The impact of Type 1 Diabetes on skeletal muscle fuel substrate storage and ultrastructure in rodents and adult humans

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Abstract

Type 1 diabetes (T1D) is the result of the autoimmune-mediated destruction of the pancreatic beta-cells leading to the inability to produce insulin sufficiently and, in turn, regulate blood glucose levels. Abnormal levels of blood glucose, specifically hyperglycemia, have been linked to many diabetic complications, with Brownlee proposing decreased GAPDH activity and the resultant increase in four main pathways as the mechanism(s) leading to these complications. Though skeletal muscles play a major role in glucose uptake, they are believed to be relatively protected against these complications as they are able to regulate their glucose uptake. However, evidence is accumulating that skeletal muscles are adversely affected in T1D, particularly with respect to their mitochondrial function. This led us to consider that the skeletal muscles of those with T1D would experience substrate overload (high intracellular lipids and recurrent, high levels of intracellular glucose), which would initiate a negative spiral whereby substrate excess would damage mitochondria - leading to an impaired ability to utilize these substrates - further worsening the substrate overload. Therefore, the objective of this study was to investigate glycogen and intramyocellular lipid (IMCL) content in the muscles of mice and humans with T1D, as well as the potential downstream effects in the form of post-translational modifications (PTMs), mitochondrial content, and lipofuscin accumulation. The Akita T1D mouse model was used to assess substrate overload in uncontrolled diabetes, whereas human participants were used to investigate substrate overload in the presence of insulin therapy. Assessment of glycogen and IMCL content revealed no difference between controls and diabetic cohorts in both the rodent and human study, indicating the lack of substrate overload. Post-translational modifications did not significantly change between Akita and wild-type mice; however, there was a main effect of diabetes on acetylation levels within Akita mice. Lastly, most mitochondrial properties, except

for subsarcolemmal pixel density, did not differ either between diabetic and non-diabetic subjects in the human study. Thus, despite mitochondrial complex impairments in diabetic subjects, its extent was not significant enough to cause alterations to the mitochondria as a whole and result in mitochondrial degradation and lipofuscin formation.

This study has provided novel insight into the metabolic properties of skeletal muscle during diabetes. Although there was no indication of substrate overload, diabetes still resulted in some changes to PTM levels and mitochondrial pixel density. However, the effects of these changes did not significantly alter the muscle and resulted in pathway impairments of those that were studied. This could be due to an adaptive mechanism in mice, although future studies are needed to confirm this hypothesis. In the human study, healthy, well-controlled individuals could explain why there was hardly any difference seen, suggesting that controlling glycemic levels was imperative in preventing diabetic complications in muscle.

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Declaration

Most experiments and subsequent analyses were carried out and performed by Maria Nguyen for the completion of this thesis. As follows, in the rodent study, Maria was responsible for assisting either Dr. Hawke, Dr. Rebalka or Cynthia Monaco during the mice harvest, preparing the samples and appropriate reagents, and conducting the oil-red-o (ORO), periodic-acid Schiff's (PAS) stain and western blot experiments and subsequent analysis. In the human study, Maria assisted during the pre-biopsy tests, tissue collection process, and aftercare of the participants. The human PAS stain images presented in this thesis were provided by Athan Dial, while the electron microscopy images were taken and provided by Cynthia Monaco and Grace Grafham. Maria completed the human PAS stain and electron microscopy images analysis for the glycogen, mitochondrial, and intramyocellular lipid (IMCL) data presented.

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List of Abbreviations

ADP - adenosine diphosphate
AGEs - advanced glycation endproduct
ATP - adenosine triphosphate
BMI - body mass index
BSA - bovine serum albumin
BCA - bicinchoninic acid assay
β-cells - beta cells
β-HAD - β-hydroxyacyl-CoA dehydrogenase
CPT-1 - carnitine palmitoyltransferase 1
CPS1 - carbamoyl phosphate synthetase I
DHAP - dihydroxyacetone phosphate
EM - electron microscopy
FABPpm - plasma membrane-associated fatty acid-binding protein
DAG - diacylglycerol
DNA - deoxyribonucleic acid
FA - fatty acid
FADH2 - flavin adenine dinucleotide
FAT/CD36 - fatty acid translocase CD36
FATP - fatty acid transport protein
ETC - electron transport chain
F1,6BP - fructose-1,6-bisphosphate
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GFAT - glutamine:fructose-6-phosphate amidotransferase
GLUT - glucose transporters
GTP - guanosine-5'-triphosphate
G3P - glyceraldehyde-3-phosphate
G6P - glucose-6-phosphate
HEK - human embryonic kidney
HFD - high-fat diet
HMGCS2 - 3-hydroxy-3-methylglutaryl-CoA synthase 2
IMCL - intramyocellular lipid
IMF - intermyofibrillar
IMM - inner mitochondrial membrane
KAT - lysine acetyltransferase
MDH - malate dehydrogenase
MEF - mouse embryonic fibroblasts
NADH - nicotinamide adenine dinucleotide
NADPH - nicotinamide adenine dinucleotide phosphate
NAD+ - nicotinamide adenine dinucleotide
NO - nitric oxide
eNOS - nitric oxide synthase
OGT - O-Glc-NAc transferase
OMM - outer mitochondrial membrane
ORO - oil-red-O
OXPHOS - oxidative phosphorylation
O-Glc-NAc - β-N-acetylglucosamine
PARP - poly (ADP-ribose) polymerase
PAS - Periodic acid Schiff's

PDH - pyruvate dehydrogenase
PKC - protein kinase C
ROS - reactive oxygen species
ROUT – robust regression and outlier removal
SDH - succinate dehydrogenase
SIRT - sirtuins
SOD1 - superoxide dimutase 1
SOD2 - superoxide dimutase 2
SS - subsarcolemmal
STZ - streptozotocin
TA - tibialis anterior
TBS-T - tris-buffered saline-tween
TCA - tricarboxylic acid cycle
T1D - type 1 diabetes
TOM - the outer membrane complex
UDP - uridine diphosphate
UDP-GlcNAc - uridine diphospho-*N*-acetylglucosaminem
VDAC - voltage-dependent anion channel

Review of Literature

Skeletal Muscle

Skeletal muscles collectively are the largest organ in the human body by mass and make up their own organ system; the muscular system. They are named so because most of the muscles in the system are involved in moving the bones of the skeleton. With around 700 individual muscles, they work together to stabilize body position, produce movement, regulate organ volume, and generate heat.[1] Skeletal muscles are responsible for all voluntary actions within the body and as such, are under the control of the somatic nervous system. Each skeletal muscle is made up of muscle fibers, nerve fibers, blood vessels, and connective tissue. They can differ in terms of size, shape, and arrangement of fibers.[1] The muscle fibers, which are the cells of the muscle, are made up of myofibrils that are rich in sarcomeres, the primary contractile unit of a muscle fiber. Sarcomeres make up the basic machinery required for a muscle contraction and result in the striated pattern associated with skeletal muscle.[2] They are organized into repeated structures containing overlapping actin thin filament and myosin thick filaments. This overlap in actin and myosin allows the filaments to slide against each other during a muscle contraction and results in the shortening of the sarcomere.[2]

Skeletal muscle fibers can differ in terms of their fiber types as some fibers can vary in myoglobin content, contraction and relaxation speed, fatigability, and how adenosine triphosphate (ATP), the primary energy substrate of the cell, is produced. Muscle fibers with a higher myoglobin content are called red muscle fibers, while those with lower content are called white muscle fibers.[1] Additionally, skeletal muscle fibers that rely more heavily on aerobic pathways to generate ATP are referred to as oxidative. In contrast, those that preferentially utilize anaerobic glycolysis are referred to as glycolytic.[3] Muscle fibers can also be classified

based on the speed of ATP hydrolysis by ATPase in the myosin head. Based on these criteria, muscle fibers classification can fall under three types: slow oxidative, fast glycolytic, and fast oxidative glycolytic fibers. Slow oxidative fibers have the highest content of myoglobin and are red in colour. They also have the most mitochondria, making them more suited for aerobic respiration and as such, are oxidative.[1] However, their rate of ATP hydrolysis is rather slow, hence the "slow" in its name. Slow oxidative fibers have a much slower rate of contraction but are more resistant to fatigue. On the other hand, fast glycolytic fibers contain low myoglobin and mitochondrial content but are high in intracellular glycogen.[1] They mainly utilize glycolysis to produce ATP, and because of their ability to rapidly hydrolyze ATP, they can contract much more quickly and forcefully. However, this also means that they fatigue more easily.[1] Lastly, fast oxidative glycolytic fibers are an intermediate between the two and are commonly the largest fiber. Features that they share with slow oxidative fibers are their higher myoglobin content and ability to generate ATP via aerobic processes, making than more resistant to fatigue than fast glycolytic fibers.[1,3] But like glycolytic fibers, they can also utilize glycolytic pathways and have a high glycogen content.[3] Additionally, the speed of ATPases in fast oxidative glycolytic muscle fibers are much quicker, and as a result, they have a quicker rate of contraction than slow oxidative fibers.[1]

Each muscle fiber type is adapted for specific functions. Slow oxidative fibers are involved in maintaining posture and are more adaptive for prolonged endurance exercise.[1] Fast glycolytic fibers are more adaptive for movements that require a burst of strength such as weightlifting, while the intermediate between them, i.e. fast oxidative glycolytic fibers, are more suited for activities such as walking and sprinting.[1] Although they each have their niche and role

in muscle movement, most skeletal muscle contains all three fiber types, albeit in different proportions based on the function of the muscle.

Cellular Respiration

Muscle contractions and force production are energy-intensive and, as such, require ATP production via anaerobic and/or aerobic pathways. Anaerobic respiration or glycolysis takes place in the cytoplasm and is the process of splitting glucose into two pyruvate molecules to be used in downstream respiration associated pathways.[4,5] Glycolysis is separated into two phases; the energy-requiring and the energy-releasing phase. The energy-requiring phase uses two ATP molecules in order to add two phosphate groups to the 6-carbon glucose. Phosphorylation and rearrangement of glucose results in the formation of fructose-1,6-bisphosphate (F1,6BP).[4] The instability of F1,6BP facilitates its division into two three-carbon molecules, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). Although G3P can be readily used in the next phase of glycolysis, DHAP requires its conversion to G3P first with the help of the enzyme triose-P isomerase.[5] The next phase of glycolysis is energy-releasing, as G3P undergoes further modification to form the end product, pyruvate. Through the series of reactions, one nicotinamide adenine dinucleotide (NADH) and two ATP molecules are generated per G3P.[4] Since two G3P are produced per glucose molecule, the overall pathway results in the formation of 4 ATP and 2 NADH molecules, as well as two 3-carbon pyruvates. However, it is important to note that because two ATP molecules are used up in the energy-requiring phase, the net number of ATP produce from glycolysis is two.[4] These end products of glycolysis are then shuttled into the mitochondria, where it undergoes more chemical reactions to generate the majority of ATP.

The mitochondria are highly metabolic organelles bound by a double membrane, a porous outer mitochondrial membrane (OMM), and a vastly impermeable inner mitochondrial membrane (IMM).[5] The OMM is composed of a phospholipid bilayer embedded with voltage-dependent anion channels (VDAC) and translocase of the outer membrane complex (TOM), which permits the free diffusion of molecules or transport of larger proteins, respectively.[4] The IMM is largely impermeable and provides a physical barrier that encloses the mitochondrial matrix to ensure it is kept separate from the cytosol. It is folded into cristae, which allows for an increase in surface area, and consequently, metabolic activity. The intermembrane space is the area between the two membranes.[4,5]

The mitochondria are primarily responsible for ATP production through numerous metabolic pathways, the most common of which are the Krebs or tricarboxylic acid (TCA) cycle and oxidative phosphorylation. These processes are aerobic and, as such, require oxygen. The TCA cycle occurs in the mitochondrial matrix and utilizes metabolic intermediates from carbohydrates, proteins, and lipids.[4] Through glycolysis and activity of pyruvate dehydrogenase, or beta-oxidation, the formation of acetyl-CoA, the most common intermediate, can be used in the TCA cycle. Acetyl-CoA undergoes a series of chemical reactions that results in the production of carbon dioxide (CO_2), guanosine-5'-triphosphate (GTP), NADH, and flavin adenine dinucleotide ($FADH_2$).[4,5] CO_2 is a waste by-product and, as such, is released from the cell. However, the latter products are all involved in energy production. Even though GTP can be used for energy immediately, reducing equivalents NADH and $FADH_2$, are involved in downstream oxidative phosphorylation associated energy production.

Oxidative phosphorylation (OXPHOS) utilizes the reducing equivalents in the electron transport chain (ETC) to create a proton gradient that drives ATP production. The ETC is located

on the IMM and consists of a chain of reducing enzymes. The electrons gain from NADH and $FADH_2$ are passed through a series of complexes starting from complex I or II, depending on the electron donor.[4] NADH passes its electrons to complex I, also known as NADH dehydrogenase, while $FADH_2$ starts at the second complex or succinate dehydrogenase. The electrons from both complexes are then transferred to ubiquinone (Q) and pass down the next two complexes, III (cytochrome bc_1 complex) and IV (cytochrome C oxidase).[4] Electron transfer ends with the formation of water, as oxygen acts as the final electron acceptor. The series of redox reactions and the transfer of electrons drive proton efflux to the intermembrane space, creating an electrochemical gradient. When protons re-enter the matrix and flow down the concentration gradient, its passage through the ATP synthase complex, a motor that drives the unfavourable addition of phosphate to adenosine diphosphate (ADP), results in the formation of ATP.[4,5]

Through the course of ATP production, leakage of electrons from complex I and III can result in the partial reduction of oxygen and generation of superoxide, a form of reactive oxygen species (ROS). Under normal conditions, the superoxide is converted to hydrogen peroxide (another form of ROS) by superoxide dismutase 1 and 2 (SOD1 & SOD2) and subsequently removed.[5,6] Generally, ROS are considered a by-product of cellular metabolism and have been linked in certain signaling pathways to serve as signaling molecules. However, high levels of ROS, such as times of high substrate load and thus high substrate oxidation, are detrimental to the mitochondria and cell itself. As high substrate oxidation leads to oxidative stress resulting in deoxyribonucleic acid (DNA) damage and activation of apoptotic pathways.[5,6]

Substrate Uptake and Storage

In order for the mitochondria to have access to energy substrates to produce ATP, glucose and lipids must first enter the cell and/or be readily available within the cell. Hence the uptake and incorporation of substrates into tissue are highly crucial in the production of energy. Glucose uptake occurs primarily through a family of membrane proteins called glucose transporters or GLUT.[7] Skeletal muscles specifically incorporate and utilize GLUT1 & GLUT4 transporters. GLUT1 is constitutively activated and plays a vital role in low-level basal glucose entry; however, its concentration within skeletal muscle is relatively low, where it is localized primarily to the muscle cell periphery.[7,8] Even so, changes in GLUT1 concentration and expression on the plasma membrane has been shown to correlate directly with glucose availability.[8] Therefore, high glucose concentration induces an increase in GLUT1 expression.

Although GLUT1 is present in skeletal muscles, the majority of glucose transporters present on the plasma membrane are GLUT4 and is hence responsible for the majority of glucose uptake.[7] In the resting state, glucose incorporation and metabolism are determined by GLUT4, which serves as the rate-limiting enzyme. Glucose entry via GLUT4 differs from GLUT1 in that it is activated through two mechanisms: insulin-stimulated or contraction-mediated.[10] Insulin is one of the two main hormones involved in maintaining glucose homeostasis. Insulin secretion occurs when blood glucose levels are elevated to stimulate glucose uptake in cells. Glucose uptake serves to lower blood glucose levels while supplying the muscle with the fuel it needs for future contractions.[10,14] Stimulation of GLUT4 via insulin or contractions triggers its translocation from intracellular vesicles to the plasma membrane, where it facilitates glucose entry. The importance of GLUT4 in glucose uptake is demonstrated in rodent models, where the

loss of the transporter is linked to insulin resistance, and its overexpression is shown to increase insulin sensitivity and glucose tolerance in both wild-type and diabetic mice.[11-13]

At times when ATP levels are sufficient, excess glucose can be stored as glycogen to reduce circulating glucose concentration. In fact, skeletal muscles account for the majority of glycogen stores in tissues.[10] The conversion of glucose to glycogen or glycogenesis is regulated by the enzyme glycogen synthase and concentration of glucose-6-phosphate (G6P) – the first intermediate in glycolysis.[4] Glycogen synthase is responsible for adding individual units of glucose to growing glycogen chains and, as such, serves as the main enzyme involved in glycogenesis. Its activity is directly dependent on G6P and insulin levels. Therefore, at times of elevated blood glucose, insulin release and increases in G6P concentration synergistically activate glycogen synthase and promote glycogenesis.[4]

Although some lipids can readily cross the hydrophobic plasma membrane, larger fatty acids (FA) must enter the muscle through fatty acid transporters. Within skeletal muscles, numerous transporters facilitate the entry of fatty acids, including fatty acid translocase (FAT/CD36), plasma membrane-associated fatty acid-binding protein (FABPpm), and fatty acid transport protein 1 and 4 (FATP1 and FATP4).[15] Fatty acids that enter skeletal muscles can undergo beta-oxidation to produce substrates for the TCA cycle or accumulate within the muscle and form intramyocellular lipids (IMCL). IMCL, which are found in higher numbers in type 1 muscle fibers, are fatty acid stores that serve as an intracellular supply of energy for prolong exercise.[16] Its role as skeletal muscle's energy supply is supported by IMCL depletion during prolonged submaximal exercise and enhanced IMCL content in trained individuals.[16-18] In addition to increasing levels of IMCL, endurance athletes adapt to have larger IMCL reserves

that are closer in proximity to their mitochondria.[19,20] It is hypothesized that this adaptation increases the accessibility and efficiency of mitochondrial fat use.

However, increases in IMCL also occur when plasma free fatty acid or dietary fat levels are elevated, indicating that fat storage in skeletal muscle also transpires when fat availability is high.[15] IMCL, in this case, are located more sporadically within the muscle fiber. In obesity, FA uptake is upregulated; however, there is no concomitant increase in fatty acid oxidation capacity.[21] In fact, many enzymes involved in fatty acid oxidation have been shown to be altered with obesity. Enzymes such as carnitine palmitoyltransferase I (CPT-1), β-hydroxyacyl-CoA dehydrogenase (β-HAD), citrate synthase, and cytochrome oxidase, which are involved in mitochondrial fatty acid translocation, β-oxidation, Krebs cycle, and ETC, respectively, have all displayed reduced activity.[21,22] Moreover, in those with type 2 diabetes and obesity, the overall activity of the electron transport chain in skeletal muscle has been shown to be reduced compared to healthy individuals. Even when mitochondrial content is taken into account the difference in ETC activity is still seen.[22]

It is hypothesized that since obesity is associated with increases in IMCL accumulation, this can result in a lipid burden on the mitochondria.[15,23] Due to the rise in FA load and rate of beta-oxidation, the subsequent increase in oxidative stress causes mitochondrial impairments that result in incomplete substrate breakdown. Evidence of mitochondrial impairments in obesity include reduced content and altered/deteriorated morphology, with the mitochondria appearing smaller and more swollen, which eventually results in mitophagy.[24-26] Incomplete degradation of FA occurs as the TCA cycle cannot keep up with lipid breakdown.[15,23] As a result, FA by-products such as acylcarnitine derivatives accumulate within the tissue, where it may play a role in insulin resistance, although the precise mechanism has yet to be elucidated.[21,22]

Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic disease mediated by the autoimmune destruction of insulin-producing beta cells (β-cells) of the pancreas. The autoimmune-mediated destruction of β-cells, and consequently, loss of insulin production, severely impairs the body's internal homeostatic mechanisms (feedback loops) that function to keep blood glucose concentrations within the euglycemic range (4-11mM).[27,28] As such, following a meal, with little to no insulin production and subsequent release, patients are left in a state of hyperglycemia and hypoinsulinemia that can become fatal if continuously left untreated. As a result, an exogenous source of insulin such as that from subcutaneous injections is required in order to restore euglycemia.[28] The use of exogenous insulin requires constant monitoring of glucose levels, and even when insulin therapy is tightly regulated, patients with T1D still experience daily bouts of periodic dysglycemia, leading to many diabetic complications later in their lives.

Brownlee's Theory of Diabetic Complications

Complications that can arise as a result of hyperglycemia include nephropathy – kidney failure, neuropathy – nerve damage, retinopathy – eye damage, and vasculopathy – vascular damage.[29] Brownlee proposes that these selective tissues and cells (for example, retina endothelial cells, renal glomerulus mesangial cells, peripheral neurons and Schwann cells) are affected and damaged in diabetes because they are unable to regulate glucose transport and, therefore, cannot adequately maintain appropriate intracellular glucose concentrations amid hyperglycemia.[29,30] High influx of glucose negatively alters cellular function through four proposed mechanisms: activation of the protein kinase C (PKC) pathway, increase flux through

the polyol and hexosamine pathways, and increase production of advanced glycation end products (AGE).[29]

During periods of hyperglycemia, the polyol pathway is responsible for converting glucose to sorbitol via the enzyme aldose reductase. Sorbitol is then oxidized to fructose. Conversion of glucose to sorbitol requires nicotinamide adenine dinucleotide phosphate (NADPH).[31] However, this is problematic as NADPH is an essential cofactor in the production of glutathione, an important intracellular antioxidant. Without glutathione available to reduce intracellular ROS, cells become more susceptible to oxidative damage. Therefore, with diabetes, increase flux through the polyol pathway leads to decreases in glutathione production, increasing reactive oxygen species within the cell, resulting in cellular damage.[29-31]

Hyperglycemia can also induce massive ROS production through the diacylglycerol (DAG) - protein kinase C (PKC) (DAG–PKC) signaling pathway in cells that are unable to prevent glucose influx.[30] Elevated glucose levels increase DAG synthesis, a critical activating cofactor of protein kinase C. PKC, which is involved in gene expression, increases the production of vasoconstrictor endothelin-1, transforming growth factor and plasminogen activator inhibitor-1, while decreasing vasodilator producing endothelial nitric oxide (NO) synthase (eNOS).[30,32] These changes in enzyme levels result in abnormal blood flow in those with diabetes. In addition to this, activation of PKC due to high blood glucose can also negatively modify diabetic vasculature by causing capillary and vascular occlusion, vascular permeability angiogenesis, and expression of pro-inflammatory genes.[30] Aside from its negative impact on blood vessels, PKC also plays a role in oxidative stress by stimulating NADPH oxidase to produce more reactive species.[29,30]

High ROS levels and intermediates of carbohydrate metabolism - such as glyceraldehyde-3-phosphate - enhance protein glycation during periods of hyperglycemia. Glycation is the non-enzymatic reduction of sugar resulting in the chemical modifications of lysine or arginine residues of proteins.[30] Further oxidation reactions result in the formation of advanced glycation end products (AGEs), an irreversible chemical modification that can either (1) form cross-links with other residues in the peptide or proteins, or (2) undergo additional chemical reactions to give rise to multiple different AGEs.[33] Since the discovery of glycated hemoglobin in diabetes, AGEs have been negatively implicated in diabetic complications, as increase accumulation of AGEs in body tissues can change enzymatic activity, reduce ligand binding, alter protein half-life and modify immunogenicity.[30]

Akin to glycation, increased flux through the hexosamine pathway during times of high glucose concentration can negatively affect tissues that cannot adequately maintain their intracellular glucose concentration through the alteration of enzyme structure and function. In the process of breaking down glucose, the glycolytic intermediate fructose-6-phosphate is diverted into the hexosamine pathway, where it is converted to glucosamine-6-phosphate.[30,34] Fructose-6-phosphate amidotransferase (GFAT) then converts glucosamine-6-phosphate into uridine diphosphate (UDP) N-acetyl glucosamine. [34] The resultant product, N-acetyl glucosamine can be conjugated onto proteins, altering protein function and, subsequently, gene expression. This will be discussed in more detail under the post-translational modification subheading on page 15.

The four pathways mentioned above are elevated in diabetes and are proposed to stem from decreased glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, which Brownlee hypothesizes as the unifying mechanism behind diabetic complications.[30] Brownlee articulates that increase ROS production in diabetes not only negatively affects protein and gene expression

but can also induce DNA strand breaks. DNA damage activates the DNA repair enzyme, poly (ADP-ribose) polymerase (PARP).[4] PARP produces ADP-ribose, which can accumulate in the cell during periods of hyperglycemia, leading to ADP-ribosylation of GAPDH.[4,30] This modification decreases GAPDH activity, and as a consequence, results in the accumulation of glycolytic intermediates.[30] Clearance of intermediates occurs through diversion into the upstream pathways discussed earlier (the polyol, PKC, AGEs and hexosamine pathway). As mentioned, and specified by Brownlee, activation of these pathways and increase AGEs result in diabetic complications commonly seen in tissues incapable of regulating their glucose influx.[30] However, based on Brownlee's theory, tissues capable of controlling glucose transport, such as skeletal muscles, should not be negatively affected by these pathways during periods of hyperglycemia.

Skeletal Muscle in Relation to Type 1 Diabetes

As previously mentioned**,** skeletal muscle is, by mass, the largest organ in the human body. As highly metabolic organs, skeletal muscles require an ample supply of energy. During bouts of elevated physical activity, insulin-independent glucose uptake in skeletal muscles can increase up to 50-fold in order to maintain the sufficient energy needed.[35] Postprandially, 75% of insulin-dependent glucose clearance occurs through skeletal muscle.[36] Given the skeletal muscle's extensive role in glucose homeostasis, it has been termed the largest glucose "sink" in the human body. Therefore, the health of the muscle is immensely crucial in maintaining glycemic control and the overall well-being of the individual. Unfortunately, studies have detected intramuscular differences in people with diabetes when compared to controls.[28,37-39]

Metabolically, lack of sufficient insulin results in reduced glucose uptake, and as such, cells shift substrate reliance to fatty acids in order to meet their energy demands.[1] Those with

diabetes also display impairments in muscle structure and function which includes reduced muscle size (atrophy) and strength as well as decreased maximal isometric force production. Increase muscle fatigability is also present in diabetes and is more prominent in those with poor glycemic control and longer duration of the disease.[28,37] At the ultrastructural level, patients with type 1 diabetes display an increase in the number and size of intermyofibrillar autophagic remnants, as well as reduced mitochondrial size, myofiber and myofibrillar diameter, and displaced A- and I- bands. [37, 39] And within the mitochondria of skeletal muscle, Monaco *et al* noted impairments in complex II and increases in ROS formation from complex III.[38]

The decline of muscle health and consequently, its metabolic capabilities diminish its ability to adapt to large fluctuations in glucose. Therefore, it will be interesting to see how glucose uptake, utilization, and storage are affected by these changes in muscles. Since insulin is responsible for stimulating glucose uptake and glycogenesis, it is hypothesized that individuals with T1D will have reduced levels of glucose entry and, as a result, will have a lower glycogen content within their skeletal muscle than healthy individuals. Moreover, as glucose uptake may be limited, in order to meet the energy demands of the muscle, an alternative energy source such as lipids is required.[40] Therefore, it will also be interesting to explore how IMCLs are being stored and utilized within skeletal muscles, especially in relation to their localization to mitochondria. Due to the increase reliance on lipids, it is hypothesized that IMCLs will lie more adjacent to the mitochondria so that they are readily available for use.

Dyslipidemia and Substrate Overload

In addition to dysglycemia, patients with T1D have been shown to exhibit dyslipidemia.[42] Since cells are unable to meet their energy demands from glucose due to the absence of insulin-stimulated uptake, they must find an alternate energy source such as plasma free fatty acids.[40] As such, adipose cells undergo lipolysis resulting in the production and release of free fatty acids into the circulation.[41] Markers of dyslipidemia include hypertriglyceridemia and decreased HDL levels, which are commonly present in poorly controlled T1D cases.[42] Recurring episodes of dysglycemia and dyslipidemia over prolonged periods can lead to the development of many diabetic complications.[44] We hypothesize that if the body is in a dyslipidemic and dysglycemic state when insulin is administered, it will result in excessive glucose entry on top of the already present high intramyocellular lipid within the muscle due to dyslipidemia, and in turn, can cause substrate overload. Substrate overload in muscle has yet to be proven; however, it is expected that it will result in an increase in ROS production via the mitochondria, NO synthase and NADPH oxidase.[45-48] As the mitochondria adapt to increases in substrates by increasing their oxidation, a larger number of electrons are leaked from the electron transport chain (ETC), resulting in elevated ROS formation and oxidative stress.[49] Furthermore, this increase load on the mitochondria can lead to mitochondrial dysfunction, further exacerbating oxidative stress and affecting the mitochondria's ability to carry out its function. Increases in oxidative stress leads to oxidative damage of cellular proteins and DNA, which in turn indirectly alters GAPDH activity.[30] This results in the activation of subsequent pathways involved in diabetic complications. A schematic representation of substrate overload can be seen in steps 1-6 of Figure 1, which is presented in the "Post-translational modification in diabetes" section on page 21.

Post-Translational Modifications

Post-translational modifications (PTMs) are chemical alterations made to proteins following their biosynthesis. These modifications occur through enzymatic or non-enzymatic attachments of specific chemical groups to amino acid side chains, resulting in altered protein structure and function.[51] These conformational changes affect metabolic pathways by increasing/decreasing the catalytic activity of enzymes, causing protein misfolding and/or aggregation, and recruiting other protein binding partners.[50] In addition to this, PTMs are involved in regulating protein expression through epigenetic changes.[34,51] As such, PTMs are a widespread mechanism that are used to regulate numerous cellular pathways.[52] The diverse functions of PTMs are reflected in the myriad of chemical modifications that proteins can undergo, including glycosylation, acetylation, succinylation, and ubiquitination.

Glycosylation

Glycosylation is the enzymatic addition of sugar moieties. Of specific interest to this study is the modification of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) onto serine and threonine residues of proteins.[53] This reaction is catalyzed by the enzyme *O*-GlcNAc transferase (OGT).[53] OGT uses uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), an end product of the hexosamine pathway, as an energy source and substrate to transfer the GlcNAc moiety onto target proteins. O-GlcNAcylation of proteins has been implicated in regulating transcription and translation, as well as protein degradation.[53] Furthermore, this PTM has been demonstrated to have a role in signal transduction and apoptosis.[53] Given its vast role in the regulation of numerous pathways, it would follow that *O*-GlcNAcylation occurs in all subcellular locations,

including the nucleus, cytosol, and mitochondria. As previously discussed, high levels of glucose in cells unable to regulate glucose influx cause increase flux through the hexosamine pathway, resulting in a greater production of UDP-GlcNAc.[30] This leads to more N-acetyl glucosamine modification of proteins, such as transcription factor Sp1. As a transcription factor, Sp1 increases the expression of two enzymes – transforming growth factor- β1 and plasminogen activator inhibitor-1.[9,30,54] Activation of these enzymes causes vasculopathy in patients, a known complication in T1D. Increase *O*-GlcNAcylation is also linked to reduced glucose-stimulated insulin release, and in certain rodent models, insulin resistance.[54] We postulate that diabetic hyperglycemia activates the hexosamine pathway and leads to altered protein structure and function as described above within skeletal muscles when insulin is present.

Acetylation

Acylation is the addition of acyl groups to proteins. One of the most common and widely studied form of acylation is acetylation. Acetylation can modify around 80-90% of eukaryotic proteins through the addition of an acetyl-group.[52,55] Proteins that are targeted for acetylation include transcription factors, receptors, and metabolic enzymes. As follows, acetylation regulates numerous processes such as epigenetics, DNA transcription, and other immunological and metabolic pathways.[55] Lysine acetylation, in particular, is involved in regulating energy storage and expenditure.[56] This regulation occurs through the reversible addition of an acetyl group from acetyl-CoA to the lysine residues of proteins. The addition of the acetyl moiety abolishes the positive charge that was previously present, resulting in a neutral residue which in turn affects protein abundance, activity, or availability to substrates in either a positive or negative manner.[57] Protein acetylation occurs via the enzymatic help of lysine acetyltransferases (KAT). On the

other hand, the removal of the moiety occurs through lysine deacetylases (KDAC), such as nicotinamide adenine dinucleotide (NAD+) dependent protein sirtuins (SIRT), specifically SIRT1 and SIRT3.[56] In diabetes, protein acetylation has been shown to alter metabolic enzymes within the kidneys. In a study conducted by Kosanam *et al*, gene ontology analysis has revealed that three glycolytic enzymes and six out of the eight TCA cycle proteins are differentially acetylated in rat kidneys injected with streptozotocin (STZ).[57] The modified glycolytic proteins include pyruvate carboxylase, GAPDH, and triosephosphate isomerase. In the TCA cycle, citrate synthase, malate dehydrogenase (MDH), isocitrate dehydrogenase, fumarate hydratase, *cis*-aconitase, and succinate dehydrogenase are differentially acetylated.[57] Although the effects of acetylation on these enzymes are only partially known, modification of MDH has been shown to increase its activity in liver cells. In contrast, acetylation of GAPDH prompts its translocation to the nucleus, preventing substrate interaction within cells.[58,59]

Succinylation

Succinylation, like acetylation, is an acyl post-translational modification. It is the reversible addition of a succinyl group to lysine residues of proteins.[60] The addition of the acidic succinate group to lysine residues imparts a negative charge on the previously positively charged lysine, disrupting any ionic interactions with other negatively charged molecules.[60] Taken together with the change in charge, the bulky nature of succinate (approximately 100Da) can substantially influence protein structure and function.[61] Succinylation of proteins are more prevalent in the mitochondria and has been implicated in regulating carbamoyl phosphate synthetase I (CPS1), pyruvate dehydrogenase (PDH), succinate dehydrogenase (SDH), and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2).[62-65] Currently, there is no known

mitochondrial enzyme that mediates the transfer of succinyl-CoA to lysine and hence performs the lysine succinyl-transferase activity. Therefore, succinylation most likely occurs non-enzymatically, indicating that its reaction rate depends on the abundance of succinyl-CoA, pH, and other protein parameters.[66,67] In favour of this idea, the inherently high pH within the mitochondrial matrix along with the abundance of succinyl intermediate may facilitate the spontaneous, non-enzymatic succinylation of proteins, and hence corresponds to the high levels of succinylated mitochondrial proteins.[67] Although there is no known modifying enzyme for succinylation, it, like acetylation, is also regulated by the NAD+ dependent family of silent information regulators, sirtuins, and in this case, SIRT5. SIRT5 is a desuccinylase that is localized primarily to the mitochondria.[63,68] Desuccinylation of proteins can have opposite effects depending on the enzyme modified. In mouse embryonic fibroblasts (MEF) and human embryonic kidney (HEK) cells, studies have demonstrated inhibition of PDH and SDH activity upon desuccinylation.[60] On the other hand, within the liver and skeletal muscle, SIRT5 activity is shown to activate enzymes involved in regulating fatty acid oxidation and ketogenesis.[69,70] Therefore, it is believed that SIRT5 regulation favours fatty acid oxidation over pyruvate usage and as such, plays a role in mitochondrial substrate preference. Accordingly, in SIRT5$^{-/-}$ mice model, loss of SIRT5 regulation and, in turn, increase succinylation results in metabolic impairments with mice displaying diminished liver fatty acid oxidation in the fasted state.[70]

Ubiquitination

In addition to altering protein structure and function, as described above, PTMs are involved in signaling protein degradation through the ubiquitin-proteasome pathway.[4] Ubiquitin is a highly conserved post-translational modification. But unlike the PTMs mentioned above,

ubiquitin is a short polypeptide sequence that is 76 amino acids long.[4] The process of protein degradation via the ubiquitin-proteasome pathway involves three enzymes, E1, E2, and E3, which facilitate the activation, transfer, and ligation of ubiquitin onto proteins. The ubiquitination of proteins occurs at the N-terminal lysine of targeted proteins. Ubiquitin marked proteins are then subject to degradation via the 26S proteasome.[50,71] Protein turnover is essential in maintaining normal cellular functioning and protein metabolism. However, impairments in this pathway may result in abnormal turnover rates. The inability to properly mark specific proteins for degradation can lead to toxic protein accumulation and result in a disease state.[54] Diseases that are associated with the buildup of proteins include Alzheimer's and Parkinson's disease, both of which have had detrimental effects on the individual's well-being and demonstrates the importance of maintaining proteostasis.[71,72] Therefore, PTMs play a vital role in maintaining cellular activity; not only is it involved in regulating protein levels but it also determines the protein's structure and ability to carry out its function.

Post-Translational Modifications in Diabetes

Although the PTMs present in diabetes have been described in the earlier sections of this literature review, it is important to summarize and highlight their relation to diabetes and its associated complications, all of which are presented in Figure 1. Physiologically, during periods of increased substrate availability, proteins can be non-enzymatically modified with the abundant substrate. Additionally, a modifying enzyme can be activated in response to elevated substrate concentration.[52] In diabetes, both processes occur. Hyperglycemia-induced ROS elevation results in the nonenzymatic glycation of proteins and enzymatic activation of PARP and OGT.[45,73] Activation of the enzymes, PARP and OGT result in increased ADP-ribosylation of GAPDH and

N-acetyl glucosamine glycosylation of proteins, respectively.[45,48, 73-74] These chemical alterations negatively affect the health outcomes of diabetic patients by causing nephropathy, neuropathy, retinopathy, cardiomyopathy, and vasculopathy.[30]

Aside from the modifications mentioned in the section above, PTMs remain largely unexplored in diabetes. In particular, PTMs caused by insulin therapy and resulting substrate overload in skeletal muscles has yet to be explored. As previously mentioned, Monaco *et al* has seen a decrease in complex II (succinate dehydrogenase) activity in those with T1D.[38] This decrease in complex II activity can result in the accumulation of TCA intermediates such as succinyl- and acetyl-CoA within the mitochondria, which in addition to the low pH within the matrix may prompt the succinylation and acetylation of mitochondrial proteins.[67] As such, it is hypothesized that the succinylome and acetylome profiles of those with diabetes will differ compared to controls. Additionally, since modification via succinylation, acetylation, and ubiquitination all transpire on the lysine residues of proteins, acyl modification may prevent proteins from being tagged and degraded. This can explain the increase in autophagic remnants that is unaccompanied by a change in the level of autophagic proteins seen in diabetic muscles by Monaco *et al*, as prevention of degradation results in increased debris accumulation.[38] Ergo, we hypothesize that the increase in autophagic remnants is due to impairments caused by acyl modifications of proteins rather than impairments in the autophagic proteins themselves. The changes in mitochondrial complex function and subsequent changes in PTMs discussed are visually represented in steps 7-9 of Figure 1.

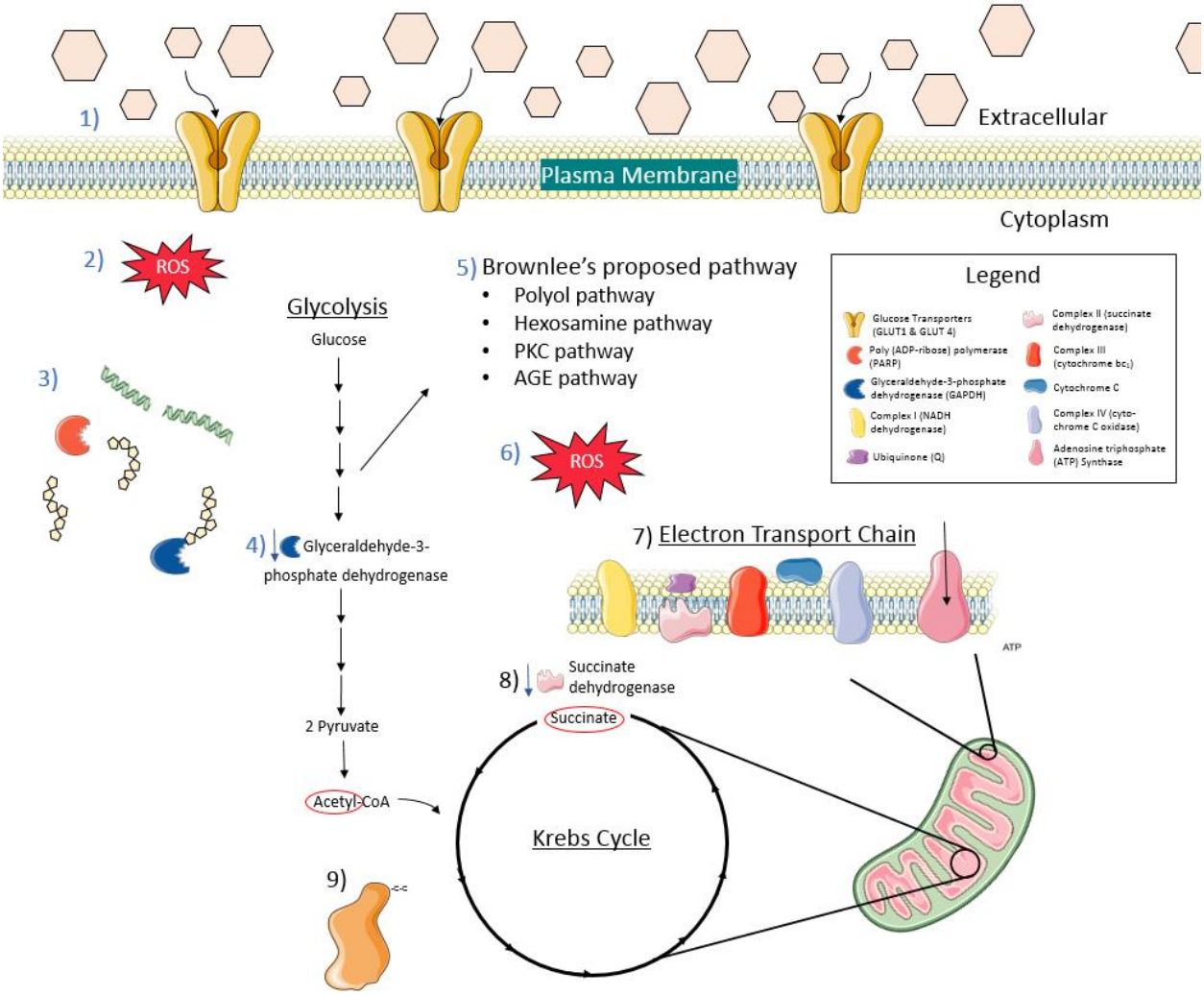

Figure 1: Proposed metabolic effects of hyperglycemia in skeletal muscle. 1) Hyperglycemia and insulin treatment induces glucose influx in skeletal muscle. 2) Increased intracellular glucose concentration results in increase reactive oxygen species (ROS) production as cells increase respiration. 3) Elevated levels of ROS cause deoxyribonucleic acid (DNA) strand break and activation of poly (ADP-ribose) polymerase (PARP). 4) In the process of DNA repair, PARP produces ADP–ribose, which can then be modified onto glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby decreasing its activity. 5) Decreased GAPDH activity results in increased glycolytic intermediates that are diverted into upstream (Brownlee's proposed) pathways. 6) Increased influx in the polyol, hexosamine, protein kinase C (PKC), and advanced glycation endproduct (AGE) pathways exacerbate oxidative stress within the cell. 7) Oxidative stress negatively alters mitochondrial function and, consequently, the enzymatic activity of enzymes within the mitochondria, such as complex II (succinate dehydrogenase). 8) Decreased mitochondrial enzyme activity causes a buildup of tricarboxylic acid (TCA) intermediates that can be 9) modified onto nearby proteins, further altering enzyme activity within the mitochondria. Steps 1-6, highlighted in blue, are mentioned in Brownlee's theory of diabetic complication in relation to cells that are unable to regulate their intracellular glucose concentration.

Lipofuscin

Lipofuscin is a fluorescent pigment generated due to the incomplete lysosomal degradation of lipids, proteins, and carbohydrates. They are produced as a result of oxidative stress. The buildup of oxidative stress oxidizes macromolecules, and as a consequence, during degradation, they become immune to lysosomal hydrolysis, making them resistant to degradation via autophagy, lysosomal enzymes, and proteasomal systems.[75] Thus, preventing further breakdown and resulting in the accumulation of incompletely degraded products within lysosomal compartments of postmitotic cells such as neurons and skeletal muscles.[75,76] Since the mitochondria is a known ROS manufacturer; it is hypothesized to play a role in lipofuscin formation. The "mitochondrial-lysosomal axis theory of aging" assumes that lipofuscin aggregates are composed of damaged mitochondria that are not completely broken down.[76] This results in a dangerous cycle that further exacerbates lipofuscin formation whereby lysosomal dysfunction alters mitochondrial structure and function, which in turn causes more lysosomal impairments due to increase mitochondrial ROS.[76,77]

Although compelling, there is currently limited direct experimental evidence on the "mitochondrial-lysosomal axis theory of aging," however, a study by Sohal *et al.* has demonstrated the association of lipofuscin in times of oxidative stress.[78] In this study, rat cardiac myocytes and human glial cells given pro-oxidants display an increased rate of lipofuscin accumulation while those given antioxidants show a reduced rate of accumulation, providing evidence of ROS's role in lipofuscin formation. And thereby, also alludes to lipofuscin as a potential marker for oxidative stress. Likewise, due to its accumulation with increasing age in postmitotic cells, it is also considered a hallmark of aging.[75] Because lipofuscin is known to be correlated with age, it has been hypothesized to be pathological in nature. Evidence of this are

studies that have shown large lipofuscin accumulation in degenerative diseases as a result of impairments in lysosomal function.[78] In STZ mouse models of diabetes, increase lipofuscin accumulation is present in the trigeminal neurons, subgranular zone, and granular cell layer. The STZ treated mice also exhibits phenotypes similar to diabetic encephalopathy with reduce hippocampus proliferation and mild neurodegeneration of the dentate gyrus.[79-81]

Considering the potentially harmful nature of lipofuscin, the accumulation of ROS, and subsequently lipofuscin in aging, it poses a unique question on how metabolic disorders may affect lipofuscin accumulation. Specifically, whether T1D, a metabolic disorder that has been shown to increase ROS production, as well as contain impairments in degradation, will result in any changes to lipofuscin within the muscle. Additionally, as substrate overload increases mitochondrial burden and further exacerbates lipofuscin pathogenesis due to elevated ROS production, it will be interesting to examine the effects of lipofuscin on the overall health of the individual's muscle as they age. These experiments will be crucial in understanding diabetes and the influence of skeletal muscle health on diabetes treatment.

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The impact of Type 1 Diabetes on skeletal muscle fuel substrate storage and ultrastructure in rodents and adult humans

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Abstract

Type 1 diabetes (T1D) is the result of the autoimmune-mediated destruction of the pancreatic beta-cells leading to the inability to produce insulin sufficiently and, in turn, regulate blood glucose levels. Abnormal levels of blood glucose, specifically hyperglycemia, have been linked to many diabetic complications, with Brownlee proposing decreased GAPDH activity and the resultant increase in four main pathways as the mechanism(s) leading to these complications. Though skeletal muscles play a major role in glucose uptake, they are believed to be relatively protected against these complications as they are able to regulate their glucose uptake. However, evidence is accumulating that skeletal muscles are adversely affected in T1D, particularly with respect to their mitochondrial function. This led us to consider that the skeletal muscles of those with T1D would experience substrate overload (high intracellular lipids and recurrent, high levels of intracellular glucose), which would initiate a negative spiral whereby substrate excess would damage mitochondria - leading to an impaired ability to utilize these substrates - further worsening the substrate overload. Therefore, the objective of this study was to investigate glycogen and intramyocellular lipid (IMCL) content in the muscles of mice and humans with T1D, as well as the potential downstream effects in the form of post-translational modifications (PTMs), mitochondrial content, and lipofuscin accumulation. The Akita T1D mouse model was used to assess substrate overload in uncontrolled diabetes, whereas human participants were used to investigate substrate overload in the presence of insulin therapy. Assessment of glycogen and IMCL content revealed no difference between controls and diabetic cohorts in both the rodent and human study, indicating the lack of substrate overload. Post-translational modifications did not significantly change between Akita and wild-type mice; however, there was a main effect of diabetes on acetylation levels within Akita mice. Lastly, most mitochondrial properties, except

for subsarcolemmal pixel density, did not differ either between diabetic and non-diabetic subjects in the human study. Thus, despite mitochondrial complex impairments in diabetic subjects, its extent was not significant enough to cause alterations to the mitochondria as a whole and result in mitochondrial degradation and lipofuscin formation.

This study has provided novel insight into the metabolic properties of skeletal muscle during diabetes. Although there was no indication of substrate overload, diabetes still resulted in some changes to PTM levels and mitochondrial pixel density. However, the effects of these changes did not significantly alter the muscle and resulted in pathway impairments of those that were studied. This could be due to an adaptive mechanism in mice, although future studies are needed to confirm this hypothesis. In the human study, healthy, well-controlled individuals could explain why there was hardly any difference seen, suggesting that controlling glycemic levels was imperative in preventing diabetic complications in muscle.

Introduction

Type I Diabetes (T1D) is a chronic disease caused by the autoimmune-mediated destruction of pancreatic beta-cells, leading to insufficient insulin production.[1, 2] Lack of insulin results in abnormal fluctuations of blood glucose that, in the long term, may lead to detrimental complications such as vasculopathy, neuropathy, and cardiovascular disease.[3] These complications are hypothesized by Brownlee to arise due to diabetes-associated elevation in reactive oxygen species (ROS) within cells incapable of regulating their level of glucose entry.[3] Excess levels of ROS can lead to deoxyribonucleic acid (DNA damage), which results in the activation of poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme.[3,4] In the process of

DNA repair, PARP produces adenosine diphosphate (ADP)-ribose, which, in turn, can be post-translationally modified onto glyceraldehyde-3-phosphate dehydrogenase (GAPDH), decreasing its activity.[4] Decreased GAPDH activity results in the accumulation of glycolytic intermediates that are diverted and cleared through other upstream pathways, including the polyol, protein kinase C (PKC), hexosamine, and advanced glycation endproduct (AGE) pathway.[3,4] Increased flux through the polyol and PKC pathways further exacerbates cellular oxidative stress, while elevated activity in the hexosamine and AGE pathway increases protein modification, negatively affecting their function.[3]

Since T1D is a disorder affecting glucose concentration, it is vital to explore organs and pathways involved in regulating glucose when studying the disease. Such organs include skeletal muscle, which is not only the largest organ by mass but also contributes significantly to the clearance of blood glucose.[1, 5-6] Skeletal muscle is a highly metabolic organ and, as such, accounts for up to 80 percent of whole-body glucose clearance.[7] Glucose uptake in muscle occurs via glucose transporters, GLUT1 (non-insulin mediated) and GLUT4 (insulin mediated).[8,9] Cellular glucose can be stored in the form of glycogen or utilized for adenosine triphosphate (ATP) production in order to meet the muscle's metabolic demands. In addition to glucose, skeletal muscle can also utilize lipids as an energy substrate. Lipids can enter the cell via fatty acid transporters and, in excess, will accumulate to form intramyocellular lipids (IMCL) within the muscle.[10,11,13] Increased IMCL can have positive and negative effects on the individual depending on its location to mitochondria and the individuals' physical activity level.[10, 12-15]

In T1D, skeletal muscle has been shown to exhibit structural and functional differences. For example, skeletal muscle has been found to be reduced in size (atrophy), strength, and maximal isometric force production.[16,17] Evidence of displaced A- and I-bands, decreased

mitochondrial size, and reduced myofiber and myofibrillar diameter are also present in diabetic skeletal muscle.[17,18] Moreover, Monaco *et al*, has demonstrated that skeletal muscle exhibits increased autophagic remnants, decreased mitochondrial complex II activity, and increased mitochondrial complex III ROS production.[19] Metabolically, as T1D is a disease that affects cellular glucose uptake in specific cells, certain adaptations such as increasing utilization of lipids must also occur for the cell to produce sufficient energy and carry out its designated task.[20] Therefore, these structural and functional changes to skeletal muscle in diabetes bring to light the potential of exploring its effects on the way glucose and lipids are utilized and stored under different conditions, especially in the presence or absence of insulin.

Diabetes derived lack of insulin stimulation in skeletal muscle results in increased reliance on free fatty acids as a substrate for energy production. Lipolysis of adipose tissue and subsequent increase in circulating free fatty acids, however, can result in dyslipidemia on top of the already present dysglycemia.[21,22] In this circumstance, when insulin is administered, the high levels of lipids within the cell along with the high influx of glucose may result in "substrate overload," which may negatively affect the muscle fiber and tissue as a whole as it increases the metabolic burden on the cell.

It is hypothesized that "substrate overload" causes an increase in oxidative stress, which will result in various unfavourable outcomes, three of which are explored herein; increases in 1) ROS and glycosylation, 2) PTMs due to mitochondrial impairments, and 3) lipofuscin accumulation. Firstly, oxidative stress associated DNA strand break and activation of PARP will ultimately result in the activation of the four pathways Brownlee proposed to be involved in diabetic complications.[3,4] Thereby increasing protein modification such as glycosylation and glycation, as well as further exacerbating oxidative stress within the cell. Secondly, it is

hypothesized that increases in ROS and intracellular substrate concentration will lead to mitochondrial dysfunction as the mitochondria attempts to adapt to the increase load by increasing their respiration but ultimately ends up overwhelmed. The overwhelmed mitochondrial complexes will be adversely impaired and, in turn, increase ROS production. Decreases in mitochondrial function and, consequently, the tricarboxylic acid (TCA) cycle may result in the accumulation of acyl intermediates. These intermediates (succinyl- and acetyl-coA), in turn, can be modified onto the lysine residues of proteins. Acyl modification of proteins will not only alter protein activity but may also prevent their degradation, as the ubiquitination of proteins also occurs on the lysine residue and, as such, may no longer be able to take place. Accordingly, this decrease in protein degradation will result in the accumulation of autophagic remnants seen by Monaco et al.[19] Lastly, as deduced from the "mitochondrial-lysosomal axis theory of aging," increases in ROS will result in the accumulation of lipofuscin, a potentially pathological auto-fluorescent pigment formed by the incomplete lysosomal degradation of proteins, lipids, carbohydrates and damaged mitochondria.[23-25]

In conclusion, since T1D affects the cells' ability to uptake glucose, and since skeletal muscles are highly involved in the clearance of glucose, it is important to investigate skeletal muscle properties to determine their impact on the health outcome of those with T1D. Therefore, properties, including the utilization and storage of glucose and lipids under certain conditions, while taking into account the differences that occur when insulin is administered, are vital to explore to further our understanding of diabetes. Additionally, it is crucial to examine the effect of insulin-stimulated substrate overload on proteins, mitochondria, and degradation/autophagic pathways.

Methods

Animal Handling and Tissue Collection

Akita$^{+/-}$ (Ins2^{Akita}) mice were originally purchased from Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME, USA). The Akita mice strain is a model for type 1 diabetes in which a mutation in the insulin 2 gene results in incorrect protein folding and subsequent pancreatic toxicity. A breeding colony was established from wild-type and Akita$^{+/-}$ mice to give rise to successive generations featuring littermates of genotypes; Akita$^{+/-}$ and wild-type. Akita mice developed the diabetic phenotype around four weeks of age, with the males displaying more severe symptoms than females. Therefore, only male mice were used in this study. Wild-type and Akita mice were provided enrichment material and fed a chow diet (Research Diet, D1245K Rodent Diet: energy (kcal/g) from protein (20%), fat (10%), carbohydrate (70%)). Mice were given ad libitum access to water and food. Animals were housed at 21°C with 50% humidity and a 12h/12h light-dark cycle. Experiments were approved by the McMaster University Animal Research Ethics Board in accordance with the Canadian Council for Animal Care guidelines. At four weeks of age, mice underwent a glucose tolerance test, and those with blood glucose levels >14mM were determined diabetic (Akita$^{+/-}$), whereas those with glucose levels <14mM were considered wild-type (WT). At 11-14 weeks of age, WT and Akita mice were randomly assigned to fasted or fed groups prior to their sacrifice. The fasted mice were fasted for 12 hours before they were harvested. During the harvest, tissues including the heart, liver, quadriceps, tibialis anterior (TA), and gastrocnemius plantaris were collected, weighed, and stored at -80°C for future analyses. Prior to freezing, the TA was fixed in optimal cutting temperature (O.C.T) compound (Leica Biosystems, Richmond, IL).

Histochemical Staining – Periodic acid Schiff's (PAS) stain for glycogen content

TA cross-sections were cut 7μm thick and fixed with Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 10 minutes. Following fixation, sections were stained with 0.1% periodic acid (Sigma, St. Louis, MO, USA, 375810) in distilled water and subsequently, neat Schiff's reagent (Sigma, 3952016) for 10 minutes each. Slides were then washed under lukewarm running tap water for another 10 minutes before application of coverslip. Negative controls were first incubated with 0.5% α-amylase (Sigma, A6255) in distilled water for 10 minutes before fixation via Carnoy's solution. The following steps were all completed as described above. Imaging and analysis of sections were undertaken with the Nikon 90i microscope and Nikon NIS-Elements ND2 software (Melville, NY, USA). Muscle fibers were circled manually for analysis of density indicative of glycogen content. Glycogen content was determined as the average of the summed density relative to fiber area of the 100 fibers circled.

Histochemical Staining – Oil Red O (ORO) staining for intramyocellular lipid content

TA muscles were sectioned at 10μm and given time to air dry before incubation with 1.5 parts ORO (Sigma, O0625) in 1 part distilled water for 10 minutes. Sections were then washed under running tap water for 30 minutes. Scanning and analysis of sections were performed using a Nikon 90i microscope and Nikon NIS-Elements ND2 software (Melville, NY, USA). Muscle fibers were circled manually for subsequent analysis of lipid content. Lipid content was determined as the average density of 100 fibers over the total area circled.

Protein Characterization and Analysis – Western Blot

Muscle tissue was homogenized in lysis buffer, and protein concentration was determined using bicinchoninic acid assays (BCA). Western blots were completed using the standard procedure. Each well of a 12% SDS-PAGE gel was loaded with 20µL of lysate containing 20µg of proteins (final concentration of 1µg/µL). Only 10µL of lysate was loaded for the positive liver control. The running apparatus for the western blot was set to 100V for 20 minutes and increased to 120V for another 30 minutes. Gels were transferred to a PDVF membrane, with transfer conditions of 90 minutes at 90V. The transfer was completed at 4°C with a stir bar and ice pack placed within the apparatus. Upon completion of the transfer, membranes were stained with amido-black and imaged using the MBI Lab Equipment Fusion Fx7 (Kirkland, PQ, Canada). Membranes were then blocked with 5% bovine serum albumin (BSA) in 1X tris-buffered saline-tween (TBS-T) (20mM tris base, 150mM NaCl, and 0.1% (v/v) Tween-20). Primary antibodies (pan-lysine succinylation – 1:500, PTM Biolabs, Hangzhou, China, PTM-401; O-GlcNAc – 1:1000, Cell Signaling, Danvers, MA, USA, 9875; Acetyl lysine – 1:1000, Abcam, Cambridge, United Kingdom, ab80178; Ub (P4D1) – 1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-8017) were incubated overnight at 4°C along with constant rocking. On the next day, membranes were washed three times for 10 minutes in 1X TBS-T, after which the appropriate horseradish peroxidase secondary antibodies were applied at dilutions of 1:10, 000 (anti-mouse IgG HRP 7076S, anti-rabbit IgG HRP 7074S – cell signaling). Upon three 10 minute washes with 1X TBS-T, a chemiluminescent reagent (Biorad 1705061, Mississauga, ON, Canada) was used to visualize the bands which was exposed using the MBI Lab Equipment Fusion Fx7

(Kirkland, PQ, Canada). Band intensity was determined through Image J (National Institutes of Health, MD) as the area under the curve and adjusted to the amido band intensity at 47kDa.

Electron Microscopy Imaging

Muscle biopsies from the vastus lateralis of human participants were taken by Dr Tarnopolsky, and the samples were fixed in 4% paraformaldehyde and 1.5% glutaraldehyde. Muscle sections were placed on copper grids and contrasted with uranyl acetate and lead citrate. The JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) located at McMaster University was used to view and capture non-overlapping images of the prepared muscle sections. Images were captured at magnifications ranging from 10,000x to 15,000x and used for analysis. Analysis of images were performed with the Nikon NIS-Elements ND2 software (Melville, NY, USA) where mitochondria, IMCL, and lipofuscin were manually circled based on the criteria set in Supplementary Table 1. The mitochondrial or lipid area, content, and density, as well as lipofuscin count, were recorded, and their location within the fiber was taken into account (i.e. located within the intermyofibrillar (IMF) or subsarcolemmal (SS) region of the muscle). All data obtained were analyzed relative to the total fiber area seen of the muscle region. A total of 8 IMF and SS regions were analyzed for each participant, and the cumulative average result was taken for the analyzed property of interest.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software), and significance was determined via unpaired t-test or two-way ANOVA as appropriate. Potential significant differences between fasted or fed wild-type and Akita mice were determined using a

two-way ANOVA. Unpaired t-test was used to test significance between control and diabetic subjects in the human study. Outliers were determined via the robust regression and outlier removal (ROUT) method. For data that lacked a normal distribution, outliers were removed, and the Mann-Whitney test was then performed instead. Correlations were also performed on the participants within the study. Data was considered moderately correlated if $0.3 < r < 0.7$. The data were presented as the mean ± standard error of the mean (SEM). Data were considered significant (*) when the calculated P-value was <0.05.

Results:

Fasted mice displayed reduced levels of glycogen compared to fed mice in both control and Akita groups.

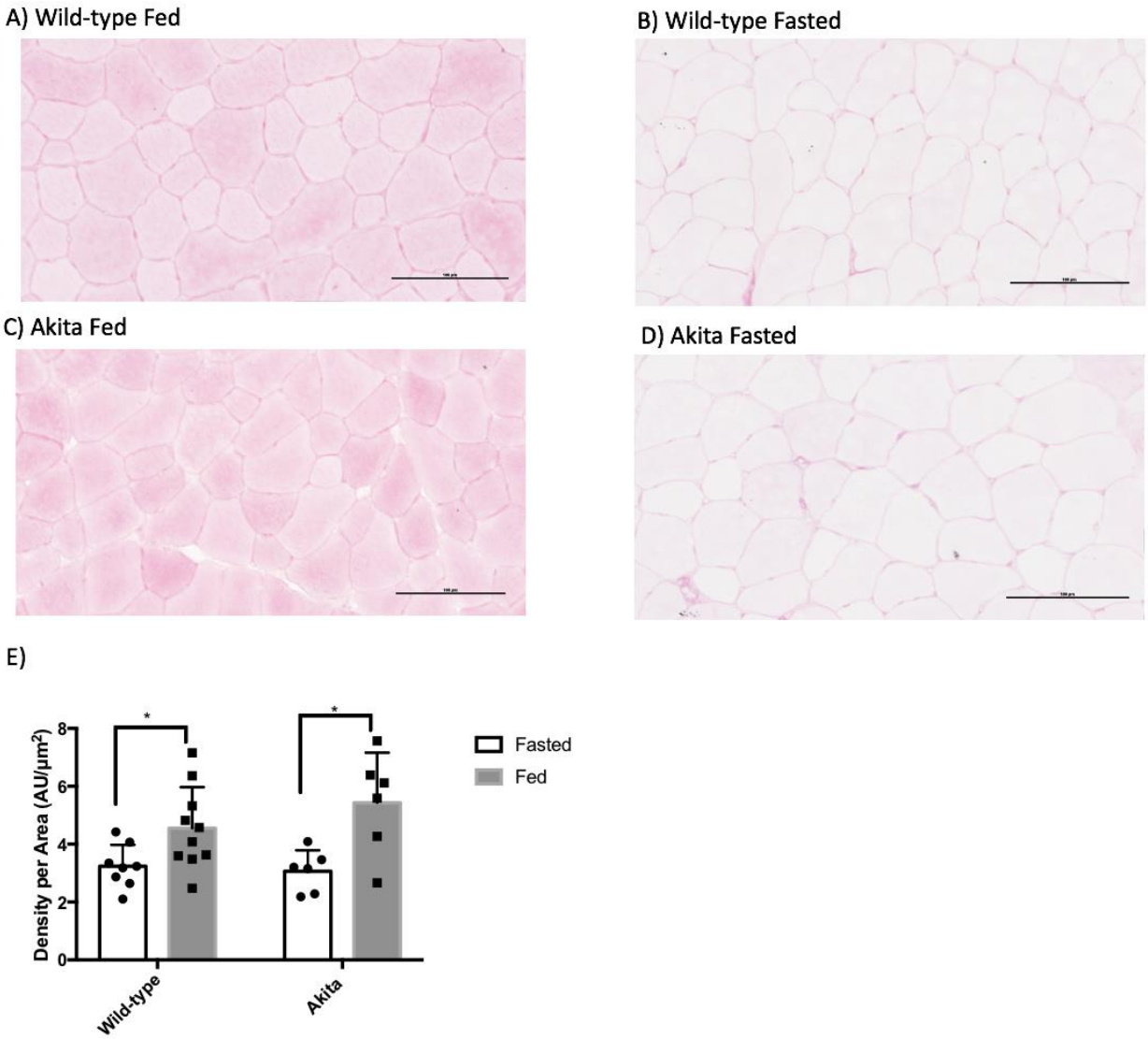


Figure 1: Periodic Acid- Schiff (PAS) Stain revealed shifts in glycogen content among different diet states but not between male wild-type and Akita models. Stain was completed on 8 wild-type fasted, and 10 wild-type fed mice. Akita fasted and fed groups with 6 mice each were also stained for glycogen content. Representative PAS stain of A) wild-type fed, B) wild-type fasted, C) Akita fed, and D) Akita fasted tibialis anterior. E) Fasted mice contained, on average lower levels of glycogen compared to their fed counterparts. Scale bars were set at 100µm (p-value > 0.05).

The PAS stain was completed to assess the level of glycogen within the muscle of diabetic mice and as a possible indicator of substrate overload. Eight and ten wild-types fasted and fed respectively, and 6 Akita fed and fasted mice' tibialis anterior were sectioned and stained. All mice were between 11-14 weeks of age. Thus, Akita mice experienced 8-10 weeks of uncontrolled diabetes at the time of harvest. A two-way ANOVA revealed that Akita fasted mice had a statistically lower average glycogen content compared to the Akita fed samples. The same significant trend was seen in wild-type fasted and fed samples, with fasted mice having a lower glycogen content. Analysis of the α-amylase treated sections indicated that there was no difference in the average density between samples (Supplementary Figure 1).

Lipid content in both the control and Akita groups were comparable and were independent of diet state.

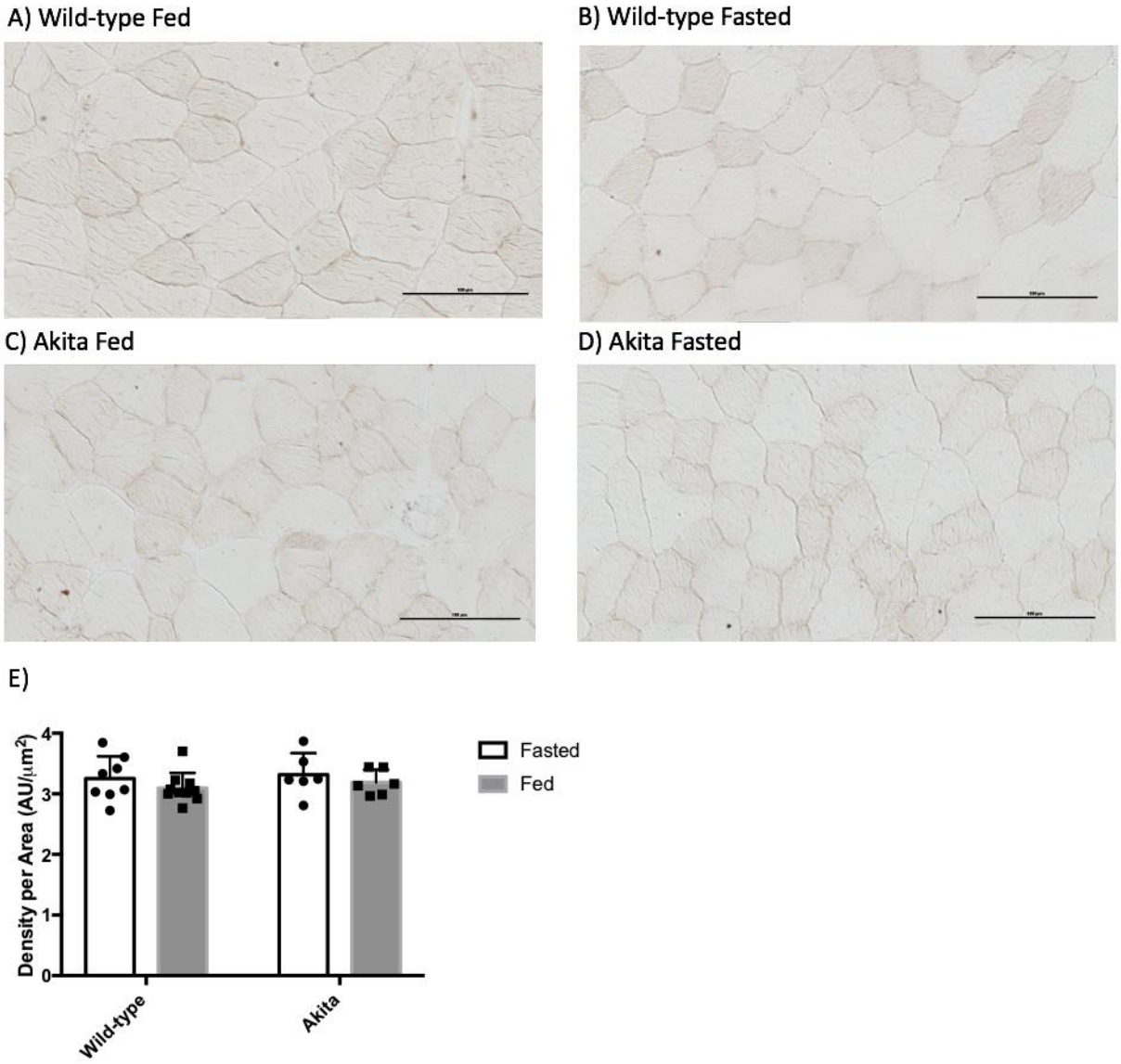


Figure 2: Absence of lipid content difference between male wild-type and Akita mice in both the fed and fasted state. Wild-type groups n = 8 (fasted) & 10 (fed), Akita group n = 6 (fasted & fed). Representative image of A) wild-type fed, B) wild-type fasted C) Akita fed, D) Akita fasted stained with Oil-Red O. E) Analysis of lipid content showed no difference between control and disease groups or diet states. Scale bars were set at 100μm (p-value > 0.05).

In addition to the PAS stain, Oil-red O was used to determine IMCL levels as a potential measure of substrate overload. Therefore, the same samples used for the PAS stain (i.e. 10 and 8

wild-type mice fed and fasted respectively, and 6 Akita fed and fasted mice) were also used to analyze lipid content. Analysis of 100 fibers per muscle section and subsequent performance of two-way ANOVA showed no difference in lipid content between the four different groups (p-value > 0.05).

Control and Akita mice did not exhibit any significant differences in post-translational modification.

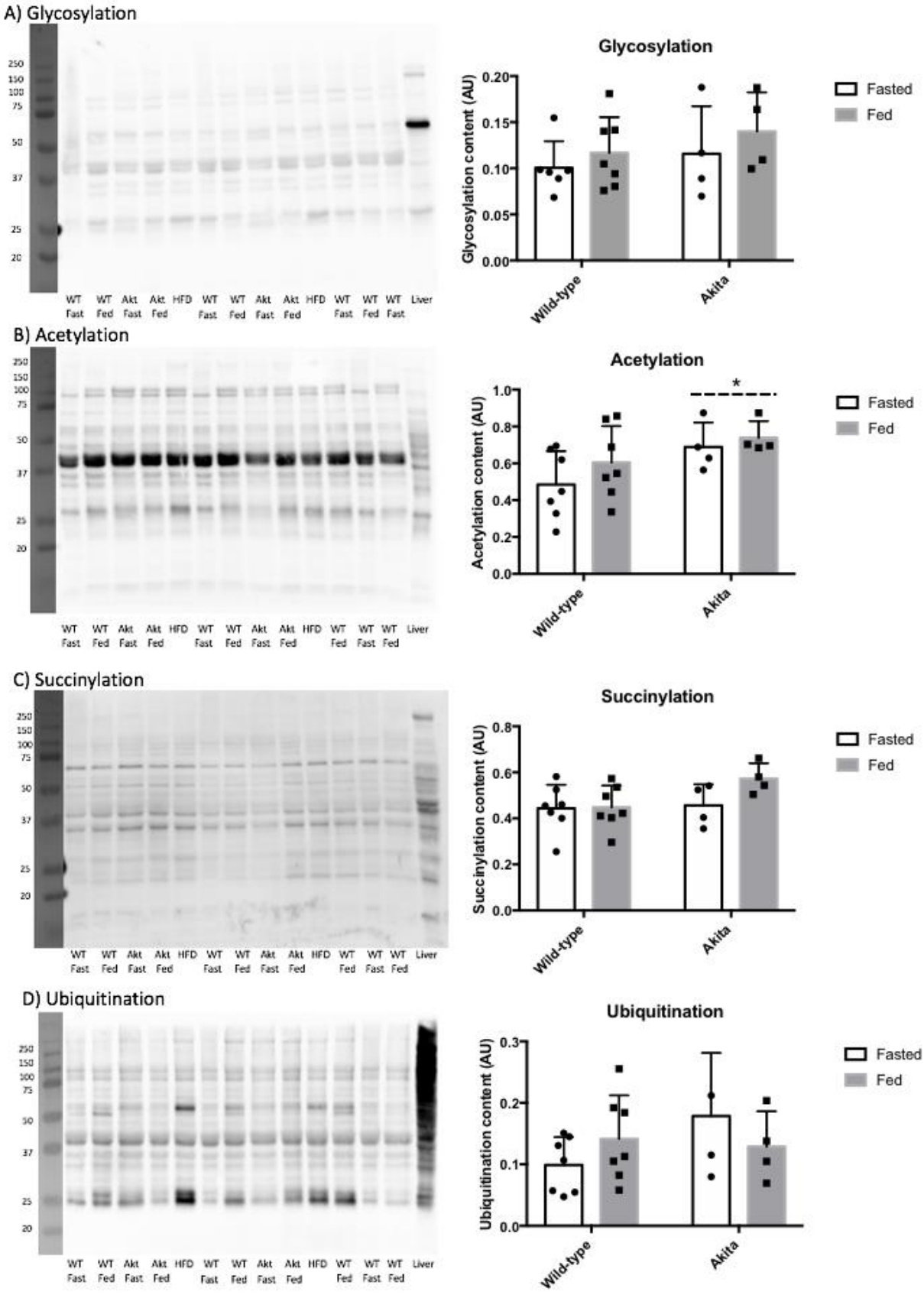

A) Glycosylation
250
150
100
75
50
37
25
20
WT Fast
WT Fed
Akt Fast
Akt Fed
HFD
WT Fast
WT Fed
Akt Fast
Akt Fed
HFD
WT Fast
WT Fed
WT Fast
Liver
Glycosylation
Glycosylation content (AU)
0.20
0.15
0.10
0.05
0.00
Wild-type
Akita
Fasted
Fed
B) Acetylation
Acetylation
Acetylation content (AU)
1.0
0.8
0.6
0.4
0.2
0.0
*
C) Succinylation
Succinylation
Succinylation content (AU)
0.8
0.6
0.4
0.2
0.0
D) Ubiquitination
Ubiquitination
Ubiquitination content (AU)
0.3
0.2
0.1
0.0

Figure 3: Post-translational modifications were not significantly altered between diets or control vs. type 1 diabetic (T1D) groups. Male groups examined included wild-type (WT) fasted (n = 7) or fed (n = 4-7); fed mice were further divided into groups provided chow (n = 7) or a high fat diet (HFD) (n = 4). Akita mice were either fasted (n =4) or fed a chow diet (n =4). Lysate containing 20µg of proteins were loaded at volumes of 20µL for muscle tissues and 10µL for liver samples. The liver served as a positive control to ensure proper detection of PTMs. It was expected that the HFD muscle would also serve as a positive control and thus both were omitted from any statistical analysis. Western blot and analysis of pan post-translational modifications of A) glycosylation, B) acetylation, C) succinylation, and D) ubiquitination revealed that levels of PTMs did not significantly differ between their respective groups. However, a main effect of diabetes on acetylation was present and is represented by the dashed horizontal bar (p-value < 0.05). Analysis of individual bands can be found in supplementary figure 2-5.

In order to determine whether hyperglycemia and potentially substrate overload affected PTM levels, immunoblotting for pan-ubiquitination, succinyl-lysine, acetyl-lysine, and O-GlcNAc was performed on the rodent samples. Groups analyzed included fed and fasted wild-type (n = 7) and Akita (n =4) mice. In addition to the groups mentioned, an experimental group including four wild-type mice fed a high-fat diet (HFD) for eight weeks was also included. Since the HFD group was only included to serve as a positive control and for the purpose of performing a two-way ANOVA, the HFD group was omitted from further statistical analysis. Statistical analysis via two-ANOVA revealed that there was no statistically significant difference in PTM between any of the groups tested. Diabetes was found to however, exhibit a main effect on the levels of acetylation when Akita fed and fasted groups were combined. Analysis of Individual bands can be found in supplementary figures 2-5.

The level of glycogen in T1D subjects were comparable to that of control subjects.

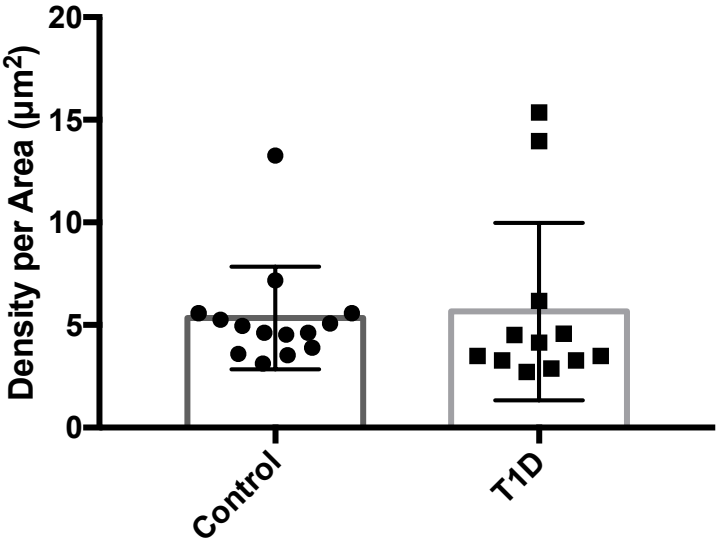
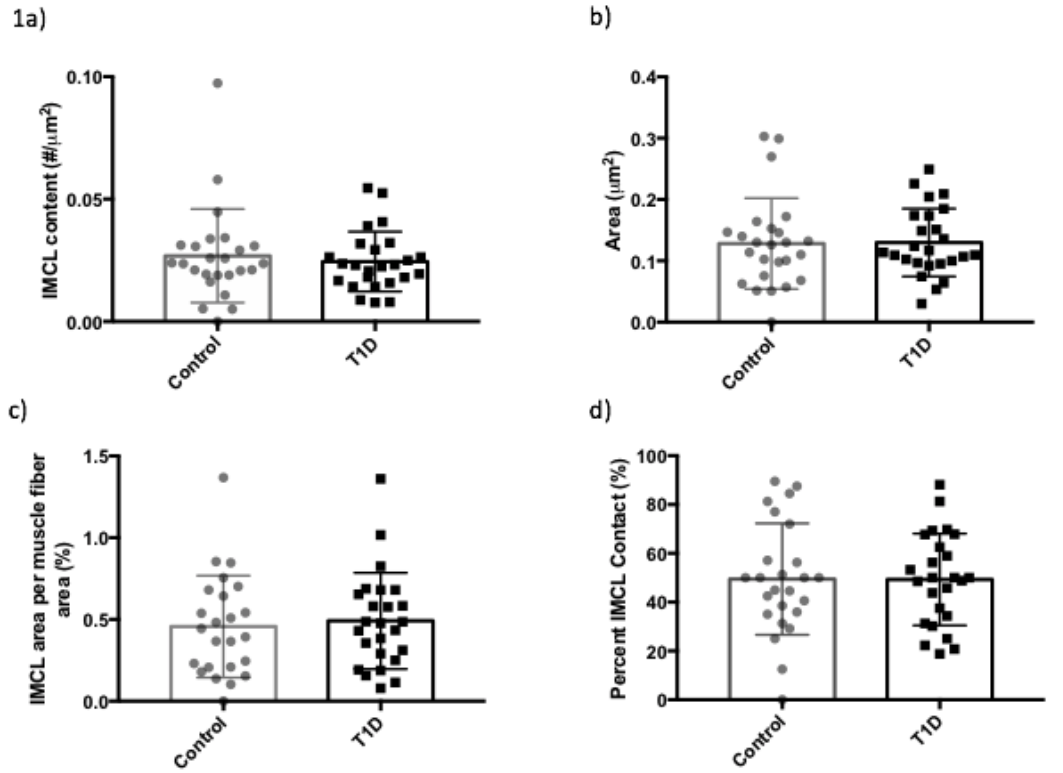


Figure 4: Insulin treated type 1 diabetic (T1D) subjects exhibited comparable levels of glycogen to control subjects. A Periodic Acid- Schiff (PAS) stain was performed on the vastus lateralis biopsy samples from 14 control and 12 type 1 diabetic participants. Computational analysis via NIS-elements AR analysis and independent t-test indicated that there was no significant difference in glycogen content between groups ($p > 0.05$).

The PAS stain was completed on the vastus lateralis muscle sections from human subjects in order to determine how glycogen content would differ with insulin treatment, as well as to examine differences in glycogen between those with controlled diabetes and non-diabetic individuals. Samples from a total of 14 control and 12 participants with diabetes were used in the experiment. Participants involved ranged from 30-80 years of age. An independent t-test revealed no significant difference in glycogen content between the control and T1D groups (Figure 4). Even when groups were split based on sex, both groups contained similar levels of glycogen.

IMCL did not differ significantly between control and T1D groups.



Subsarcolemmal Region

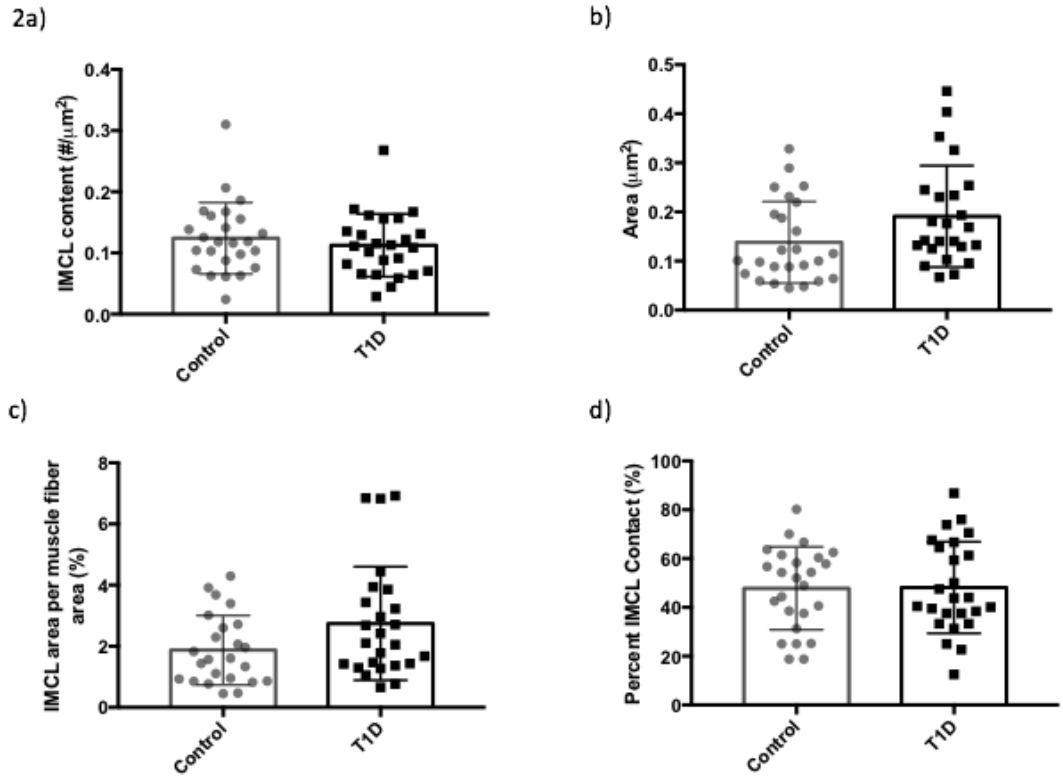


Figure 5: Type 1 diabetic (T1D) groups displayed intramyocellular lipid (IMCL) properties that closely resembled control groups. Electron transmission microscopy images of 25 control and 25 T1D participants were evaluated to determine IMCL properties. Images were taken at 12 000X – 15 000X and calibrated before analysis. Within 1) the IMF region, IMCL a) content, b) area, c) density, and d) percent mitochondrial contact did not differ significantly between groups. Likewise, 2) the SS region of the control and T1D groups did not differ in IMCL a) content, b) area, c) density, and d) percent mitochondrial contact either. ($p > 0.05$).

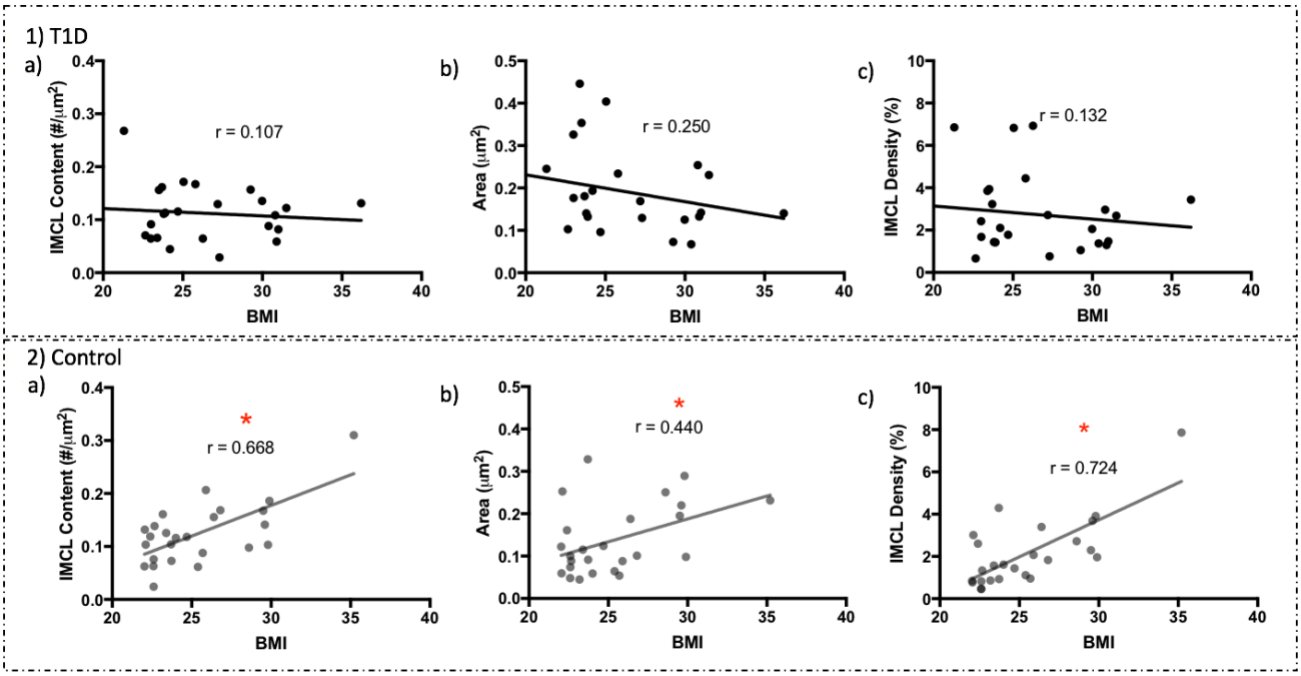


Figure 6: Intramyocellular lipids (IMCL) within the subsarcolemmal region of diabetic skeletal muscle were independent of body-mass index (BMI). 1)Diabetic participants (n = 25, black points) displayed an absence of correlation between a) IMCL content, b) size, and c) density despite increases in BMI. However, a positive correlation was present in 2) control matched subjects (n = 25, grey points) as increasing BMI was seen with increasing a) IMCL content, b) size, and c) density.

Images of 25 control and 25 T1D participants ranging from the ages of 18-80 were taken via electron microscopy and analyzed for IMCL properties such as area, content, density, and percent mitochondrial contact. For each participant, a total of 8 images per region were analyzed. An independent t-test revealed that there was no significant difference in IMCL content, size, density, and percent mitochondrial contact between groups within the IMF region (Figure 5).

Similar to the IMF region, there was no difference in the IMCL properties analyzed in the SS region. Furthermore, there was no significant difference when groups were split into their respective sex in either muscle regions. Additionally, in both the IMF and SS regions of those with diabetes, age, BMI, and duration of diabetes were not correlated with IMCL area, content, density, and percent mitochondrial contact. However, increases in IMCL content, size, and density were seen with increased BMI in the SS region of control matched subjects that were not seen in the diabetic participants (Figure 6).

Control and T1D groups displayed similar mitochondrial properties and lipofuscin levels.

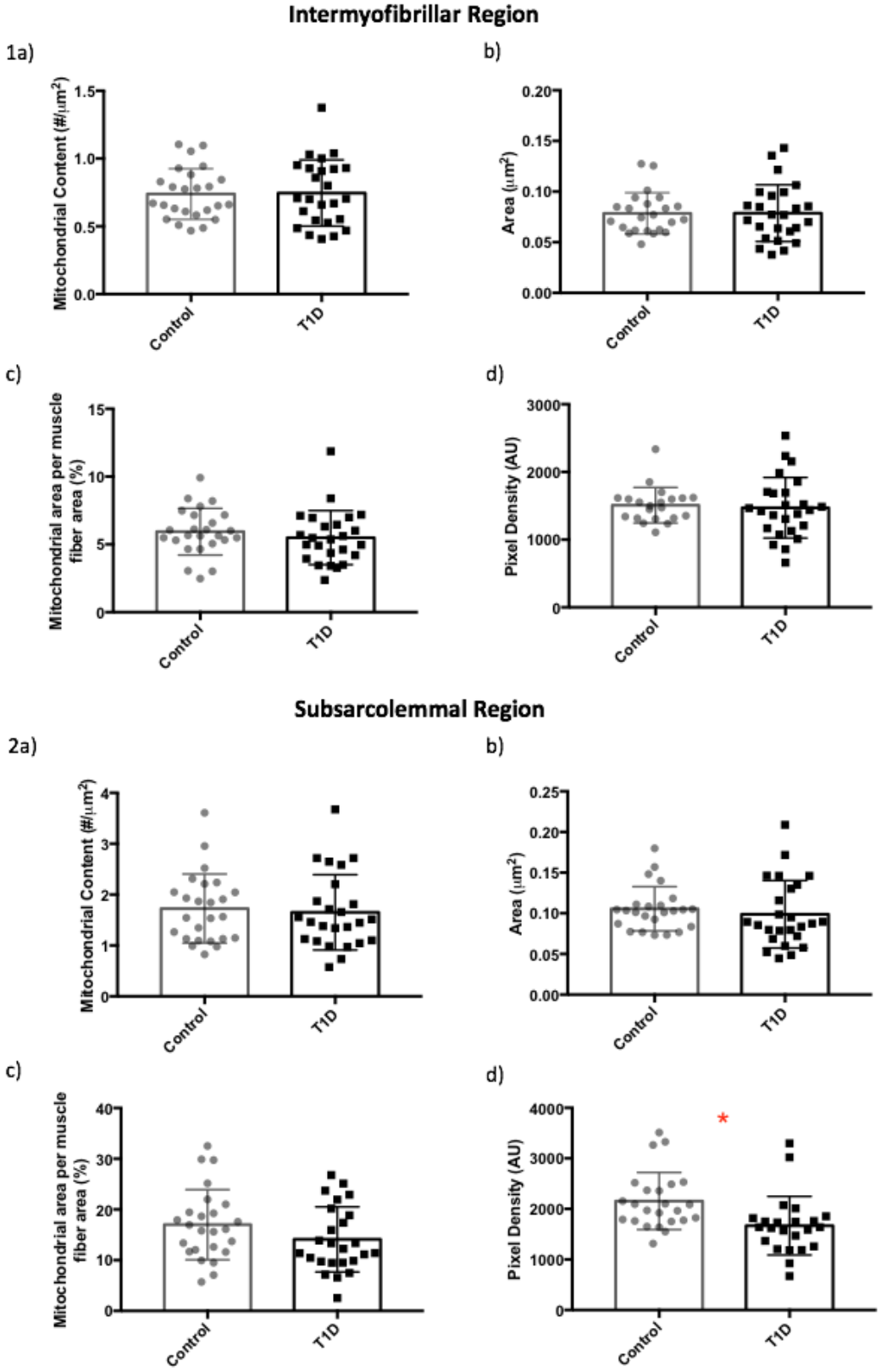


Figure 7: Most mitochondrial features were not different between control and type 1 diabetic (T1D) groups. Electron microscopy images from control group (n = 26) and T1D group (n = 26) were analyzed at 12 000X – 15 000X. 1) The intermyofibrillar (IMF) region of T1D subjects were comparable to controls groups in mitochondrial a) content, b) area, c) mitochondrial density, and e) pixel density. Both groups also displayed similar mitochondrial properties within 2) the subsarcolemmal (SS) region, as the mitochondrial a) content, b) area, and c) density of diabetic subjects did not differ from controls. However, the control group in the subsarcolemmal region displayed a higher e) pixel density than the diabetic group. ($p > 0.05$)

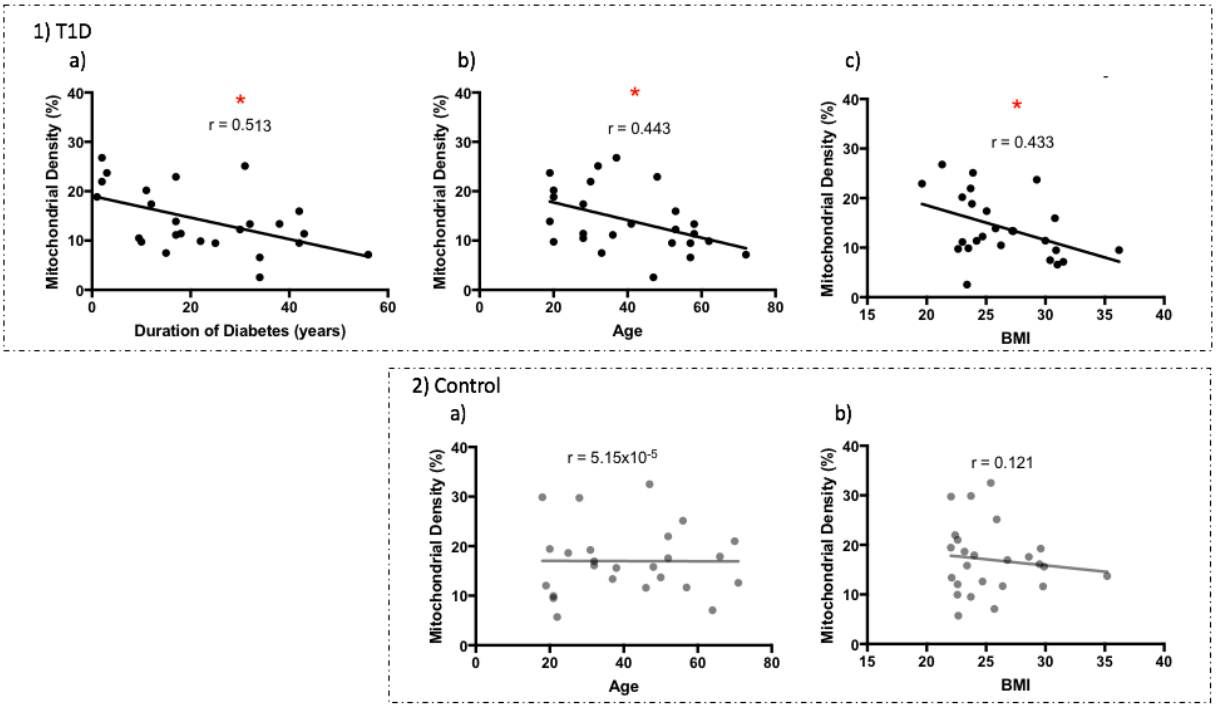


Figure 8: Diabetes negatively affected subsarcolemmal (SS) mitochondrial density. Moderate negative correlations are present between mitochondrial density and 1a) duration of diabetes, b) age, and c) BMI in those with diabetes (n = 25, black points) that are not present in the control sample. 2) Control matched subjects (n = 25, grey points) displayed variable mitochondrial density in relation to a) age, and b) BMI.

Akin to IMCL analysis, transmission electron microscopy images from 25 control and 25 T1D participants ranging from the ages of 18-80 were used for mitochondrial and lipofuscin analysis. With the exception of pixel density, the mitochondrial content, density, and average area did not differ between the two groups in both IMF and SS regions (Figure 6). Pixel density within the IMF region was comparable between control and diabetic groups, but within the SS region, pixel

density was found to be higher in the control group than the diabetic group. Moreover, when split between sexes, females differ in average pixel density, with the control group having a higher IMF pixel density than the T1D group. In the IMF region, age was moderately correlated with the mitochondrial size (Supplementary Figure 8). On the other hand, BMI, age, and duration of diabetes demonstrated a moderate negative correlation with SS mitochondrial density in those with T1D (Figure 8). Duration of diabetes was also found to be moderately correlated to SS mitochondrial content (Supplementary Figure 8). Analysis of lipofuscin revealed that it was present in subjects with T1D at levels comparable to controls (Figure 9). Additionally, both control and diabetic groups displayed increased levels of lipofuscin with increasing age (Figure 10).

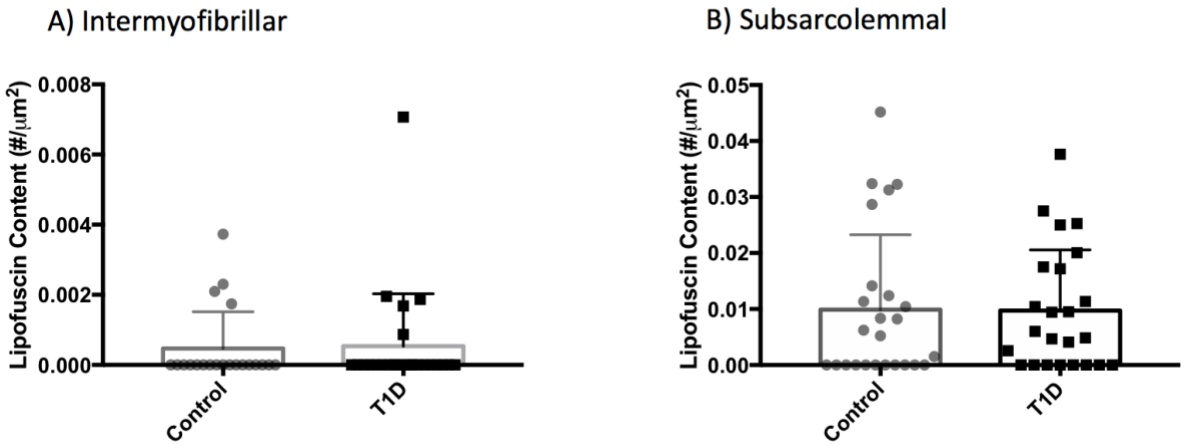


Figure 9: Accumulation of lipofuscin did not significantly differ between control and type 1 diabetic (T1D) groups. Control n = 25, T1D n = 25, Electron microscopy (EM) images were taken and analyzed at 12,000X- 15,000X. Lipofuscin levels were not statistically different among groups within the A) intermyofibrillar (IMF) or B) subsarcolemmal (SS) region. ($p > 0.05$).

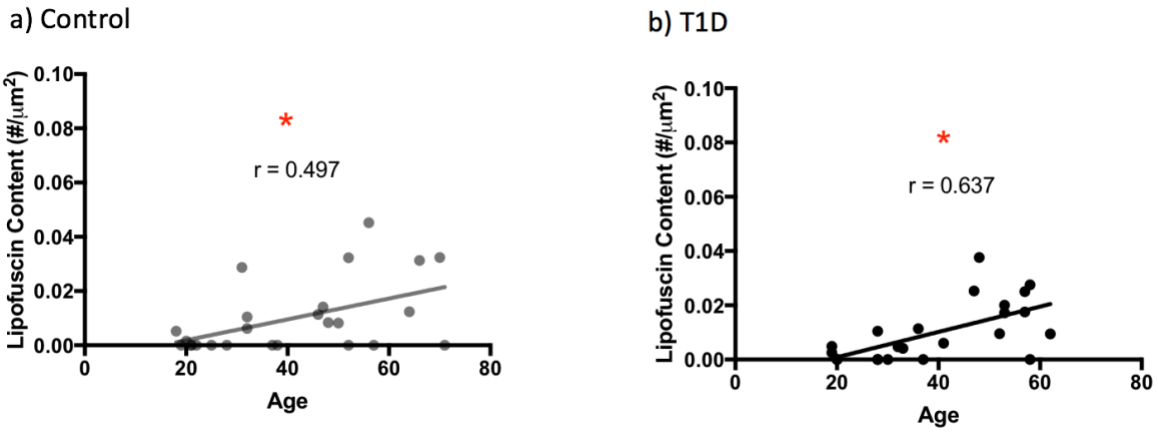


Figure 10: Levels of lipofuscin increased with increasing age. Lipofuscin levels demonstrated a positive linear relationship with increasing age in both the a) control (grey points) and b) diabetic groups (black points) composed of 25 well-matched participants each. ($p < 0.05$).

Discussion

The current experiments aimed to explore whether the diabetic environment modified substrate storage and posttranslational modifications in skeletal muscle in an effort to explain the impaired metabolism displayed in those with type 1 diabetes. As Brownlee mentioned, the high concentration of blood glucose in diabetes resulted in an increased influx of glucose into tissues that are unable to maintain their intracellular glucose concentration, resulting in diabetic complications.[3,4] However, with the administration of insulin, the high level of blood glucose would be driven into insulin-sensitive tissues, such as the muscle (Figure 11), and thus, the muscle would be expected to experience a rise in intracellular glucose concentration.[26,27] Elevated intracellular glucose had been implicated as the mechanism behind diabetic complications in other cell types.[4] Hence, it was imperative to explore whether this drastic drop in blood glucose and resulting expected increase of glucose within the muscle would adversely affect skeletal muscle as it does in other tissues. In extension of this, the study was undertaken to

determine whether recurrent increases in intracellular glucose could explain the skeletal muscle dysfunction previously demonstrated in those with Type 1 Diabetes.[19] Therefore, in order to examine this, the study was divided into two parts, the first of which was conducted in rodents and the second in human muscle biopsy samples.

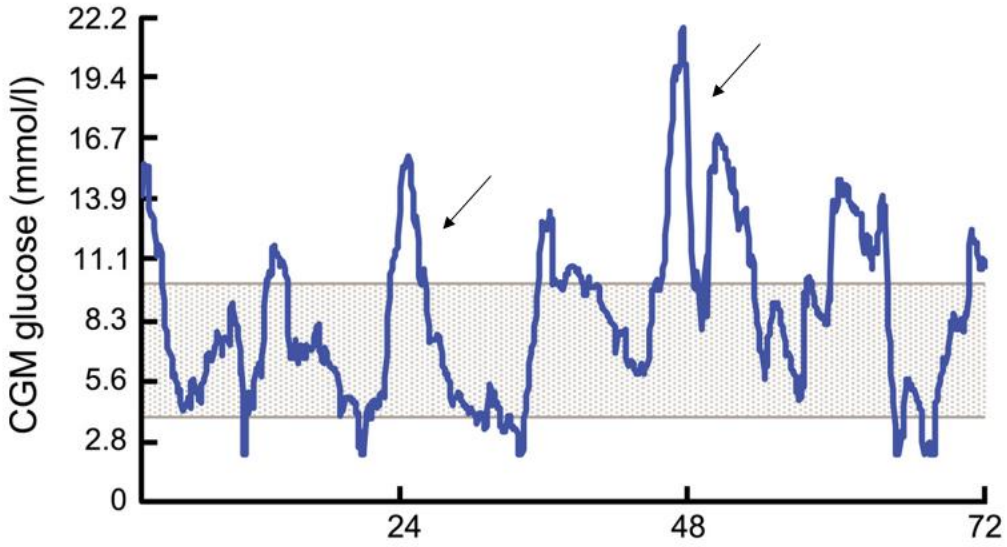


Figure 11: Continuous glucose monitor (CGM) tracing of a sample diabetic patient over the course of 72 hours. Diabetic individuals typically experience large blood glucose fluctuations that are outside the ideal glucose range highlighted by the shaded area. Insulin administration results in a large drop in blood glucose (arrow) caused by a concomitant rise in intracellular glucose within the muscle and other tissues. Image was obtained from Brown S.A *et al*, 2019.[28]

The first part of the project was conducted on mouse models with no insulin administration. The experiments focused on identifying whether muscles, an organ system that has the ability to regulate glucose transport, were also affected by hyperglycemia. Specifically, if hyperglycemia alone could induce increased intracellular glucose or substrate overload within the muscle as it did in other cell types, such as endothelial and mesangial cells.[3] Moreover, the

study sought to investigate whether these changes in substrate concentration would manifest as changes in protein post-translational modification and in turn, affect the protein's function, ultimately impairing the muscle fiber. By examining mouse models without insulin, we were able to investigate the effects of hyperglycemia on substrate storage in muscle and determine certain baseline properties present in uncontrolled diabetes, such as the utilization of substrates under different diet states, that would not be easily feasible in human studies.

In order to explore substrate overload in these models, ORO and PAS stains were chosen to assess lipid and glycogen content, respectively, as an indicator of substrate levels. It was hypothesized that Akita mice, which are incapable of producing sufficient insulin, would have less glycogen content and in turn, rely more on lipids to meet their energy demands. This increasing reliance on lipids would be demonstrated by elevated IMCL levels. However, the ORO and PAS stain analysis revealed no difference in wild-type and Akita mice models independent of their fed or fasted state. Contrary to our hypothesis, these results suggested that glucose could enter the diabetic skeletal muscle by means other than insulin-stimulated glucose uptake. In a study by Dimitrakoudis *et al,* the glucose transporters, GLUT4 (insulin-dependent) and GLUT1 (insulin-independent), were found at ratios of 3.5:1 on the plasma membrane during normal physiological conditions.[30] However, in STZ diabetic induced rats, the number of GLUT4: GLUT1 shifted to 1.5:1 due to decreased GLUT4 and increased GLUT1 expression.[30] This increase in dependency on GLUT1 transporters as a means to deliver glucose could explain the comparable levels of glycogen seen within Akita and wild-type mice. Regarding the mice's diet state, results from the PAS stain showed that the fasted group had a lower average glycogen content than its fed counterpart, suggesting that glycogen was being used routinely as a fuel

source for the muscle. In the fasted state, breakdown of glycogen is required as an alternative method of maintaining glucose supply; hence the reduced glycogen levels observed.[29]

Interestingly, fasted mice had comparable levels of lipids as their fed counterpart. In consideration of the PAS stain, it was possible that the fasted mice were preferentially utilizing their glycogen stores. Thus, once the glycogen stores and other available substrates such as free fatty acids are depleted, there may be a marked reduction in lipid content as they shift to utilizing IMCL as a fuel source; however, future studies are needed in order to determine whether this occurs. A lack of change in mice muscle triglyceride following 16 hours of fasting was also noted in the study by Heijober *et al.*[31] Instead, in fasted mice, there were noticeable decreases in hepatic and muscle glycogen and increased hydrolysis of adipose triglyceride, so as to provide circulating glucose and FFA to other organs.[32] Overall, both the PAS and ORO stain did not indicate differential shifts in substrate storage and utilization due to diabetes, nor did they indicate any signs of substrate overload as both glycogen and lipid content were comparable between groups and diet status.

Although substrate overload was not present in the untreated Akita mice, we were still interested in whether there were any differences in post-translational modifications due to hyperglycemia. Of specific interest to us, was the glycosylation of proteins, since *O*-GlcNAcylation of protein was found to be elevated in diabetes due to increased influx through the hexosamine pathway – a component that Brownlee proposed to be involved in the pathogenesis of diabetic complications.[3] Immunoblotting and subsequent analysis showed no significant difference between any of the groups, indicating the absence of hexosamine pathway upregulation in diabetic mice and indirectly lack of glycolytic substrate overload.

Succinylation and acetylation of proteins were also explored because it was hypothesized that both modifications would be elevated during times of substrate overload. The succinyl- and acetyl- groups required for the aforementioned modification of proteins are both intermediates in the TCA cycle. As such, it was hypothesized that following insulin administration, in conjunction with decreased complex II activity, as seen in those with type 1 diabetes, acetyl and succinyl would be elevated within the mitochondria.[19] The high concentration of these intermediates would result in their non-enzymatic addition to the lysine residues of metabolic proteins nearby. This would be especially true for succinyl-CoA as the low pH of the mitochondrial matrix would promote the succinylation of mitochondrial proteins.[31,33-37] Ubiquitination was also explored because in the same study by Monaco *et al*, increased autophagic debris unaccompanied by a change in autophagic protein levels were found within the skeletal muscle of diabetics.[19] As succinylation, acetylation, and ubiquitination all occur on the lysine residue of proteins; it was hypothesized that the increase in autophagic remnants were due to impairments of degradation caused by succinylation and acetylation of proteins rather than the impairments of the autophagic proteins themselves. In what is hypothesized as a result of protein modification cross-talk, acyl modification of the lysine N-terminus could prevent proteins from being ubiquitinated and subsequently degraded, resulting in debris accumulation. Analysis of western blots revealed no significant difference in the levels of post-translational modifications between Akita and wild-type mice and their respective fed and fasted groups for any of the modifications studied. However, when Akita fasted and fed mice were grouped, it revealed a main effect of diabetes on acetylation levels, with Akita mice displaying higher acetylation than wild-type mice. The effects of acetylation are still relatively unknown as it can affect different proteins in completely opposite ways. However, with regards to GAPDH, a key

enzyme in diabetic complications, it has been shown to decrease GAPDH activity by preventing substrate accessibility through nuclear translocation.[38,39] This, in turn, would cause accumulation of glycolytic intermediates, which would increase flux through the polyol, hexosamine, PKC, and AGE pathway, resulting in diabetic complications. A decreased rate of glycolysis in T1D has been demonstrated in many tissues, including the liver and adipose tissue.[33]

Since the acetyl intermediate required for acetylation of protein could be produced in both mitochondrial and cytosolic compartments via numerous pathways that are based on nutrient availability, acetylation is considered to be more widespread.[40] This was also consistent with our data. When comparing the acetylation levels to the other protein modifications studied, acetylation was found to be the most abundant, especially in comparison to glycosylation and ubiquitination. As such, and as within this study, changes in acetylation may be more evident due to the greater degree to which it takes place. Taking this into consideration, the degree and extent of occurrence throughout the cell could explain why there were no differences in succinyl-protein modifications. Succinylation, which was hypothesized to modify mitochondrial proteins, might not have been visually apparent since immunoblotting was completed on whole muscle lysate. Considering that mitochondrial proteins would only make up a fraction of the total protein pool, using whole muscle lysate could have masked the potential differences in mitochondrial PTMs. Thus, future fractional studies are required in order to confirm these results fully.

However, within the current study, individual bands were also analyzed to account for the potential masking of differences due to excess exposure of abundant unaltered proteins and are shown in Supplementary Figure 2-5. Despite no changes in the overall levels of PTMs, there were individual protein bands that displayed differential levels of post-translational

modifications from their matched counterparts. The modified proteins that were differentially acetylated and ubiquitinated from their wild-type counterpart were around 37kDa and 100kDa for acetylation and between 50-75kDa for ubiquitination, indicating that diabetes played a role in altering these proteins. Both acetylation and ubiquitination were elevated with regards to their respective proteins in diabetes. The acetylated protein present at 37kDa was predicted to be GAPDH, and as previously mentioned, acetylation of GAPDH ultimately reduced its activity.[39] And although the result of acetylation on the proteins present at 100kDa was not known, ubiquitination of proteins would mark them for degradation.[29] Thus, within diabetes, elevated degradation of proteins between 50-75kDa could have occurred. Regrettably, the total effects of the modifications in diabetes are still unknown as identification of the specific proteins were outside the scope of our study. As such, in addition to experiments on specific subcellular fractions, protein identification should be considered in order to determine the specific and functional changes diabetes plays on proteins.

Immunoblotting for pan-posttranslational modification was challenging as the appearance of numerous bands brought into light the potential possibility of detecting nonspecific bands, which could therefore alter the data collected. Although an HFD group was included within the experiment to serve as a positive control, it was not shown to be differentially elevated as was expected from the literature.[41,42] Therefore, in order to verify that the banding patterns present in Figure 3 were indeed due to the correct detection of the modification of interest and not due to non-specific binding of proteins, a negative test was performed in which the blot was only incubated in either mouse or rabbit secondary antibody (Supplementary Figure 7). From the different banding patterns present between the mouse and rabbit secondary test blots, it was concluded that the contribution from nonspecific binding of secondary antibodies was negligible

in the appearance of the bands. Moreover, the differences between the liver and muscle tissue within blots, and among the primary probed experiment blots incubated with either mouse or rabbit secondary antibody (Figure 3 and Supplementary Figure 7), further supports that the blots presented are indeed representative of the overall PTM levels of interest within the tissue and thus can be trusted.

Therefore, in light of the current data collected, one would consider that the lack of difference in PTMs between wild-type and Akita mice could be due to the lack of substrate overload. This was supported by the ORO and PAS stain, as Akita mice matched their wild-type counterparts in lipid and glycogen content in both the fed and fasted state. As the hypothesis was based on substrate availability during times of excess - when these substrates were more likely to be added onto proteins - the lack of abnormally high substrate required to increase protein modification could explain why there were no differences seen between the two groups. The absence of substrate overload was understandable as insulin was not administered in these rodent models. Thus the muscle was not stimulated to uptake the excess circulating glucose. Therefore, insulin treatment should be considered when designing future experiments. Since there was no difference in the overall levels of ubiquitination and most post-translational modifications in Akita mice, it would suggest that the increased autophagic remnants seen in the Monaco *et al* study was not due to the prevention of proteins from being ubiquitinated, as we had hypothesized.[19] Therefore, other degradation pathways should be explored in order to understand the mechanism behind the increased autophagic remnants seen in diabetic muscle. However, it is important to note that there were two main differences between our study and the study by Monaco *et al*.[19] The latter was conducted on insulin-treated humans. In contrast, our study was

conducted on rodents without any use of insulin, making the results in this study uncertain in terms of its applicability to the mechanism behind increased autophagic remnants in humans.

The second part of the study involved examining well-controlled diabetic participants in order to determine the effects of diabetes on muscle. In this study, muscle samples of diabetic participants from the ages of 18-80, undertaking regular insulin therapy, were analyzed for potential signs of substrate overload. Substrate overload was hypothesized to occur with insulin treatment as insulin would cause a large influx of glucose - comparable to the hyperglycemia induced influx seen in endothelial and mesangial cells, resulting in diabetic complications - in skeletal muscle. Moreover, it was hypothesized that substrate overload due to the presence of exogenous insulin would cause a metabolic burden on the mitochondria, leading to increased ROS production and subsequent adverse effects. Of specific interest to us was the development of ROS-associated mitochondrial and lysosomal dysfunction, as elevated oxidative stress has been shown to adversely affect the mitochondria and lysosomal degradation pathways.

Similar to the rodent experiment, a PAS stain was performed on the vastus lateralis collected from human muscle biopsies to determine whether intramuscular glycogen content differed in the presence of insulin. However, no differences were seen in glycogen content between insulin-treated and control subjects. Although the muscle would experience a greater flux of glucose following insulin therapy, it was not deprived of glucose for prolonged periods of time. Thus, it was not required to adapt, unlike with the Akita mice. Therefore, as the participants in this study were all relatively healthy and took proper care of their glycemic levels,

there was no need to increase GLUT1 transporters as a compensatory mechanism. As such, there was no increase in glycogen content, which was hypothesized to occur when increased GLUT1 and insulin therapy were both present. Taking this into consideration, it would then follow that the Akita mice represented the more extreme case of diabetes and consequently uncontrolled hyperglycemia. Therefore, given that there was no difference in the glycogen content of untreated mice, it was reasonable that there would be no differences in the glycogen content of insulin-treated T1D participants who were less extreme in their conditions. These findings were in agreement with a study Standl *et al* in which no difference in glycogen content was observed between diabetic and control participants.[43] Interestingly, Standl *et al* also saw that those with poorly controlled diabetes displayed reduced glycogen content and increased lipid levels, which was in line with what was predicted to occur in the rodent models.[43] Given the fact that the Akita mice did not display different levels of glycogen or lipids despite having uncontrolled diabetes, this might indicate that Akita mice were not the best models for replicating diabetes in humans.

Studies from Bergstrom *et al* and Maehlum *et al* also saw no differences in glycogen levels within the muscle of diabetic patients.[44-45] Maehlum *et al* further demonstrated that intramuscular glycogen recovery following exercise in T1D participants treated with insulin occurred at the same rate as in non-diabetic participants.[45] Like Standl *et al,* reduction in intramuscular glycogen levels were only seen in people with untreated diabetes. In accordance with that, Roch-Norlund *et al* found an association between muscle glycogen content and the severity of the disease.[46] These investigators also showed that three days of insulin treatment rapidly increased muscle glycogen levels.[46] Considering that all participants included in this study were insulin-treated and had an average HbA1C of 7.46%, it was reasonable that we did not see any differences in muscle glycogen content. While it was interesting to note that in our

current study, glycogen content did not demonstrate any statistically significant correlation with HbA1C, including participants with uncontrolled diabetes or a greater HbA1C may reveal an association with glycogen levels.

In addition to intramuscular glycogen content, intramyocellular lipids were also measured. Although glycogen content did not change, IMCL levels could differ between groups because lipid entry into skeletal muscle relied less on insulin stimulation.[10] To test this hypothesis, electron microscopy images from human participants were analyzed for IMCL content. Additionally, the percent contact of IMCL to mitochondria was examined to determine whether skeletal muscles relied more heavily on lipids to meet their energy demands. In some cases of diabetes, lipid levels had been shown to be elevated and result in insulin resistance, in what is called double diabetes.[47] Moreover, in a study by Standl *et al*, the concentration of muscle triglycerides per gram of muscle protein was significantly elevated in poorly controlled diabetics compared to well-controlled and non-diabetics.[43] In these subjects, increased reliance on triglycerides during exercise was also seen. However, in the current study, no differences were found. IMCL content in both the IMF and SS region of the vastus lateralis did not differ between the two groups. This was corroborated by a study by Nadeau *et al*, in which no differences in IMCL content were seen in the soleus and tibialis anterior of those with diabetes.[48] Within the current study, IMCL density and average size also showed no difference between groups and, therefore, the percent IMCL-mitochondrial contact was also similar between the two groups. As previously mentioned, increased mitochondrial and IMCL contact was hypothesized to occur as an adaptation to increase fat accessibility and usage within the mitochondria.[15] Because there was no indication of increased reliance on lipids in the T1D group, it was reasonable that the percent contact of IMCL and mitochondria did not differ from the controls.

Like glycogen content, this lack of difference between groups could be due to the fact that the participants in the study had well-controlled diabetes, were physically active, and overall healthy.

Surprisingly, BMI was not correlated with any of the IMCL properties that were assessed in T1D subjects in the SS region, indicating that IMCL was independent of BMI. It was expected that with increasing BMI, IMCL content, density, and size would also increase, as was seen in the subsarcolemmal region of control subjects. This lack of association in T1D could suggest the preferential usage of lipids over other substrates within the muscle or alternative storage of fat in other tissues such as adipose tissues. Preferential utilization of fatty acids has been demonstrated in the heart of those with diabetes.[54] On the other hand, insulin has been implicated in increased fat accumulation due to its anabolic nature - inhibition of protein catabolism, stimulation of lipogenesis, and reduction of basal metabolic rate.[1,55,56] Increased hip to waist ratio and increased prevalence of obesity in those with diabetes supported the storage of lipids in adipose tissue as opposed to skeletal muscle.[55,56]

Since IMCL and glycogen content can often fluctuate and are transient characteristics, we decided to assess mitochondrial content, area, density, pixel density, and lipofuscin count as a chronic measure of substrate overload. The analysis was completed on the same EM images used to analyze IMCL. As mentioned, Monaco *et al* saw decreased mitochondrial complex II activity and increased complex III ROS production; therefore, we were curious whether this change would initiate increases in mitochondrial content and/or density to compensate for functional impairments.[19] Additionally, mitochondrial area and pixel density were examined to determine whether there was the presence of mitochondrial swelling and abnormal cristae arrangement. In

the study by Monaco *et al,* most of these parameters were also analyzed, and no differences were seen in the mitochondrial content, density, and size. However, the participants that partook in that study were only 18-30 years old, whereas the current study included participants ranging from 18-80 years old. Therefore, this large age range might reveal some differences that were not seen in the study by Monaco *et al.*[19] Yet, through EM analysis of eight images per subject, it was determined that there was no significant difference in mitochondrial content, area, density, and pixel density. As IMCL and glycogen content did not indicate substrate overload, it was not surprising that there was no change in mitochondrial content and most of the other properties analyzed either despite the impairments in complex II. Mitochondrial content was also reported to be comparable between diabetic and non-diabetic subjects in the studies by Heyman *et al,* Wallberg-Henriksson *et al* and Harmer *et al.*[57-59] As mentioned, the lack of difference in mitochondrial properties (i.e. content, density, and area) correlated with the previously mentioned study by Monaco *et al,* where the same results were found.[19] This suggested that even though there was a change in mitochondrial complex function, it does not manifest at the level that required compensatory adaptations, i.e. increased mitochondrial content.[19] Of all the mitochondrial properties analyzed, only SS pixel density differed, with the control group having a higher pixel density than the diabetic group. Pixel density detected the darkness of the area analyzed, so an area with more dark regions would have a higher pixel density reading, or oppositely, an area with more light regions would have a lower pixel density output. In the context of the mitochondria, a lower pixel density would indicate less cristae density, such as in the case of mitochondrial swelling or abnormal cristae arrangement. The presence of abnormal cristae within diabetic subjects was seen in the Monaco *et al* study, where electron tomography (3D) images revealed irregular cristae arrangement.[19] Despite the fact that our current study did

not further examine whether the change in pixel density resulted from abnormal cristae or mitochondrial swelling, the presence of either or indicates a problem with the mitochondria in diabetes**.** As the cristae houses the respiratory chain complexes, changes to the cristae arrangement and surface area could adversely affect the mitochondria's metabolic ability.[60] While on the other hand, mitochondrial swelling could induce mitochondrial outer membrane rupture and, in turn, leakage of pro-apoptotic proteins, activating cell death.[61]

Although, for the most part, there were no differences in the mitochondrial properties assessed, it could have resulted from the large age range of the subjects, which could end up masking the diabetes-associated complications present in older adults. Thus, this was accounted for by performing correlations on the mitochondrial properties assessed. Mitochondrial density within the SS region displayed a moderate negative correlation with age, duration of diabetes, and body mass index (BMI) in those with type 1 diabetes. Increased age, BMI, and duration of diabetes were associated with reduced mitochondrial size per area. Age in the absence of diabetes was shown to be associated with reduced mitochondrial density and therefore, might be a confounding factor.[62-64] Even so, it was not appropriate to rule out the adverse effects of diabetes on mitochondrial density given the negative correlation with duration of diagnosis. As such, mitochondrial density was likely negatively affected by diabetes. Although, the extent of its effect is unknown. When age and mitochondrial density correlations were examined in control matched participants, no correlation was seen. However, because the participants in the study were tightly matched, this might indicate that the length of diabetes had a more substantial effect on mitochondrial density than age. Even when comparing BMI to mitochondrial density in the control group, there was no correlation seen, unlike that of the diabetic group, which further strengthens the notion of diabetes having a strong negative effect on mitochondrial density

(Figure 8). The absence of a correlation between age/BMI and mitochondrial density of control participants in this study could be caused by the variability seen between the traits analyzed. This could be due to the limited number of participants, especially in the older age group, within the study and would likely be solved by increasing the sample size.

Lipofuscin, an aging-associated pigment, was also analyzed within our study as impaired mitochondrial function and increased autophagic remnant could suggest an inability to clear damaged mitochondria.[19,65] And according to the "mitochondrial-lysosomal axis theory of aging," impairments in lysosomal pathways caused by mitochondrial ROS resulted in the accumulation of lipofuscin composed of damaged mitochondria.[24,25] This created an ongoing cycle where lysosomal dysfunction in turn, caused mitochondrial damage, which exacerbated ROS production, resulting in more lipofuscin accumulation.[24] Thus, given the phenotype seen in diabetic muscles, it was expected that lipofuscin levels would be elevated. However, our measures showed no differences in lipofuscin within the vastus lateralis of control and T1D groups.

A proponent of the "mitochondrial-lysosomal axis theory of aging" was increase oxidative stress. Although it was shown that there was elevated ROS production from complex III in skeletal muscle by Monaco *et al*, it might not be significant enough to contribute to lipofuscin production and accumulation.[19] The absence of excess levels of ROS is supported by the previous experiments in which there were no indication of substrate overload that would further aggravate oxidative stress within the tissue. Moreover, in the current study, most mitochondrial properties did not deviate from the control. Of particular relevance, mitochondrial pixel density, indicative of mitochondrial swelling or abnormal cristae, was the same as control subjects within the IMF region, suggesting that there was no cumulative mitochondrial damage.

Although the SS region's pixel density did deviate from the controls, the fact that lipofuscin levels did not differ could imply that the extent of abnormal cristae or swollen mitochondria did not accrue enough damage to lead to mitochondrial degradation. Thus, it would then follow that increased lipofuscin accumulation would not occur given the muscle environment – lack of extensively damaged mitochondria and extreme oxidative stress. Moreover, as lipofuscin levels did not change, it could suggest that the increased autophagic remnants seen in the study by Monaco *et al* was not completely due to an inability to clear damaged mitochondria properly.[19] However, although there was no cumulative damage seen, the mitochondrial complex dysfunction and differing pixel density in diabetic subjects indicate the potential for future manifestation of overall mitochondrial damage and deterioration, and in turn, lipofuscin accumulation. Additionally, the positive correlation between increased age and lipofuscin accumulation demonstrated in both control and diabetic subjects within this study further lends power to the possibility of future lipofuscin formation (Figure 10). This is likely the case as many studies have demonstrated an association between lipofuscin build-up with increasing age.[24,25] Although the study aimed to take this into account by including participants ranging from the ages of 18-80, there was a higher proportion of participants in the younger - middle-aged group, which may have resulted in the lower levels of lipofuscin seen. Even so, given the data collected, there seems to be no indication that diabetes played a role in lipofuscin accumulation. However, in order to fully confirm these findings, the inclusion of more participants in the older age group and longer-term studies are needed to determine whether lipofuscin accumulation occurs at a different rate or number in skeletal muscle of those with T1D.

In conclusion, there was no difference in glycogen and IMCL storage or utilization between diabetic and non-diabetic rodents or human subjects. Moreover, no noticeable effects of substrate overload were demonstrated in diabetes. Future studies are required to determine why no differences were seen in the rodent studies. However, the lack of difference in the human studies might be due to the combined effects of having well-controlled diabetes and the muscles' properties that granted it more protection against hyperglycemia. Overall, the data suggested that controlling glycemic levels was crucial in preventing changes to the muscle and other adverse complications in diabetes.

Limitations and Future Directions

The limitations of the rodent portion of the study included the small sample size for each group (n = 4-10). Additionally, there were no insulin injected groups, which made it difficult to draw conclusions on how insulin treatment would affect glycogen and lipid content. Therefore, although the data collected from those stains were interesting and revealed insights into the metabolic status of those rodents especially in terms of their diet state, they did not reveal substrate overload occurring within the tissue. Since substrate overload was defined as an increased influx of substrate either following insulin administration or when animals were fed an HFD, it was difficult to determine whether substrate overload occurred in diabetes without the insulin-treated group. As such, future plans consist of increasing the power of the study and including the appropriate groups. In doing so, this would also provide an opportunity in which we could track glucose's pathway and examine how it differs with or without insulin treatment through the use of radiolabelled glucose. It would also be vital to investigate GLUT levels in

order to determine whether the lack of difference in glycogen content could be accounted for by increased GLUT1 concentration. In addition to that, future studies should include fractionation experiments in order to isolate mitochondrial proteins and examine differences in PTMs.

The second portion of the study focused on the human participants ranging from ages 18-80. Due to the large age range, an increase in participants would be needed in order to confirm the findings of this study. In fact, the 18-30 age group was made up of 32% of the participants, whereas only 12% of participants were in their 60-80's. This difference in the proportion of age ranges could heavily skew the data towards the middle age group and in turn, provide misrepresented results. As demonstrated, age plays a role in certain outcomes, especially in relation to lipofuscin. Another factor that should be considered for future studies is how to track participants' glycemic levels. The participants within the study were mostly well-controlled with their diabetes and had a cumulative average HbA1C of 7.46%. HbA1C provides an overall depiction of average blood sugar over a duration of approximately three months. However, many factors could affect daily glycemic levels that would not show up within the HbA1C results. Therefore, more measures should be taken to control for these factors, or a more detailed account of their glycemic levels should be taken. In line with this, even though a food log of the participants' meals were recorded within the current study, their difference in diets (i.e. keto or paleo diet) could affect blood glucose concentration as well as the glycogen and lipid content of the individual.[64-69] As such, it would be optimal for future studies to design pre-planned meals that have allocated specific amounts of carbohydrates and lipids for each meal. Lastly, as alluded to in the discussion, the Akita mouse model does not seem to accurately replicate diabetes in humans. Even if the mice were provided exogenous insulin, once-per-day injections or pellet treatment would not fully represent the effects of numerous insulin administration in diabetic

humans. Therefore, it would be essential to repeat the post-translational modification experiments with human tissue before officially ruling out our hypothesis on the mechanism behind increased autophagic remnants.

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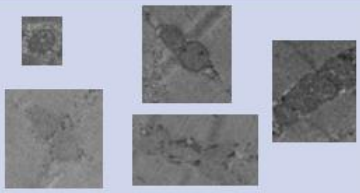
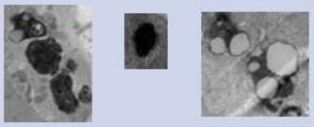
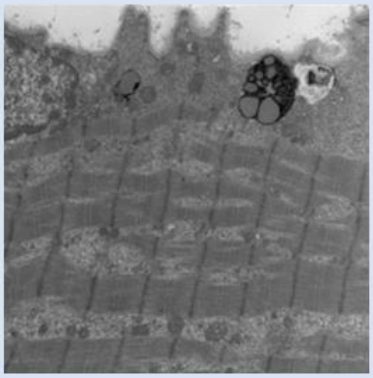
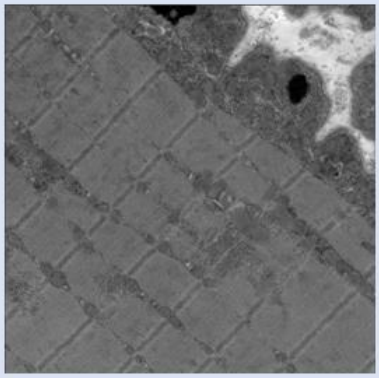
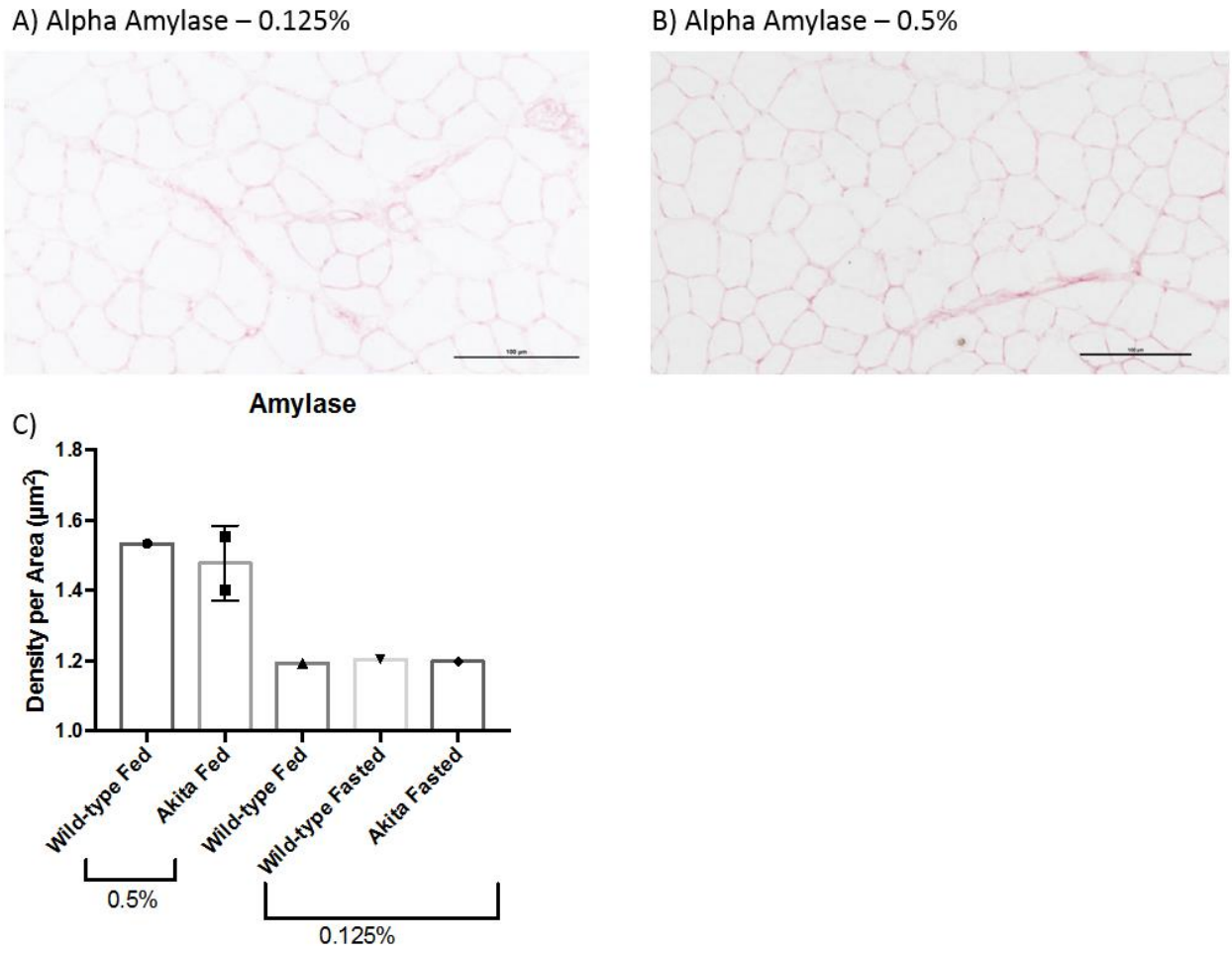
Appendix

Supplementary Tables

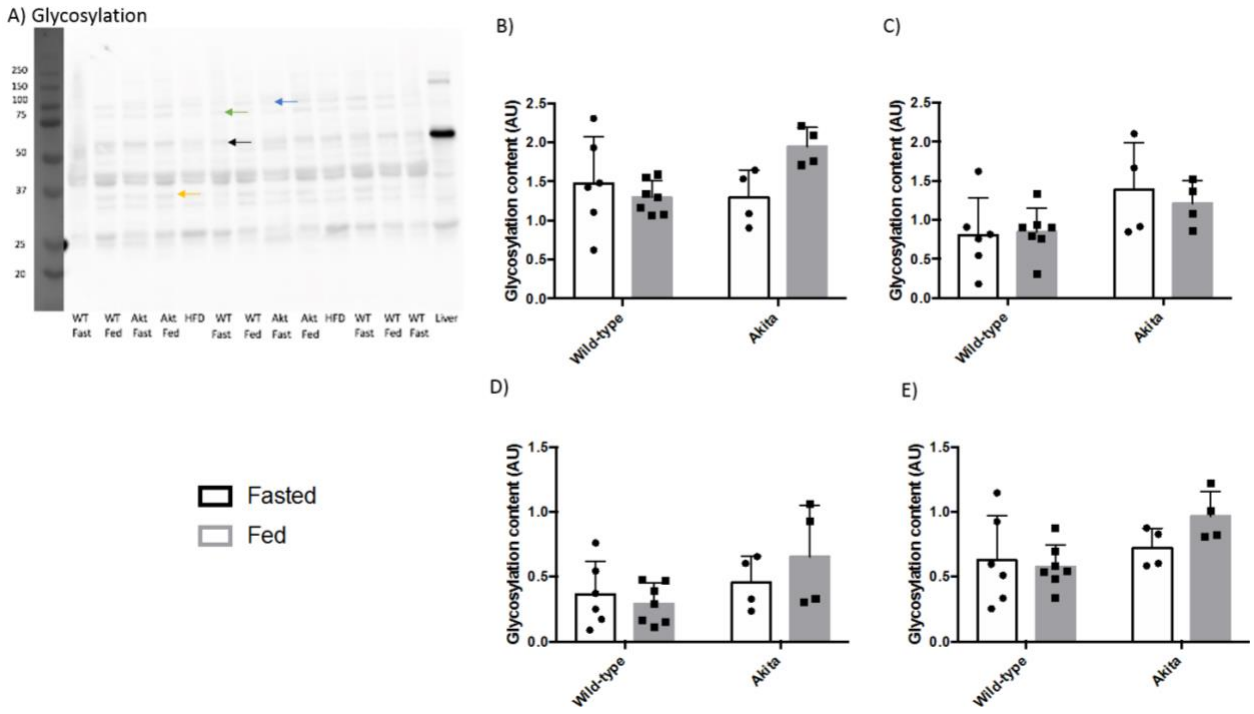
Supplementary Table 1: Method of identification of intramyocellular lipids (IMCL), mitochondria, and lipofuscin for electron microscopy (EM) analysis. Cropped images of the item of interest are presented in the sample images section, along with their description. The sample electron microscopy image section contains sample EM images with all three items (mitochondria, IMCL, and lipofuscin) present. EM images were taken at 12, 000X or 15, 000X and calibrated during analysis.
*In order to be counted, it must satisfy at least one of the criteria listed under its category.

	Visual Description	Sample Images
Mitochondria[19, 69-72]	• Circular or oval in shape • Located near triad region • Double membrane and cristae are present, if it is not visible the mitochondria should appear darker and uniform in colour • Is different in colour/texture from the surrounding muscle region	
Lipid[70,72-74]	• White on the outside with mixed grey-black colours within • Bright white in colour • Grey in colour and a different shade from mitochondria	
Lipofuscin[25,75-76]	• Very dark region • Enclosed by a membrane	
Sample Electron Microscopy Image		
		

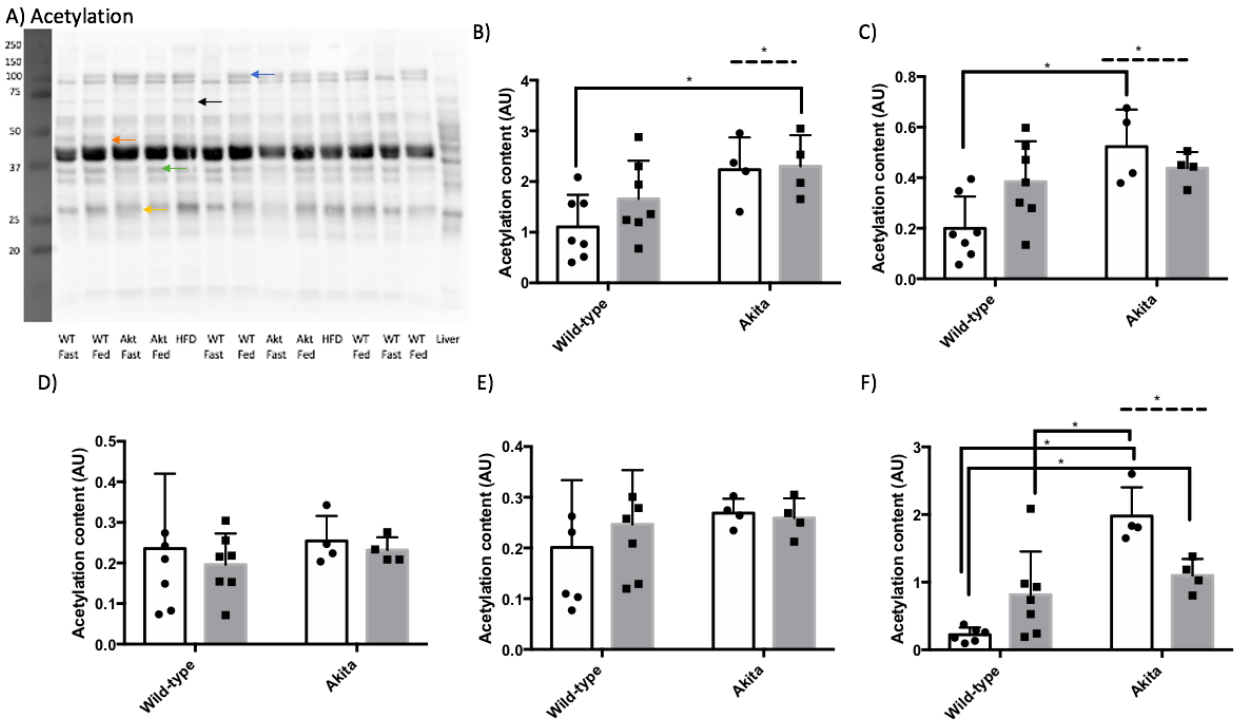
Supplementary Figures



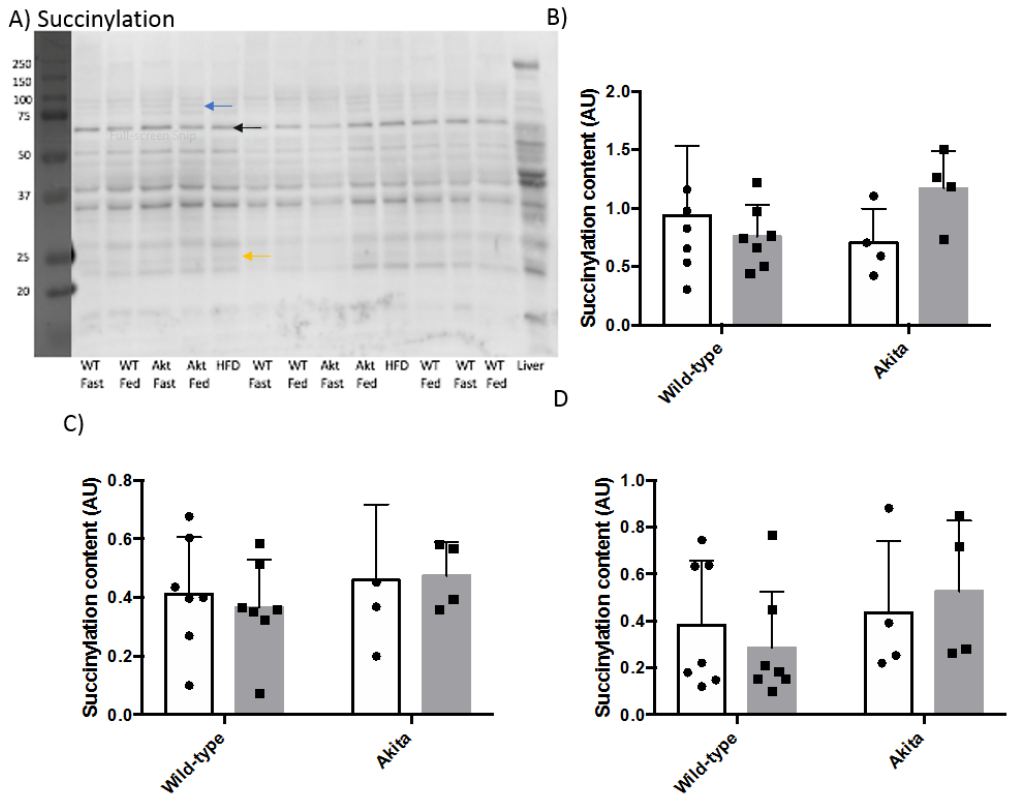
Supplementary Figure 1: Alpha-amylase treated PAS sections displayed the same average density. Alpha-amylase treated section at A) 0.5% in suspension form, and B) 0.125% in solid form. C) Analysis of 100 fibers in the alpha-amylase treated sections revealed that fibers exhibited the same average density indicating that mucosubstances contributed the same density independent of disease and diet state, and could be omitted from PAS stain analysis. Scale bars were set at 100µm.



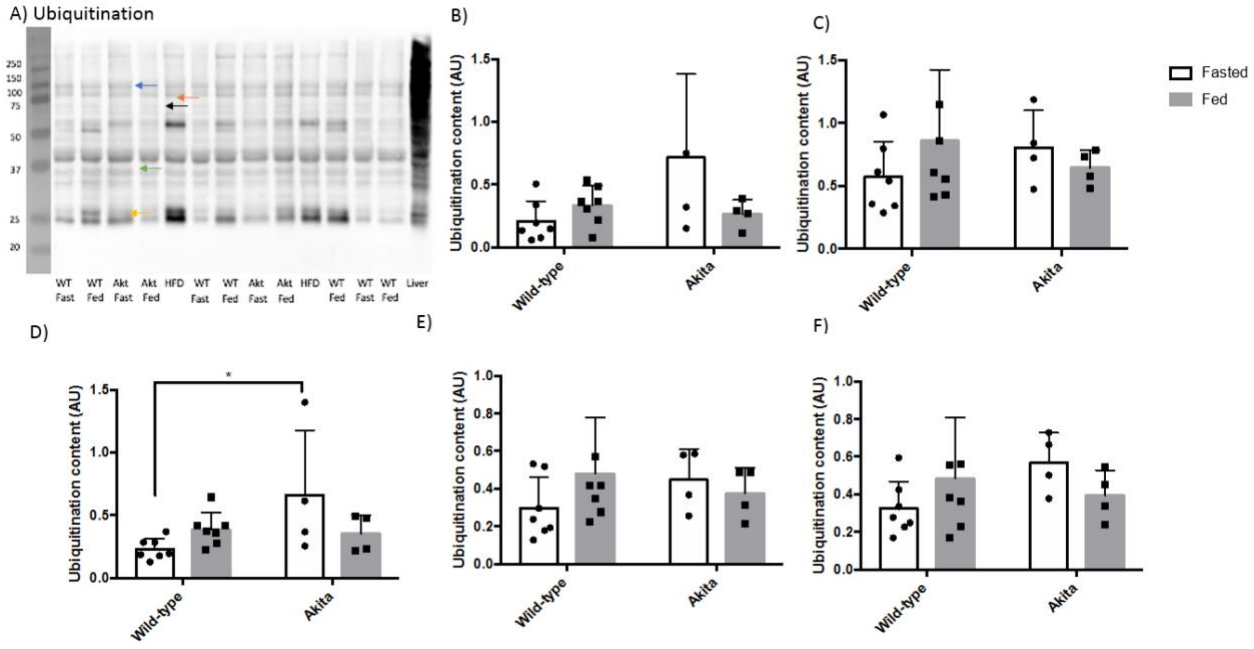
Supplementary Figure 2: Pan Glycosylation western blot and analysis of individual bands. Male groups analyzed included wild-type fed (n = 7) and fasted (n = 7), and Akita fed (n =4) and fasted (n = 4). A) Western blot probed with anti-O-GlcNac. Analysis of band at B) a bit greater than 37kDa (indicated by the yellow arrow), C) in-between 50-75kDa (indicated by black arrow), D) 75kDa (indicated by the green arrow), and E) 100kDa (indicated by the blue arrow).



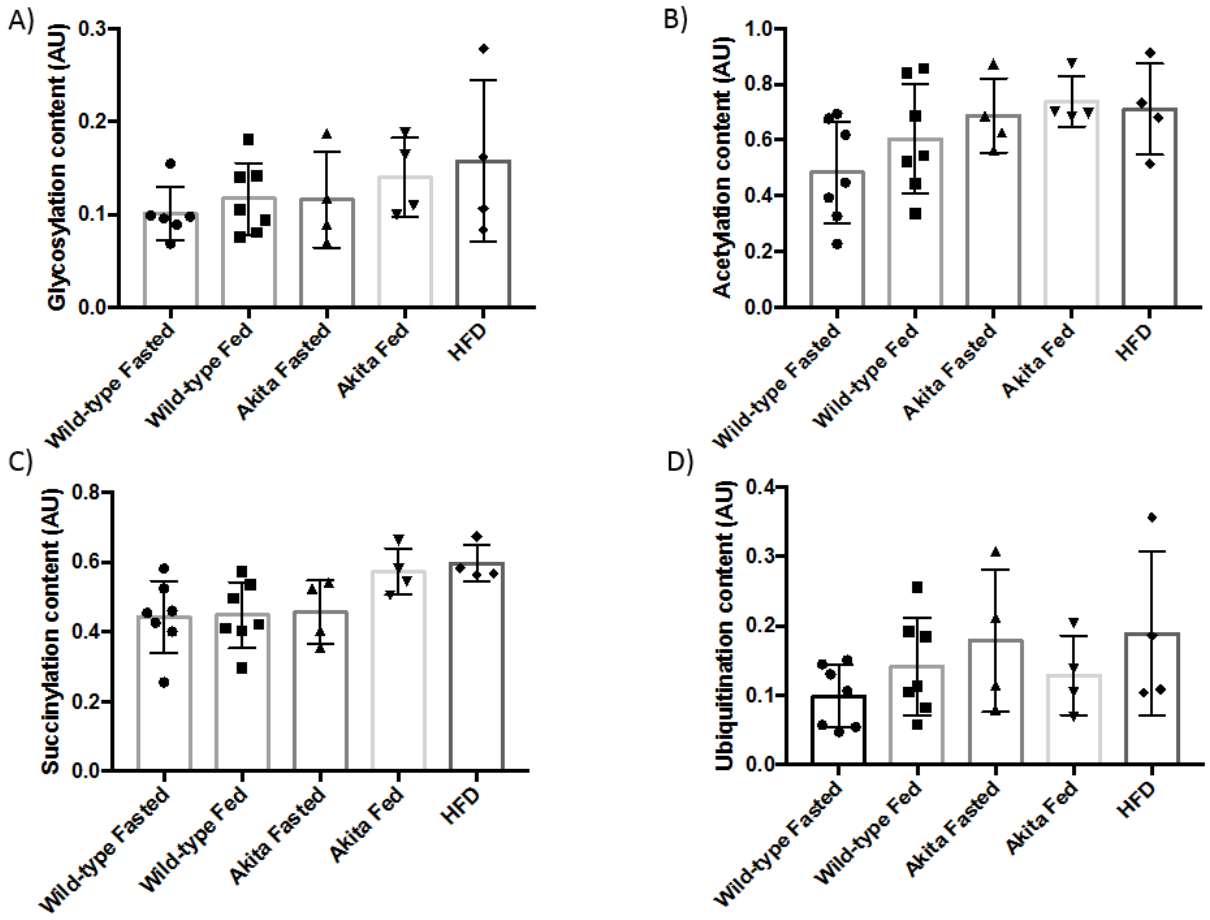
Supplementary Figure 3: Pan Acetylation western blot and analysis of individual bands. Male groups analyzed included wild-type fed (n = 7) and fasted (n = 7), and Akita fed (n =4) and fasted (n = 4). A) Western blot probed with anti-acetyllysine. Analysis of band at B) a bit above 25kDa (indicated by the yellow arrow), C) at 37kDa (indicated by the green arrow), D) a bit below 50kDa (indicated by the orange arrow), E) in-between 50-75kDa (indicated by black arrow), and F) 100kDa (indicated by the blue arrow). The dashed horizontal line represented a main effect of diabetes. ($p < 0.05$).



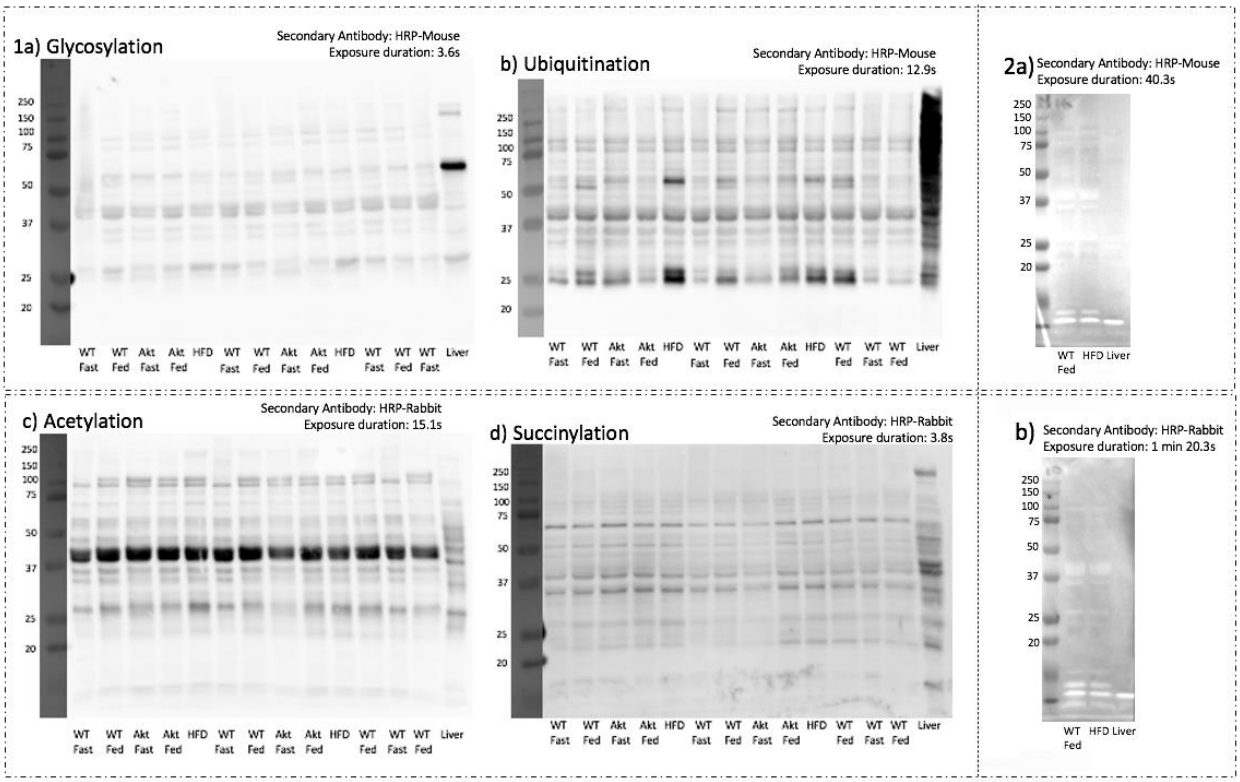
Supplementary Figure 4: Pan Succinylation western blot and analysis of individual bands. Male groups analyzed included wild-type fed (n = 7) and fasted (n = 7), and Akita fed (n =4) and fasted (n = 4). A) Western blot probed with anti-succinyllysine. Analysis of band at B) 25kDa (indicated by the yellow arrow), C) in-between 50-75kDa (indicated by black arrow), and D) in between 75-100kDa (indicated by the blue arrow).



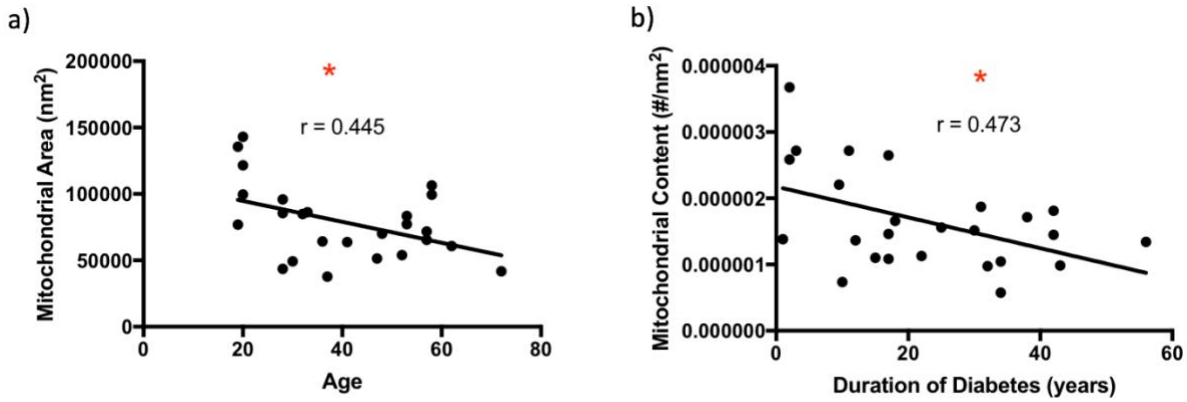
Supplementary Figure 5: Pan Ubiquitination western blot and analysis of individual bands. Male groups analyzed included wild-type fed (n = 7) and fasted (n = 7), and Akita fed (n =4) and fasted (n = 4). A) Western blot probed with anti-ubiquitination. Analysis of band at B) a bit above 25kDa (indicated by the yellow arrow), C) 37kDa (indicated by the green arrow), D) in-between 50-75kDa (indicated by black arrow), E) 75kDa (indicated by the orange arrow), and F) 100kDa (indicated by the blue arrow).



Supplementary Figure 6: Validation of high-fat diet (HFD) as a positive control for post-translational modification. Rodents used include seven wild-type mice fed a chow diet or fasted, four wild-type mice fed an HFD, and four Akita mice fed a chow diet or fasted 12 hours prior to harvest. One-way ANOVA revealed that the post-translational modification levels of 1a) glycosylation, b) acetylation, c) succinylation, and d) ubiquitination in the HFD groups did not differ from any other groups indicating that the HFD group could not serve as a positive control. ($n > 0.05$).



Supplementary Figure 7: Verification of primary antibody specificity and low non-specific binding of secondary antibodies. Muscle and liver tissue displayed distinct banding patterns within the same blot. Comparison of 1a) glycosylation and b) ubiquitination blots showed different banding patterns despite both being incubated with horseradish-peroxidase (HRP) - mouse antibody. HRP- rabbit conjugated secondary blots; c) acetylation and d) succinylation, also displayed different banding patterns suggesting that the banding pattern present was due to the binding of primary antibodies rather than non-specific secondary antibody binding. In order to further verify that non-specific binding of secondary antibody was not present, a negative blot was performed in which the primary antibody was omitted. Negative control of 2a) HRP-mouse and b) HRP-rabbit blots displayed some light bands similar to their respective pan-posttranslational modification blots. However, given the band's lightness and longer exposure time required with the negative control, it indicated that non-specific binding of secondary antibody contributed very little to the pattern seen on the pan-posttranslational modifications blots and gave strength to the accuracy of the blots.



Supplementary Figure 8: Correlations between mitochondrial properties and type 1 diabetic (T1D) participant characteristics. (n = 25 for each group, 8 electron microscopy images were analyzed for each participant). Moderate negative correlations between a) age and intermyofibrillar (IMF) mitochondrial area, and b) duration of diabetes and subsarcolemmal (SS) mitochondrial content were present in diabetic subjects. ($p < 0.05$).