyzed using GraphPad Prism 8 software and were initially tested for normal distribution using the Shapiro–Wilk test of normality. The ROUT test was used to identify outliers. Data that did not follow normal distribution were log transformed.

Based on previously published work (1, 10, 12), an a priori hypothesis was established that control women and men would differ in mitochondrial respiratory function and thus we could not strictly compare absolute measures between men and women with T1D. To determine if T1D truly affected women and men differentially, the T1D data were also expressed as the change from the average control values of their respective sex (i.e., delta). A Student's unpaired, two-tailed t test was used to compare differences between women and men within the control group, women and men within the T1D group, and the delta between T1D women and T1D men relative to their sex-matched control values (average value). Significance was established at $P < 0.05$.

RESULTS

Skeletal Muscle Mitochondrial Bioenergetics

ADP-stimulated mitochondrial respiration. We first investigated the ability of the mitochondria to respond to low versus high metabolic demands (i.e., low and high ADP concentrations). As can be seen in Fig. 1*A* (*left*), in the presence of complex I substrates and submaximal ADP (25 μM), the absolute rate of mitochondrial respiration was trending lower in control women ($P = 0.077$) compared with control men, whereas in T1D, it was significantly lower in women ($P = 0.043$) compared with men. This indicates a lower sensitivity of the mitochondria to ADP in women and corroborates pre-

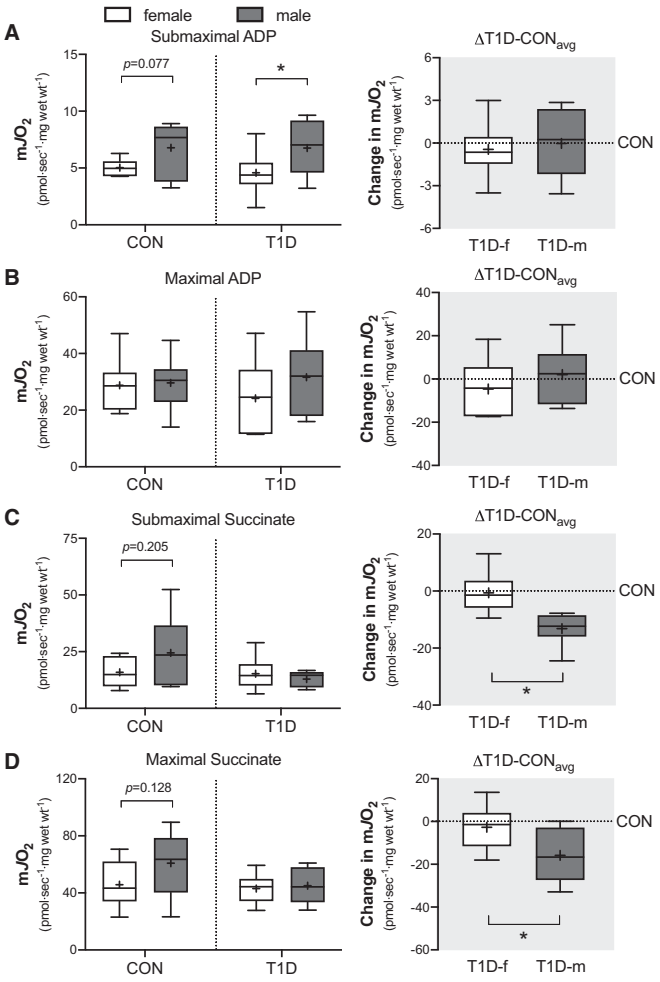

Fig. 1. Mitochondrial ADP-stimulated respiration supported by pyruvate (5 mM) and malate (2 mM) in the presence of either submaximal (25 μM) ADP (*A*) or maximal (varied 6 to 18 mM) ADP (*B*) in skeletal muscle biopsies from control (CON) and type 1 diabetes (T1D) women and men. Right panel of (*A*) and (*B*) is the absolute change in ADP-stimulated respiration from each T1D participant relative to the average of their sex-matched control (ΔT1D-CON$_{avg}$). In (*C*) and (*D*) is mitochondrial complex II respiration supported by ADP (5 mM), rotenone (0.5 μM), and in the presence of either submaximal (2 mM) succinate (*C*) or maximal (varied 22 to 30 mM) succinate (*D*). Right panel of (*C*) and (*D*) is the absolute change in complex II-supported respiration from each T1D participant relative to the average of their sex-matched control ($\triangle$T1D-CON$_{avg}$). Boxes represent the interquartile range, whiskers show the maximum and minimum, the horizontal line indicates the median and the cross indicates the mean. *$P <$ 0.05 versus women. f, female; m, male; mJO_2, mitochondrial oxygen flux; Wt, weight. n = 7–8 for CON women; n = 7 for CON men; n = 9–10 for T1D women; n = 7–8 for T1D men.

vious work that reported reductions in mitochondrial ADP sensitivity in control women compared with men (10). When the T1D data were expressed as the absolute change from the control (average values) of their respective sex (i.e., delta), no differences (P = 0.678) were observed between sexes Fig. 1*A* (*right*). In the presence of maximal ADP (i.e., oxidative capacity; >5 mM ADP), the absolute rate of mitochondrial respiration was not different between sexes in either the control (P = 0.871) or the T1D group (P = 0.242) as shown in Fig. 1*B* (*left*). In line with this, there was no difference (P = 0.295) in the delta (Fig. 1*B*, *right*). Together, these observations suggest that skeletal muscle mitochondrial oxidative capacity is similar between sexes in both control and T1D individuals.

Complex II-supported mitochondrial respiration. We next investigated complex II function by measuring mitochondrial respiration in the presence of varying succinate concentrations, maximal ADP (5 mM) and rotenone (0.5 μM). As can be seen in Fig. 1 *C* and *D* (*left*), the absolute rate of mitochondrial respiration in the presence of either submaximal (2 mM) or maximal (>20 mM) succinate was observably higher in control men compared with women (P = 0.205 and P = 0.128, respectively). In contrast, in the T1D group, the absolute rate of

mitochondrial respiration was comparable between sexes in the presence of both submaximal ($P = 0.423$) and maximal ($P = 0.685$) succinate (Fig. 1, *C* and *D*, *left*). Thus, when the delta was calculated, men with T1D displayed a greater change in mitochondrial respiration compared with women with T1D, where respiration was lower in the T1D men in the presence of both submaximal ($P < 0.001$) and maximal ($P = 0.031$) succinate (Fig. 1, *C* and *D*, *right*). Combined, this suggests that the T1D environment negatively alters mitochondrial complex II sensitivity and capacity in men compared with women.

Complex I-supported mitochondrial respiration. We next investigated complex I function by measuring mitochondrial respiration in the presence of varying pyruvate concentrations, maximal ADP (5 mM) and malate (2 mM). As shown in Fig. 2, *A* and *B* (*left*), the women and men in control group had similar absolute rates of mitochondrial respiration in the presence of submaximal (25 μM) and maximal (>6 mM) pyruvate concentrations ($P = 0.961$ and $P = 0.955$, respectively). In contrast, in the T1D group, the absolute rate of mitochondrial respiration was trending higher in men compared with women

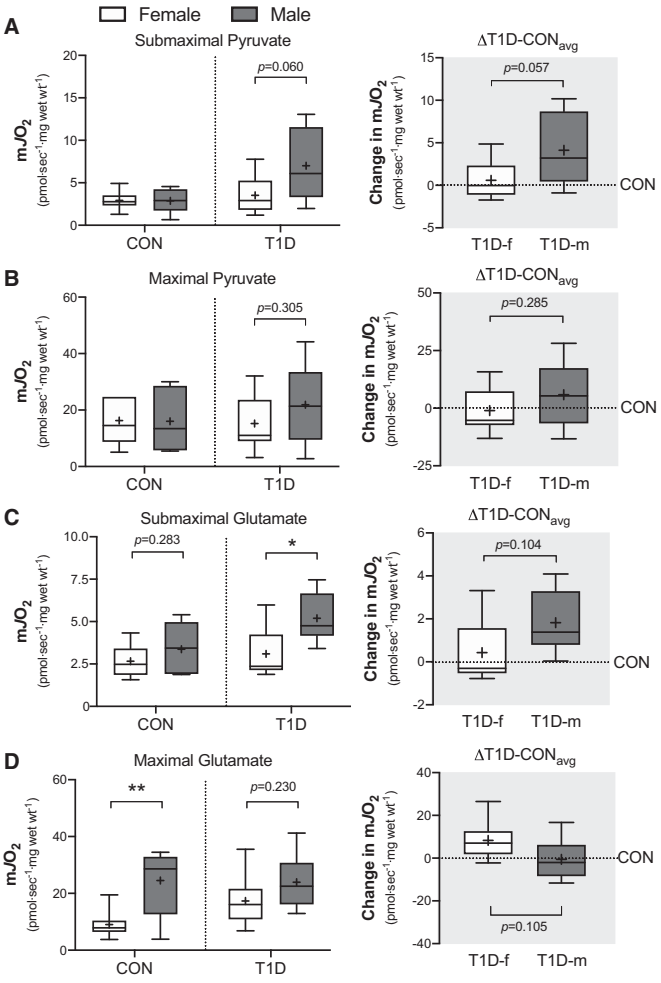

Fig. 2. Mitochondrial complex I respiration supported by ADP (5 mM) and malate (2 mM) in the presence of either submaximal pyruvate (*A*; 25 μM), maximal pyruvate (*B*; varied 6 to 38 mM), submaximal glutamate (*C*; 50 μM) or maximal glutamate (*D*; varied 4 to 20 mM) in control (CON) and type 1 diabetes (T1D) women and men. Right panel of (*A*) through (*D*) is the absolute change from each T1D participant relative to the average of their sex-matched control (ΔT1D-CON$_{avg}$). Boxes represent the interquartile range, whiskers show the maximum and minimum, the horizontal line indicates the median and the cross indicates the mean. $^*P < 0.05$ versus women, $^{**}P < 0.01$ versus women. f, female; m, male; mJO_2, mitochondrial oxygen flux; Wt, weight. $n = 6–8$ for CON women; $n = 6–7$ for CON men; $n = 8–9$ for T1D women; $n = 6$ for T1D men.

with submaximal pyruvate ($P = 0.060$) but not maximal pyruvate ($P = 0.305$) (Fig. 2, *A* and *B*, *left*). Given this, the change in submaximal pyruvate-supported respiration was trending greater in T1D men compared with T1D women ($P = 0.057$), whereas T1D men displayed greater pyruvate-supported respiration (Fig. 2*A*, *right*). The change in maximal pyruvate-supported respiration on the other hand was comparable between sexes ($P = 0.285$; Fig. 2*B*, *right*).

However, to ascertain that these observations were specific to complex I and not upstream dehydrogenases, we also measured mitochondrial respiration in the presence of varying glutamate concentrations, maximal ADP (5 mM), and malate (2 mM). In the control group, the absolute rate of respiration was similar between sexes with submaximal glutamate (50 μM; $P = 0.283$), as shown in Fig. 2*C* (*left*). However, in the presence of maximal glutamate (>4 mM), the absolute rate of respiration was increased in control men compared with women ($P = 0.007$; Fig. 2*D*, *left*). Thus, this suggests that the capacity of the enzyme glutamate dehydrogenase, but not the capacity of complex I, is increased in control men. In the T1D group, the absolute rate of respiration with submaximal but not maximal glutamate was increased in men compared with women ($P = 0.021$ and $P = 0.230$, respectively; Fig. 2, *C* and *D*, *left*). The change in submaximal and maximal glutamate-supported respiration was observably greater in T1D men versus T1D women ($P = 0.104$ and $P = 0.105$, respectively; Fig. 2, *C* and *D*, *right*), whereas T1D men had higher respiration rates. Altogether, the greater change in both submaximal pyruvate- and glutamate-supported respiration in T1D men compared with women suggests that T1D increases the sensitivity of complex I in men.

Mitochondrial H_2O_2 emission. In the control group, mH_2O_2 emission potential was similar between women and men at all sites measured, including: complex I driven by succinate ($P = 0.600$; Fig. 3*A*, *left*), complex I driven by pyruvate and malate ($P = 0.389$; Fig. 3*B*, *left*), complex III driven by antimycin A ($P = 0.412$; Fig. 3*C*, *left*), pyruvate dehydrogenase complex (PDC) driven by rotenone and pyruvate ($P = 0.242$; Fig. 3*D*, *left*), and mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH) driven by glycerol-3-phosphate ($P = 0.421$; Fig. 3*E*, *left*). On the other hand, in the T1D group, mH_2O_2 emission potential was lower ($P = 0.004$) at complex I (succinate driven) in women compared with men (Fig. 3*A*, *left*) but remained similar at complex I when driven by pyruvate and malate ($P = 0.867$; Fig. 3*B*, *left*), complex III ($P = 0.645$; Fig. 3*C*, *left*), PDC ($P = 0.606$; Fig. 3*D*, *left*), and mG3PDH ($P =$

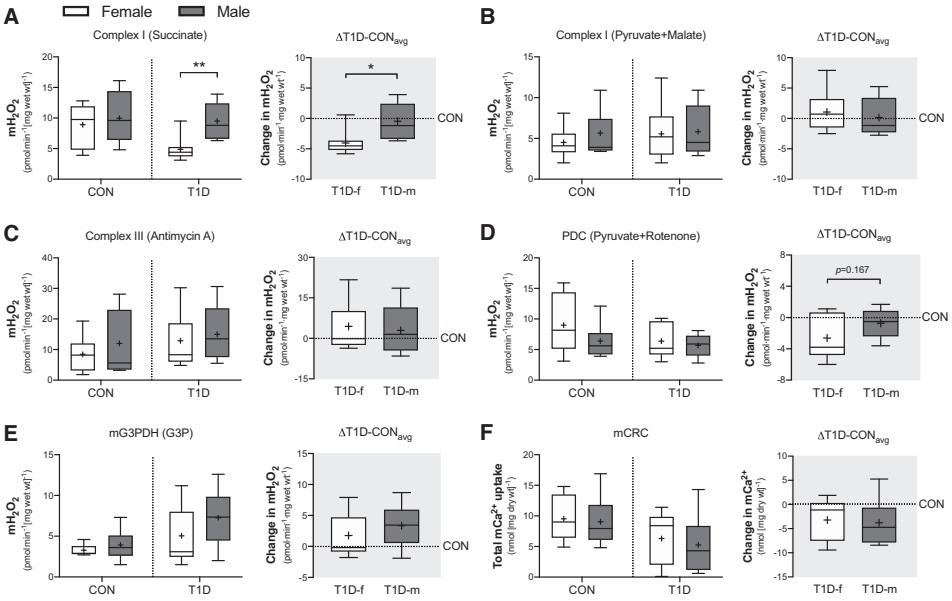

Fig. 3. Skeletal muscle site-specific mitochondrial hydrogen peroxide (mH_2O_2) emission and susceptibility to opening of the permeability transition pore in control (CON) and type 1 diabetes (T1D) women and men. (*A*) Complex I-derived mH_2O_2 (10 mM succinate). (*B*) Complex I-derived mH_2O_2 (10 mM pyruvate and 2 mM malate). (*C*) Complex III-derived mH_2O_2 (2.5 μM antimycin A). (*D*) Pyruvate dehydrogenase complex (PDC)-derived mH_2O_2 (10mM pyruvate and 0.5 μM rotenone). (*E*) Mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH)-derived mH_2O_2 (20mM glycerol-3-phosphate). (*F*) Mitochondrial Ca^{2+} retention capacity (mCRC). Right shaded panel in (*A*) through (*F*) is the absolute change from each T1D participant relative to the average of their sex-matched control (ΔT1D-CON_{avg}). Boxes represent the interquartile range, whiskers show the maximum and minimum, the horizontal line indicates the median and the cross indicates the mean. $^*P < 0.05$ versus women, $^{**}P < 0.01$ versus women. f, female; m, male; Wt, weight. $n = 7–8$ for CON women; $n = 6–7$ for CON men; $n = 8–10$ for T1D women; $n = 6–8$ for T1D men.

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0.195; Fig. 3*E*, *left*). Similarly, the change in mH_2O_2 emission potential was comparable at all sites ($P = 0.550$ complex I pyruvate + malate; $P = 0.740$ complex III; $P = 0.167$ PDC; $P = 0.344$ mG3PDH) except for complex I (succinate driven), where T1D women displayed a greater change in emission potential compared men ($P = 0.017$); specifically, lower mH_2O_2 emission potential (Fig. 3, *A–E*, *right*).

Mitochondrial Ca^{2+} tolerance. As can be seen in Fig. 3*F* (*left*), the ability of the mitochondria to retain Ca^{2+} (i.e., mCRC) before opening of the mitochondrial permeability transition pore (marker of susceptibility to cell death) was comparable between sexes in the control ($P = 0.822$) and the T1D group ($P = 0.634$). Similarly, the delta was not different between sexes ($P = 0.802$) (Fig. 3*F*, *right*).

Skeletal Muscle Mitochondrial Content

Protein expression of complexes I through V of the electron transport chain. To ensure that the differences in mitochondrial bioenergetics were not due to changes to the content of the individual complexes of the electron transport chain, we next measured the protein expression of complexes I through V. As shown in Fig. 4*A*, in the control group, no differences were observed in complex I ($P = 0.474$), complex II ($P = 0.689$), complex III ($P = 0.676$), complex IV ($P = 0.435$), or complex V ($P = 0.786$). Similarly, in the T1D group, no differences were observed in complex I ($P = 0.512$), complex II ($P = 0.774$), complex III ($P = 0.752$), complex IV ($P = 0.849$), or complex V ($P = 0.775$). As expected, the delta was also not different between sexes (Fig. 4*B*) [complex I ($P = 0.198$), complex II ($P = 0.564$), complex III ($P = 0.523$), complex IV ($P = 0.490$), or complex V ($P = 0.588$)]. Combined, these data suggest that skeletal muscle mitochondrial content is similar between sexes irrespective of T1D status.

Conclusions

This study is the first to reveal site-specific differences in skeletal muscle mitochondrial respiratory function and mH_2O_2 emission between young adult women and men with T1D that have moderately well-controlled glycemia. Importantly, unexpectedly, these findings indicate that T1D alters skeletal muscle mitochondrial respiratory function to a greater extent in men compared with women despite women exhibiting a higher

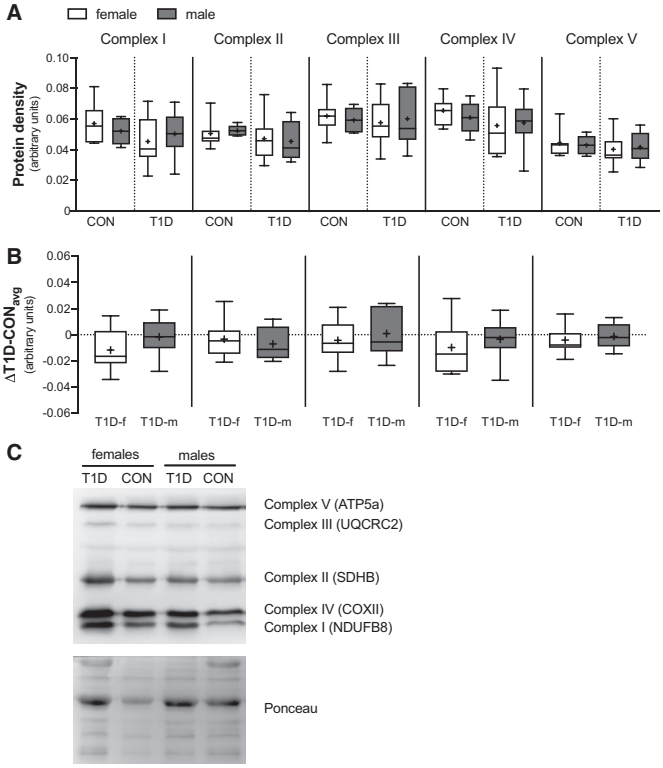

Fig. 4. Skeletal muscle mitochondrial content in control (CON) and type 1 diabetes (T1D) women (f) and men (m). (*A*) Protein expression of the individual complexes of the electron transport chain (I through V) were individually quantified by densitometry. (*B*) Protein density absolute change from each T1D participant relative to the average of their sex-matched control ($\triangle$T1D-CON_{avg}). (*C*) Representative immunoblot of Complex I (~18 kDa), Complex II (~29 kDa), Complex III (~48 kDa), Complex IV (~22 kDa) and Complex V (~54 kDa) and Ponceau stain for loading control. Boxes represent the interquartile range, whiskers show the maximum and minimum, the horizontal line indicates the median and the cross indicates the mean. ATP5a, ATP synthase subunit alpha 5; COXII, cytochrome oxidase subunit II; NDUFB8, NADH:ubiquinone oxidoreductase subunit B8; SDHB, succinate dehydrogenase [ubiquinone] iron-sulfur subunit B; UQCRC2, ubiquinol-cytochrome c reductase core protein 2. $n = 7$ for CON women; $n = 5$ for CON men; $n = 10$ for T1D women; $n = 8$ for T1D men.

hemoglobin A_{1c} (Table 1). This study also extends our current understanding of sexual dimorphism and similarities in mitochondrial bioenergetics in young, healthy people (control).

Sexual dimorphism in skeletal muscle metabolism has long been identified in the healthy (control) population, where women rely on fat oxidation to a greater extent than men during prolonged exercise (14). It was not until more recently that studies began exploring the possibility of specific differences between women and men in humans with respect to skeletal muscle mitochondrial phenotype and oxidative capacity. Although disparate results exist between these studies (1, 10, 12), owing in part to different methodologies and protocols employed, these reports nonetheless led us to reflect on our previous study that systematically assessed mitochondrial bioenergetics in both women and men with T1D compared with controls (11). Thus, by increasing subject number, we sought to identify whether T1D differentially impacts skeletal muscle mitochondria in women compared men. Our experimental approach was similar to that of Miotto et al. (10), and, consistent with this work, we observed lower mitochondrial ADP-stimulated respiration at submaximal ADP but not maximal ADP concentrations (oxidative capacity) in women compared with men in both the control and T1D groups. This indicates that *1*) mitochondria are less sensitive to ADP at rest in women (control and T1D) and *2*) T1D does not differentially impact ADP-stimulated respiration between sexes. We also observed significantly lower complex I-supported respiration in control women compared with control men when stimulated with maximal glutamate concentrations but not maximal pyruvate concentrations, suggesting sexual dimorphism in upstream dehydrogenase capacity. In the T1D group, when the delta was calculated from absolute rates of mitochondrial respiration, men displayed lower complex II-supported respiration (both submaximal and maximal succinate) but greater complex I-supported respiration in the presence of either submaximal glutamate or submaximal pyruvate concentrations compared with women. Combined, these observations suggest enhanced complex I sensitivity/respiratory function in men with T1D, potentially as compensation for deficits in complex II function.

Additionally, we demonstrate for the first time that in healthy controls, mCRC and site-specific mH_2O_2 emission are generally similar between women and men. In people with T1D, mCRC was also similar between sexes, whereas sex differences in mH_2O_2 emission rates were observed at complex I (succinate driven), where women demonstrated lower emission rates compared with men. These findings guide future investigations to examine the impact of these altered emission rates on antioxidant, cellular oxidative stress, and redox signaling networks related to metabolic homeostasis. For example, women may be particularly capable of preventing oxidative stress in T1D through the combined effects of lower H_2O_2 emission kinetics seen at complex I (succinate driven) in the present study and the greater and higher expression and content of antioxidants in women reported in other studies [e.g., increased glutathione levels and superoxide dismutase (7)].

Lastly, it is important to highlight that these sex-specific alterations in mitochondrial bioenergetics in T1D could impact how women and men with T1D respond and adapt to exercise and/or exercise training (e.g., metabolic responses to exercise), which, in turn, has the potential to greatly influence glucose homeostasis between the sexes during and after exercise (16). For example, the greater reliance on carbohydrate oxidation, and hence glycogen, reported in healthy and T1D men compared with women during exercise can increase the likelihood of postexercise hypoglycemia in T1D men. Therefore, alterations in mitochondrial respiratory function would not only reduce cellular ATP and hence, exercise efficiency, but also likely further increase reliance on glycolysis and, consequently, further depletion of glycogen stores. Ultimately, we expect this would amplify the risk of postexercise hypoglycemia in T1D men. Future studies are certainly warranted to address these important questions.

Altogether, our study revealed sexual dimorphism in mitochondrial bioenergetic adaptations to T1D that, to our surprise, were not worse in women compared with men and highlights the need for larger cohort studies with an expanded age range to fully tease out sex differences in skeletal muscle metabolism. Given the importance of skeletal muscle to our physical and metabolic wellbeing, maintaining the health of this organ system is of primary importance in this metabolic disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.M.F.M., R.L., C.G.P., and T.J.H. conceived and designed research; C.M.F.M., C.A.B., M.C.H., S.V.R., and C.G.P. performed experiments; C.M.F.M., C.A.B., C.G.P., and T.J.H. analyzed data; C.M.F.M., M.C.H., C.G.P., and T.J.H. interpreted results of experiments; C.M.F.M. prepared figures; C.M.F.M. and T.J.H. drafted manuscript; C.M.F.M., C.A.B., M.C.H., S.V.R., R.L., C.G.P., and T.J.H. edited and revised manuscript; C.M.F.M., C.A.B., M.C.H., S.V.R., R.L., C.G.P., and T.J.H. approved final version of manuscript.

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CHAPTER 4 — Considering type 1 diabetes as a form of accelerated muscle aging

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PREFACE

Author contributions:

CMFM, MAG and TJH drafted the review article outline. **CMFM** wrote the initial draft. **CMFM** designed the figures. **CMFM**, MAG and TJH edited the manuscript and collaboratively worked in refining the final draft of the manuscript.

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ARTICLE

Considering Type 1 Diabetes as a Form of Accelerated Muscle Aging

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MONACO, C.M.F., M.A. GINGRICH, and T.J. HAWKE. Considering type 1 diabetes as a form of accelerated muscle aging. *Exerc. Sport Sci. Rev.*, Vol. 47, No. 2, pp. 98–107, 2019. *Recent evidence reveals impairments to skeletal muscle health in adolescent/young adults with type 1 diabetes (T1D). Interestingly, the observed changes in T1D are not unlike aged muscle, particularly, the alterations to mitochondria. Thus, we put forth the novel hypothesis that T1D may be considered a condition of accelerated muscle aging and that, similar to aging, mitochondrial dysfunction is a primary contributor to this complication.* **Key Words:** type 1 diabetes, aging, skeletal muscle health, atrophy, metabolism, muscle strength, mitochondrial dysfunction

Key Points

- Emerging evidence in humans indicates that type 1 diabetes (T1D) impairs skeletal muscle health (*e.g.*, mass, function, metabolism).
- Impairments to skeletal muscle health in T1D are, in many ways, similar to that observed in the muscle of aged individuals, but are occurring at a younger age in T1D.
- Mitochondrial dysfunction has been implicated in the deterioration of skeletal muscle health in aging healthy adults.
- Adolescent/young adults with T1D display dysfunctional skeletal muscle mitochondria despite being recreationally active and having moderately well-controlled glycemia.
- We hypothesize that mitochondrial dysfunction is a primary mediator of the accelerated muscle aging phenotype in those with T1D.

INTRODUCTION

Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune-mediated destruction of the insulin-producing pancreatic beta cells, resulting in little to no insulin production. Approximately 1.5 million North Americans currently live with T1D, and evidence indicates that T1D prevalence is on the rise worldwide (1). Multiple daily subcutaneous insulin injections/insulin pump therapy, frequent blood glucose tests, and careful dietary monitoring (*e.g.*, carbohydrate counting) are currently the standard of care for those with T1D. Although these therapeutic strategies allow for treatment of T1D, they are not a cure, and the limitations of exogenous insulin therapy lead to chronic, recurrent bouts of dysglycemia, dyslipidemia, and, ultimately, insulin resistance. These three factors have been identified as the major contributors to the development of long-term diabetic complications, including neuropathy, nephropathy, and cardiovascular disease (2).

Surprisingly, the impact of T1D on skeletal muscle has received little clinical attention despite the importance of skeletal muscle to our physical and metabolic well-being. There is, however, accumulating evidence of structural, functional, and metabolic alterations to the skeletal muscle of both rodent models and humans with T1D, changes that appear to present before the clinical onset of many other diabetic complications (see (3) and (4) for more detailed and extensive reviews). In many ways, these alterations to the "health" of skeletal muscle are consistent with alterations observed in aged muscle including declines in muscle mass and strength, as well as dysregulation of glucose, lipid, and protein metabolism, albeit at a considerably younger age. Particularly noteworthy, at the cellular level, are the similarities in the structural and metabolic alterations to mitochondria in both conditions whereby the mitochondria appear to have a reduced oxidative capacity (5–10), increased capacity to produce reactive oxygen species (ROS) (10,11), and an increased susceptibility to opening of the mitochondrial permeability transition pore (mPTP) (10,12). Of clinical significance though is the fact that these cellular changes are occurring at a younger age in those with T1D (<30 yr old in T1D vs ~50–80 yr old in otherwise healthy individuals). With this as our backdrop, we put forth the novel hypothesis that T1D recapitulates a condition of accelerated skeletal muscle aging and that, similar to the decline in muscle health with increasing age, mitochondria are a primary mediator of this accelerated muscle aging phenotype. It is thus the purpose of

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this review to discuss the recent findings that support this novel hypothesis and identify the gaps in the literature that may be used to direct future studies to improve the healthy lifespan of those living with T1D.

SIMILARITIES BETWEEN AGED AND T1D SKELETAL MUSCLE

Muscle Mass

As skeletal muscle ages, there is a progressive reduction in mass and function (sarcopenia). The loss of skeletal muscle mass typically begins around 50 yr of age and continues to gradually decline by ~2% each year (13). This leads to an increased risk of frailty, physical disability, chronic metabolic disease, and mortality (14). Aged skeletal muscles have been associated with a decline in myofiber number and size (atrophy), and studies have reported that the decrease in myofiber cross-sectional area is fiber-type specific, with type II muscle fibers being on average 10%–40% smaller in the elderly compared with young adults (15). In contrast, type I muscle fibers seem to remain largely unaffected (16). These progressive changes to aging skeletal muscle are further exacerbated by 1) the "anabolic resistance" observed in elderly people, where muscle protein synthesis has a blunted responsiveness to nutritional (*e.g.*, protein-rich diet) and exercise interventions (*e.g.*, resistance exercise), causing a loss in the ability of muscle to maintain its protein mass (17); and 2) the impairments to muscle regeneration that exist in the elderly, with considerable evidence supporting reductions in muscle stem (satellite) cell content and activation in this population (for review see (18)). As a result, these alterations make it considerably more difficult for the elderly to recover from insults to the muscle (*e.g.*, prolonged periods of disuse, injury, or reduced daily step counts) and exacerbate the loss of muscle mass with increasing age.

A loss of muscle mass also has been reported in those with T1D. Before the discovery of insulin, patients with T1D were cachectic and only lived 1 to 2 yr after diagnosis. Although the introduction of insulin therapy has rescued T1D patients from this debilitating syndrome and early mortality, the few studies conducted to date have reported an overall reduction of myofiber size/muscle volume/cross-sectional area in those newly diagnosed (19) and in middle-aged (35–50 yr old) persons with T1D compared with controls (Andersen's work reviewed in (4)). Interestingly, this deficit in muscle volume/cross-sectional area in adults with T1D was present even in those without neuropathy but was amplified with presence of neuropathy (4).

The evidence is less equivocal in children and adolescents with T1D after a period of insulin therapy. Many studies report no differences in lean body mass (measured by either dual energy X-ray absorptiometry or magnetic resonance imaging) compared with matched controls or normative values (9,20–22). However, evidence is accumulating that this maintenance of muscle mass is evident only in those with "good" glycemic control (23,24); an observation consistent with insulin's suppression of protein degradation pathways and upregulation of anabolic pathways, as well as the reported aberrations in the growth hormone-insulin-like growth-factor-I axis correlated to poor glycemic control (25). Given that those with T1D are not euglycemic all day everyday, we would speculate that periods of disrupted protein balance are impacting the ability to achieve optimal muscle mass though further study in this area is greatly needed. The importance of a healthy and optimal muscle mass with increasing age cannot be overstated (17). As mentioned previously, our limited understanding of the temporal impact of T1D on skeletal muscle mass is an area where future studies are necessary.

Whether fiber-type specific attenuations in fiber cross-sectional area may exist in the muscles of patients with T1D also is inconclusive. To the best of our knowledge, only two studies have examined this in detail after the introduction of the more intensive insulin therapies still in use today. Specifically, Andreassen *et al.* (26) observed a greater frequency of type II (glycolytic) fibers and larger fiber diameters (both type I and type II) in distal muscles (gastrocnemius) of 16 T1D adults (10 with neuropathy, 6 without) in comparison with patients with type 2 diabetes (T2D) and controls. Interestingly, no relation between neuropathy and fiber diameter/fiber-type proportion was found, and no difference in the number of capillaries per fiber was observed, suggesting intrinsic impairments as a result of T1D (rather than the result of capillary rarefaction, for example). Fritzsche *et al.* (27) also reported greater frequency of type II fibers, albeit in young adults with T1D: however, muscle fiber cross-sectional area was not measured. Thus, it is possible that the increased number of glycolytic fibers resulted from increased atrophy, leading to an increased number of smaller fibers rather than an expansion in the number of functional type II fibers, affecting quantification.

As mentioned previously, an inability to properly repair from muscle damage has been reported in older adults and has been linked to reductions in muscle size/mass (28). A loss of muscle satellite cells in aged muscle also has been identified as a contributing factor to the decline in muscle mass with advancing age (28). Although no muscle regeneration studies have been undertaken in humans with T1D, rodent models of T1D indicate that their muscles are more prone to damage (29) and exhibit delayed regeneration (30). What is known, however, is that both T1D human (~18–21 yr old) and mouse skeletal muscles demonstrate a significant reduction in satellite cell content (29) consistent with aged skeletal muscle.

Muscle Function

In otherwise healthy adults, the age-related changes to muscle eventually manifest in functional limitations and disability, adversely impacting activities of daily living and quality of life. Decreased muscle strength (maximal muscle force) and power (product of force and velocity of muscle contraction) are common features seen in aging and sarcopenia, with the rate of loss of muscle isometric force being ~1%–2% per year and muscle power being 3%–4% per year (31). Considering that the majority of activities of daily living rely on muscle power (*e.g.*, raising from a chair or climbing stairs) versus muscle strength, the greater power decline that occurs with advanced age is likely the fundamental cause of increased risk of falls and vulnerability to injury reported in the older population (32).

Similar to aged muscle, T1D has been demonstrated to compromise skeletal muscle function. In fact, the decline in function seems to begin early in life (and disease progression) as multiple studies performed in children with T1D have observed reduced muscle strength and power and increased

fatigability compared with their counterparts (33–36); however, this is not universally reported (reviewed in (4)). Nonetheless, this remains an important observation for two reasons: 1) the impairments in muscle function in youth indicate the skeletal muscle dysfunction is a primary diabetic complication (rather than secondary to other complications such as neuropathy) and 2) as T1D is a chronic disease, the manifestation of impaired muscle health early in T1D could mean an earlier development of sarcopenia in those with this disease. Support for this comes from studies in adults with T1D that demonstrate that both isometric and isotonic maximal force production are decreased compared with controls (4,37). With advancing age, the reductions in skeletal muscle strength become more closely associated with the severity of neuropathy in those with T1D (4,26,37). Therefore, similar to the loss of function seen in aging muscle, muscle strength/power decreases with increasing T1D duration; however, this decline becomes more accelerated with the development of neuropathy (particularly in the distal limb musculature). Akin to the age-related declines in functionality, over 70% of people with diabetes (both T1D and T2D) report difficulty with routine physical activities and diabetes alone was associated with two to three times increased odds of suffering from disability (38).

Muscle Metabolism

The changes in skeletal muscle mass and function that occur with increasing age also are accompanied by deteriorations in metabolism, including dysregulation in glucose, lipid, and protein metabolism.

The evidence to date generally supports age-related reductions in the protein synthesis pathways, specifically decreases in Akt/PKB-mTORC signaling, as well as age-related increases in the protein degradation pathways (*e.g.*, ubiquitin proteasome system), all of which inevitably impact skeletal muscle mass and promote wasting (reviewed in (39,40)). In T1D, our understanding of changes in protein metabolism is extremely limited. To the best of our knowledge, no studies to date have investigated the protein synthesis (*e.g.*, mTORC) and degradation (*e.g.*, ubiquitin) pathways at the molecular level in those with T1D. Thus, it is unknown if the changes seen in protein signaling in older, nondiabetic adults are comparable with adults with T1D. Our limited understanding of protein metabolism in T1D stems largely from the whole-body level studies conducted in adults (~30 yr old) by Nair *et al.* (41–43). These researchers found that protein metabolism in T1D seems to be largely dependent on insulin therapy and glycemic control. For instance, poor T1D management has been shown to cause an increase in both whole-body protein breakdown and protein synthesis, with breakdown rates far exceeding synthesis rates, resulting in a net protein loss. More recent work revealed that this loss largely stems from proteins within skeletal muscle (44). In contrast, good glycemic control and adequate insulin injections seems to only allow for protein conservation (via reductions in protein breakdown) (43). Thus, similar to aging muscles, T1D seems to negatively impact protein metabolism. Taken together, it is clear that studies are urgently warranted to not only elucidate the impact of T1D on protein metabolism in youth and elderly T1D individuals, but also whether aging adults with T1D develop anabolic resistance similar to non-T1D aging adults, and if so, at what age and timepoint in disease duration.

With respect to fuel metabolism, key enzymes in glucose metabolism, including hexokinase (HK), lactate dehydrogenase (LDH), and citrate synthase (CS), all have been shown to have lower activity with advanced age (45). In addition, aging seems to downregulate transcripts important for lipid transport and oxidation in human skeletal muscle (46), which likely explains the increased presence of intramyocellular lipids (IMCL) in older adults (46). Importantly, the combination of decreased fat oxidation and decreased mitochondrial oxidative phosphorylation (discussed in detail hereinafter) seen in aging muscle can cause incomplete oxidation of fatty acids, which in turn can lead to increased ROS production and the accumulation of toxic lipid metabolites in the muscle cells, and consequently, interference with muscle contraction (discussed previously) and the insulin signaling pathway (46,47). Not surprisingly, older individuals are more likely to develop insulin resistance, resulting in a further impact on the metabolic capacities of skeletal muscle, including muscle protein turnover.

Although T1D is inherently a metabolic disease characterized by impaired glucose handling, some of the aforementioned impairments with aging also are seen within the muscle of individuals with T1D, but at a much younger age. In particular, early studies have reported lower HK activity in adults with T1D (~29 and 32 yr old) compared with non-T1D controls (48,49). In contrast, younger adults with T1D (~21 yr old) seem to have normal HK activity (50), suggesting that there may be a sensitive threshold for the onset of glycolytic impairments in T1D muscle. LDH activity also has been reported to be elevated in T1D muscle (48,49), and this may be a result of decreased pyruvate dehydrogenase activity (48) or mitochondrial dysfunction. To the best of our knowledge, only one study has measured CS in humans with T1D (~32 yr old), and no differences were reported (49). Although there are disparities in which key metabolic enzymes are affected with aging and T1D, insulin resistance still develops in those with T1D and importantly, this seems to occur early in disease progression (*e.g.*, adolescence) (9). Consequently, the mechanisms that lead to declines in insulin sensitivity are not fully understood in T1D, but similar to muscles of older adults, the muscles of those with T1D have been reported to exhibit greater IMCL content (51), particularly in those who are greater than 30 yr old and in poor glycemic control (20,52). The increased IMCL content is very likely the result of a reduced mitochondrial oxidative capacity (7–10), similar to aging, in association with increased free fatty acid (FFA) delivery resultant from adipose tissue insulin resistance, which has been well characterized in those with this disease (20,23,53,54). We therefore speculate that muscles in those with T1D exhibit an increased presence of toxic lipid metabolites, similar to aged skeletal muscle, resulting in impaired insulin signaling and ultimately impaired muscle metabolism, including the muscle protein synthesis and degradation pathways.

MITOCHODNRIAL DYSFUNCTION AS THE UNDERLYING MECHANISM FOR ACCELERATED MUSCLE AGING IN T1D: A "MITO-CENTRIC" HYPOTHESIS

Mitochondria are commonly referred to as the "powerhouse of the cell" because of their essential role in the production of the high-energy molecule adenosine triphosphate (ATP). However,

mitochondria have many other vital roles in muscle cells including ROS production/signaling, Ca^{2+} homeostasis, and the regulation of programmed cell death (apoptosis). This makes them indispensable organelles for the regulation of skeletal muscle mass, function, and metabolism. It is thus not surprising that mitochondrial dysfunction has long been considered to be centrally implicated in the aging process and age-related deteriorations to skeletal muscle health. Although we acknowledge that there is still some areas of debate within this highly complex area, owing in part to the nature of methodologies used for interrogating mitochondrial function, as well as the age and level of physical activity of subjects studied (we refer the readers to (55) for more detail), the weight of evidence in aged human muscle supports reductions in mitochondrial oxidative capacity (5,6), reductions in mitochondrial respiration at physiological adenosine diphosphate (ADP) concentrations (11), increased mitochondrial ROS production (11), and increased sensitization of the mPTP (12). Thus, mitochondrial dysfunction seems to be strongly implicated in the continuum of age-induced skeletal muscle dysfunction. The following sections will concisely discuss basic mitochondrial physiology, how mitochondrial dysfunction is implicated in deteriorations to skeletal muscle health, and the evidence supporting our novel hypothesis that mitochondria are a primary mediator of accelerated muscle aging in T1D (Fig. 1).

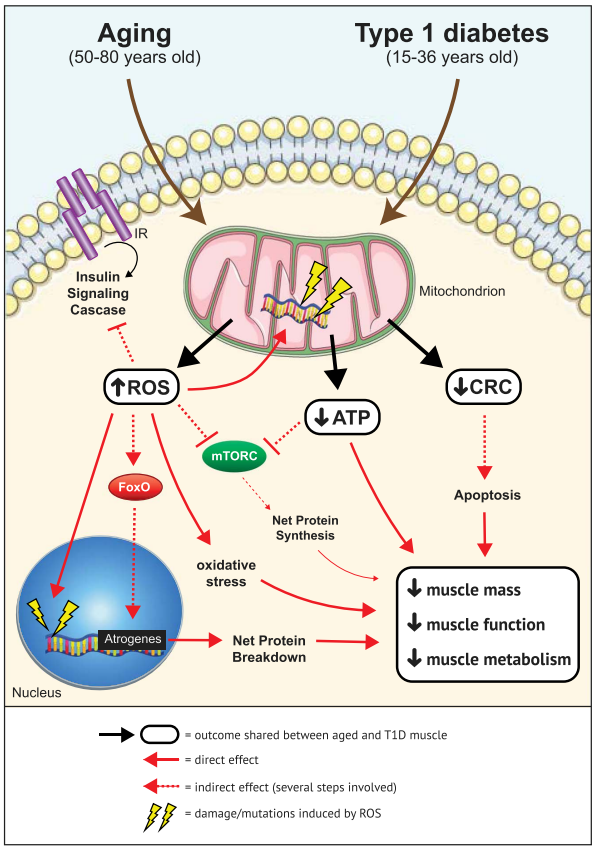

Figure 1. Proposed mechanism by which accelerated muscle aging occurs in adolescent/young adults with type 1 diabetes (T1D). Similar to aging muscle, mitochondrial dysfunction in 15-36 year-old individuals with T1D results in increased mitochondrial reactive oxygen species (ROS) emission poten-tial, decreased mitochondrial energy production (ATP) and decreased mitochondrial calcium retention capacity (CRC). The increased ROS emission poten-tial can directly impact mitochondrial and nuclear DNA and cause oxidative stress. It can also indirectly enhance the expression of atrogenes via activation of FoxO and reduce rates of protein synthesis indirectly via inhibition of mTORC. Increased ROS emission potential has also been shown to interfere with the insulin signaling cascade and induce insulin resistance. Interference with the insulin signaling cascade, and reductions in mitochondrial ATP, can both cause reductions in mTORC, further impacting muscle protein synthesis. Additionally, reductions in ATP can lead to reduced muscle function and inevitably impair muscle metabolism. Reductions in CRC increases the potential for cell death via apoptosis. Thus, mitochondrial dysfunction is a primary contributor to impaired skeletal muscle health in those with T1D. FoxO, forkhead box; IR, insulin receptor; mTORC, mammalian target of rapamycin complex.

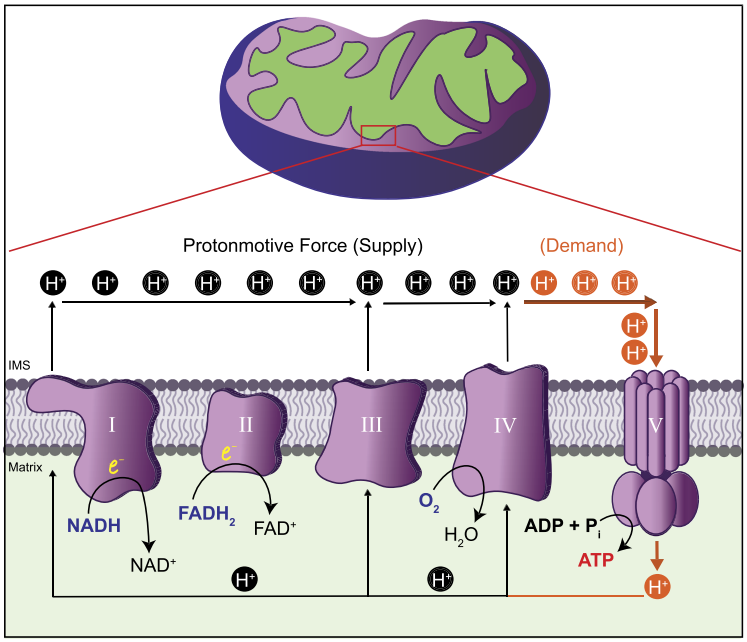

Figure 2. Schematic representation of mitochondrial oxidative phosphorylation. The electron carriers NADH and $FADH_2$, derived from the metabolism of carbohydrates and lipids, donate electrons (e-) to complex I and complex II, respectively. The electrons are then passed down in a series of redox reactions until they reach the final electron acceptor, oxygen (O_2), producing water (H_2O). Free energy is released during the transfer of electrons, which is captured and used by complexes I, III, and IV to pump protons (H^+) from the matrix into the IMS against an electrochemical gradient to create the protonmotive force (the "energy supply"). The protonmotive force, or membrane potential, in turn can drive the production of ATP from ADP (the "energy demand") and inorganic phosphate (P_i) by driving protons back into the matrix through complex V.

Brief Overview of Mitochondrial Physiology

Mitochondrial respiration

Mitochondria produce ATP via the mechanism of oxidative phosphorylation (also referred to as mitochondrial respiration). As depicted in Figure 2, a series of specific proteins (complexes I through V) located in the inner mitochondrial membrane, termed the electron transport chain (ETC), catalyze the oxidation of respiratory substrates (electron carriers) NADH and $FADH_2$, generated during the metabolism of carbohydrates (glycolysis) and lipids (B-oxidation). Electrons are then transferred into the ETC and, through a series of redox reactions, are passed down from complex I or complex II all the way to complex IV until they reach the final electron acceptor, oxygen. Free energy is released during these chemical reactions, which is captured and used to "pump" protons across the mitochondrial inner membrane and into the intermembrane space (IMS) against an electrochemical gradient. This creates a proton-motive force, also known as the mitochondrial membrane potential ($\Delta\Psi$), that in turn drives protons back into the matrix through the ATP synthase (complex V), rotating a part of the enzyme that drives the phosphorylation of ADP into ATP.

Mitochondrial ROS production

Approximately 2% of the total oxygen consumed during "normal" oxidative phosphorylation results in the production of ROS due to natural electron leakage from the ETC. These leaked electrons are highly unstable and react quickly with nearby oxygen to produce oxygen free radicals, such as the superoxide radical ($O_2^{\bullet}$-) and hydroxyl radical ($^{\bullet}OH$), as well as nonradicals, such as hydrogen peroxide (H_2O_2). The mitochondrial matrix and cytoplasm are equipped with enzymatic (*e.g.*, glutathione and catalase) and nonenzymatic (*e.g.*, vitamin E) antioxidant defenses to counterbalance these oxidants. At low physiological levels, ROS acts as an important signaling molecule for various processes, including muscle contraction, cell proliferation, and cell adaptation to exercise training. However, when ROS levels exceed antioxidant capacity (as may occur with excess substrate delivery, dysfunctional mitochondria, or reduced antioxidant defences), it becomes pathological and causes oxidative stress.

Mitochondrial Ca^{2+} uptake

Mitochondria also contain a Ca^{2+} uniporter that allows Ca^{2+} flux into the matrix, a process that is essential for ATP production and for regulating muscle contractile function and programmed

cell death. The influx of Ca^{2+} into the matrix not only contributes to shaping of the sarcoplasmic Ca^{2+} transients but also simultaneously stimulates mitochondrial energy production by activating select Ca^{2+}-sensitive mitochondrial dehydrogenases (56). This ensures that adequate energy is provided to support muscle contraction. Moreover, mitochondria can aid in the buffering of cytoplasmic Ca^{2+} by chelating excess levels of this ion with inorganic phosphate in its matrix. However, Ca^{2+} overload or the combination of excess Ca^{2+} and damaged mitochondria can induce opening of the mPTP. mPTP opening collapses the $\Delta\Psi$ and leads to decreased ATP production, disruption of ionic homeostasis, and swelling. As the mitochondrial swells, the cristae of the inner mitochondrial membrane begin to unfold and the increased pressure on the outer mitochondrial membrane causes it to rupture and releases proapoptotic factors that activate caspase cascades in the cytosol to initiate cellular fragmentation and ultimately, cell death.

Mitochondrial Dysfunction and the Impact on Skeletal Muscle Health

At this point in time, the term mitochondrial dysfunction is ill-defined owing to the multiplicity of mitochondrial functions in a cell, as described previously, as well as the fact that it remains unclear as to whether altered mitochondrial function reflects a physiological adaptation, a pathological maladaptation, or simply a pathological phenomenon. For the purpose of this review, mitochondrial dysfunction will entail any abnormality in the key physiological roles (*e.g.*, respiration, ROS, Ca^{2+} uptake) of a mitochondrion (*i.e.*, intrinsic mitochondrial function).

Depending on the metabolic state of the cell, mitochondria regulate skeletal muscle mass, function, and metabolism via either the activation of anabolic or catabolic signaling pathways, some of which feed-forward to myonuclei to either upregulate or downregulate the expression of genes important for muscle protein synthesis/degradation, muscle contraction, and substrate oxidation (reviewed in (57)). For example, excess ROS production, caused by either 1) nutrient excess, 2) a combination of nutrient excess and reduced mitochondrial respiration, 3) damaged mitochondria, 4) depleted antioxidant defenses, or 5) a combination of these factors, can induce the expression of atrogenes via activation of the JNK/FoxO signaling pathway as well as the activation of endoplasmic reticulum stress, which in turn suppress protein synthesis and promote protein degradation, and hence, muscle atrophy (57). Excess mitochondrial ROS also can lead to opening of the mPTP and thus increase apoptotic potential. Opening of the mPTP also can be triggered by impairments in the ability of the mitochondria to retain excess levels of Ca^{2+} due to failure of cytosolic Ca^{2+} homeostasis and ionic disturbances. Irrespective of the cause, the resultant increase in apoptosis invariably promotes muscle atrophy. Increased mitochondrial ROS also can lead to nuclear and mtDNA mutations/deletions, protein damage (including ETC enzymes), and lipid peroxidation — in other words, oxidative stress — all of which can directly and indirectly impact muscle mass, function, and metabolism. For instance, damage to enzymes of the ETC caused by ROS can lead to even greater ROS production/damage, creating a vicious cycle that perpetuates mitochondrial dysfunction and apoptotic cell death. Mitochondrial dysfunction, specifically impaired mitochondrial respiration, can lead to the accumulation of incompletely oxidized substrates and toxic metabolites, which have been implicated in impaired muscle contraction (57). Furthermore, impaired mitochondrial respiration not only drives mitochondrial ROS production but also increases the AMP/ATP ratio, which in turn leads to the activation of the energy sensor molecule AMP-activated protein kinase (AMPK). Increased AMPK activity inhibits anabolic pathways, including muscle protein synthesis by inhibiting mTOR and directly phosphorylating FoxO3 (see Fig. 1).

Mitochondrial Dysfunction in Skeletal Muscle of Individuals With T1D

Mitochondrial energy production

Although dramatic efforts have been put toward interrogating mitochondrial function in T2D over the past two decades, surprisingly, little efforts have been made in the area of T1D. To our knowledge, the first assessment of mitochondrial function in individuals with T1D was undertaken in 2003. Crowther *et al.* (7) used the noninvasive *in vivo* technique ^{31}phosphorous-magnetic resonance spectroscopy (^{31}P-MRS) to measure the rate constant of phosphocreatine (PCr) resynthesis, a measure that infers *in vivo* mitochondrial oxidative capacity, in ~36-yr-old men with an HbA1c of <7.0% (*i.e.*, well-controlled T1D) and disease duration of ~18 yr (no neuropathy reported). They observed a slower rate of PCr recovery after a 30-s bout of isometric contraction (70% of maximal voluntary contraction (MVC)) compared with matched controls. Importantly, this occurred irrespective of level of physical activity in the T1D individuals (four sedentary and three recreationally active), indicating 1) men with T1D in their thirties already have a reduced muscle mitochondrial oxidative capacity despite having well-controlled glycemia and 2) being recreationally active with T1D was not sufficient to prevent attenuations in muscle mitochondrial respiratory function. Despite these important findings, the next studies to interrogate mitochondrial function in T1D were not until 8 yr later. Using the same technique, Kacerovsky *et al.* (8) interrogated the ability of insulin to stimulate flux through mitochondrial ATP synthase (fATP) during a hyperinsulinemic-euglycemic clamp in ~36-yr-old men and women with well-controlled T1D (HbA1c, 6.8%) and disease duration of ~17 yr. They found that insulin failed to stimulate fATP in the T1D group compared with matched controls, suggesting abnormal mitochondrial oxidative metabolism. However, this may not have been an intrinsic mitochondrial defect *per se* as these participants also had an ~50% lower whole-body glucose disposal compared with controls, indicating the presence of insulin resistance, which could account for the attenuation in fATP. In contrast, Item *et al.* (58) reported no differences in the rate of PCr recovery after a 30-s bout of isometric contraction (85% of MVC) in untrained ~27-yr-old women with an HbA1c of 7.6% and disease duration of 13 yr. Taken together, these studies indicate that the degree of muscle mitochondrial dysfunction in T1D may depend on age and sex. However, more recent work contradicts these findings. Cree-Green *et al.* (9) interrogated mitochondrial oxidative capacity and the rates of mitochondrial oxidative phosphorylation (*i.e.*, time taken to convert ADP to ATP) in sedentary/recreationally active adolescents (~15 yr old) with an HbA1c of ~8.2% (*i.e.*, moderately

controlled for adolescents with T1D) using ^{31}P-MRS. After a 90-s bout of isometric contraction (70% of MVC), they found that mitochondrial oxidative capacity was significantly lower in adolescents with T1D compared with matched controls, similar to Crowther *et al.*'s (7) findings, and they also found a delay in the recovery of ADP (*i.e.*, slower rates of mitochondrial oxidative phosphorylation). Therefore, the evidence to date, albeit limited, highlights skeletal muscle mitochondrial dysfunction in individuals with T1D, a complication that appears to occur early in diabetes progression.

Although ^{31}P-MRS is undoubtedly advantageous for capturing *in vivo* ATP kinetics under physiological conditions, a limitation of this technique is the lack of resolution into the underlying changes responsible for impairments in mitochondrial function. This limitation can be overcome using *in vitro* techniques such as high-resolution respirometry and permeabilized myofiber bundles. We recently used this technique to interrogate mitochondrial function in young adults with T1D under moderate glycemic control (HbA1c, 7.9%) (10). Specifically, it was found that young, physically active men and women (~26 yr old) with T1D (duration, ~15 yr) had an ~20% reduction in mitochondrial oxidative capacity compared with matched controls, consistent with the aforementioned *in vivo* work by Crowther *et al.* (7) and Cree-Green *et al.* (9). In addition, significant attenuations in the sensitivity and respiratory capacity of complex II of the ETC, but not complex I, reveal for the first time site-specific deficiencies in the mitochondrial ETC from muscle of young adults with T1D. Importantly, these impairments in mitochondrial respiratory function occurred in the absence of changes in mitochondrial content (measured by electron microscopy and Western blot) and capillary density, implying that these mitochondrial impairments were intrinsic to the mitochondria and not secondary to oxygen/energy supply or mitochondrial content.

Mitochondrial ROS production/oxidative stress

Early work by Brownlee (reviewed in (59)) demonstrated that hyperglycemia leads to enhanced mitochondrial ROS production in tissues that cannot effectively regulate glucose uptake, including endothelial, mesangial, and Schwann cells, thereby increasing the susceptibility to oxidative stress and ultimately cellular damage/complications. Since then, it has largely been thought that tissues that can regulate glucose transport (*i.e.*, the insulin-sensitive tissues: skeletal muscle, adipose tissue, and liver) are not susceptible to damage inflicted by hyperglycemia in T1D. In fact, until recently, it remained unknown whether T1D impacts mitochondrial ROS production/oxidative stress in these insulin-sensitive tissues in humans. Using permeabilized myofibers, the capacity of five different sites within mitochondria to emit H_2O_2 in the skeletal muscle of young men and women with and without T1D was assessed (10). It was found that mitochondrial H_2O_2 emission (production minus scavenging) was significantly elevated at complex III in T1D compared with matched controls. Although only one site of enhanced ROS emission was detected, complex III has been demonstrated to be the main producer of ROS within the ETC (60), and thus further study is needed to fully elucidate the importance of elevated ROS from complex III and whether this manifests in an oxidative stress within the skeletal muscle of those with T1D. Furthermore, establishing whether the increase in mitochondrial H_2O_2 emission potential in T1D muscle is the result of attenuations in anti-oxidant defenses (as has been reported in aged muscle (57)) or dysfunction at complex III is necessary. In particular, a critical outcome of excess ROS/oxidative stress includes DNA and protein damage and apoptotic cell death, as previously mentioned, which can lead to a decrease in muscle function by damaging proteins critical for muscle contraction as well as a decrease in muscle size by reducing fiber number and selectively removing individual targeted myonuclei (57).

Mitochondrial Ca^{2+} uptake

Opening of the mPTP is favored by mitochondrial Ca^{2+} overload and high concentrations of ROS leading to programmed cell death. An increased susceptibility to opening of the mPTP has been documented in aged muscle (12). To date, we (10) are the only group that has demonstrated that skeletal muscle mitochondria in those with T1D have significant reductions in Ca^{2+} retention capacity (CRC) compared with matched controls, highlighting increased apoptotic potential and mitochondrial Ca^{2+} overload in human T1D skeletal muscle, further supporting the mitochondrial dysfunctional in this tissue with T1D. Clearly, future work in this area is needed as the role of mitochondria in intracellular Ca^{2+} handling continues to gain appreciation.

SUMMARY OF EVIDENCE SUPPORTING ACCELERATED AGING IN THE MUSCLES OF THOSE WITH T1D

This article discussed how the "health" of aged muscle, including decreased muscle mass and function and dysregulated metabolism, in otherwise healthy older adults is in many ways similar to that of the muscle from individuals with T1D of a younger age. Specifically, the loss of muscle mass has been observed in young, newly diagnosed patients with T1D (19), in adolescents with T1D that are in poor glycemic control (61), and in individuals with T1D in their fifth decade of life (age 40 to 49) (4,37), declines that typically begin in the sixth decade (age 50 to 59) in otherwise healthy individuals. The presence of neuropathy has been shown to amplify this difference (4,26,37). Similarly, decrements in muscle strength with T1D have been reported to occur in childhood (34), continue into adolescence (33–36), and seem to remain significantly lower by middle-age (~50 yr old) even in the absence of secondary complications (*e.g.*, neuropathy), compared with matched counterparts (4,37). Metabolic dysregulation is inherent to T1D, and thus it is not surprising that some of the metabolic changes observed in aged muscle, including altered HK activity (45,48,49) and reduced mitochondrial oxidative phosphorylation (5–11), also occurs early in T1D (less than 36 yr old vs greater than 50 yr old). Altogether, the evidence in the human T1D literature (albeit incomplete) led us to postulate that T1D is a disease that accelerates the aging of skeletal muscle.

Although the pathogenesis behind this accelerated skeletal muscle dysfunction is undoubtedly multifactorial, the combination of the vital role of mitochondria for skeletal muscle health and the work of us and others (7–10) led us to consider impairments to mitochondria as a primary mediator of this phenotype similar to that reported in aging muscle (5,6,11,12). Specifically, the age-related attenuations in mitochondrial respiration

(11) and oxidative capacity (5,6), increased mitochondrial ROS production (11), and impaired mitochondrial Ca^{2+} handling (12) reported in older adult muscle mimic that which we recently observed in ~26-yr-old, physically active adults with T1D. These early deficits to skeletal muscle mitochondria in T1D adults also occurred in the absence of changes in mitochondrial content and vascularity, suggesting an intrinsic dysfunction as a result of T1D. However, we acknowledge that although both aging and T1D share similar skeletal muscle characteristics, it is likely that the underlying etiology of mitochondrial dysfunction remains different between both conditions. For instance, there is a strong genetic component to the aging process including accumulation of DNA mutations as well as an inability to repair DNA and to properly synthesize proteins (55). Whether this occurs in T1D muscle is still unknown (to our knowledge). In contrast, T1D is a disease characterized by a hyperglycemic and hypoinsulinemic *milieu*, and thus it seems plausible that substrate overload (both glucose and lipids) as a result of repeated subcutaneous insulin injections, which bypass the typical liver-first canonical pathway (reviewed in (3)), is leading to increased glycation and posttranslational modifications of skeletal muscle proteins, thereby impacting function. It is clear that there remains considerable work to be done in this area to prove our mitocentric hypothesis of accelerated muscle aging in T1D, particularly if we are to develop adjuvant therapies for improving the quality of life and healthy lifespan of those currently living with this chronic disease.

FUTURE PERSPECTIVES: WHY IT IS TIME FOR T1D-SPECIFIC EXERCISE GUIDELINES

The systemic benefits of exercise training cannot be disputed. Improvements (adaptations) to skeletal muscle have largely been considered to be the primary mediator of the positive whole-body effects of exercise. Of particular relevance with respect to this article is the potent ability of exercise training to significantly improve skeletal muscle health in older adults. Indeed, resistance training has garnered considerable attention in the past decade because of accumulating evidence in both older men and women showing that resistance exercise increases muscle mass (62) and myofiber size (63), improves muscle quality and functional abilities (62), reduces age-related attenuations in muscle strength, and increases in muscle fat infiltration (64), as well as prevents muscle insulin resistance (65).

Although the cellular mechanisms responsible for the adaptations of skeletal muscle to exercise training are multifactorial and remain to be fully elucidated, it has long been known that exercise induces mitochondrial biogenesis (66). An increase in the generation of new mitochondria results in the muscle not only becoming more efficient at using substrates to synthesize ATP, and hence more resistant to fatigue, but also at maintaining cellular homeostasis. In more recent years, there has become a greater appreciation and interest in exercise-mediated degradation of damaged/dysfunctional mitochondria, and hence, mitochondrial turnover, a process that is crucial for maintaining a healthy mitochondrial pool and ultimately homeostasis of cellular processes (67). Consequently, few studies to date have sought to interrogate this in aged human muscle. Although contradictory findings exist in aging-associated impairments in mitochondrial biogenesis and degradation, both aerobic and resistance exercise have been suggested to induce mitophagy in aged muscle (reviewed in (67)). As such, exercise training is most likely an effective means of stimulating mitochondrial turnover, and hence, maintaining a healthy mitochondrial pool (*i.e.*, improving mitochondrial function) in aged skeletal muscle.

Despite the fact that evidence has existed as early as 1977 depicting aberrant mitochondria at the ultrastructural level in the muscle of adults with T1D (68), and that since 2003 there has been evidence for mitochondrial functional deficits in ~30-yr-old T1D men (7), no studies to date have sought to investigate whether impairments to mitochondrial biogenesis/mitophagy exist in T1D muscle. As previously mentioned, we

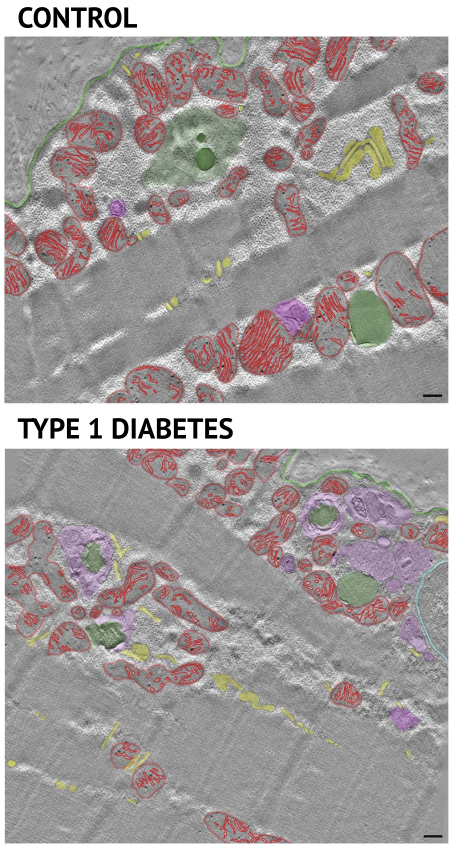

Figure 3. Representative electron tomography images of skeletal muscle from young adults with type 1 diabetes (T1D) and without (control). An increased presence of autophagic remnants (highlighted in magenta) were more frequently observed in both the subsarcolemmal and intermyofibrillar regions of the T1D muscle compared with control in addition to irregularities in the organization of the mitochondrial cristae (highlighted in red). Green highlighting, lipid droplets; light green highlighting, sarcolemma; yellow highlighting, triads/sarcoplasmic reticulum; cyan highlighting, nucleus. Scale bar, 200 nm.

(10) recently demonstrated for the first time that skeletal muscle mitochondrial content is not different in physically active, young men and women with T1D compared with matched counterparts, suggesting that mitochondrial biogenesis is not impaired in T1D. We did, however, observe ultrastructural abnormalities, similar to those reported in 1977, that would imply impairments in mitochondrial turnover in T1D (10) even with the use of more intensive insulin therapies. Specifically, electron tomography analysis revealed not only mitochondria with morphological defects, including both a loss of cristae and abnormal organization of remaining cristae, but also an increased presence of autophagic debris/remnants in both the subsarcolemmal and intermyofibrillar regions of the muscle (Fig. 3), suggestive of impairments in clearance of damaged organelles. This is of utmost concern and of clinical significance because, as mentioned earlier, these participants were young, physically active adults (~26 yr old) whose moderate to vigorous activity levels exceeded current exercise guidelines set forth by most major national diabetes associations (which are similar to the exercise guidelines of those without T1D). Thus, despite exceeding current exercise guidelines, this was not sufficient to fully maintain a healthy skeletal muscle mitochondrial pool.

Taken together, if our proposed novel hypothesis holds true, then increasing the balance of time spent resistance-training may prove fundamentally important in T1D, if we are to consider how impactful these activities are for improving the skeletal muscle health in older adults, as discussed previously. With that said, future studies are clearly needed to define the duration, intensities, and types of exercise (*e.g.*, high-intensity interval training) necessary for those with T1D to optimize their skeletal muscle health. It is expected that this in turn would reduce the development of secondary complications, disability, and ultimately premature mortality.

CONCLUSIONS

Despite the limited studies on the impact of T1D on human skeletal muscle health, the recent work from our lab (10,29) and others (7–9,48–50) has demonstrated structural and metabolic impairments in the muscle of individuals with T1D, at both the tissue and cellular levels, across all age groups studied — adolescence to middle-age — and suggest that glycemia and duration of diabetes is not a major determinant of these deficiencies. Importantly, the bulk of these studies were conducted in recreationally active T1D individuals who met/exceeded current exercise guidelines, emphasizing the urgent need to coordinate muscle analysis with various exercise training regiments in those with T1D with the goal to correct, and ideally improve, muscle's metabolic health and ultimately quality of life and lifespan.

Acknowledgments

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CHAPTER 5 — Normal to enhanced intrinsic mitochondrial respiration in skeletal muscle of middle- to older-aged women and men with uncomplicated type 1 diabetes

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PREFACE

Author contributions:

CMFM, AGD, and TJH conceived and designed the experiments. CGRP, VL and MAT contributed to the conception of experiments. **CMFM** wrote the manuscript. **CMFM** performed all experiments with assistance from IAR and JPN. **CMFM**, AGD, MN, IAR, JPN and LVT analysed data. **CMFM**, CGRP, and TJH interpreted data. MAT performed muscle biopsies and venous blood draw. **CMFM,** AGD, and LVT assisted with muscle and blood sample collection and processing. All authors edited the manuscript.

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Normal to enhanced intrinsic mitochondrial respiration in skeletal muscle of middle- to older-aged women and men with uncomplicated type 1 diabetes

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ABSTRACT

AIMS/HYPOTHESIS: This study interrogated mitochondrial respiratory function and content in skeletal muscle biopsies of healthy adults between 30 and 72 years old with and without uncomplicated type 1 diabetes.

METHODS: Participants (12/9 women/men) with type 1 diabetes (48±11 years of age), without overt complications, were matched for age-, sex-, BMI-, and level of physical activity to participants without diabetes (control) (49±12 years of age). Participants underwent a Bergström biopsy of the *Vastus lateralis* to assess mitochondrial respiratory function using high-resolution respirometry and citrate synthase activity. Electron microscopy was used to quantify mitochondrial content and cristae (pixel) density.

RESULTS: Mean mitochondria area density was 27% lower (p = 0.006) in participants with type 1 diabetes compared to controls. This was largely due to smaller mitochondrial fragments in women with type 1 diabetes (-18%, p = 0.057), as opposed to a decrease in the total number of mitochondrial fragments in men with diabetes (-28%, p = 0.130). Mitochondrial respiratory measures, whether estimated per mg of tissue (i.e., mass-specific) or normalised to area density (i.e., intrinsic mitochondria function), differed between cohorts, and demonstrated sexual dimorphism. Mass-specific mitochondrial oxidative phosphorylation (OXPHOS) capacity with the substrates for complex I and complex II (C_{I+II}) was significantly lower (-24%, p = 0.033) in women with type 1 diabetes compared to controls, whereas mass-specific OXPHOS capacity with substrates for complex I only ($C_{I\ pyr}$ or $C_{I\ glu}$) or complex II only ($C_{II\ succ}$) were not different (p > 0.400). No statistical differences were found in mass-specific OXPHOS capacity in men with type 1 diabetes compared to controls despite a 42% increase in $C_{I\ glu}$ OXPHOS capacity (p = 0.218). In contrast, intrinsic C_{I+II} OXPHOS capacity was not different in women with type 1 diabetes whereas in men with type 1 diabetes it was 25% higher (p = 0.163) compared to controls. Men with type 1 diabetes also demonstrated higher intrinsic OXPHOS capacity for C_{I+II} (+25%, p = 0.163), $C_{I\ pyr}$ (+50%, p = 0.159), $C_{I\ glu}$ (+88%, p = 0.033) and $C_{II\ succ}$ (+28%, p = 0.123), as well as higher intrinsic respiratory rates with low (more physiological) concentrations of either ADP, pyruvate, glutamate, or succinate (p < 0.01). Women with type 1 diabetes had higher (p < 0.003) intrinsic respiratory rates with low concentrations of succinate only. Calculated aerobic fitness (PWC_{130}) showed a strong relationship with mitochondrial respiratory function and content in the type 1 diabetes cohort.

CONCLUSION: In uncomplicated middle- to older-aged adults with type 1 diabetes, we conclude that skeletal muscle mitochondria differentially adapt to type 1 diabetes and demonstrate sexual dimorphism. Importantly, these cellular alterations were significantly associated with our metric of aerobic fitness (PWC_{130}) and preceded notable impairments in skeletal mass and strength.

RESEARCH IN CONTEXT

What is already known about this subject?

- Aging adults with diabetes are at greater risk of geriatric syndromes, particularly those implicated with skeletal muscle dysfunction, including frailty, falls and fractures, and physical disability, which can impact glycaemic management and lead to early complications.

- Mounting evidence indicate that type 1 diabetes may cause deficits in skeletal muscle mitochondria early in life (and disease progression), including lower mitochondrial oxidative capacity and site-specific deficiencies in the individual mitochondrial complexes.

What is the key question?

- Do abnormalities in skeletal muscle mitochondria exist in relatively healthy adults with type 1 diabetes (no overt complications) that are beyond young adulthood?

What are the new findings?

- In skeletal muscle of uncomplicated middle- to older-aged adults with type 1 diabetes, alterations to mitochondrial OXPHOS capacity, substrate sensitivity, and ultrastructure are present and demonstrate sex differences.

- Mitochondria content (area density) was lower in the type 1 diabetes cohort despite comparable body composition (fat and lean body mass) and calculated aerobic fitness, with women having smaller mitochondria and men having fewer mitochondria.

- Compared to matched counterparts, intrinsic mitochondrial OXPHOS capacity and respiratory response to low concentrations of ADP and complex I-specific substrates were generally normal in women, but enhanced in men, with type 1 diabetes, and this was positively correlated with our metric of aerobic fitness in both sexes.

How might this impact on clinical practice in the foreseeable future?

- Considering the well-demonstrated importance of mitochondria to muscle health, and the well-established role of muscle in our overall well-being, the findings in this study cohort not only further stress the importance of aerobic fitness in those with type 1 diabetes, but also the clear need for exercise guidelines that are sex-specific in this population.

KEYWORDS: older adults; mitochondria, oxidative phosphorylation; skeletal muscle; type 1 diabetes; aerobic fitness

INTRODUCTION

For the first time in history, people with type 1 diabetes on intensive insulin therapy are living into the later decades of life [1, 2]. However, aging adults with diabetes are at higher risk for the occurrence of geriatric syndromes, particularly those associated with deteriorations in skeletal muscle including frailty [3], falls/fractures [4, 5], and functional disability [6, 7]. These occurrences lead to decreased independence/ quality of life [8], higher rates of institutionalization [9], additional co-morbidities [8], and consequently, premature mortality [9]. The cellular mechanisms underlying deficits to skeletal muscle in type 1 diabetes, particularly in older age, remains largely unknown.

Mitochondria dysfunction has long been considered to be a primary mediator of age-related muscle decline [10]. In older people without diabetes, declines in *in vivo* mitochondrial oxidative capacity are associated with reduced gait speed [11], muscle strength and quality [12], resting metabolic rate [13], and insulin sensitivity [14]. Though considerable variability in mitochondrial (dys)function exists amongst adults of the same age (predominantly due to variability in aerobic fitness), the literature generally supports an age-dependent downward trend in skeletal muscle mitochondrial function and content [15, 16].

Evidence exists that mitochondrial dysfunction occurs early in type 1 diabetes, and hence, at an considerably earlier age in life than those without type 1 diabetes (reviewed in [17, 18]). We recently reported a decrease in skeletal muscle mitochondrial respiratory function in physically-active young adults with type 1 diabetes, despite no change in mitochondrial content [19], suggesting intrinsic mitochondrial deficiencies. However, the deficits in mitochondrial oxidative capacity may not be a universal phenomenon in those with type 1 diabetes as some have reported no differences between control and young adults with type 1 diabetes [20–22].

With an aging population of type 1 diabetes adults on the horizon, understanding whether deficits in muscle mitochondria exist, persist, or worsen beyond young adulthood is imperative. Therefore, we employed a cross-sectional study interrogating mitochondrial respiratory function and ultrastructure in skeletal muscle biopsies of adults between 30 and 72 years of age with uncomplicated type 1 diabetes. We hypothesized that women and men with uncomplicated type 1 diabetes have reduced muscle mitochondrial respiratory function and content compared to matched controls.

METHODS

Recruitment and Participant Characteristics

Females and males (hereafter referred to as women and men), with uncomplicated type 1 diabetes between 30 and up to 80 years old were recruited from the McMaster University campus area and surrounding cities in Southwestern

Ontario, Canada. Type 1 diabetes participants were closely matched with participants without diabetes (control group) for age (± 4 years), sex (self-identified male or female), body mass index (BMI) (± 4 kg m^{-2}) and physical activity levels. The latter was assessed by participant's self-reported leisure physical activity type and time. We excluded persons diagnosed with prediabetes and type 2 diabetes; statin-induced myopathy or myalgia; heart disease; severe hypertension; stage 3 neuropathy; severe retinopathy; used assistive walking devices; used corticosteroids and NSAIDs; smoked or used tobacco products. The study was reviewed and approved by the Hamilton Integrated Research Ethics Board (HiREB #5344), in accordance with the Declaration of Helsinki. All participants (n = 42) gave verbal and written informed consent.

Participant characteristics are presented in Table 1, including PWC_{130} and MVC. There were no significant differences in any characteristics except for HbA_{1c} which was significantly higher in type 1 diabetes compared to control. A neurological examination of the peripheral nervous system by a neuromuscular clinician revealed similar scores between groups except for ~20% of participants with type 1 diabetes, who had evidence of a mild sensory neuropathy (decreased vibration more than mild and/or absent ankle reflexes). No study participants demonstrated severe or even moderate peripheral neuropathy by examination, nor was there any evidence of proximal limb motor deficit.

In both the control and type 1 diabetes group, ~63% of women were premenopausal (n = 7/8 for control/type 1 diabetes), and ~37% of women were postmenopausal (n = 4 each group). In the control group, 43% reported using hormonal contraceptives (birth control or intrauterine device) compared to 50% in the type 1 diabetes (n = 3 and 4, respectively). Postmenopausal women (n = 5/4 for control/type 1 diabetes) did not declare use of hormone replacement therapy except for 1 in the type 1 diabetes group. Menstrual cycle was not controlled for in this study.

Characteristics specific to the type 1 diabetes cohort are presented in Table 2. On average, diabetes duration was 28 (±13) years and age of diagnosis was 19 (±10) years old. Diabetes duration was significantly longer (p = 0.027) in men (36 ± 11 years) than women (23 ± 13 years). Two participants self-reported symptoms of diabetes-related complications [neuropathy (n=1) and mild retinopathy/neuropathy (n=1), respectively], though neurological examination by a neuromuscular clinician did not confirm these self-reports, as mentioned earlier.

Study Protocol

Participants visited the laboratory twice with 7-9 days between visits. All participants were instructed to consume a standardized meal 1.5-2 hours prior to their visits and refrain from

Table 1. Participant Characteristics

	Combined		Women		Men	
	CON (*n* = 21)	T1D (*n* = 21)	CON (*n* = 12)	T1D (*n* = 12)	CON (*n* = 9)	T1D (*n* = 9)
Age (years)	49 ± 12	48 ± 11	47 ± 10	45 ± 10	51 ± 15	52 ± 13
Weight (kg)	75.8 ± 12.9	78.3 ± 16.3	71.5 ± 14.1	73.2 ± 16.2	81.4 ± 9.0	85.2 ± 14.4
Height (cm)	169 ± 12	170 ± 8	162 ± 10	166 ± 6	179 ± 5	176 ± 7
BMI (kg/m^2)	26.4 ± 3.3	26.9 ± 4.7	27.1 ± 3.7	26.5 ± 5.6	25.5 ± 2.5	27.5 ± 3.4
Lean mass (kg)	49.2 ± 11.9	49.8 ± 10.4	40.5 ± 6.0	42.8 ± 5.0	60.7 ± 6.5	59.1 ± 8.2
Lean mass (% BW)	64.9 ± 11.3	64.6 ± 10.1	57.9 ± 8.4	60.8 ± 10.6	74.2 ± 7.1	69.7 ± 6.9
Fat mass (kg)	23.7 ± 10.5	25.3 ± 11.6	28.1 ± 10.3	26.9 ± 13.4	17.9 ± 7.9	23.2 ± 8.9
Fat mass (% BW)	64.9 ± 11.3	64.6 ± 10.1	57.9 ± 8.4	60.8 ± 10.6	74.2 ± 7.1	69.7 ± 6.9
HbA_{1c} (mmol/mol)	**33.8 ± 4.0**	**56.9 ± 8.3*****	**34.5 ± 2.9**	**57.1 ± 7.6*****	**32.8 ± 5.2**	**56.8 ± 9.5*****
HbA_{1c} (%)	**5.2 ± 0.4**	**7.4 ± 0.8*****	**5.3 ± 0.3**	**7.4 ± 0.7 *****	**5.2 ± 0.5**	**7.3 ± 0.9*****
PWC130 (watts)	127.3 ± 62.9	104.3 ± 49.8	94.6 ± 38.3	87.4 ± 41.5	167.2 ± 65.5	133.3 ± 52.4[a]
PWC130 (watts/LM)	2.5 ± 0.8	2.2 ± 0.9	2.2 ± 0.7	2.1 ± 1.0	2.7 ± 1.0	2.3 ± 0.8
MVC (N:m)	226 ± 68	212 ± 78	186 ± 48	175 ± 47	279 ± 53	261 ± 85
MVC (N:m/leg LM)	26.1 ± 3.6	24.9 ± 5.5	26.3 ± 3.2	24.1 ± 5.2	25.8 ± 4.3	26.0 ± 5.9

All variables were measured and/or calculated except for age, which was based on participant self-report. CON, control; BMI, body mass index; LM, lean mass; MVC, maximal voluntary contraction; SD, standard deviation; T1D, type 1 diabetes. An unpaired, two-tailed Student's *t* test was used to compare variables between the control and type 1 diabetes groups in all the participants (combined) as well as within each respective sex (women and men, respectively). ***P<0.001 CON vs T1D. [a]Methodological issues with heart rate monitors caused a loss of data from 2 male participants with type 1 diabetes for this measurement (*n*=7 for T1D men).

Table 2 Characteristics specific to the type 1 diabetes participants

	Combined (*n* = 21)	Women (*n* =12)	Men (*n* = 9)
Disease duration (years)	28 (13)	**23 (13)**	**36 (11) ***
Age of diagnosis (years)	19 (10)	22 (11)	16 (8)
Total daily insulin dose (units/day)	47.0 (25.4)	38.5 (16.6)	59.2 (31.6)
Total daily insulin dose (units/kg/day)	0.56 (0.22)	0.49 (0.16)	0.67 (0.26)
Using CSII (n, %)	11 (52%)	6 (50%)	5 (56%)
Using MDI (n, %)	10 (48%)	6 (50%)	4 (44%)
Using CGM (n, %)	14 (67%)	8 (67%)	6 (67%)
Using FGM (n, %)	7 (33%)	4 (33%)	3 (33%)
Self-reported complications, high blood pressure (n, %)	3 (14%)	1 (8%)	2 (22%)
Self-reported complications, neuropathy (n, %)	2 (10%)	1 (8%)	1 (11%)
Self-reported complications, retinopathy (n, %)	1 (5%)	1 (8%)	0 (0%)

Characteristics above were based on participant self-report. CGM, continuous glucose monitoring; CSII, continuous subcutaneous insulin infusion; FGM, flash glucose monitoring; IQR, interquartile range; MDI, multiple daily injections; SD, standard deviation. An unpaired, two-tailed Student's *t* test was used to compare variables between women and men with type 1 diabetes. *P<0.05 women vs. men.

caffeine, alcohol, and moderate to vigorous exercise for at least 24 hours before each visit.

Visit 1. Body height and weight were recorded to calculate BMI while body composition was assessed via dual-energy X-ray absorptiometry (DEXA; GE Healthcare, Toronto, ON, Canada). Muscle strength was assessed by isometric maximal voluntary contraction (MVC) of the right leg (unless otherwise specified) using an isokinetic dynamometer (Biodex System 3; Biodex Medical Systems, Shirley, NY, USA). Participants were instructed to contract their quadriceps isometrically as hard as they could for 5 sec and to rest for 30 sec. This was repeated 3 times with the highest recorded value used. Aerobic fitness was assessed by the PWC_{130} cycling test. Participants were provided with a wrist-based heart rate (HR) watch (Polar M430) and instructed to cycle at a cadence between 50 and 70 revolutions min^{-1} and maintain it for 10 min. Baseline power was set at 25 watts and thereafter adjusted according to the watch HR data. The goal was to carefully adjust workload in order to steadily increase HR to 130 beats min^{-1} (bpm) by 8 min into the cycling test (with cadence being constant). For the remaining 2 min, power was slightly adjusted if HR began to exceed 135 bpm. Power and HR were recorded every 15 sec during the entire fitness test in order to generate a standard curve of power output (x-axis) versus HR (y-axis). The slope of the standard curve was used to calculate the power output at 130 bpm (i.e. PWC_{130}) and was also normalized to lean body mass (LBM) (Table 1).

Visit 2. A Bergström needle sample of *Vastus lateralis* muscle was obtained under local anaesthetic (2% lidocaine, 0.005 mg/ml epinephrine) [23]. A portion of the sample muscle (~15-20 mg) was immediately placed in ice-cold BIOPS [19] for subsequent *in vitro* assessment of mitochondrial respiratory function. Approximately 5 mg of muscle was placed in ice-cold 2% glutaraldehyde [buffered with 0.1 M sodium cacodylate (pH 7.4)] to preserve the muscle ultrastructure for transmission electron microscopy (TEM) analysis. A blood sample was obtained using venepuncture for HbA_{1c} analysis at the McMaster Core Facility laboratory.

Mitochondrial bioenergetics in permeabilized muscle fibres

The freshly obtained muscle sample in ice-cold BIOPS was divided into small muscle bundles (~1.5-5 mg wet weight) for the preparation of permeabilized muscle fibres (PmFBs), as previously described [24]. Following this, the

PmFBs were weighed and washed in buffer Z [in mmol/l: 105 K-MES, 30 KCl, 10 KH_2PO_4, 5 $MgCl_2 \cdot 6H_2O$, 1 EGTA and 5 mg ml^{-1} BSA (pH 7.1)] until measurements were initiated (<20 min).

Mitochondrial respiratory kinetics were assessed using the Oroboros Oxygraph-2K (Oroboros Instruments, Corp., Innsbruck, Austria) similar to our previous study [19]. The chambers were filled with 2 mL buffer Z supplemented with 20 mmol/l creatine [25] and 5 µmol/l blebbistatin, and experiments were undertaken at 37ºC with constant stirring at 750 rpm and mild hyperoxygenation (250-275 µmol/l O_2).

ADP-stimulated respiratory kinetics were determined through ADP (A5258; Sigma) titrations in the presence of 5 mmol/l pyruvate (P2256; Sigma), 2 mmol/l malate (M1000; Sigma) and 10 mmol/l succinate (S2378; Sigma) to recruit both complex I and complex II (C_{I+II}). Standard pyruvate and glutamate (GLU303; BioShop, Burlington, ON, Canada) titrations including 2 mmol/l malate and 5 mmol/l ADP, were performed to determine complex I kinetics ($C_{I\,pyr}$ and $C_{I\,glu}$, respectively). Complex II kinetics was determined through standard succinate titrations in the presence of 10 µmol/l rotenone (R8875; Sigma) and 5 mmol/l ADP ($C_{II\ succ}$). Mitochondrial LEAK respiration (state II) was measured in the presence of saturating complex I substrates (5 mmol/l pyruvate and 2 mmol/l malate) and in the absence of adenylates (i.e., ADP). In doing so, any change in respiration is largely considered to be the result of increased/decreased coupling of OXPHOS to ATP synthesis due to proton re-entry into the matrix through either uncoupling mechanisms or H^+-dependent transporters that have been altered [26]. In other words, herein, mitochondrial LEAK respiration refers to the *in vitro* measure of mitochondria respiration compensating for proton leak across the inner mitochondrial membrane [27]. Integrity of the mitochondria (outer mitochondrial member) was verified by the addition of 10 µmol/l cytochrome *c* (cat no. 192-10; Lee Biosolutions, Maryland Heights, MO, USA). Any experiments that had a >10% increase in respiration after the addition of cytochrome *c* were excluded.

All respirometry data were normalised per mg wet weight (from here on, referred to as mass-specific mitochondrial respiration); however, this does not discern whether the function per mitochondria has changed or whether mitochondrial content per mg wet weight has changed. Thus, we further normalised each participant's respiration rates to their own unique mitochondrial content (area density, discussed below). Importantly, since mitochondrial content was determined in a subset of participants (n = 18/17 for control and type 1 diabetes, respectively), the intrinsic mitochondrial respiration data does not include all participants in the study, only those we could normalise to (from here on, referred to as intrinsic mitochondrial respiration).

Transmission electron microscopy

TEM Facility staff at McMaster University Medical Centre processed the fresh muscle samples fixed in 2% glutaraldehyde, as described previously [28]. Mitochondria area density, size, number per muscle area, and cristae (pixel) density were quantified in a subset of control (n = 18) and type 1 diabetes (n = 17) participants by Nikon Imaging Software (NIS)-Elements AR (v4.6; Melville, NY, USA) using representative micrographs, as previously described in detail [19].

Citrate synthase assay

The maximal activities of citrate synthase (CS) were determined as previously described [29]. In brief, muscle homogenate was added to a cuvette containing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (D1830; Sigma-Aldrich), acetyl CoA (A2181; Sigma-Aldrich), Tris-HCl buffer (pH 8.1) and incubated at 37 °C. Oxaloacetate (O4126; Sigma-Aldrich) was then added to initiate the reaction. Absorbance was measured continuously at 412 nm for 2 min at 37 °C using a UV-Vis spectrophotometer (Cary Bio-300; Varian Inc., Palo Alto, Calif., USA), and the slope between 30 s and 90 s was used for calculations. Enzyme activity was measured in duplicate and CS maximal activity (expressed as nmol min^{-1}·mg) was expressed relative to total protein measured with a BCA Assay Kit (Pierce, Rockford, IL, USA).

Statistics

Statistical analyses were performed using GraphPad Prism 8 software. All data were initially tested for normal distribution (D'Agostino-Pearson omnibus test) and equal group variance (Brown-Forsythe variance test). The specific analyses used are defined in the figure legends. Statistical significance was established at $p \leq 0.05$.

RESULTS

At study initiation, a design to interrogate sex differences was not considered. However, recent work demonstrating sexual dimorphism in human skeletal muscle mitochondrial bioenergetics [30–32] made it critical to present control versus type 1 diabetes data as follows: combined (all participants irrespective of sex); women (females only); and men (males only).

Skeletal muscle mitochondria ultrastructure

Mean mitochondrial area density was significantly lower (p = 0.006) in the type 1 diabetes group (-27%; Fig. 1b) versus controls. Women with type 1 diabetes demonstrated an ~20% decrease (p=0.110) in density while men with type 1 diabetes demonstrated an ~35% decrease (p=0.033) (Fig. 1b). In women, this was largely due to a decrease (p=0.057) in mitochondrial fragment size (-18%; Fig. 1c) and not mitochondrial fragment number per muscle area (-9%, p=0.141) (Fig. 1d). In men, lower mitochondrial density was largely due to

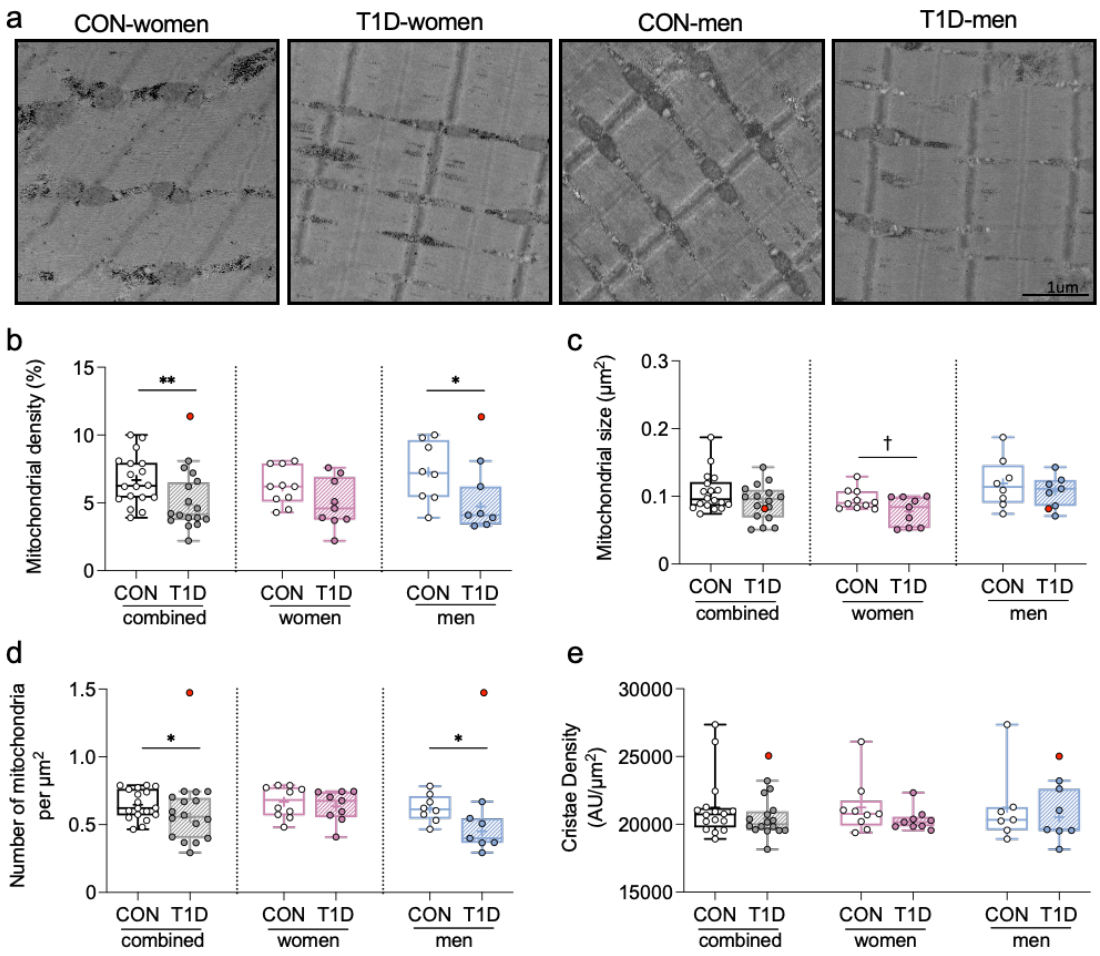

Figure 5.1 Quantification of mitochondria content and characteristics using representative micrographs captured by TEM.

Since TEM provides 2-dimensional images, mitochondrial content was determined by calculating the percentage mitochondrial area "density" (mean mitochondrial size x number of mitochondrial fragments per tissue area, expressed as % area of muscle [Tarnopolsky 2007]) in a subset of participants with type 1 diabetes (n = 16) and without type 1 diabetes (n = 18). Mitochondrial cristae density was also determined by calculating pixel density of each mitochondrion whereby a lower pixel density is indicative of swollen mitochondria or reduced cristae surface area. (a) Representative micrographs, Scale bar, 1 µm. (b) Mitochondrial density was significantly lower (**p = 0.006) in combined type 1 diabetes versus control. Compared to controls, women with type 1 diabetes had observably lower (p = 0.110) density while men with type 1 diabetes had significantly lower (*p = 0.033) density (c). Compared to controls, individual mitochondrion size was observably lower (†p = 0.056) in women but not men with type 1 diabetes. (d) The number of mitochondrial fragments per unit area was significantly lower in combined (*p = 0.048) and men (*p = 0.014), but not women (p = 0.549), with type 1 diabetes. (e) Mitochondrial cristae density was not statistically different in type 1 diabetes. The data was analysed using an Unpaired Student's t test (two-tailed). One male participant with type 1 diabetes was a significant outlier using the ROUT test in Graphpad prism, depicted as a red circle, and was removed from all statistical analyses in this figure. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. AU, arbitrary units; CON, control; T1D, type 1 diabetes.

decreased (p=0.130) mitochondrial fragment number per muscle area (-28%; Fig. 1d) compared to mitochondrial size (-9%, p=0.380) (Fig. 1c). Mitochondrial cristae density (measured by pixel density) was not different between cohorts irrespective of sex (Fig. 1e).

Skeletal muscle mitochondrial OXPHOS capacity

Citrate Synthase Activity. No difference was found in CS activity between cohorts in a subset of participants (n = 14) [combined (-18%, p=0.265), women (-17%, p=0.088), men (+14%, p>0.999)] suggesting that TCA cycle was not impaired in this cohort (Fig. 2a).

Complex I and II supported respiration. Mitochondrial C_{I+II} OXPHOS capacity was comparable between cohorts for both mass-specific (-11%, p=0.165) and intrinsic (+11%, p=0.217) respiration (Fig. 2b,c). Separated by sex, mass-specific C_{I+II} OXPHOS capacity was significantly lower in women (-24%, p=0.033) but not men with type 1 diabetes (+5%, p=0.397) (Fig. 2b,c). In contrast, intrinsic C_{I+II} OXPHOS capacity was not different in women with type 1 diabetes (+5%, p=0.378) while in men it was 25% non-significantly higher (p=0.163) (Fig. 2b,c).

Complex I supported respiration. To determine whether alterations exist at the level of complex I or upstream dehydrogenases, specifically PDH (pyruvate dehydrogenase) and GDH (glutamate dehydrogenase), $C_{I\ pyr}$ and $C_{I\ glu}$ OXPHOS capacity was also measured as both substrates generate NADH that supports complex I activity. No differences were found between groups with mass-specific $C_{I\ pyr}$ OXPHOS capacity [combined (-8%; p=0.572), women (-14%; p=0.404), men (+1%; p=0.970;)] or $C_{I\ glu}$ OXPHOS capacity between groups [combined (-3%; p=0.853), women (-12%; p=0.404)] except in men with type 1 diabetes where a 42% non-significant increase was measured (p=0.218) (Fig. 3a,c).

When normalised to mitochondria content, $C_{I\ pyr}$ and $C_{I\ glu}$ OXPHOS capacity were non-significantly higher (both +27%, p>0.201) in type 1 diabetes (Fig. 3b,d). When separated by sex, we observed a non-significant 50% increase (p=0.159) in intrinsic $C_{I\ pyr}$ OXPHOS capacity and a significant 88% increase (p=0.033) in intrinsic $C_{I\ glu}$ OXPHOS capacity in men with type 1 diabetes only (Fig. 3b,d).

Complex II supported respiration. No differences were found in mass-specific $C_{II\ succ}$ OXPHOS capacity between groups [combined (-6%; p=0.502), women (-9%; p=0.475), men (-3%; p=0.868)] (Fig. 3e). Normalised to mitochondria content, $C_{II\ succ}$ OXPHOS capacity was 25% higher in the type 1 diabetes cohort (p=0.058) (Fig. 3f). However, both women and men with type 1 diabetes demonstrated a non-significant 23% (p=0.268) and 28% (p=0.123) increase, respectively, in intrinsic $C_{II\ succ}$ OXPHOS capacity (Fig. 3f).

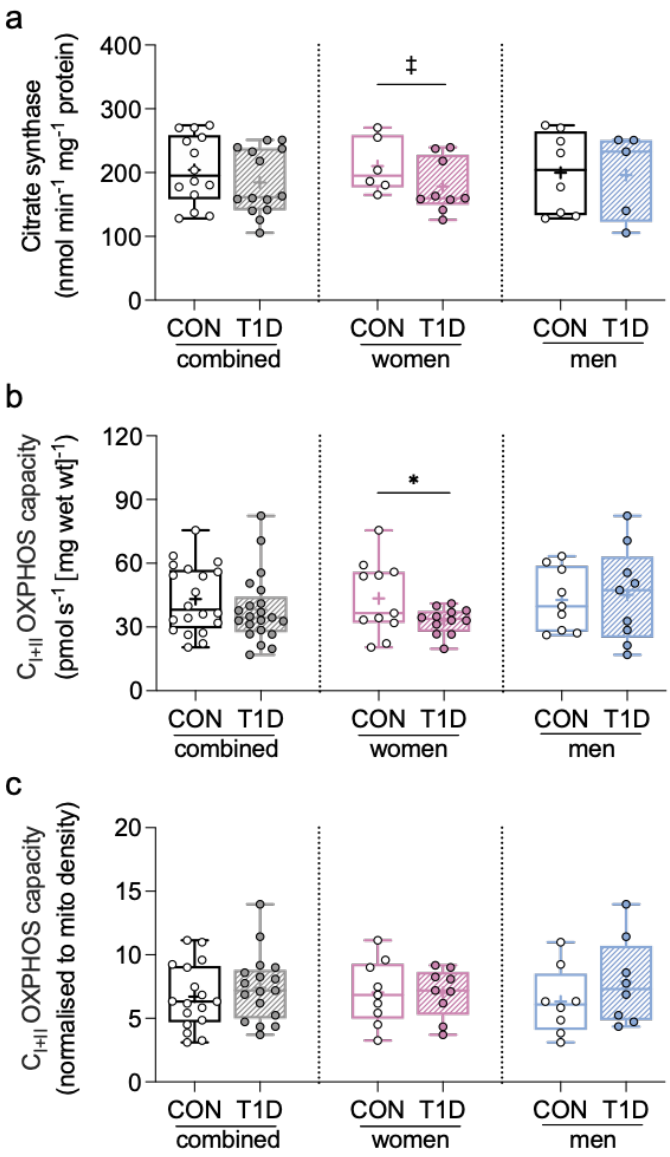

Figure 5.2 Skeletal muscle mitochondrial CS activity and oxidative phosphorylation (OXPHOS) capacity supported by saturating complex I and complex II substrates (C_{I+II} OXPHOS capacity).

(a) CS activity was observably lower (‡p = 0.088) in women with T1D only. Respiration assays were performed in PmFBs, supported by saturating complex I (pyruvate + malate) and complex II (succinate) substrates, and normalised by initial muscle wet weight (pmol s-1 [mg wet weight]-1) and by mitochondrial area density – i.e. intrinsic mitochondria respiration. (b) Mass-specific C_{I+II} OXPHOS capacity was significantly lower (*p = 0.042) in women, but not men, with type 1 diabetes compared to controls. (c) Intrinsic C_{I+II} OXPHOS capacity was not different between cohorts irrespective of sex. A Student's unpaired t test (one-tailed) was used to analyse the data. One control female participant was not included in the analyses due to methodological issues. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. CON, control; T1D, type 1 diabetes; wt, weight.

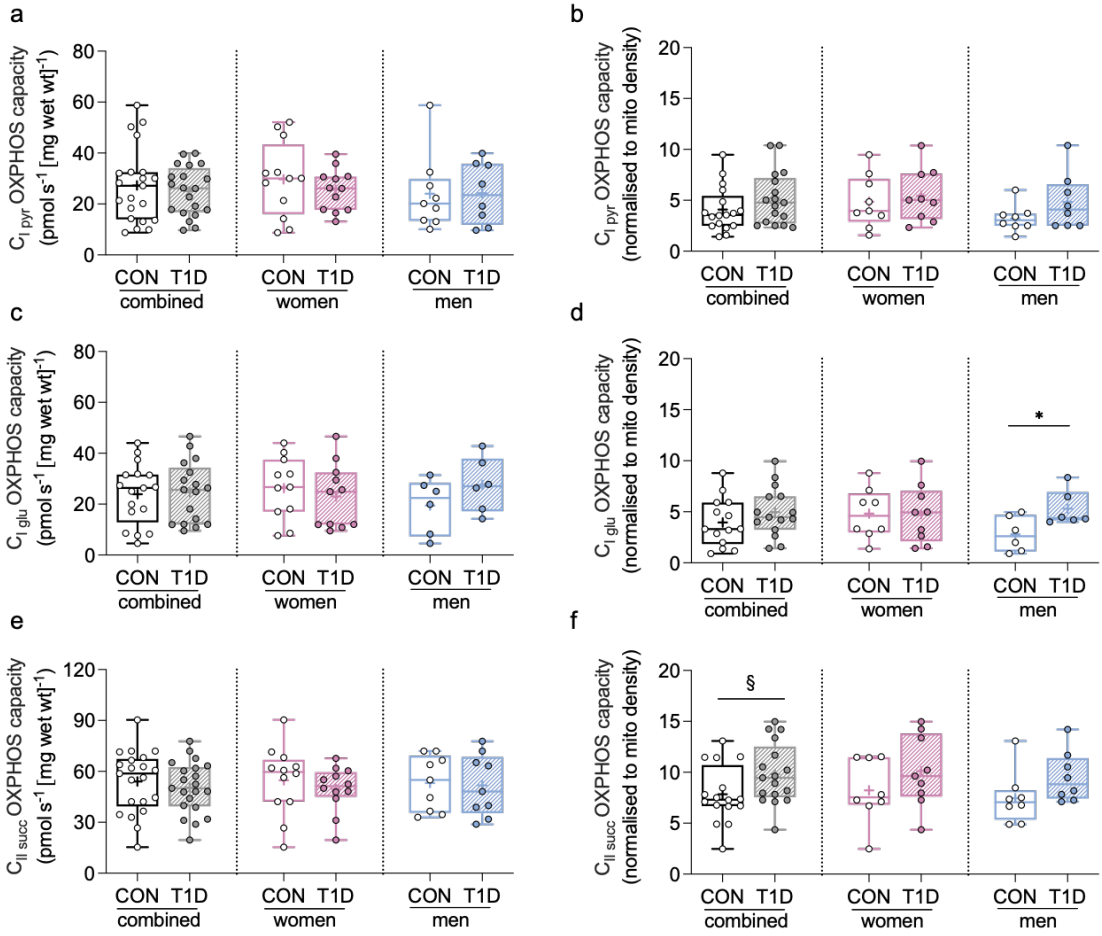

Figure 5.3 Skeletal muscle OXPHOS capacity of complex I supported by pyruvate ($C_{I\ pyr}$) or glutamate ($C_{I\ glu}$) and complex II supported by succinate ($C_{II\ succ}$) in PmFBs.

Respiration assays were normalised by the initial muscle wet weight (pmol s-1 [mg wet weight]-1) or by mitochondrial area density (i.e. intrinsic mitochondria function), depicted on the left and right, respectively. Neither (a) mass-specific nor (b) intrinsic CI pyr OXPHOS capacity was different between cohorts. One male participant with type 1 diabetes was not included in the analyses due to methodological issues. While (c) mass-specific CI glu OXPHOS capacity was observably higher in men with type 1 diabetes compared to control men ($p = 0.220$), only (d) intrinsic CI glu OXPHOS capacity was significantly higher in men with T1D (*$p = 0.033$). As a result of methodological issues, data was not obtained for n = 4 control (1 women, 3 men) and n = 4 type 1 diabetes (1 women, 3 men) participants. Only (f) Intrinsic CII succ OXPHOS capacity was observably higher (§$p = 0.057$) in the type 1 diabetes cohort. A Student's unpaired t test (two-tailed) was used to analyse all the data. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. CON, control; T1D, type 1 diabetes; wt, weight.

Skeletal muscle mitochondrial respiratory response to low substrate concentrations

ADP. The ability of mitochondria to respond to changing energy demands (i.e., range of sub-saturating ADP concentrations) was interrogated. ADP was titrated at increasing physiological concentrations considered to be representative of conditions of sedentary/low physical activity to moderate/high physical activity and beyond. Collectively, mass-specific submaximal ADP-stimulated respiration was similar (p=0.230) between cohorts (Fig. 4a). When separated by sex, women with type 1 diabetes demonstrated a reduction in ADP-stimulated respiration (main effect p<0.001) while men were non-significantly higher (main effect p=0.095) (Fig. 4c,e). In contrast, intrinsic submaximal ADP-stimulated respiration was significantly higher (main effect p=0.002) in type 1 diabetes (Fig. 4b), however no statistical difference was found in women (main effect p=0.474) whereas a significant increase was found in men (main effect p=0.001) (Fig. 4d,f).

Pyruvate and glutamate. We also interrogated the response of complex I and/or upstream dehydrogenases to low (i.e., sub-saturating) concentrations of pyruvate or glutamate. No differences were found between groups for mass-specific submaximal pyruvate-supported respiration [combined (-8%; p=0.258), women (-12%; p=0.545), men (-2%; p=0.894)] (Fig. 5a,c,e) or submaximal glutamate-supported respiration [combined (+9%; p=0.232), women (-0.7%; p=0.956), men (+30%; p=0.197)] (Fig. 6a,c,e). In contrast, intrinsic pyruvate and glutamate submaximal respiration were both significantly increased (main effect p = 0.003 and p = 0.030, respectively) in the type 1 diabetes group cohort (Fig. 5b and Fig. 6b). Only men with type 1 diabetes showed a significant increase in both pyruvate- (main effect p=0.005) and glutamate-supported (main effect p=0.012) respiration (Fig. 5f and Fig. 6f).

Succinate. The response of complex II to low concentrations of succinate was also interrogated. No significant differences were found in mass-specific submaximal succinate-supported respiration between groups [combined (-6%; p=0.335), women (-2%; p=0.813), men (+17%; p=0.149)] (Fig. 7a,c,e). In contrast, intrinsic submaximal succinate-supported respiration was significantly increased in the type 1 diabetes group (main effect p<0.001), and this was consistent in both women and men (main effect p=0.003 and p<0.001, respectively) (Fig. 7b,d,f).

Skeletal muscle mitochondrial coupling

Collectively, mitochondrial LEAK respiration was 40% higher (p=0.007) in type 1 diabetes compared to controls (Fig. 8a). When separated by sex, LEAK respiration was not different in women with type 1 diabetes (+8%; p=0.608) while in men, LEAK respiration was 82% greater (p=0.002) (Fig. 8b,c) compared to controls.

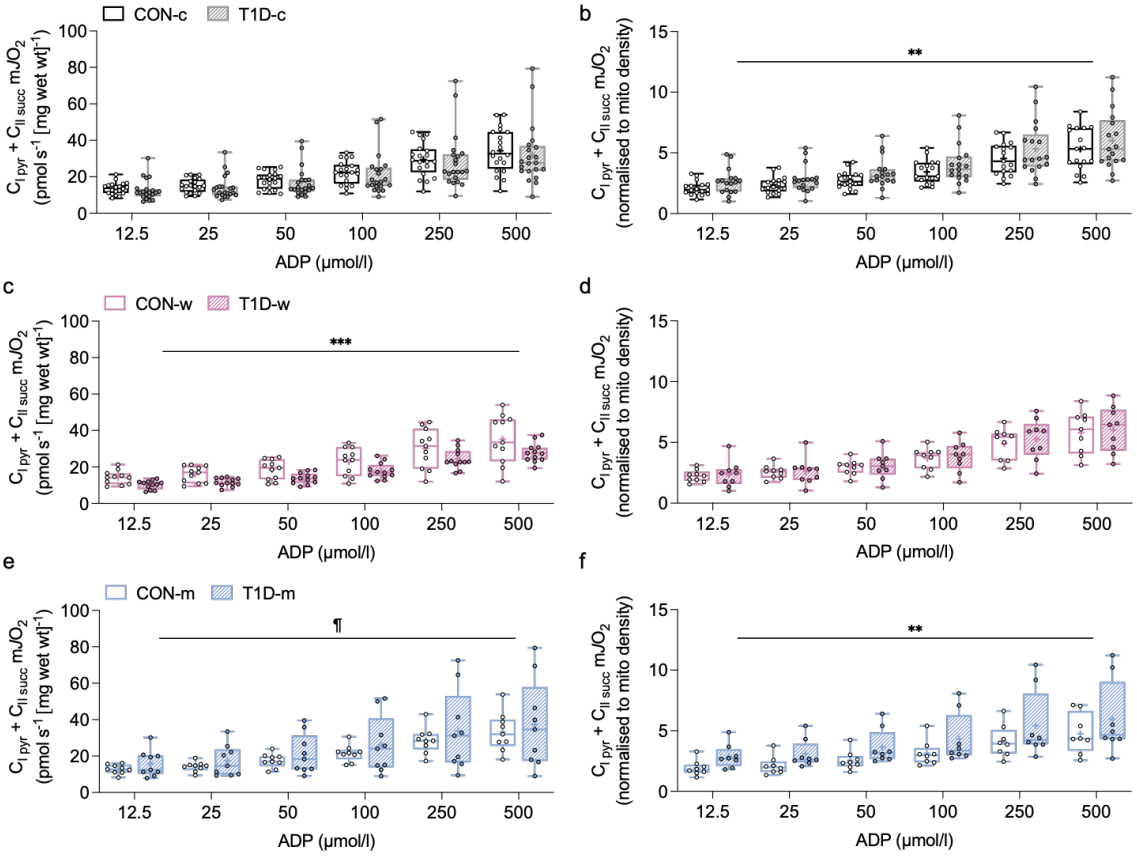

Figure 5.4 Complex I and complex II-supported mitochondrial respiration stimulated by subsaturating ADP concentrations (CI pyr + CII succ mJO2) in PmFBs.

Respiration assays were normalised by initial muscle wet weight (pmol s-1 [mg wet weight]-1) and by mitochondrial area density (i.e. intrinsic mitochondria function) depicted on the left and right, respectively. Compared to controls, mass-specific ADP-stimulated respiration showed a main effect for lower rates (***p < 0.001) in type 1 diabetes for women (c) and observably higher rates (¶p = 0.095) in men with type 1 diabetes (e). However, intrinsic ADP-stimulated respiration showed similar rates in type 1 diabetes for women (d) compared to controls, whereas a main effect for higher respiration rates were found in type 1 diabetes for combined (**p = 0.002) and men (**p = 0.001) (b and f, respectively). A two-way ANOVA was performed to determine a main effect of disease state. One control female participant with type 1 diabetes was not included in the analyses due to methodological issues. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. c, combined; CON, control; m, men; T1D, type 1 diabetes; w, women; wt, weight

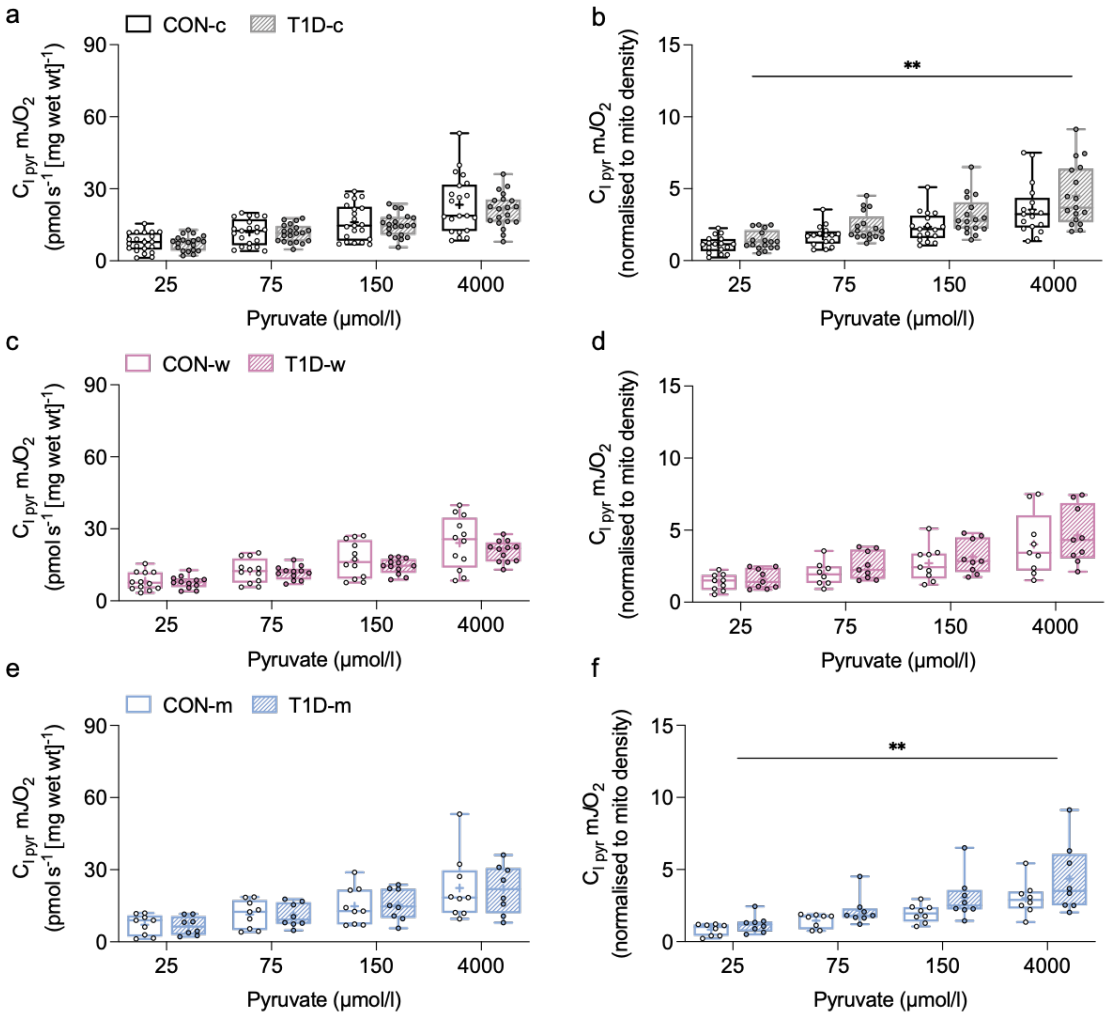

Figure 5.5 Mitochondrial respiration stimulated by subsaturating pyruvate concentrations ($C_{l\ pyr}$ mJO_2) in PmFBs.

Respiration assays were normalised by initial muscle wet weight (pmol s-1 [mg wet weight]-1) and by mitochondrial area density (i.e. intrinsic mitochondria function) depicted on the left and right, respectively. No statistical differences were found ($p > 0.050$) in mass-specific mitochondrial respiration between cohorts (a, c, and e). Intrinsic pyruvate-stimulated respiration showed a main effect for higher rates in type 1 diabetes for combined (**$p = 0.003$) and men (**$p = 0.005$) but not women ($p = 0.118$) (b, d, and f). A two-way ANOVA was performed to determine a main effect of disease state. One control female participant and one male participant with type 1 diabetes were not included in the analyses due to methodological issues. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. c, combined; CON, control; m, men; T1D, type 1 diabetes; w, women; wt, weight.

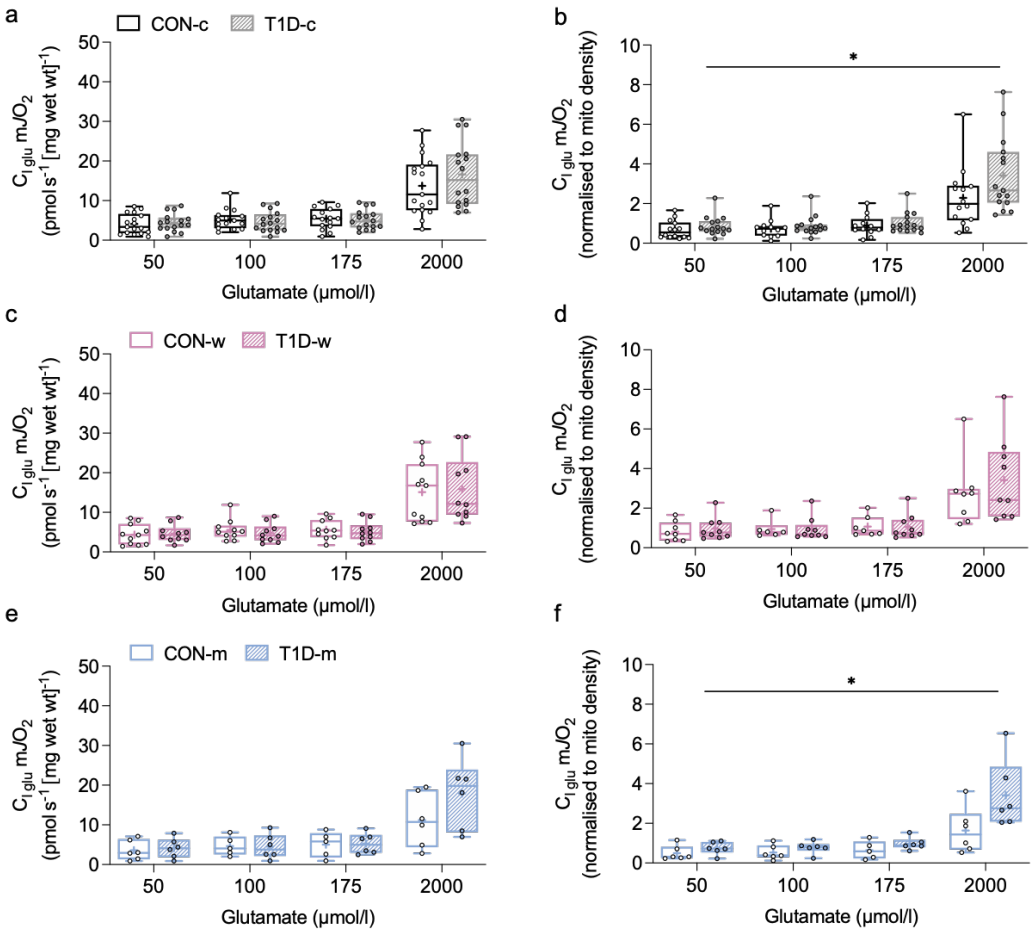

Figure 5.6 Mitochondrial respiration stimulated by subsaturating glutamate concentrations ($C_{l\,glu}$ mJO_2) in PmFBs.

Respiration assays were normalised by initial muscle wet weight (pmol s-1 [mg wet weight]-1) and by mitochondrial area density (i.e. intrinsic mitochondria function) depicted on the left and right, respectively. No statistical differences ($p > 0.050$) were found in mass-specific mitochondrial respiration between cohorts (a, c, and e). Compared to controls, intrinsic glutamate-stimulated respiration showed a main effect for higher rates in type 1 diabetes for combined (*$p = 0.030$) and men (*$p=0.012$) but not women ($p = 0.438$) (b, d, and f). A two-way ANOVA was performed to determine a main effect of disease state. As a result of methodological issues, data was not obtained in 4 control participants (1 women, 3 men) and 4 participants with type 1 diabetes (1 women, 3 men). Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. c, combined; CON, control; m, men; T1D, type 1 diabetes; w, women; wt, weight.

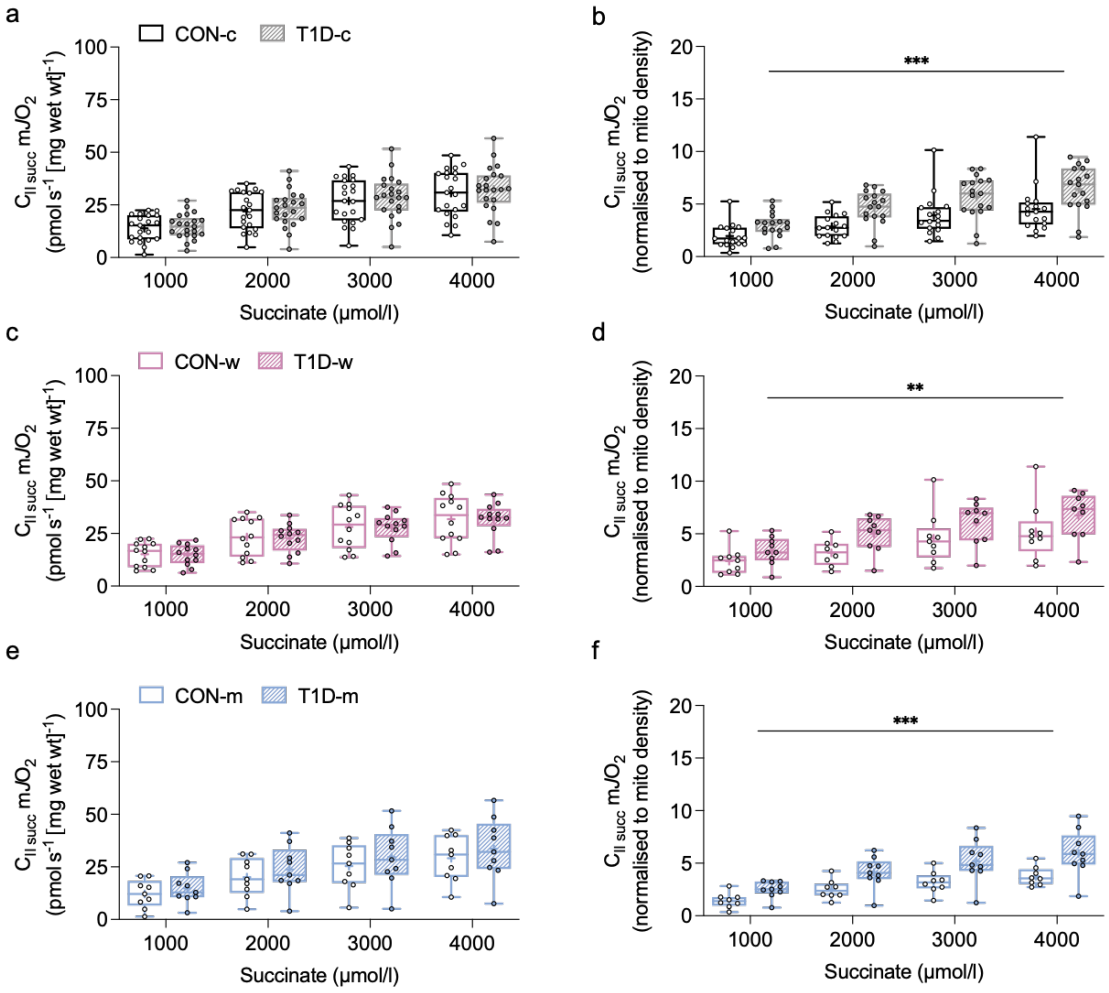

Figure 5.7 Mitochondrial respiration stimulated by subsaturating succinate concentrations ($C_{II\ succ}$ mJO_2) in PmFBs.

Respiration assays were normalised by initial muscle wet weight (pmol s-1 [mg wet weight]-1) and by mitochondrial area density (i.e. intrinsic mitochondria function) depicted on the left and right, respectively. No statistical differences ($p > 0.050$) were found in mass-specific mitochondrial respiration between cohorts (a, c, and e). Compared to controls, intrinsic succinate-stimulated respiration showed a main effect for higher rates in type 1 diabetes for combined (***$p < 0.001$), women (**$p = 0.003$), and men (***$p < 0.001$) (b, d, and f). A two-way ANOVA was performed to determine a main effect of disease state. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. c, combined; CON, control; m, men; T1D, type 1 diabetes; w, women; wt, weight.

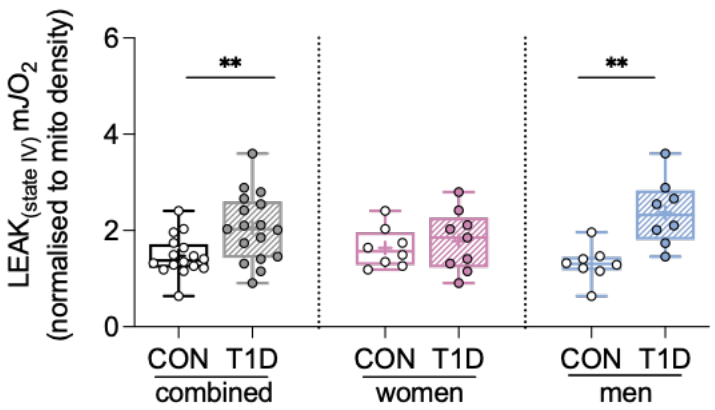

Figure 5.8 Intrinsic mitochondrial LEAK (state II) respiration.

Mitochondrial respiration was measured in the absence of ADP but presence of saturating complex I substrates (pyruvate+malate) to drive (maximize) proton leak-dependent respiration (i.e., absence of adenylates). Compared to controls, LEAK respiration (i.e. state II) was significantly higher with type 1 diabetes for combined (**p = 0.007) and men (**p = 0.002) but not women (p = 0.408). A Student's unpaired t test (two-tailed) was used to analyse the data. One control female participant was not included in the analyses due to methodological issues. Boxes represent the interquartile range, whiskers show the maximum and minimum, horizontal solid line indicates the median, and the cross indicates the mean. CON, control; T1D, type 1 diabetes; wt, weight.

Correlation Analyses

Figure 9 contains a customised heat map summarizing the correlational analyses performed in the type 1 diabetes cohort, separated into women (Fig. 9a) and men (Fig. 9b), between metrics that we considered clinically-relevant and the mitochondrial respiratory measures undertaken.

DISCUSSION

This is the first study to interrogate mitochondrial respiratory function and content in muscle biopsies of adults with uncomplicated type 1 diabetes with a wide age span (30-72 years old) in comparison to strictly-matched controls (age, sex, BMI, and self-reported physical activity levels). The success in our matching strategy was supported by the comparable LBM, muscle strength, and calculated aerobic fitness between cohorts. Despite the similarities in these clinically-relevant metrics, mitochondrial OXPHOS capacity, mitochondrial respiratory response to low ("physiological") substrate concentrations, and ultrastructure were altered with type 1 diabetes and demonstrated sexual dimorphism. Specifically, at the whole-muscle level, we found mitochondrial OXPHOS capacity and submaximal ADP respiration were significantly lower in women, but not men, with type 1 diabetes compared to controls. We also found mitochondrial density to be significantly lower in women and men with type 1 diabetes; with decreased mitochondrial fragment size in women and reduced number of mitochondrial fragments in men being the primary drivers of the decreased density. When respiration rates were normalised to mitochondrial density, the mitochondrial respiratory deficiencies in women with type 1 diabetes disappeared, whereas in men with type 1 diabetes, we found significant increases in mitochondrial OXPHOS capacity and mitochondrial respiratory rates in response to low concentrations of either ADP, pyruvate, glutamate or succinate, indicating normal and enhanced intrinsic mitochondrial respiratory function between sexes, respectively. Altogether, these novel findings reveal differential adaptations of muscle mitochondria to type 1 diabetes in middle- to older-aged women and men without overt diabetes complications.

Previous work from our group and others have reported decrements in muscle mitochondrial respiration in young people with type 1 diabetes. This includes attenuations in *in vivo* and *in vitro* muscle oxidative capacity [19, 30, 33–35], decreased submaximal ADP-stimulated respiration [19, 30], decreased complex II submaximal and maximal respiration [19, 30], and decreased complex IV activity [22]. However, these findings are not consistent across all studies, as some groups did not observe differences in mitochondrial oxidative OXPHOS capacity [20, 22] and complex II function [22], with only deficiencies in complex IV function reported [22]. These *in vitro* studies also

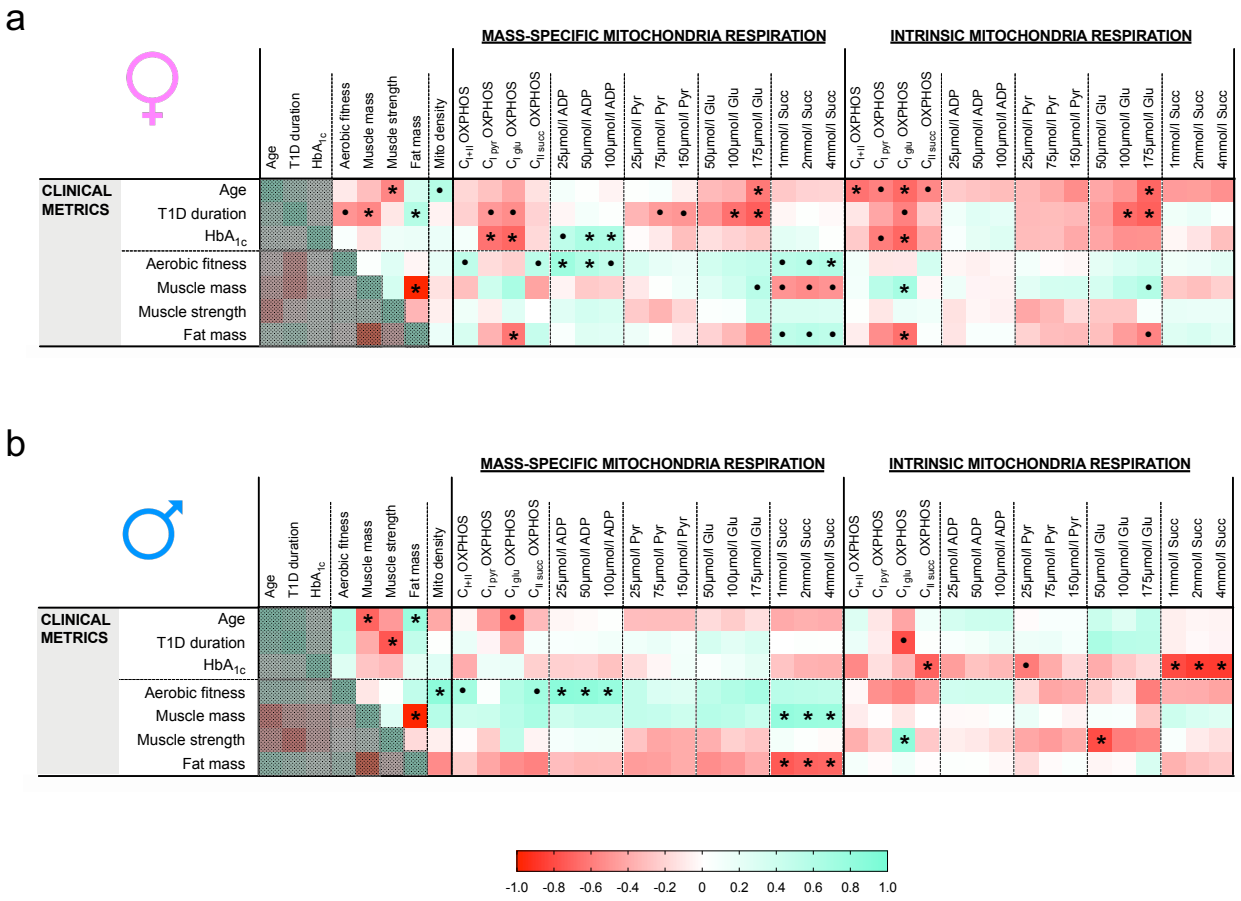

Figure 5.9 Heat map of correlations between mitochondrial measures performed and metrics of clinical relevance in women (a) and men (b) with type 1 diabetes.

The heat map shows the computed Pearson r correlations using GraphPad prism. Positive correlations are given in blue/green shade, and negative correlations in red shade, with intensity indicating the magnitude of the correlation. *p ≤ 0.050 and ●p ≤ 0.120. Aerobic fitness, muscle mass, muscle strength, and fat mass refer to PWC_{130}, % LBM, MVC, and % FM, respectively. Glu, glutamate; Pyr, pyruvate; Succ, succinate; T1D, type 1 diabetes. Refer to previous figures for respective n sizes.

measured markers of mitochondrial content and reported no differences in either mitochondrial area density [19], protein expression of complexes I to V [19, 30], or CS activity [22], indicating intrinsic mitochondrial respiratory deficiencies occur early in the progression of type 1 diabetes in human muscle. While informative, the bulk of studies to date have studied adolescents and young adults, making it unclear if older adults with type 1 diabetes exhibit similar or worsened muscle mitochondria dysfunction. In this study of older adults with type 1 diabetes that are relatively healthy, we found normal CS activity and normal or enhanced intrinsic mitochondrial respiratory function, which was also observably different between sexes. Women with type 1 diabetes had normal intrinsic mitochondrial C_{I+II} OXPHOS capacity, submaximal ADP respiration, and $C_{I\ pyr}$ and $C_{I\ glu}$ submaximal respiration, whereas intrinsic $C_{II\ succ}$ submaximal respiration was significantly higher. In contrast, men with type 1 diabetes tended to have increased intrinsic mitochondrial C_{I+II} OXPHOS capacity, significantly greater $C_{I\ glu}$ OXPHOS capacity, increased mitochondrial ADP submaximal respiration and increased $C_{I\ pyr}$, $C_{I\ glu}$, and $C_{II\ succ}$ submaximal respiration, respectively. While these novel findings contradicted our working hypothesis [17] and the current literature available in young adults with type 1 diabetes, they provide for the first-time invaluable insight and evidence that muscle health, as it pertains to mitochondria function, can generally be preserved in adults with type 1 diabetes with increasing age and disease duration.

Enhanced intrinsic mitochondrial respiratory function has been previously reported in type 2 diabetes muscle in spite of many studies suggesting normal or dysfunctional mitochondria in adults with type 2 diabetes. Specifically, Larsen and colleagues found higher intrinsic mitochondrial substrate sensitivity to glutamate and succinate in biopsies from middle-aged adults with type 2 diabetes compared to age- and BMI-matched controls [36]. While the mechanisms remain to be fully delineated, some studies suggest this is a compensatory effect and/or adaptation during the progression of diabetes [36, 37]. Interestingly, in the streptozotocin (STZ)-induced type 1 diabetes rodent model, Larsen and colleagues also found intrinsic mitochondrial OXPHOS capacity to be elevated in soleus, and to a varying degree in plantaris, and this occurred in parallel with reduced mitochondrial content [38], similar to our present work. Since insulin resistance is also a feature of type 1 diabetes, the enhanced intrinsic mitochondrial response to physiological substrate concentrations and/or capacity can be a compensatory mechanism for decreased glucose flux into the cell because of insulin resistance. However, this is strictly speculative at this time. We did measure the protein expression of mitochondrial pyruvate carrier 1 and 2 (MPC1, MPC2) in a subset of participants but found no statistical difference between groups (ESM Fig. 1).

Another potential explanation for the sexual dimorphism and altered mitochondrial respiratory function in type 1 diabetes is fibre type differences. While muscle fibre type was not measured, we have preliminary data that (compared to controls) women with type 1 diabetes display a greater proportion of type-IIX and type-I fibres, while men exhibit more type IIX and reduced type I fibres. Both men and women with type 1 diabetes have less type-IIA fibres. As type-II fibres have greater substrate sensitivity than type-I fibres, it may be that the differential fibre type expression between men and women with type 1 diabetes explain, in part, the sex differences in mitochondrial respiratory function observed herein.

Interestingly, we did not find significant differences in mitochondrial respiratory function and ultrastructure between pre and postmenopausal women irrespective of type 1 diabetes (ESM Fig. 2). Altogether, if we compare these study findings to our previous work in younger cohorts with type 1 diabetes [19], we propose that in otherwise healthy women and men with uncomplicated type 1 diabetes entering their 4th decade of life and beyond, intrinsic mitochondrial respiratory function does not worsen, but rather, differentially adapts to the type 1 diabetes environment to preserve mitochondrial health, and ultimately, muscle metabolic health. Indeed, this a significant stray from our initial hypothesis of accelerated muscle aging in those with type 1 diabetes [17], but if we consider the complexity and heterogeneity of type 1 diabetes, what remains is clear is that there is significant research needed to be done in the area, beginning with larger cohort studies in both sexes.

In the present study, there was the collective ~27% decrease in mitochondrial content (i.e., area density) in the type 1 diabetes cohort. This in contrast to previous work and is intriguing considering that our participants were strictly-matched, had comparable physical activity levels, and scored similarly on the aerobic fitness test. Indeed, it's important to recognize that PWC_{130} is not a direct measure of maximal aerobic capacity (VO_2max), but rather submaximal aerobic capacity. Thus, we cannot negate the possibility that our type 1 diabetes cohort had a lower maximal aerobic capacity compared to matched controls, and a lower mitochondrial content as a result. On the other hand, a similar aerobic capacity between those with/without type 1 diabetes, as is suggested by our data, would indicate deficits in the mechanisms regulating skeletal muscle adaptations to increased physical activity/exercise in type 1 diabetes (i.e., mitochondrial biogenesis/degradation/quality control). In STZ rodent models, but not type 2 or insulin-resistant models, a decrease in PGC-1a expression, and consequently reduced transcription of mitochondrial genes, has been reported [39]. Insulin deprivation in adults with type 1 diabetes (and thus hyperglycaemia) reduces rates of mitochondrial ATP production and the expression of OXPHOS genes [40]. A

recent study in STZ-induced diabetes also found reductions in OXPHOS capacity (but not intrinsic OXPHOS capacity), and reduced protein expression of PGC1a and proteins of the ETC and substrate transport (VDAC, ANT) with acute insulin deprivation [41]. Interestingly, we did not find differences in ANT1 or ANT2 protein expression between sexes in a subset of participants with type 1 diabetes (ESM Fig. 1). Thus, while speculative, perhaps after more than two decades of exogenous insulin treatment concurrent with muscle insulin resistance, mitochondria undergo numerous adaptations to ensure adequate redox balance and ATP supply, similar to what has been previously suggested in type 2 diabetes [36–38].

PERSPECTIVES AND CONCLUSIONS

Advances for type 1 diabetes care, while not a cure, are allowing those with this chronic disease to live consistently longer than ever before. However, the literature indicates that persons with diabetes are more likely to suffer from fatigue, weakness, and frailty with increasing age compared to controls [51]. Given deficits in muscle mitochondria are well known to trigger catabolic signalling cascades and promote atrophy and loss of muscle strength [44], the present findings provide, for the first time, evidence that alterations to mitochondrial respiratory function and ultrastructure are occurring in middle-to-older aged adults with type 1 diabetes. Our findings also indicate that the mitochondrial changes may be an adaptation/compensation to the pathological consequences of type 1 diabetes (i.e., dysglycemia and hyper/hypoinsulinemia) and/or the loss of mitochondrial content. It is important to note that the participants recruited were active (recreationally to very active), mostly resided in a university-dwelling community, had at least a post-secondary education, and, if had type 1 diabetes, were well controlled and knowledgeable on the importance of a healthy lifestyle for managing their disease. Thus, the results of this study should be restricted to these demographics and support the dire need for further research into other demographics with type 1 diabetes, including those who have poor glycaemic management (~83% of youth and ~79% of adults with type 1 diabetes [45]), are sedentary, and/or have unhealthy body weight (~60% of adults with type 1 diabetes are overweight/obese [46]).

Taken together, as we continue to uncover how muscle adapts to the type 1 diabetes environment in humans, what remains clear is that women and men are differentially impacted at the cellular level, and future studies must not only consider sex in the treatment and management of type 1 diabetes, but also the importance of regular exercise/physical activity to maintain skeletal muscle metabolic health across the lifespan.

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Duality of interest

None to report.

Data Availability

All relevant data are included in this article.

Contribution statement

CMFM, AGD, and TJH conceived and designed the experiments. CGRP, VL and MAT contributed to the conception of experiments. CMFM wrote the manuscript. CMFM performed all experiments with assistance from IAR and JPN. CMFM, AGD, MN, IAR, JPN and LVT analysed data. CMFM, CGRP, and TJH interpreted data. MAT performed muscle biopsies and venous blood draw. CMFM, AGD, and LVT assisted with muscle and blood sample collection and processing. All authors edited the manuscript and provided final approval of the version to be published. TJH is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of data and the accuracy of the data analysis.

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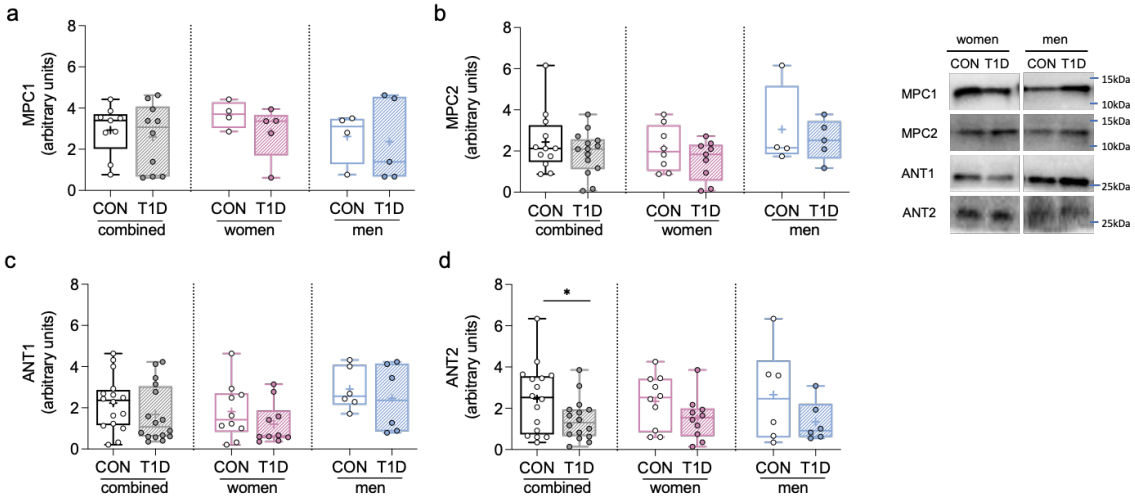
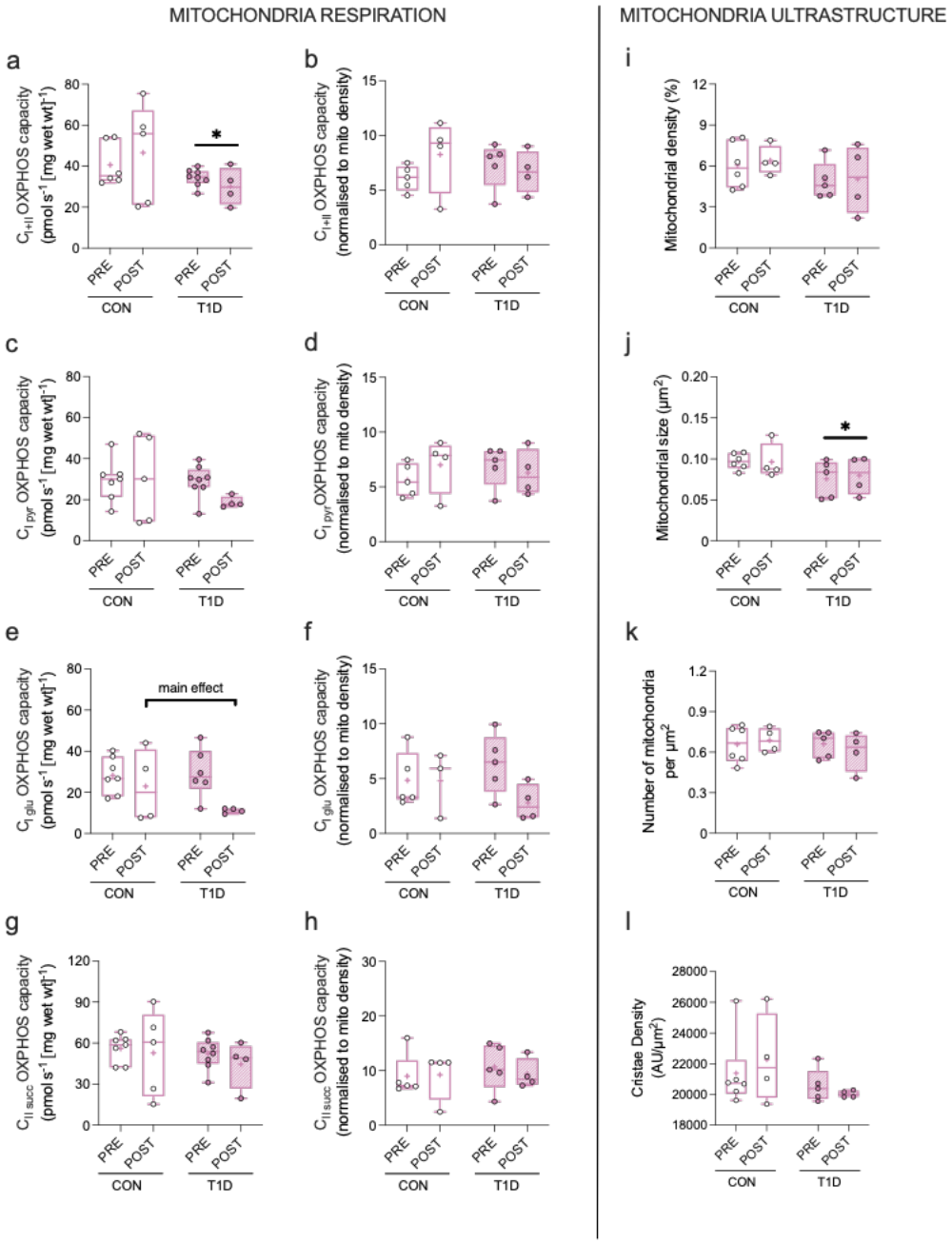


ESM Fig 5.1 Protein expression of MPC1/2 and ANT1/2 measured by Western blot in a subset of samples.

Mitochondrial pyruvate carrier (MPC) 1 and 2 expression were not statistically different between groups. Adenine nucleotide translocase (ANT) 1 and 2 expression were also not statistically difference except for ANT2 in combined. While also observably lower in women and men with T1D, this did not reach statistically significance. Methodology used has been previously published [19]. MCP1 (Cell Signaling mAB#14462, 1:1000); MCP2 (Cell Signaling mAB#46141, 1:1000); ANT1 (Abcam, ab110322, 1:1000); ANT2 (Cell Signaling, mAB#14671, 1:1000). CON, control; T1D, type 1 diabetes.



ESM Fig 5.2 Mitochondria respiration (a to h) and ultrastructure (i to l) in premenopausal (PRE) and postmenopausal (POST) women with type 1 diabetes (T1D) and without T1D (control. CON). Data was analysed using a two-way ANOVA. A main effect of post-menopausal was observed in (e) only. While there were observably lower values in T1D, this did not reach statistical significance.

CHAPTER 6 — OVERALL CONCLUSIONS

6.1 Significance of Studies and Conclusions

After nearly a century since insulin was discovered, there still exists no cure for T1D, and current treatment and management of this complex disease remains a major challenge and burden (Harding *et al.*, 2019). While it is well understood that achieving good metabolic control reduces the risk of macro and microvascular complications in people with T1D (Nathan *et al.*, 2005; Cleary *et al.*, 2006), current data from the T1D Exchange registry indicates that ~80% of youth and adults with T1D fail to achieve the Canadian and American Diabetes guidelines of $HbA_{1c} < 7\%$ (Foster *et al.*, 2019). Moreover, achieving near-normal glycemia with intensive insulin therapy is still associated with a two-fold greater risk of mortality (all-cause) in people with T1D compared to counterparts without diabetes (Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) Study Research Group, 2016). Current treatments available for the T1D population are not sufficient to prevent diabetes complications and early mortality.

As discussed in Chapter 1 of this thesis, skeletal muscle is an attractive candidate to target as an adjuvant therapy in people suffering from T1D because of its remarkable plasticity and its role in our physical and metabolic well-being. For instance, a healthy skeletal muscle is associated with increased survival and recovery rates from disease (van Venrooij *et al.*, 2012; Landi *et al.*, 2017). In addition, it is well known and appreciated that physical activity and exercise are a powerful and highly effective therapy to improve musculoskeletal fitness/health

(Cartee *et al.*, 2016), which in turn is associated with numerous whole-body benefits, including increased insulin sensitivity, improved glucose homeostasis, functional independence in older age, and longevity (Garber *et al.*, 2011) – all known to be negatively impacted by T1D.

Unfortunately, the limited evidence available to date indicates that skeletal muscle mass, function, and metabolism are negatively affected by T1D in humans (i.e., diabetic myopathy), including loss of muscle mass and function at the time of diagnosis (reviewed in (Krause *et al.*, 2011)), a shift towards anaerobic glycolysis as a fuel source, and mitochondrial deficiencies (during both submaximal and near-maximal levels of exercise). The latter is of particular interest as it's been reported as early as adolescence, and hence, early in disease progression. Further, the reported mitochondrial dysfunction in T1D adolescence was also found to be tightly associated with muscle insulin resistance but not acute or chronic glycemia, serum FFAs, or IMCLs (Cree-Green *et al.*, 2015). While is well known that a relationship exists between insulin resistance and mitochondrial dysfunction in human muscle (Millstein *et al.*, 2018), this study shows for the first time that early in the progression of T1D, a relationship between skeletal muscle mitochondrial dysfunction and insulin resistance exists, which may have a critical role in the development of diabetes complications (predominantly cardiovascular), exercise impairments, and the occurrence of geriatric syndromes (e.g., premature frailty, falls, fractures, functional disability) (Wong *et al.*, 2013; Yang *et al.*, 2016; Fan *et al.*, 2016; Aguayo *et al.*, 2019). As such, the clinical relevance and

importance of understanding skeletal muscle mitochondria, and hence, the underlying cellular mechanisms of diabetic myopathy, in T1D cannot be overstated.

Thus, this dissertation focused on understanding the cellular mechanism(s) underlying the metabolic impairments of skeletal muscle previously reported in humans with T1D measured by *in vivo* methodology ^{31}P-MRS (revised in more detail in Chapter 1 and Chapter 4). Specifically, this dissertation sought to comprehensively interrogate skeletal muscle mitochondrial bioenergetics and ultrastructure across the lifespan of adults with T1D (18 and up to 80 years old), including sex differences. The compiled series of studies herein makes for the largest muscle biopsy sampling of *Vastus lateralis* to have ever been undertaken yet in humans with T1D, and the first to employ gold-standard *in vitro* methodology for the investigation of mitochondrial bioenergetics – that is, high-resolution respirometry in permeabilized muscle fibres – in the T1D population.

Broadly, the findings presented in this document reveal differential mitochondrial adaptations in response to the T1D environment in skeletal muscle biopsies. In study 1 of this thesis (Chapter 2), we observed reduced mitochondrial sensitivity and capacity to oxidize carbohydrate-derived substrates, greater capacity to generate ROS, and greater capacity to trigger cell death in a cohort of young adults (26 ± 4 years old) with T1D (duration 15 ± 8 years) with moderately well-managed glycemia (HbA_{1c} 7.9 ± 1.5%) and who met/exceeded current

Canadian and American physical activity guidelines (201 ± 169 min). The percent decline in mitochondrial OXPHOS capacity was comparable to that reported in adolescents with T1D using *in vivo* methodology, ^{31}P-MRS (Cree-Green *et al.*, 2015), suggesting for the first time that high-resolution respirometry in permeabilized muscle fibres is consistent with *in vivo* muscle measurements in T1D humans. However, by sampling muscle in our study, we identified that the reductions in mitochondrial oxidative capacity are unlikely to be due to reductions in mitochondrial content or muscle fibre capillary density, but rather intrinsic dysfunction. We did not find differences in ETC protein expression of complexes I to V or in mitochondria area density (ultrastructure). Thus, it is plausible to speculate that the intrinsic dysfunction found to occur early in T1D progression is due to protein modifications (e.g., glycation, glycosylation, succinylation, etc.) and/or damage. The latter is indirectly supported by our measure of greater mitochondrial ROS emission combined with greater susceptibility to trigger cell death in our T1D cohort. While speculative, and part of one of our lab hypotheses discussed in Chapter 1 and 4, repeated subcutaneous bolus insulin injections in T1D causes muscle substrate overload, which leads to protein modifications and/or oxidative stress/damage. This hypothesis is well-supported in other tissues that cannot efficiently regulate glucose transport inside the cell when exposed to hyperglycemia such as capillary endothelial cells, kidney mesangial cells and neurons and Schwann cells in peripheral nerves (Brownlee, 2005). Certainly, the work herein opens many avenues for future studies.

In study 2 (Chapter 3), we observed sex differences in mitochondrial bioenergetics in a similar cohort of T1D women (26 ± 5 years) and T1D men (25 ± 4 years). Compared to the female participants with T1D, the male participants with T1D had lower mitochondrial complex II-supported respiration rates in the presence of submaximal and maximal succinate concentrations (i.e., reduced complex II sensitivity and capacity) but had a tendency towards greater complex I-supported respiration rates in the presence of submaximal and maximal concentrations of carbohydrate-derived substrates pyruvate or glutamate. Interestingly, we found that mitochondrial content and expression of the individual ETC proteins were not different between sexes with T1D; thus, we propose that complex I is compensating for functional deficiencies in complex II to maintain energy production in males with T1D. Despite these sex differences in mitochondrial respiration rates in T1D, site-specific mitochondrial ROS emission was not significantly different between sexes except for succinate-driven H_2O2 emission, which was significantly lower in the T1D female participants. This finding is in stark contrast to our hypothesis that T1D women would have greater ROS emission than T1D men and also contrasts previous work that found increased oxidative stress and reduced antioxidant defenses in women with T1D (31 ± 8 years of age, T1D duration 7 ± 3 years) compared with matched T1D men (measured in plasma) (Marra *et al.*, 2002). Indeed, as this is the first investigation of sex differences in mitochondrial bioenergetics in human T1D muscle, the mechanisms behind these sex differences are largely unknown. Considering that

sex hormones, particularly estrogen, regulate mitochondrial function and biogenesis (Ventura-Clapier *et al.*, 2017, 2019), perhaps the mechanism(s) involves changes in sex hormones. For example, some studies report lower estrogen and greater testosterone in women with T1D (Codner, 2008; Codner & Cassorla, 2009) while some have shown lower testosterone in men with T1D (Holt *et al.*, 2014). Understanding sex differences in a chronic metabolic disease like T1D is complicated by many factors, including: (1) the variability in age of diagnosis (e.g., prepuberty versus puberty versus early adulthood), (2) the variability in disease duration, (3) the treatment and management of the disease (e.g., multiple daily insulin injections compared to SCII with an insulin pump, episodes of diabetes burnout (diabetes distress), diabulimia, and major depression, just to name a few), (4) excess weight or obesity, and of course, (5) overall lifestyle (e.g., sedentary, active, exercise-trained). Therefore, while still in its infancy, it is very likely that women and men with T1D require, and would greatly benefit from, divergent adjuvant therapies to improve and maximize their healthspan.

In study 3 (Chapter 5), we observed normal to enhanced mitochondrial substrate sensitivity and oxidative capacity in a cohort of middle- to older-aged women and men (48 ± 11 years) with T1D (duration 28 ± 13 years) and moderately well-controlled glycemia (HbA_{1c} 7.4 ± 0.8%) that self-declared as being recreationally active. Mitochondrial content was lower with T1D in this cohort, which was largely a result of smaller mitochondria in women compared to a smaller number of mitochondria in men. As mentioned repeatedly throughout this body of

work, no studies, to the best of our knowledge, have interrogated mitochondrial bioenergetics and ultrastructure before in this age span, and thus it is impossible at this time to compare our findings to other T1D human work. The enhanced function is certainly intriguing and contrasts the hypotheses of our previously published review papers (Chapter 1 and Chapter 4) and of this dissertation as a whole. What remains clear is that skeletal muscle mitochondria in adults with T1D differentially adapt across the lifespan. While strictly speculative, considering the positive relationship between aerobic fitness (PWC_{130}) and various measures of mitochondria function found herein, the importance of living a recreationally active incorporated with exercise training is obligatory for *preserving* muscle mitochondria in this population.

In conclusion, this represents the first body of work to comprehensively investigate skeletal muscle bioenergetics and ultrastructure in adults with T1D compared to strictly matched controls. When we consider the link between mitochondria dysfunction, skeletal muscle deficits, and health outcomes/survival, the importance of maximizing skeletal muscle mitochondrial health early in people living with T1D cannot be overstated, especially with respect to ensuring a prolonged healthspan.

6.2 Future Research

This research has provided mechanistic insight into the metabolic health of skeletal muscle in adults with T1D. However, as this is the first body of human cellular work in this area of research, there are inevitably several questions that have been raised that warrant further investigation.

Firstly, since 33% of adults with T1D meet the recommended daily exercise guidelines (McCarthy *et al.*, 2016), the findings herein cannot be generalized to the entire T1D population. Thus, an obvious question comes to mind: what is the impact of T1D on skeletal muscle mitochondrial bioenergetics and ultrastructure in sedentary adults with T1D? When we consider the importance of physical activity and exercise towards a healthy mitochondrial pool in skeletal muscle, it seems reasonable to speculate that sedentary adults with T1D, irrespective of biological sex, have greater deficits in mitochondrial bioenergetics and ultrastructure than what we found. In turn, is this cellular mechanism associated with reduced muscle function, greater insulin resistance and as a result, difficulty managing glycemia? This could be studied by repeating the study presented in Chapter 2 but with the following amendments: (1) Euglycemic-hyperinsulinemic clamp to measure insulin sensitivity and determine the degree of insulin resistance; (2) 90 days of data from a Continuous Glucose Monitoring (CGM) device and physical activity tracker (e.g., Fitbit) to draw correlations with glycemic management (e.g., % time spent in range of 4.0 to 10.0 mM, % time spent above > 10.0 mM, % time spent < 3.9 mM) and time spent being active (e.g., % time spent in various zones of HR_{max}); (3) Body

composition assessment via DEXA (gold-standard) to draw correlations with body fat and lean body mass (i.e., muscle mass); (4) Muscle function measures (e.g., MVC, fatiguability, stability). This additional information would help establish potential relationships between the mitochondrial adaptations in T1D muscle and muscle insulin resistance, time spent in range versus outside of range, physical fitness, body fat, body muscle mass, and muscle function. This information is invaluable for identifying adjuvant therapies (e.g., exercise prescriptions) that will maximize skeletal muscle health in the T1D population, and ideally, in turn, prevent or delay the onset of diabetes complications and ensure an extended healthspan.

Second, there is increasing evidence that sex differences exist in the burdens of managing diabetes and the development of complications (Peters & Woodward, 2018). Women with T1D struggle more with daily glucose control, have greater insulin resistance (Millstein *et al.*, 2018), and as a result are at greater risk for cardiovascular complications and premature mortality than men with T1D (Huxley *et al.*, 2006, 2015). While the mechanisms of skeletal muscle insulin resistance are still debated, mitochondria dysfunction remains a common denominator, specifically excess ROS and/or accumulation of toxic lipids/intramyocellular lipids (IMCLs) due to lower oxidative capacity (Fisher-Wellman & Neufer, 2012). Since women preferentially metabolizes lipid substrates (Tarnopolsky *et al.*, 2007), particularly during exercise, one of many unanswered questions: what is the impact of T1D on mitochondrial bioenergetics supported by lipid- rather than carbohydrate-derived substrates in women and men with T1D,

and how does this differ between sexes? A plausible hypothesis is that skeletal muscle substrate overload is heightened (specifically lipid uptake and oxidation) in the female sex with T1D due to inherent biological differences in fuel selection and preference between sexes, which consequently leads to greater ROS emission, protein modification/damage, reduced oxidative capacity, greater IMCL/toxic lipid species, and consequently insulin resistance. Thus, a future study could expand from our current work in Chapter 3 to: (1) include a larger cohort of young women and men with T1D (18 to 30 years old) (2) determine insulin resistance (euglycemic-hyperinsulinemic clamp) (3) include additional measures of control: phase of menstrual cycle, use of contraceptives, CGM and physical activity tracker (e.g., Fitbit watch). High-resolution respirometry assays would be performed in permeabilized muscle fibres with lipid substrate palmitoyl-CoA (P-CoA). This would also apply to spectrofluorometric analysis of H_2O_2 emission in muscle fibres. Protein expression of fatty acid transporters at the plasma membrane (e.g., FAT/CD36) and within mitochondria (e.g., CPT) and other important metabolite transporters (e.g., ANT, VDAC, mtCK, UCP3), all of which would further complement functional assays. Ideally, muscle mass and function measures would also be captured to investigate relationships between cellular alterations and muscle phenotype.

Thirdly, our understanding of skeletal muscle mitochondrial bioenergetics in older people with T1D is next to none. While study 3 herein provides for the first-time metabolic insight into muscle metabolism in adults between 30 and 72 years

old, our study cohort was relatively healthy and free of diabetes complications (which is not representative of the majority of the current T1D population), and underpowered to evaluate the impact of increasing age and disease duration on muscle mitochondrial bioenergetics. Thus, future studies are certainly warranted in sedentary adults with T1D in their fourth decade of life and beyond and that have suboptimal glycemic management. A good starting point would be interrogating mitochondrial function by a less invasive, but robust methodology: ^{31}P-MRS. One of the many reasons being that not only is this methodology less invasive, which facilitates participant enrolment, but it also has been shown to exhibit both accuracy and precision with high-resolution respirometry in *vastus lateralis* muscle biopsies from healthy adults (Layec *et al.*, 2016). As such, we could determine whole-muscle mitochondrial function *in vivo* in a large cohort of adults with and without T1D (> 30 years old) binned by each decade of biological age in adults greater than 30 years old (e.g., 30 to 39 years old, 40 to 49 years old, etc.). Similar to the proposed studies above, glycemia, physical activity, insulin sensitivity, etc., would also be considered in addition to sex and co-morbidities (including the presence of diabetes complications). This would provide a meaningful amount of clinically relevant information (as correlations can be determined) that could be used to not only identify biomarkers of skeletal muscle health in T1D, but also the required evidence to improve our current guidelines for the management and treatment of T1D (in particular, physical activity and exercise). In turn, the latter

would help healthcare professionals better educate and guide people with T1D, especially those that want to maximize their health and live a healthy lifespan.

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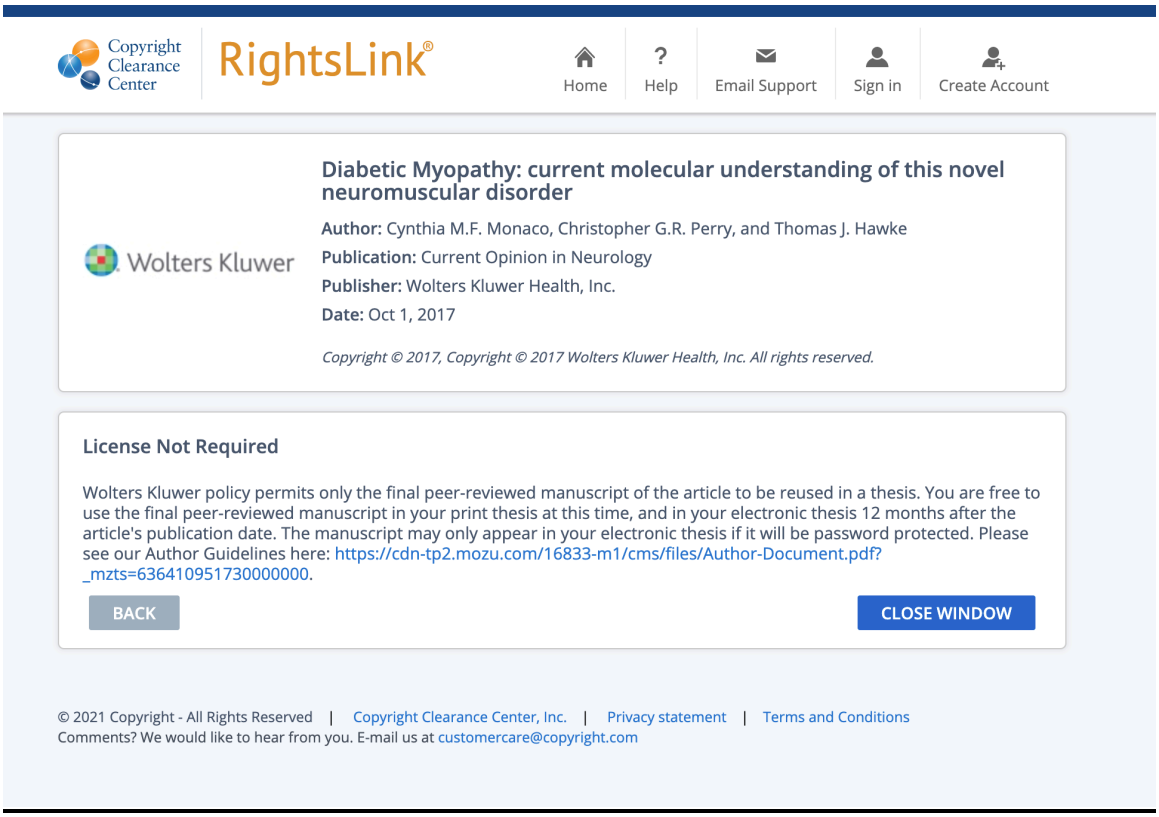

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Author: Cynthia M.F. Monaco, Christopher G.R. Perry, and Thomas J. Hawke
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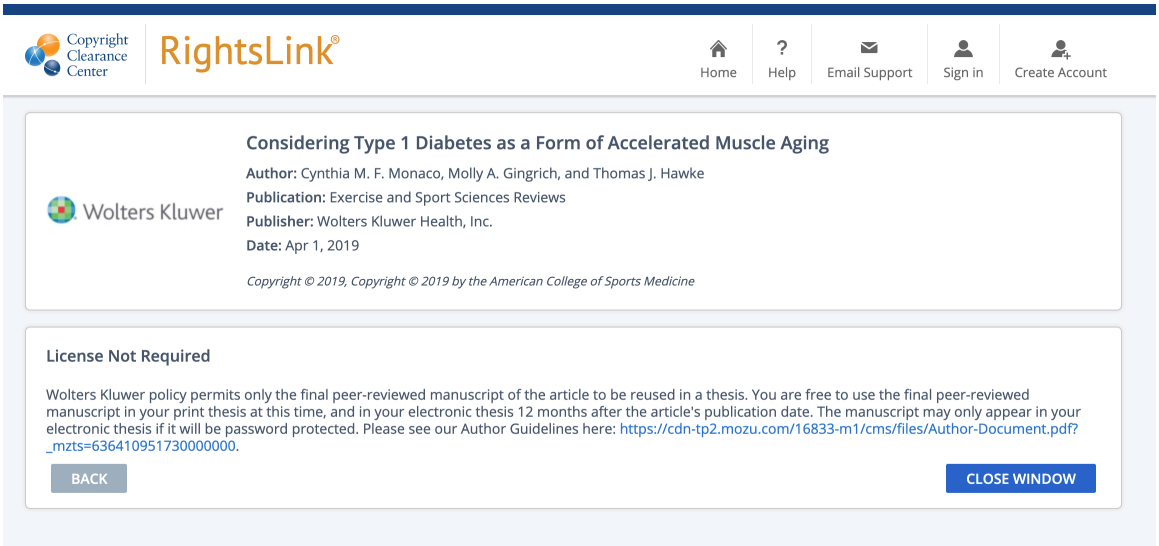

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Considering Type 1 Diabetes as a Form of Accelerated Muscle Aging

Author: Cynthia M. F. Monaco, Molly A. Gingrich, and Thomas J. Hawke
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APPENDIX B:

Alterations in mitochondrial functions and morphology in muscle and non-muscle tissues in type 1 diabetes: implications for metabolic health

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SYMPOSIUM REPORT

Alterations in mitochondrial functions and morphology in muscle and non-muscle tissues in type 1 diabetes: implications for metabolic health

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Edited by: Philip Atherton

Abstract

We recently made the observation that there are significant alterations to the ultrastructure and functions of mitochondria in skeletal muscle of people with type 1 diabetes (T1D). These alterations are proposed to lead to decreased energy production in skeletal muscle during exercise and thus may contribute to the impaired aerobic exercise capacity reported in some people with T1D. This Symposium Review summarizes the evidence that similar alterations also occur in the mitochondria present in organ systems outside skeletal muscle in people with T1D, and that this may contribute to the development and progression of the known complications of T1D, which eventually lead to the reported premature mortality.

KEYWORDS
bioenergetics, IDDM, insulin-dependent diabetes mellitus, insulin resistance, mitochondria, ROS, T1D, type 1 diabetes, unified theory

1 | INTRODUCTION

Type 1 diabetes (T1D) is a complex, autoimmune-mediated metabolic disease characterized by hyperglycaemia and insulin deficiency. Millions of people worldwide are afflicted with T1D (Xu et al., 2018), and the prevalence has consistently risen over the last decade (You & Henneberg, 2016). Insulin therapy continues to be the primary treatment option for people with T1D, and while advances in technology (e.g. insulin pumps and continuous glucose monitors) have facilitated the clinical management of this disease, the unfortunate reality is that diabetes complications (e.g. cardiomyopathy, neuropathy, nephropathy, retinopathy) still develop and remain the major cause of morbidity and premature mortality in this population (Mameli et al., 2015). Thus, it is clear that insulin therapy is not a cure, and if we are to improve the healthy lifespan of people living with T1D, greater research efforts are urgently needed towards unravelling the mechanisms underlying the development of diabetes complications in multiple organs and tissues.

Mitochondria are unique and highly dynamic organelles that regulate a variety of cellular functions and processes in nearly all cell types. These include energy supply to the cell in the form of ATP, production and emission of reactive oxygen species (ROS), Ca^{2+} homeostasis and cell death. Mitochondria also demonstrate tissue specificity due to the fact that the vast majority of mitochondrial proteins are derived from the nuclear genome and most tissues inherently have different metabolic and energetic demands (Ventura-Clapier, Kuznetsov, Veksler, Boehm, & Anflous, 1998). It is thus not surprising that alterations in mitochondrial morphology and functions, commonly termed mitochondrial *dysfunction*, accompany various human diseases, including myopathies, neurodegeneration, ageing and diabetes (Wallace, 1999). However, it is important to note that the term mitochondrial dysfunction is ill-defined as it remains unclear

whether the changes in mitochondria, and hence cellular function, that accompany diseases are simply a physiological adaptation or a pathological maladaptation of the mitochondria. These include impairments in electron transport chain (ETC) activity, decreased ATP synthesis, increased or excessive ROS, shifts in metabolic substrate utilization, defects in mitochondrial dynamics (e.g. mitochondrial shape/size) and loss of cristae density, just to name a few. Maintaining mitochondrial homeostasis is thus critical for cell survival, which largely depends on the balance between mitochondrial biogenesis, fission and fusion, and mitophagy. This symposium review gives an overview of the evidence of impaired mitochondrial functions and/or morphology in people with T1D across various organ systems, which may be a key mechanism in the pathophysiology of diabetes complications.

New Finding

- **What is the topic of this review?**
  Evidence of impaired mitochondrial functions and/or morphology in people with type 1 diabetes across various organ systems.
- **What advances does it highlight?**
  Impairments to mitochondrial functions and morphology may be a primary mechanism underlying the pathophysiology of various complications in people with type 1 diabetes.

2 | TYPE 1 DIABETES: IMPACT ON SKELETAL MUSCLE AND MITOCHONDRIA

In rodents, T1D is known to lead to abnormalities in skeletal muscle morphology/ultrastructure, functions and metabolic health (i.e. insulin resistance) (Krause, Riddell, & Hawke, 2011). While less understood, studies in humans with T1D also suggest impairments in skeletal muscle mass, function and metabolism (see previous reviews: Krause et al., 2011; Monaco, Perry, & Hawke, 2017; Monaco, Gingrich, & Hawke, 2019). Although these impairments in skeletal muscle are not considered life-threatening (and as a result are often overlooked clinically), diabetic myopathy is likely the primary reason underlying the greater risk of sarcopenia, exercise intolerance, mobility limitations, physical disability and frailty that has been reported, especially with increasing age, in those with diabetes relative to those without (Yanase, Yanagita, Muta, & Nawata, 2018). In fact, we recently postulated that T1D recapitulates a condition of accelerated muscle ageing, with many of the deficiencies that occur in aged muscles already being present in individuals with T1D but at a significantly earlier age (Monaco, Gingrich, & Hawke, 2019). Indeed, while the underlying mechanisms are undoubtedly multifactorial, there is evidence to suggest that similar to ageing, alterations in mitochondria are associated with the observed reductions in aerobic exercise capacity and overall skeletal muscle metabolic health that occurs in some people with T1D (Monaco et al., 2019).

Along these lines, we have recently discovered that skeletal muscle mitochondria in young adults with T1D have irregularly organized cristae at the ultrastructural level, decreased ability to produce ATP in response to increasing energy demand (ADP), increased complex III-derived ROS emission and increased susceptibility to opening of the mitochondrial permeability transition pore (mPTP) in permeabilized muscle fibre bundles (Monaco et al., 2018). Other groups, but not all (see Monaco et al., 2019), have also found reductions in skeletal muscle mitochondrial oxidative phosphorylation in adolescents and adults with T1D utilizing ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS), and this may be linked to insulin resistance, potentially through altered redox signalling (Fisher-Wellman & Neufer, 2012). Insulin resistance will invariably impact muscle mass and function through an imbalance between protein synthesis and degradation (Hebert & Nair, 2010). Moreover, in ageing rodent studies, increases in ROS have been associated with accumulation of oxidized proteins and altered proteostasis, leading to denervation of motor units, and consequently, loss of muscle innervation, muscle fibre atrophy and loss of muscle function (discussed in more detail in Vasilaki et al., 2017). Opening of the mPTP plays an important role in the regulation of programmed cell death, and hence muscle fibre degeneration (i.e. muscle mass/size), via the release of pro-apoptotic factors from mitochondria. Altogether, it remains to be investigated whether these mechanisms also operate in people with T1D.

3 | TYPE 1 DIABETES: BEYOND SKELETAL MUSCLE

3.1 | Cardiac tissue mitochondria

The cardiac muscle is one of the most oxidative and energetically demanding tissues in the body. To put this into perspective: mitochondria occupy nearly 30–40% of the cell volume in the heart, compared to <10% in skeletal muscle, and they rely mainly on fatty acids to generate and supply the large amounts of ATP required for the beating heart (i.e. fatty acid oxidation) (Ponsot et al., 2005). In other words, cardiac mitochondria are highly adapted for the continuous provision of ATP to sustain daily contractile activity. In T1D, studies have shown that some individuals have abnormal left ventricle (LV) function despite the absence of major coronary artery disease or hypertension (Shivu et al., 2010). This indicates that the T1D environment itself may directly impact the cardiac tissue (through mechanisms such as unrecognized microvascular disease, autonomic neuropathy, oxidative stress and impaired cardiac energetics).

With respect to this review, two studies, to the best of our knowledge, have interrogated cardiac energetics in humans with T1D. Utilizing ^{31}P-MRS imaging to quantify the high-energy phosphates of the LV, Metzler et al. (2002) found that compared to age-matched healthy male controls, men with T1D (36 years old; HbA_{1c} 7.6%; 4–42 years' history of T1D) have a 13% reduction in the

phosphocreatine (PCr) over β-ATP concentration (PCr/ATP) ratios, a commonly used measure to characterize the *in vivo* energy status of the heart. Shivu et al. (2010) found that newly diagnosed men and women with T1D (32 years old; HbA_{1c} 7.4%; 0–6 years' history of T1D) have a 31% reduction in PCr/ATP ratios whereas men and women with longer duration of T1D (33 years old; HbA_{1c} 8.3%; 11–25 years' history of T1D) have a 41% reduction compared to matched controls. We acknowledge that many factors are at play in determining the PCr/ATP equilibrium, including rates of mitochondrial oxidative phosphorylation, microvascular dysfunction and the presence of cardiac fibrosis. The latter refers to the fact that people with T1D are known to have increased myocardial fibrosis (Armstrong et al., 2017), an observation that would imply that their cardiomyocytes have to work at a higher workload per cell to compensate for the smaller LV volume fraction. This would not only lead to an inability in maintaining the PCr/ATP ratio, but also a lower maximal cardiac output and $\dot{V}_{O_2\,max}$. Nonetheless, without further experiments and studies, these assessments, for the moment, provide indirect evidence for the concept of impaired cardiac mitochondrial bioenergetics *in vivo* in T1D.

It is interesting to note that ischaemic preconditioning does not appear to protect atrial samples of adults with T1D from ischaemia–reperfusion injury compared to those without diabetes (Ghosh, Standen, & Galiñianes, 2001; Hassouna et al., 2006). These studies suggest that the mechanism underlying this failure to precondition may involve mitochondrial dysfunctions, specifically dysfunction of the mitochondrial K_{ATP} channels as pharmacological manipulation of these channels failed to protect the T1D samples as opposed to the non-diabetic samples from ischaemia–reperfusion injury. It is thought that this leads to mitochondrial depolarization, superoxide production and, ultimately, an inability of the cardiomyocytes to respond to preconditioning.

3.2 | Nervous tissue mitochondria

The central nervous system also has an immense metabolic demand. Similar to cardiac muscle, neurons rely heavily on oxidative metabolism for high energy supply. In a resting state (i.e. non-stimulated condition), approximately 60% of mitochondria-derived ATP is expended on the maintenance of ionic gradients/signalling processes across the cell membranes (Silver & Erecińska, 1998). During enhanced neural activity, this percentage increases even more. In T1D, there is a growing appreciation of an underlying role of impairments in mitochondria in cells of the nervous system (neurons, glial and Schwann cells). However, the evidence to date has been in rodent models or cell culture models of hyperglycaemia/T1D. With this caveat in mind, there are reports that mitochondrial trafficking and functions may be abnormal, particularly within distal axons. A number of studies supporting mitochondria's role in diabetic neuropathy (DN) have come from Feldman's group, who have observed that hyperglycaemia induced mitochondrial biogenesis and an increase in mitochondrial fission, thereby creating numerous small, damaged mitochondria within neurons (Feldman, Nave, Jensen, & Bennett, 2017). More recently, an emerging idea in the field of DN has been proposed: an imbalance in the mitochondrial redox state as a result of disorders in transferring energy between axons and their supporting glia cells (for more details see Feldman et al., 2017). The interplay and inter-dependence of multiple cell types within the nervous system for survival is well appreciated and it is the hope that future studies will uncover a strong relationship between deficits in energy metabolism in supporting cell types and axonal loss in the nervous system.

Within the context of human T1D neuropathy, studies of mitochondrial ultrastructure have been undertaken and mitochondrial dysfunctions inferred from these observations (Kalichman, Powell, & Mizisin, 1998; Schmidt et al., 1997). Importantly, the majority of these ultrastructure studies were performed in T1D patients with clinically diagnosed neuropathy, so the relevance of this work (with respect to mitochondrial dysfunctions as a mediator of neuropathy) is limited. Some early work by Reske-Nielsen, Harmsen, and Vorre (1977) included one newly diagnosed adult (3 weeks of diabetes) and three adults with longer duration diabetes (24–32 years of diabetes). In the motor plate of the newly diagnosed adult, no abnormalities in mitochondria were reported in the Schwann cells, axon or synaptic clefts. However, the motor end plates exhibited an increased presence of lipid bodies/lipofuscin, which may be indicative of an early mitochondrial impairment (e.g. reductions in mitochondrial respiration causing accumulation of lipids in the cells). In contrast to the newly diagnosed, the longer duration group exhibited reductions (and, in one case, a loss) in mitochondrial content within the sarcoplasma around the synaptic clefts consistent with the aforementioned changes in mitochondrial biogenesis. Sural nerve biopsies from T1D individuals with neuropathy revealed enlarged and swollen mitochondria and cristae effacement in the Schwann cells as well as tightly aggregated mitochondria in post-synaptic dendrites (Kalichman et al., 1998; Schmidt et al., 1997). To the best of our knowledge, whether mitochondrial ATP production and ROS are altered in neural tissue of people with T1D remains to be established.

3.3 | Kidney tissue mitochondria

Though it may be underappreciated, renal tissue is second only to cardiac tissue with respect to energy demands and mitochondrial content (Bhargava & Schnellmann, 2017). This largely stems from the fact that many of the kidney's primary functions, including waste removal from blood, nutrient reabsorption, and electrolyte and fluid balance, rely on ion gradients generated by the Na^+–K^+-ATPase. The abundance of normal mitochondria in renal tissue therefore ensures sufficient ATP supply for the ion gradients, and hence, normal kidney function. Ultimately, impairments in the renal mitochondria with T1D should result in obvious, measurable deficits. While evidence of a mitochondrially mediated basis for diabetic nephropathy has only recently been reported in humans (discussed below), the findings in many ways are consistent with the body of evidence in T1D rodent models. In these rodent studies, isolated kidney mitochondria displayed gradual and progressive reductions in ATP, reductions in the individual expression and activity of the ETC proteins, increased fragmentation (i.e. defective mitochondrial dynamics), and increased

capacity for opening of the mPTP, which correlated with mitochondrial H_2O_2 emission (Coughlan et al., 2016; Dugan et al., 2013). Importantly, some of these changes preceded the development of renal lesions, suggesting that alterations in mitochondrial phenotype are a primary cause of diabetic kidney disease, at least in T1D rodents.

Similar to rodent studies, mesangial cells from human kidney cultured in high glucose (25 mM) display gradual and progressive reductions in mitochondrial respiration (ATP), increased cellular ROS (assay used was not mitochondria-specific), and reduced cell viability (indirectly suggests increased susceptibility to opening of mPTP) (Czajka et al., 2015). Interestingly, the increase in ROS occurs in parallel with an increase in mtDNA, suggesting an increase in mitochondrial content; however, further analysis in this study revealed a reduction in mtDNA transcription, increased mtDNA damage, and reduced mitophagy. In addition, mitochondria became more rounded and fragmented with increasing exposure time to high glucose. Thus, alterations in renal mitochondrial functions and morphology appears to occur prior to renal damage and as a direct result of continuous exposure to hyperglycaemia in human cells.

Similar findings have also been reported in another study albeit in proximal tubular biopsies obtained from people with diabetes (pooled T1D and T2D) and diabetic nephropathy (Zhan et al., 2018). In particular, this study found an increase in superoxide (dihydroethidium staining), cytochrome *c* release (indirectly indicates increased opening of mPTP), and perturbations in mitochondrial dynamics (decreased expression of protein markers of mitochondrial fusion, increased expression of protein markers of mitochondrial fission, increased and scattered mitochondrial fragments, and remodelled mitochondrial cristae). Mitochondrial ATP production was not interrogated in this study. Interestingly, in surrogate cells for kidney tissue (peripheral blood mononuclear cells), basal and maximal mitochondrial respiration are decreased in people with diabetes (pooled T1D and T2D) with diabetic nephropathy compared to those without diabetic nephropathy but are not different in people with diabetes without diabetic nephropathy compared to controls (Czajka et al., 2015). Consequently, without renal biopsies from people with T1D without diabetic nephropathy, the cause–effect relationship between changes in mitochondrial content/morphology and bioenergetics (respiration, ROS, mPTP) must still be defined. This will be essential in unravelling the contributions of mitochondrial dysfunctions to diabetic nephropathy development and progression in humans.

Lastly, a metabolomics study of urine from patients with and without diabetic kidney disease has also been undertaken (Sharma et al., 2013). Indeed, while not a direct assessment of mitochondrial metabolism in kidney tissue, urine metabolomics permit the measurement of a wide range of metabolites that the kidney is responsible for concentrating and excreting in the urine. Thus, urine metabolomics allows for indirect insights into biochemical pathways linked to kidney dysfunction. In this particular study, the researchers found that in people with diabetes (both T1D and T2D), a number of metabolites were linked together in a large network and virtually all of these metabolites were produced by enzymes localized to the mitochondria. It was further reported that a significant reduction of these metabolites occurred in the urine of people with diabetes (both T1D and T2D) with and without diabetic kidney disease. The reduction in these metabolites suggests that the mitochondrial enzymes responsible for producing them might also be attenuated, thereby suggesting an overall reduction in mitochondrial function in kidneys from people with diabetes.

4 | SUMMARY AND PERSPECTIVES

The intention of this Symposium Review article was to spark discussion and research ideas regarding the concept of mitochondrial 'dysfunction' as a primary mechanism in diabetes complications development and/or progression, and to summarize the limited human literature in T1D as it pertains to abnormalities in mitochondrial functions and morphology within a number of tissues. This idea was borne out of (1) our previous work in skeletal muscle (Monaco et al., 2018), which demonstrated the presence of clear mitochondrial deficiencies, both *in vivo* and *in vitro*, in adults with T1D prior to any notable impairments in functionality; and (2) the pathways which Brownlee's 'unified theory' of diabetes complications (Brownlee, 2005) identified as 'damaging' may be expandable to more tissues, with the concept that the mitochondria-derived superoxide may be the result of hyperglycaemia or impairments to mitochondrial bioenergetics (by mechanisms yet to be elucidated). We acknowledge the limitations that are currently present in the data supporting this expansion of the unified theory, particularly in human studies investigating the mechanisms leading to cardiovascular disease, nephropathy and neuropathy in T1D, as there are ethical limitations to *in vivo* tissue sampling (biopsies). However, recent advances with non-invasive methods such as ^{31}P-MRS are expected to open opportunities to further test this line of enquiry as the need for clinically attenuating the development and progression of diabetes complications cannot be understated.

5 | CONCLUSIONS

The research described in this review suggests that mitochondria are negatively impacted by the T1D environment in skeletal, cardiac, nervous and kidney tissues in people with T1D through mechanisms yet to be fully elucidated. A consistent finding across the majority of these tissues is the impairment in mitochondrial dynamics (e.g. increased fragmented mitochondria) and bioenergetics, specifically reductions in mitochondrial respiration (i.e. ATP production) and increases in ROS, which are worsened by the presence of diabetes complications (Figure 1). As such, the authors expect that the widespread energy depletion (potentially a result of altered mitochondrial dynamics) likely goes hand in hand with elevated mitochondrial ROS, especially during repeated bouts of hyperglycaemia. This likely culminates in oxidative stress and damage to cells and, consequently, development of diabetes complications across a variety of tissues.

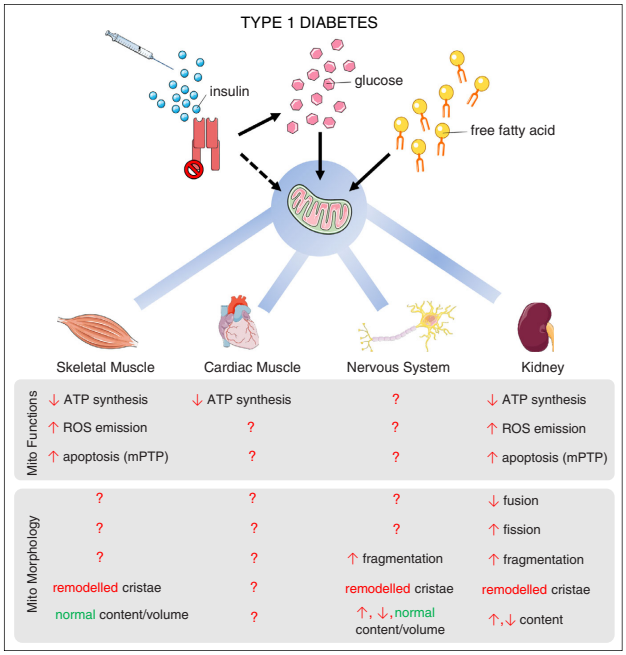

FIGURE 1 Our current understanding of the impact of T1D on mitochondrial functions and morphology across various tissues in people. Dyslipidaemia, dysglycaemia, hyperinsulinaemia (from subcutaneous insulin injections) and insulin resistance are characteristic of T1D and are considered to be the primary contributors in the development of diabetes complications. At the cellular level, these characteristics can lead to substrate overload (e.g. glucose toxicity) and decreased or poor insulin signalling, all of which can directly or indirectly impact mitochondria. Alterations in mitochondrial functions and morphology accompany various human diseases, including myopathies, neurodegeneration, ageing and diabetes (Wallace, 1999). It is thus our working hypothesis that impairments to mitochondria may be a primary cellular mechanism underlying the pathophysiology of various complications in people with T1D. Unfortunately, there exists a very finite amount of human literature on the impact of T1D on mitochondria in skeletal muscle, heart, nervous system and kidneys. What is currently known is listed under each respective tissue. The question marks indicate that no studies to date have interrogated this measure in people with T1D. The inclusion of multiple arrows and 'normal' means that this measure differed across various studies or differed due to disease status and presence of secondary complications

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COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

C.M.F.M., C.G.R.P. and T.J.H.: conception and design of research, drafting, editing and revision of manuscript. T.J.H. approved the final version of the manuscript and is the guarantor of this work. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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