Effect of Semaglutide on Skeletal Muscle Mass During Weight Loss in Diabetic Rats

KIAN TAHERKHANI

A THESIS SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF REQUIREMENTS

FOR THE DEGREE OF

MASTERS OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY

TORONTO, ONTARIO

July 2025

ABSTRACT

Obesity and diabetes mellitus are among the most widespread chronic conditions in modern society. The growing prevalence of obesity has prompted the exploration of various therapeutic interventions, including semaglutide, a glucagon-like peptide-1 receptor agonist (GLP-1RA). GLP-1RAs have shown promise in weight management, but GLP-1 receptors have been found in many tissues beyond the original therapeutic targets (pancreatic beta-/alpha- cells), including skeletal and smooth muscle, heart, and brain. Therefore, it is critical to study the potential effects of GLP-1 on these tissues, as potential off target effects (beneficial or detrimental) are expected. Many aspects or side effects of the medication remain underexplored, including the impact of semaglutide on skeletal muscle mass preservation during weight loss. This thesis investigates the differential effects of semaglutide administration compared to traditional caloric restriction on skeletal muscle mass, insulin signalling and markers of protein turnover in a male rat model of type 2 diabetes (T2D).

Objective: This study aims to compare the effects of semaglutide vs. traditional caloric restriction on skeletal muscle mass loss in rats with T2D, assessing whether pharmacological weight loss leads to more muscle mass depletion (i.e., relative atrophy) than weight loss from calorie restriction alone. By analyzing changes in histological markers of muscle, as well as protein markers of muscle atrophy and hypertrophy, this study seeks to determine if semaglutide accelerates muscle atrophy pathways independent of the drug's effect on reduced food intake. The study hopes to inform further research into confirming the effects of semaglutide on differential skeletal muscle mass loss and protein turnover and aims to set the stage for more detailed mechanistic studies that should help to better understand the possible implications of incretin therapies with and without strategies to preserve skeletal muscle mass and function while still promoting its vast and apparently global health effects.

Methods: Male Sprague Dawley rats (3 groups, n≈10 per group) were placed on a high-fat diet for 3 weeks and then injected with low-dose streptozotocin (35 mg/kg) to induce T2D, characterized by mild hyperglycemia in the fed state (i.e., whole blood glucose >12 mmol/L) but with some residual beta cell function to limit excessive weight loss and ketosis. After T2D induction, rats were randomized to one of three treatment groups for a period of 3 weeks: 1) controls (i.e., male rats with insulin-requiring T2D), receiving ad-libitum high-fat chow and daily insulin by injection for glucose management; 2) bi-weekly treatment with semaglutide (here-on referred to as Sema group) (dose escalation from 35 μg/kg body weight to 200 μg/kg body weight); and 3) caloric restriction (abbreviated to CR group) matching the food intake to the Sema group. All rats were administered daily insulin (glargine and regular insulins), as needed, to maintain a mild T2D phenotype. Daily food consumption, body weight, blood glucose

concentrations (fed) were monitored in all rats during the treatment period. A glucose tolerance test, via oral gavage (OGTT; 2g dextrose/kg body mass) was performed during the second week of treatment and an Insulin tolerance test (ITT, intraperitoneal injection of 8u of rapid acting insulin) was performed in the final (third) week of treatment. After 3-wks, the animals were killed via exsanguination under deep anaesthesia and select hindleg muscles were evaluated for muscle weights, fiber cross sectional areas and markers of protein turnover.

Results: By design, food intake was identical between the Sema and caloric-restricted rats, with both groups consuming ~20-30 percent less food daily, relative to controls. Through the treatment phase, body mass levels were similar in the Sema and caloric-restricted groups ($p=0.31$) but were about 13% lower than in the control group by the end of the 3rd week ($p=0.0001$). Rats in the Sema group and caloric restriction groups had reduced skeletal muscle mass at 3 weeks (by about 20%) and reduced cross sectional area, relative to controls, and there were only minor differences in mass and muscle fibre cross sectional areas between the two treatment groups in some of the muscles examined. Muscle protein analyses of AKT and RPS6 (ribosomal protein S6) activation in the tibialis anterior (TA) and gastrocnemius (GAS) muscles showed no statistical difference in the activation of phosphoinositide 3-kinase (PI3K) pathway between the groups, however, the Sema and CR groups tended to have lower activation of these markers relative to controls (ANOVA $P=0.14$ and $P=0.16$ for Akt/protein kinase B (AKT) and S6 kinase (S6) activation respectively). Finaly, fibre type distribution in the GAS muscle was also similar among the three groups.

Conclusion: In conclusion, we observed that pharmacological weight loss with semaglutide in rats with T2D resulted in around 10% relative skeletal muscle mass loss in some muscle groups

than what can be accounted for by caloric restriction alone. However, we also found no significant difference between the Sema and the CR group in skeletal muscle fibre type distribution status of key regulatory proteins for skeletal muscle protein synthesis such as AKT and S6.

TABLE OF CONTENTS

1.0 INTRODUCTION

The incidence and prevalence of obesity and type 2 diabetes mellitus (T2D) have been on a constant rise globally in the past decades and are projected to rise over the next several decades[1]. Obesity and T2D are two main chronic non-communicable diseases that are accompanied by psychological and social stigmata in the developed world, with intense focus on trying to reduce the societal impact caused by these largely "lifestyle" conditions [2]. While weight-loss medication and various weight management strategies have been around for over a century[3], the rising prevalence of these genetic and "lifestyle-related" diseases have more recently inspired pharmaceutical industries to develop novel and more effective weight loss medications [4]. Several new pharmacological agents, either approved or under investigation for obesity and/or diabetes management, are primarily aimed at weight loss via caloric restrictions but they also improve glycemic control either directly or indirectly because of fat mass loss. The latest leap in advancements is the discovery of the new class of drugs developed first in 1990s by researchers in North America and Europe, the glucagon like peptide 1 receptor agonists (GLP-1RA)[5]. With the advent of GLP-1 receptor agonists, weight loss achievable with pharmacological treatment has become ever closer to the magnitude achievable with surgical intervention[6,7], which is considerably more invasive. Inevitably, GLP-1 receptor agonists have gained widespread attention due to their effectiveness in inducing clinically meaningful weight loss (i.e., >10% body mass over weeks to months) [3], as well as ease of use (i.e., oral or only once weekly subcutaneous injections, rather than daily injectables) compared to other previously

available weight loss medications[8]. The popularity of these drugs is represented and even perhaps partly due to the mass public attention, advertisements and endorsements. In 2021, seventy one percent of antidiabetic drug sales in Canada were for the new-generation drugs, including GLP-1 agonists and Dipeptidyl Peptidase 4 Inhibitors (inhibitor of enzyme that breaks down GLP-1), which have a similar effect compared to the agonists of GLP-1R but lower in magnitude of both effects and side effects[9]. This represents a significant increase from 2010s, where GLP-1 medication had little impact on the market, to the 2020s where they accounted for 25% of all antidiabetic drug sales[9]. But while more accessibility and usage of a treatment has been deemed a scientific advancement by most professional organizations such as the American Diabetes Association[10], it does raise concern about these drugs being so widespread beyond their initial indications for glycemic control. This is because the global effects of these agents have not yet been fully explored, and the side effects of sustained treatment, such as risks for pancreatitis, bowel obstructions and risk of thyroid cancers are only now being investigated using rodent models[11,12] and from large clinical trials[13].

Weight loss interventions—particularly those involving medication or rapid weight loss through severe caloric restriction—carry the risk of reducing not only fat mass, but also lean body mass, including the loss of bone mass and skeletal muscle mass, thus impairing skeletal muscle quantity, health, and function.[14,15] Lean body mass (i.e., the weight of everything in the body except the fat) is the most widely used marker of muscle quantity in clinical trials of GLP-1 based therapies but has important drawbacks and limitations to its use, as it is only a surrogate measure of muscle mass and this metric does not provide any information on the function or health of the musculoskeletal system. GLP-1 receptor agonist-induced weight loss, like other

methods of weight loss where nutritional intake is reduced, has been associated with changes in body composition[16,17]**.** While the primary goal of every weight loss intervention is to reduce body fat mass without excessive lean mass loss, the desired results are not always achieved[18]. This has been a concern with GLP-1 usage since their introduction, a concern that doctors, pharmacists and other health professionals share[19,20]. As an additional worry, lean body mass in general, but particularly skeletal muscle mass is profoundly important for a variety of reasons in individuals with obesity and/or T2D[21]**.** Indeed, skeletal muscle is responsible for 80 to 90% of insulin stimulated glucose uptake in the body[22], and is highly metabolically active and responsible for a large portion of the body's calorie expenditures, even at rest[23]. Muscle mass is closely linked to metabolic health, insulin sensitivity, and physical function, especially in individuals with obesity or T2D, where baseline muscle quality is often already compromised[24]. It is therefore extremely important to establish strategies to maintain muscle mass at a high as possible level and minimize loss.

Despite concern that GLP-1 therapies might detrimentally affect skeletal muscle health by reducing mass or function, the relationship between GLP-1 signaling and muscle adaptation during therapy-induced weight loss remains poorly examined. In particular, as it related to this thesis, there is ongoing debate on whether GLP-1 treatment causes muscle loss or lean mass maintenance more than what would be expected from caloric restriction alone [20,25], and even debate on the functional relevance of GLP-1 receptors in muscle tissue in the first place[26]. Importantly, most clinical trials report weight loss and fat mass loss with GLP-1 therapies[13,13,27], but qualitative and quantitative analyses of skeletal muscle is lacking[16,27]. Not only are the lean body mass measures from these studies highly variable and inconsistent [20,25,28–30], but it is

doubtful that a simple measure of lean body can be used as surrogate of skeletal muscle mass and quality after the initiation of GLP-1RA therapy use[31]. While recent studies indicate that weight loss achieved through GLP-1 therapy may lead to losses in muscle mass[20,25], the magnitude of muscle loss reports suggesting that reductions can range significantly, highlighting the variability in skeletal muscle effects among individuals and/or trial designs. If the loss in lean mass is problematic from a metabolic or functional health perspective also remains unclear.

The physiological mechanisms by which GLP-1 influences skeletal muscle function and mass warrants continued investigation using animal models and carefully controlled and more detailed human physiologic trials. For example, GLP-1 is known to enhance insulin secretion and sensitivity, potentially facilitating improved glucose uptake in skeletal muscle cells[16,32]. Additionally, the impact of GLP-1 on the vascularity of skeletal muscle is an increasingly significant area of research [33,34], particularly regarding metabolic health, muscle maintenance, and glucose homeostasis. GLP-1 is known to enhance muscle microvascular recruitment, leading to improved blood flow and nutrient delivery to skeletal muscle tissues[34]. Studies indicate that GLP-1 promotes vasodilation through its actions on the endothelial nitric oxide synthesis pathway, facilitating an increase in nitric oxide (NO) production[33]. These factors are all extremely important and multifaceted, with potential effects that might not be reflected in simple lean body mass measures but reflected in muscular quality and capacity of subject to perform actions.

The effect of GLP-1 treatment on mitochondrial function and oxidative metabolism within skeletal muscle is also in its early stages**.** Emerging research indicates that GLP-1

receptor activation stimulates skeletal[35]and cardiac[36] muscle mitochondrial biogenesis and function, which could be beneficial in combating the adverse effects of weight loss on muscle mass[19,35–38]. Improved mitochondrial function in skeletal muscle supports energy expenditure and enhances overall metabolic health. Further investigating the effect and clinical significance of reductions in lean mass, and especially skeletal muscle, with GLP-1 receptor agonist induced weight-loss is important to understand both the quantity and the "quality" of the weight loss achieved.

Another area of research that needs further exploration is the possibility that GLP-1 may modulate skeletal muscle protein turnover in potentially adverse and/or advantageous ways. One pathway of particular interest, due to its importance in skeletal muscle and responsiveness to the insulin axis, is the muscle phosphoinositide 3-Kinase (PI3K)/protein kinase B (AKT) signaling pathway. This pathway is a crucial signaling cascade that mediates various cellular processes in muscle, including cell growth, metabolism, and differentiation. When stimulated by insulin and other growth factors, PI3K activates and in turn activates AKT. AKT plays a vital role in promoting muscle hypertrophy and inhibiting atrophy by phosphorylating several downstream targets, including the mammalian target of rapamycin (mTOR)[39]**.** mTOR then initiates protein synthesis by activation the ribosomal protein S6 (RPS6). AKT also inhibits the activation of many factors associated with degradation of protein and muscle. Therefore, the PI3K/AKT/mTOR pathway is crucial in regulating the balance between protein synthesis and degradation, thus maintaining muscle mass under various physiological conditions[40]. This signalling pathway is also responsible, at least in part, for muscular hypertrophy and protein synthesis following exercise[40,41]. Dysregulation of this pathway has been implicated in many

muscle atrophy and metabolic disorders, showcasing its importance in sustaining skeletal muscle health and function[42,43]. GLP-1 has been linked to the PI3K pathway in many fields as well, from altering cancer cell behaviour[44] to hepatic insulin sensitivity[45], this hormone seems to interact with the PI3K pathway. Due to the established effect of GLP-1 and PI3K pathway on topics such as neuronal repair[46] and protection from oxidative stress induced apoptosis[47], as well as its extensive influence in skeletal muscle production and degradation[40], this pathway is one that we believe has value investigating.

Another open question about incretin therapy and skeletal muscle health is whether GLP-1 treatment differentially affects skeletal muscle fibre types, and perhaps even cause a change in fibre type ratios. This is a relevant topic, considering GLP-1's role in glucose metabolism, insulin sensitivity and recruitment of vasculature in muscle. Since different muscle fibre types have different metabolic characteristics such as insulin sensitivity, adipose deposition and nutrient utilization[48,49], potential differential effects of GLP-1 on different fibre types are very plausible. A research study has shown that neonatal exposure to GLP-1, amongst many other effects, can differentially affect composition of the muscle, with changes observed in myosin heavy chain and GLUT-4 expression[50]. Moreover, the recruitment of microvasculature and improved nitric oxide production[33] observed with GLP-1 infusion suggests a mechanistic pathway that could support a shift towards oxidative muscle fibers, possibly increasing the capacity for endurance activities with prolonged therapy, as long as exercise training also occurs. While the research in this topic is lacking as well, further research has the potential to uncover many important aspects of GLP-1 that were previously unknown.

Considering the breadth of questions surrounding the GLP-1 hormone and considering that there has never been a study directly comparing GLP-1 weight loss with CR weight loss, this research hopes to provide a new angle of analysis on the effects of GLP-1 on skeletal muscle. In this research we will be investigating the difference in skeletal muscle quantity between these two conditions, and the potential differential protein expression and activation, to inquire into possible cellular pathways that might be responsible for this change.

2. LITERATURE REVIEW

2.1.1: OBESITY

Obesity, largely recognized as a chronic disease, is an extremely complicated condition. It has typically been defined by an excessively high body max index (>30), and/or excessive accumulation of adipose tissue in the body, with adverse effects on the body's homeostasis. However, new expert "commission" criteria now define *clinical obesity* as a chronic disease associated with ongoing signs or symptoms of reduced organ function, such as sleep apnea or heart failure[51]. In the expert's own words, more than simply a metabolic condition, *"obesity a chronic, systemic illness characterized by alterations in the function of tissues, organs, the entire individual, or a combination thereof, due to excess adiposity"*[51]. Clinical obesity can lead to severe end-organ damage, causing life-altering and potentially life-threatening complications including heart attack, stroke, and renal failure[52].

In addition to having detrimental impact on the heart, kidneys and other end-organs, as described by the expert Commission paper[51], obesity also has profound effects on skeletal muscle structure and function, contributing to metabolic dysfunction and impaired physical performance. Excess adiposity is associated with increased intramuscular lipid accumulation, which can interfere with insulin signaling pathways, leading to insulin resistance[53]. Additionally, obesity promotes chronic low-grade inflammation, characterized by elevated cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which contribute to muscle catabolism and reduced protein synthesis[53,54]. These changes result in decreased muscle quality and strength, even in the presence of preserved muscle mass, a condition known as "sarcopenic

obesity"[55]. What incretin therapy might do to muscle health in an obese individual is somewhat unclear (see section 2.4 below).

2.1.2: T2D

T2D is a chronic metabolic disorder characterized by hyperglycemia resulting from insulin resistance and progressive pancreatic β-cell dysfunction[56]. It is strongly associated with obesity, physical inactivity, and genetic predisposition, and its global prevalence has risen sharply in recent decades alongside obesity[57]**,** This strong association between the two condition means that T2D also shares many co-morbidities with obesity, leading to microvascular (e.g., retinopathy, nephropathy) and macrovascular (e.g., atherosclerosis) complications [56]. In individuals with T2D, target tissues such as liver, adipose, and skeletal muscle become resistant to insulin, leading to impaired glucose uptake and metabolism[58]. Over time, sustained hyperglycemia and lipotoxicity exacerbate β-cell failure, further impairing insulin secretion and intensifying metabolic dysregulation[58].

Skeletal muscle, which accounts for approximately 80% of postprandial glucose disposal, is profoundly affected by T2D[59]. Insulin resistance in skeletal muscle reduces glucose uptake and glycogen synthesis, primarily due to impaired insulin signaling pathways, including defects in insulin receptor substrate (IRS) and phosphoinositide 3-kinase (PI3K) activity[60]. Additionally, mitochondrial dysfunction and increased lipid accumulation within myocytes contribute to oxidative stress and inflammation, exacerbating insulin resistance[60]. These alterations not only compromise metabolic health but also lead to reduced muscle quality and mass, increasing the risk of sarcopenia and decreased physical function in individuals with T2D. What incretin

therapy might do to muscle health in an individual with T2D (obese or non-obese) is also unclear (see section 2.4 below).

2.2.1: GLP-1

GLP-1 is an endogenous incretin hormone secreted predominantly by intestinal L-cells in response to nutrient intake, particularly carbohydrates and fats[61]. It plays a central role in metabolic regulation by enhancing glucose-stimulated insulin secretion, inhibiting glucagon release, delaying gastric emptying, and promoting satiety[62]. These effects help maintain postprandial glucose homeostasis. GLP-1 exerts its functions via its cell surface receptor, a G protein–coupled receptor expressed not only in pancreatic β-cells but also in extra pancreatic tissues, including the brain, heart, and gastrointestinal tract[61,63]. Due to its rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4), GLP-1 has a short half-life (1-2 minutes), which led to the development of GLP-1 receptor agonists as effective treatments for type 2 diabetes and obesity[64].

GLP-1 has many effects and target tissues, therefore its direct effects and pathways are quite difficult to pinpoint down[34,37,44–46,63]. The drug is now being explored for use in various neurogenerative diseases, such as Alzheimer's disease, where weight loss and lean mass loss may be undesirable, or even problematic[65]. In line with these concerns, emerging evidence indicates that GLP-1 also has direct and indirect effects on skeletal muscle metabolism, possibly promoting a relative attrophy[25,30,37,38,50]. While the concerns about muscle wasting are addressed in depth in the following section, additional confirmed effects of GLP-1 include improved muscle glucose uptake and insulin sensitivity through systemic mechanisms, including

enhanced insulin secretion, improved microvascular perfusion and possibly increased whole body insulin sensitivity from fat mass loss[66]. Additionally, GLP-1RAs have been shown to reduce inflammation and oxidative stress, and improve mitochondrial area, number and morphology[28]**.** While the exact molecular pathways remain unclear, the fact that GLP-1 therapy does affect skeletal muscle is clear to see.

2.2.2: GLP-1 RECEPTOR EXPRESSION

The GLP-1 receptor is found on the surface of many cell types in the body. Due to the popularity of GLP-1 medication, it is critical to investigate what tissues this molecule can exert a direct effect on. GLP-1 receptor is expressed ubiquitously in human tissue, with high receptor expression found in pancreatic islets (particularly in alpha and beta cells), the central nervous system, gastrointestinal tract, heart[26], and glomerular and proximal tubular microvasculature in kidneys[67] . Immunohistochemical and mRNA studies confirm GLP-1 receptor presence in these key metabolic and regulatory organs, aligning with its roles in glucose homeostasis, appetite regulation, and cardiovascular function[68]. While many notable studies have focused on expression of GLP-1 receptors on tissues such as the pancreas and the central nervous system, comparatively not many have focused on its presence in skeletal muscle tissue. Nonetheless, presence of GLP-1 receptors in skeletal muscle has been confirmed[69], and receptor knock-down models tend to result in reduction or abolishment of the effects observed with GLP-1 treatment[70], as will be touched on in a later section.

However, due to the extremely complicated and multifaceted nature of metabolic diseases such as obesity and diabetes, and the many effects of the GLP-1 hormone, it is difficult to pinpoint a direct or indirect effect of the incretin on skeletal muscle structure and function. Likely a considerable amount of the effect that GLP-1 treatment has on skeletal muscle result from indirect systemic effects (i.e., increases in insulin sensitivity from fat loss, changes in circulating glucose concentrations, and reduced food intake due to reduced appetite), vs. direct muscle effects from GLP-1 receptor activation.

2.3.1: TREATMENT OF OBESITY

The treatment of obesity, and consequently weight loss has historically been a lucrative market, with many pharmaceutical and lifestyle intervention programs advertising their promise of fat and weight loss. Obesity has also been recognized as a major pandemic of the 21st century[71]. Despite all of this, many individuals do not continue long-term with their obesity medication[72]. This might be partially due to social stigma around the condition, and perhaps partly due to the side effects traditionally observed with obesity medication. Current pharmaceutical treatment strategies for obesity outside of GLP-1 agonists include medication to reduce fat absorption from food, or medication to reduce appetite, which are often combinations of two medications usually used for dependence and depression therapy[73].

Perhaps partly due to this, and because the weight loss effects on incretin therapy are consistent and dramatic[27], GLP-1 receptor agonists as a new class of therapy for obesity and T2D have gained immense popularity in this field[74].

2.3.2: TREATMENT OF T2D

There are various treatments for T2D, depending on the stage of disease development and the level of obesity, hyperglycemia and associated co-morbidities[75]**.** The current American Diabetes Association (ADA) Standards of Care recommend GLP-1 after initiation of other hypoglycemic agents (such as metformin) if glycemic control is suboptimal and if weight loss is a goal[76]. Incretin therapy is also now preferred over insulin therapy and also prescribed if symptomatic heart failure with preserved ejection fraction exists (ADA standards of care[76,77]). While earlier stages can be controlled with lifestyle interventions, such as weight loss with diet and exercise, many patients fail to achieve clinical benchmarks with lifestyle therapy alone[78]. T2D medication generally aims to improve glycemic control of the body, through various mechanisms. One class of medication is the sodium-glucose cotransporter-2 (SGLT2) inhibitors, which inhibits sodium-glucose transporter 2 in the kidney to reduce reabsorption of glucose, leading to excretion of excess glucose[75]. Another class of medication, the sulfonylureas work by increasing endogenous secretion of insulin in the body[75]. Two classes of medication, namely biguanides (metformin) and thiazolidinediones, are effective for increasing the sensitivity of the body's tissues to insulin [16], but biguanides can result in adverse symptoms (gastrointestinal symptoms) and thiazolidinediones can cause weight gain and have safety concerns (cardiovascular toxicity, bone fractures and edemas[79].

The GLP-1 class of medication were also originally designed for treatment of diabetes since they slow gastric emptying, augment insulin secretion and lower glucagon release to result in better glucose homeostasis at mealtime[80]. However, other beneficial effects were soon discovered, such as satiety, weight loss and enhanced cardiovascular health[27]. While concerns

about possible side effects were initially raised, such as pancreatic and biliary tract disorders, and the risk of thyroid and pancreatic cancer, evidence suggests that this class of drugs is relative safe and highly effective for people with either obesity or T2D [81]. However, many aspects of these medications, particularly with regards to long term use and side effects are still relatively underexplored.

2.4: GLP-1 AND SKELETAL MUSCLE: CONCERNS ABOUT MUSCLE WASTING?

Loss of lean mass is common after weight loss, whether induced by medicines, caloric restriction, or bariatric surgery[82]. In clinical trials of GLP-1RAs, lean body mass has been used as the marker for muscle quantity, and reductions in lean mass as a percentage of total weight lost is typically observed in many clinical trials with obese and or T2D participants[81]. This may be of particular concern if obese individuals already have sarcopenia (termed clinically as sarcopenic obesity). Body composition analyses in people with type 2 diabetes treated with GLP-1RAs have not revealed consistent evidence for disproportionate loss of lean mass or impaired muscle strength[16,29,83]. In some randomized controlled studies and in other clinical observational studies, greater reductions in lean body mass have been observed in GLP-1RA users as compared to non-users[29]. However, this would be expected as GLP-1RA users exhibit overall more weight loss as well. In terms of reductions in lean mass as a percentage of weight lost, the results vary greatly between trials, with percent lean mass reductions from as much as 40 to 60%, to as little as 15% [20,25,28–30]. With this range of results, and as research in human means a lower degree of control over diet and exercise, and a multitude of factors introducing variability

into the results, these numbers are hard to interpret. It is important to note that lean body mass includes skeletal and cardiac muscle, smooth muscle and vascular, bones, fluids and other organs and tissues, so it is at best only an indirect measure of skeletal muscle mass and health[84]. More direct measures of skeletal muscle and bone mass are important in understanding the impact of therapeutic treatment on the various components of lean mass, alongside weight loss, as lean tissue has higher metabolic rate and therefore contributes to preventing weight regain. But skeletal muscle itself is quite an important factor to consider in those who are overweight, especially in those who have T2D because of its critical role in glucose storage and utilization. Even more so for populations that are more vulnerable to losing skeletal muscle mass and are at elevated risk of sarcopenia with ageing[85].

In summary, many of the studies published investigating GLP-1RAs do not examine the mechanisms (and relevance) of muscle mass loss in pre-clinical models or in large-scale clinical trials and mechanistic studies, if they are performed, often fail to account for the reduction in nutrient intake on skeletal muscle mass and function when GLP-1Ras are introduced. Some studies claim that GLP-1RAs can improves body composition, as the weight loss induced by the drug comes more from fat tissue loss than lean mass loss[7], or that the ratio of total fat mass and total lean mass decreases with GLP-1 treatment [86], but these state the fact that less than 50 percent of the weight lost by GLP-1 treatment is lean tissue, which is expected from any treatment and does not help answer any of the concerns about excessive muscle wasting. Other studies report as much as 0.6 kg loss of appendicular lean mass per 1.8kg of fat mass reduction [83], or that a certain percentage of patients on these medications have an excess of 40 percent weight loss from lean mass alone, which can be considered unhealthy loss[17].

2.5: GLP-1 AND SKELETAL MUSCLE: PROTECTIVE EFFECT

Contrary to concerns about muscle wasting, some research articles suggest that short-term GLP-1RAs use might be beneficial for skeletal muscle health. Studies highlighted in the following sections, performed largely on isolated muscle tissue and/or rodent models, have suggested that the muscle mass loss is rather trivial and that the muscle might be more responsive to adaptation after injury or damage. The experimental conditions used to test the theory that muscle might be positively impacted by GLP-1RA range from lipid toxicity to freeze induced injury and are highlighted below.

2.5.1: IN VITRO STUDIES

Studies investigating the effect of GLP-1RA on C2C12 myotubes found that alongside some positive base effects, GLP-1 also exerts a protective effect on the myotubes under a number of conditions such as when under exposure to chemical stressors[70]**.**

Firstly, in normal growth conditions without any stressors added, Liraglutide (another GLP-1 agonist similar to semaglutide) was identified to be an inducer of C2C12 myoblast differentiation [70]. It concentration-dependently increased myosin heavy chain II expression, as well as increased myotube area, length and minimum Feret's diameter [70]. It also was shown to increase MyoD and Myogenin expression, which are myogenic factors associated with muscle building and growth [87]. These results were prevented when GLP-1 receptor (GLPR) was knocked down before treatment[70], providing further evidence of the positive effect of GLP-1 in myotube

differentiation. Liraglutide also significantly increase ATP content, as well as S phase progression and G0 exit, but not as much as when cells were given fetal bovine serum instead [70].

Secondly, in atrophy inducing conditions, liraglutide was shown to help reduce muscle wastage. Dexamethasone, nutrient-deficiency and Lipotoxic environments are all well-known inducers of muscle atrophy[88]. These conditions all caused atrophy of fibres with reduced fibre cross section and diameter, as well as increasing the expression of atrogenes including atrogin-1, MuRF1, cathepsin-L and myostatin. Liraglutide attenuated this increase[70], reducing the aforementioned atrophy markers and increasing the muscle fibre area. Evaluation of signaling pathways showed that Dex reduced phosphorylation of AKT and its downstream targets, and Liraglutide co-treatment attenuated this effect.

Another GLP-1 agonist, extendin-4 (Ex-4), was shown to have beneficial effects in both in vivo and in vitro [89]. It was shown to down regulate expression of atrophic factors in C2C12 myotubes, such as myostatin, MuRF-1 and atrogin-1 in a dose dependant manner. Ex-4 was also effective at reducing increases observed in these atrophic factor when treated with dexamethasone [89], mirroring the previously mentioned results. Myogenic factors such as MyoD and myogenin were also significantly reduced with dexamethasone treatment, but partially recovered with Ex-4[89].

2.5.2: IN VIVO STUDIES

Dexamethasone (a synthetic glucocorticoid known to result in muscle wasting) treatment in 8-week-old Sprague Dawley rats, examined over a period of ~8 weeks, resulted in reduced food

intake and a decline in body weight. Dual treatment with liraglutide, although exhibiting higher weight loss, resulted in significantly higher lean mass than the dexamethasone only group with a lower fat mass [70,89]. Dexamethasone treatment was similarly shown to increase MuRF1 and Atrogin-1 expression and reduced MyoD expression, which was partially ameliorated with liraglutide co-treatment.

Liraglutide also had similar protective effects in muscle atrophy models of ovariectomy [90] (female sprague-dawley rats), and denervation [91] (male sprague-dawley rats). To examine this, researchers cut the sciatic nerve of 8-week-old Sprague-Dawley rats and treated them with either liraglutide or vehicle. Ovariectomy and denervation are both models of muscular atrophy, which is caused by deficiency of estrogen[90] and lack of stimulus respectively[91]. A drastic reduction in muscle mass, cross sectional area and Feret's diameter (a measure of an object's size along a specific direction) was exhibited in the denervated rats, with a significant improvement in the denervated + liraglutide group. The denervated + liraglutide group also showed improvements in myogenic and atrogene balance, with increased MyoD and Myogenin, and decreased MuRF1 and Atrogin-1 [70]. In the case of the ovariectomized (abbreviated as OVX) rats, OVX muscles exhibited lower fiber cross section area (CSA) and Feret's diameter, and the fibres exhibited distortions in shape and size. These findings were attenuated in the group of OVX rats that received liraglutide injections. Liraglutide also once again reversed the OVX-mediated increase in atrogenes and decrease in MyoD and Myogenin expression.

These results were replicated in other research using different agonistic mechanisms of the GLP-1 pathway. Rats receiving Ex-4 on top of dexamethasone treatment, exhibited significant increases in lean muscle mass compared to rats only receiving dexamethasone treatment. The

dexamethasone group showed reduced mass of the gastrocnemius, tibialis anterior, quadriceps, and extensor digitorum longus muscles, which were recovered to the levels of a control group in the when EX-4 was added[89]. The tibialis anterior muscle also showed as much as a 40% reduction in its cross-sectional area in the dexamethasone group, which was recovered with a co-treatment with EX-4. Once again, increases in atrogin-1, MuRF1, myostatin, and blood urea nitrogen (BUN), which are all catabolic markers involved in muscle atrophy, occurred with dexamethasone, but were offset with Ex-4 [92]. EX-4 co-treatment also recovered tropomyosin and myosin heavy chain protein expression[92], thereby suggesting that GLP-1 RA can help reduce muscle protein loss in a high glucocorticoid environment.

Another condition in which skeletal muscle atrophy is usually observed is chronic kidney disease (CKD)[93] and kidneys have significant GLP-1 receptor expression[94]. Researchers studied the interaction of GLP-1 receptor agonist and CKD induced muscle loss on a mouse model of CKD induced through partial kidney removal. Predictably, BUN and creatinine levels were elevated in CKD mice, which are known indicators of poor kidney function[95]. EX-4 treatment reduced both BUN and creatinine levels in this murine model of kidney failure, indicating an improvement in kidney function. Gross body weight and skeletal muscle mass were reduced in CKD mice compared to sham operated mice, but these measures were largely recovered with EX-4 treatment. The fiber cross sectional area (CSA) of the tibialis anterior was also markedly reduced in CKD vehicle-treated mice but attenuated with EX-4 treatment[89]. These findings, alongside the improved grip strength observed in the mice with GLP-1 treatment, suggests a possible improvement in skeletal muscle function in this particular murine model of CKD.

GLP-1RAs have also been examined in rodent models of Duchenne muscular dystrophy. Dystrophin deficiency is a condition that causes irregularities with skeletal muscle and overall loss of muscle mass[96]. In humans, dystrophin deficiency can exhibit as mild symptoms or as very severe cases such as in Duchene's. Dystrophin deficiency causes issues with localization of cellular components, lack of essential complexes that utilize dystrophin and irregularities with satellite cells and replenishment of muscle cells[97]. Researchers investigated the effects of dulaglutide, a long acting GLP-1 agonist, on a dystrophin deficient mouse model. Dulaglutide administration did not show any difference in body weight. However, it did significantly increase the CSA of the tibialis anterior, alongside grip strength and hanging time[89]. This points to the possibility of GLP-1 sparing muscle wasting that is characteristic of dystrophin deficiency, although the mechanisms of this improvement are not clear.

Myocardial infarction has also been shown to reduce exercise capacity and muscle function in patients[98]. These abnormalities include factors such as reduced mitochondrial content and irregular energy metabolism such as disturbances in oxidative phosphorylation [36]. Since GLP-1 receptors are present on both skeletal and cardiac muscle[63], the effects of GLP-1 treatment on the cardiovascular and skeletal muscle systems is an area of interest. Dipeptidyl peptidase-4 is an enzyme that breaks down peptides and is the enzyme responsible for breaking down GLP-1 in the body, and DPP4 inhibitors are used to increase GLP-1 levels without use of direct agonists. In rodents, no significant difference was observed in the muscle mass of mice suffering from a myocardial infarction, treated with a dipeptidyl peptidase-4 inhibitor (MK-0626) or saline control. What did improve however, was the work, run distance, and run time, which were significantly recovered in MI + MK mice compared to MI mice[36]. This was

accompanied by increases in proteins regulating mitochondrial biogenesis, expression of mitochondrial complex subunits and oxidative phosphorylation capacity throughout the skeletal muscle of the mice receiving the DPP-4 inhibitor[36], recovering from deficiencies associated with MI mice receiving no DPP-4 inhibitor. The researchers believe that the positive effects of GLP-1 in the case of myocardial infarction, mostly stem from this pro-mitochondria signals leading to higher mitochondrial content and capacity per mitochondria. A shift of fibre types from glycolytic to oxidative fibre types was also observed in the mice, further strengthening this theory[36].

Based on the protective and positive effects of GLP-1 antagonists on skeletal muscle, the question of whether it can induce the regenerative drive *in vivo* is a possibility. In one experiment with rats exposed to muscle injury with freeze probe injury on the tibialis anterior, with sham operated uninjured rats serving as controls, the effects of liraglutide treatment post injury were observed. Injured muscle showed dead zones with no viable cells and greatly enhanced macrophage infiltration. Meanwhile injured muscle from rats treated with liraglutide exhibited a markedly reduced number of dead zones and macrophage infiltration, with closer to control morphology of the surviving cells. Cross section area and Feret's diameter were also significantly reduced in the injured group, and significantly recovered with liraglutide treatment[70].

Considering all of the conditions under which GLP-1 receptor agonists appear to ameliorate muscle wasting or possible assist with muscle recovery after injury, in the above animal studies, from dexamethasone treatment to chronic kidney disease, it begs the question of how GLP-1RAs might impact muscle health in settings of obesity or diabetes where muscle health can be

suboptimal. While limited research has been conducted to date on possible effects and mechanisms for alterations in muscle health, there exists some evidence that some of the possible beneficial actions of GLP-1RAs in muscle could be related to alterations in various energy utilization pathways in the myocyte, namely the AMP-activated protein kinase (AMPK) pathway [38]. Interestingly, AMPK knocked down cells seem to lose the beneficial effects of GLP-1 in terms of glucose uptake and oxidative phosphorelation[38]. Additional pathways that seem to be activated with GLP-1 receptor agonists are PI3K-AKT signaling pathways, phospholipase D signaling pathways and TCA cycle and pyruvate metabolism[38]. The activation of these pathways points to perhaps a *metabolic advantage* with GLP-1RA-treated cells, on top of the increases observed in myogenic factors (MyoD, Myogenin) as well as decreases in atrogenes. But more research is needed to draw more definitive conclusions.

2.6.1: PI3K PATHWAY: AKT, S6

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays a pivotal role in regulating protein synthesis and muscle hypertrophy in skeletal muscle (visualized in figure A). Upon activation by insulin or growth factors such as insulin-like growth factor 1 (IGF-1), PI3K catalyzes the phosphorylation of phosphatidylinositol, leading to the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This process facilitates the recruitment and activation of Akt, also known as protein kinase B. Once activated, Akt phosphorylates multiple downstream targets that are critical for protein synthesis and muscle growth, including the mammalian target of rapamycin (mTOR) pathway. mTOR acts as a central regulator in muscle metabolism and an effector of Akt signaling. Its activation leads to the phosphorylation of

ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1), which are essential for promoting mRNA translation and thereby enhancing protein synthesis.

Phosphorylated S6K1 stimulates the translation of mRNAs required for building contractile proteins within muscle fibers. Enhanced levels of phosphorylated ribosomal protein S6, a direct substrate of S6K1, indicate increased translational capacity and muscle protein synthesis. This pathway is further implicated in preventing muscle atrophy, as its downregulation is often associated with conditions such as disuse or fasting[99]. Moreover, resistance exercise has been shown to activate the PI3K/Akt/mTOR pathway significantly, underscoring its role in mediating the hypertrophic response to mechanical overload[100]. Additionally, activation of AKT inhibits the FOXO pathway, reducing the levels of downstream proteins associated with protein degradation such as Atrogin and MuRF1, which have been mentioned already.

How exactly GLP-1 and the PI3K pathway interact is not yet fully clear. Because of GLP-1's effect on insulin tolerance and secretion, it is difficult to distinguish between direct and indirect effects it might have on the PI3K pathway. There has been evidence of GLP-1 upregulating key factors in the PI3K pathway such as the IRS-1[101], as well as insulin receptor(IR) and GLP-1 receptor cross talk[102] reducing IR triggered IRS-1 degradation. More direct activation of the PI3K pathway is also suspected, as in previous research inhibiting nodes of the pathway such as GLP-1 receptor inhibition, cAMP inhibition and PI3K inhibition abolished effects of GLP-1 treatment[103].

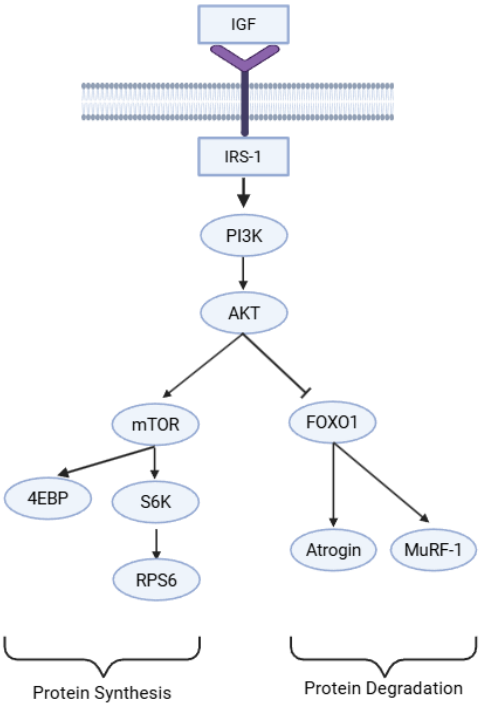

FIGURE A: PI3K/ AKT/ S6 signaling pathway visualized. Insulin-like growth factor (IGF) activates the Insulin receptor substrate (IRS) through the Insulin receptor (IR). IRS leads to the activation of phosphoinositide 3-kianse (PI3K), which turn phosphoinositide 2-phosphate (PIP2) to PIP3, in turn activating protein kinase B (AKT). AKT both activates the mammalian target of Rapamycin (mTOR) complex, as well as inhibits the fork head box O (FoxO) family of transcription factors. mTOR activation inhibits eukaryotic translation initiation factor 4E-binding protein (4EBP), allowing translation initiation factors to function without inhibition. mTOR also activates S6 kinase (S6K), which in turn activates ribosomal protein S6 (RPS6), leading to protein synthesis. On the other hand FoxO inhibition leads to further inhibition of downstream elements such as Atrophy gene-1 (Atrogin) and Muscle RING finger protein-1 (MuRF-1), a ubiquitin ligase that leads to protein degradation.

3.0 RATIONALE

3.1. RATIONALE

Both nutrient deprivation (i.e., caloric restriction) and GLP-1RAs can cause reductions in lean mass in persons with T2D. Since the later (GLP-1RA) also result in the former (reduced caloric intake), it is unclear if the results observed in clinical trials of GLP-1RA on muscle mass loss are direct or indirect and if changes to protein turnover differ between the two weight loss strategies (i.e., diet alone vs. GLP-1RA with associated caloric deficient). This study aims to address the lack of appropriate comparisons between GLP-1RA-induced weight loss and calorie restriction induced weight-loss, by clearly and directly comparing what happens to rats with established T2D in each of these conditions.

3.2. OBJECTIVES

The goal of this study is to directly compare the differences between semaglutide treatment and traditional CR weight loss treatments, particularly pertaining to the effect of weight loss on skeletal muscle mass. By analyzing changes in histological markers of muscle, as well as anabolic and catabolic protein markers of the muscle, the study seeks to determine if semaglutide accelerates muscle atrophy pathways independent of fat loss. As a secondary goal, fibre type specific parameters are also analyzed to determine if there are fibre type specific effects, or changes that affect mainly specific fibre types.

The study hopes to inform further research into confirming the effects of semaglutide on skeletal muscle and exploring possible mechanisms of this effect, performed under different conditions and with bigger sample sizes.

3.3. STUDY ENDPOINTS

The primary histological point of comparison in this study, is muscle weight and cross section area (CSA). Fibre type specific CSA is also measured, and fibre size variability and distribution are considered.

In addition to histological analysis, protein levels of key regulators of muscle atrophy and anabolism, such as AKT and Ribosomal protein S6 are measured. Furthermore, protein marker of muscular atrophy MuRF-1 is measured.

4.0 MATERIALS, METHODS AND EXPERIMENTAL DESIGN

This study was carried out by the recommendation of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2024-7).

For this protocol, we used a total of 27 male Sprague-Dawley rats (1st supplier: Charles River Laboratories, 2nd supplier: Envigo Research Models and Services, ~250-300 grams post-weaned) aged 9-10 weeks old. Rats were housed in groups of 2-3, with a 12-hour light/dark cycle. The rats were first fed on a high-fat feeding (HFF) diet for 3 weeks prior to T2D induction (see section 4.1 below) study onset to help induce insulin resistance with ad libitum access to water. Initially, rats were habituated to the vivarium for seven days before any animal handling. One to two days after T2D diabetes induction, the formal experimental treatment period started. For this, rats were allocated to one of three groups: A) a semaglutide weight loss group (Referred to as Sema, n=9); B) a caloric restricted group (Abbreviated to CR, i.e., pair fed to the Sema group; n=8). And C) A control group allowed to eat ad libitum (n=11). All three groups receive daily blood glucose checks and insulin maintenance. Groups were run in batches for practical reasons.

Note: An important issue to acknowledge here, is that due to a viral contamination of our original supplier, we were forced to order a second batch of rats from a different supplier. Although the same subspecies of rat, the differences observed in baseline weight and feeding behaviours between the two batches of rats forced us to reallocate rats equally to the three treatment groups to try and maintain as little systematic difference between the experimental

groups. Not only this led to lower N than planned for each group, but some of the data derived from the 1st batch, which consisted mostly of controls rats not given the GLP-1RA, was rendered unusable because of baseline weight differences between the groups of rats from the two suppliers to allow for direct comparison with the 2nd batch. As such, the groups are not equally match in numbers which may affect the representability of some of our study endpoints.

4.1. T2D INDUCTION

To induce overt insulin-requiring T2D, HFF rats were injected with a low-dose (35 mg/kg) streptozotocin (STZ) via the intraperitoneal route, as previously described[104]. Before STZ injection, the rats underwent an overnight fast and diabetes was confirmed the next day with a blood glucose meter and tail nick sample, and animals were moved to individual cages.

4.2. TREATMENT AND INSULIN MAINTENANCE PERIOD

After induction of T2D, all animals were checked daily with blood glucose measurements, and insulin was given as needed to maintain blood glucose in acceptable range (<15 mmol/L). Long-acting insulin glargine (Lantus, Sanofi-Aventis, Bridgewater, New Jersey), was used in the evening if necessary to achieve an expectable level of glycemia that is not associated with excessive ketosis, with the amount ranging from 1-3 Units (U-100 glargine insulin) based on the rats blood glucose reading at ~4PM (15-19.9 mmol/L: 1U; 20-24.9: 2U; >25: 3U). Blood ketone monitoring was done daily to assess insulin needs using a hand-held ketone meter (Free style Optium Neo Blood), and in the case of a high ketone reading (>1.5 mmol/L β-hydroxybutyrate), an appropriate small dose of rapid-acting insulin (1-2U, Humalog

insulin, Eli Lilly) was injected subcutaneously to reduce ketosis. To control possible hypoglycemic episodes, this bolus dose was skipped on the day of the insulin tolerance test (ITT) and the oral glucose tolerance test (OGTT) (described in more detail below).

Injections and diet matching started at day 0 of the protocol, 5 days after STZ injection (see Supplementary Figure 1 for a schematic timeline). The GLP-1 receptor agonist-treated group was injected with a subcutaneous dose escalation of semaglutide (Ozempic, Novo Nordisk) once per ~3 days, with doses escalating from 35 μg/kg body weight to 200 μg/kg body weight to limit potential gastrointestinal side effects, which is typically done in human clinical trials and for patients starting on GLP-1RA therapy. To allow for caloric intake matching between the two weight loss groups, daily food intake in the Sema group was first measured daily and used to dictate the amount of food provided for the next 24 hours to the caloric restricted (i.e., nutritional only) weight loss group. This was done to partition out (or control for) the independent effects of semaglutide *per se* on body weight and skeletal muscle from the known effects of the drug on caloric restriction. The control T2D rats received ad-libitum food for the duration of the study, in order to serve as a comparison condition where no weight loss occurs. For practical and cost reason, this study did not have non-diabetic controls with or without GLP-1PA treatment

4.3. ORAL GLUCOSE TOLERANCE TEST

An oral glucose tolerance test (OGTT) was performed in the second week of treatment for further confirmation the severity of T2D in the control group and to compare glucose responses to standardised glucose exposure in the two treatment groups. For this, rats were partially fasted overnight receiving slightly less chow than they typically consume overnight (~6-8 grams of chow when they normally consume ~10-15grams per night). Rats were given 2g/kg D-glucose (90-100% solution in water) through oral gavage and were monitored for two hours with regular blood sampling. Whole blood glucose concentrations were measured (in duplicate) using a glucose meter (Ascentia Contour Next meter) from blood taken from saphenous vein bleed at t=0-, 30-, 60-,90- and 120 minutes post gavage.

4.4. INSULIN TOLERANCE TEST (ITT)/HYPOGLYCEMIC CHALLENGE

An Insulin tolerance test (ITT) was performed on the third week of treatment to compare possible differences in whole-body insulin sensitivity between groups. For this, rats were partially fasted overnight using chow restriction (6-8 grams of chow were provided to each rat when they normally consume ~10-15 grams per night). At ~11 AM the next day, rats were injected subcutaneously with a bolus dose of a rapid-acting insulin analog (8U/kg; NovoRapid, Novo Nordisk, Mississauga, Canada). Whole blood samples were taken from the saphenous vein at t=0-, 30-, 60-,90- and 120 minutes post-insulin administration for subsequent hormone analyses. The blood was centrifuged (10 minutes at 4 degrees Celsius and 13000 RPM) for the isolation of plasma and samples were then placed on ice (see hormone analyses below).

Whole blood glucose concentrations were also monitored from a tail nick or saphenous bleeds every 15 minutes post insulin administration.

4.5. PLASMA AND TISSUE COLLECTION AND PROCESSING

As noted above, whole blood samples were collected during both the OGT test and ITT. The tubes used for saphenous blood collections were coated with EDTA to prevent coagulation. The whole blood was then centrifuged at 12,000 revolutions per minute (RPM) for ~5 minutes. Samples were frozen for future analyses (data not provided in this thesis).

On the terminal experimental day, all rats received a puromycin injection (in saline buffer) of 0.04 µmol/g body weight ~30 minutes and a branched-chain amino acid (leucine, isoleucine, valine, 2:1:1 ratio) mix gavage of 0.30 g/kg bodyweight ~15 minutes before being killed via exsanguination under deep anesthesia with inhaled isoflurane. After exsanguination, the heart, liver, pancreas, and skeletal muscles (both left and right legs: tibialis anterior, soleus, extender digitorum longus and gastrocnemius, shortened to TA, SOL, EDL and GAS respectively) were removed. The muscle tissue collected from one leg was flash-frozen in liquid nitrogen and then transferred to -80^{o}C for future use, while the tissue from the other leg was frozen with liquid nitrogen in OCT-sucrose solution for histological analysis.

4.6. HISTOLOGICAL ANALYSES

Whole muscles frozen with liquid nitrogen in OCT-sucrose solution were sectioned using a Thermo Fisher Microm HM525 NX cryostat at 20 um thickness to obtain cross sections of the muscle and collected on positive charged microscope slides. The cross section was analyzed for

cross section area, fibre type composition and specific fibre type areas. The slides were stained for the three muscle types simultaneously by combining the stains into one cocktail solution diluted in PBS. The following antibodies and concentrations were used. Primary antibody targeting MHCl (BA-F8) at 1:100 dilution– Secondary antibody targeting IgG2b (immunoglobin G2b) at 1:500 dilution, MHCIIa (SC-71) at 1:600 dilution – IgG1 at 1:500 dilution, MHCIIb (BF-F3) at 1:200 dilution – IgM at 1:500 dilution. Samples were washed in PBS solution, blocked for an hour in goat serum (Thermofischer), and incubated in primary antibodies overnight following another PBS wash. The following day slides were once again washed in PBS for 3x5 minutes before being incubated in the secondary antibodies for an hour, after which they were covered with anti-fade mounting media and imaged.

4.7. WESTERN BLOTS

Muscle samples (from GAS, TA, SOL, EDL) were homogenized in RIPA buffer, supplemented just before use with 10µL/ mL of each of protease inhibitor (Sigma Aldrich, #P8340) and phosphatase inhibitor cocktails (Sigma Aldrich, #P5726). Homogenates were then centrifuged at 1000 g for 15min at 4°C. The resulting supernatant was removed. Protein concentrations in the supernatant were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, #23225, Waltham, MA). Equal amounts of protein (~30µg) were separated on 10% or 15% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (0.2µM, BIORAD). Membranes were incubated in primary antibodies (Supplementary Table S1) overnight and in secondary antibodies (HRP-conjugated anti-rabbit (#7074) or anti-mouse (#7076), Cell Signaling Technology, Danvers, MA) for an hour before

being imaged using a Biorad ChemiDoc MP imaging machine, and quantified on the ImageLab application.

4.8. STATISTICAL ANALYSES

All values presented are presented as mean ± standard deviation unless otherwise specified. Statistical analysis and graphing of values was performed in GraphPad Prism (version 10, $p<0.05$ denoting statistical significance). For animal weights, food intake, muscle weights and protein activation analyses, one-way ANOVA with a Tukey's post hoc tests were performed. Where applicable and appropriate (comparison between the two treatment groups) T-tests were performed. For OGTT, ITT and daily blood sugar values two-way ANOVAs were performed.

5.0 RESULTS

5.1. CHARACTERISTICS OF THE RODENT MODEL

5.1.1 Glycemia, body weight and food intake

All rats were initially confirmed to have hyperglycemia (glucose ranging from 15-25 mmol/L) following STZ treatment, but then glucose levels differed over time with the onset of Sema and CR treatment (Figure 1). In this model of STZ induced T2D, we observed a significant drop in average daily blood glucose levels in both treatment groups (i.e. Sema and CR matched controls) relative to non-pair fed T2D controls, as measured both in the morning and in the evening (Figure 1, Table 1). Both treatment groups had similar levels of mild hyperglycemia (10-12 mmol/L) by the end of treatment and required very little exogenous insulin dosing (or none) to maintain glucose levels <15 mmol/L, as per protocol design (Figure 2). The non-pair fed control rats had minor reductions in hyperglycemia severity in the days following the injection of STZ and induction of T2D, but their glycemia stabilized at around 18 mmol/L by around 7 days into the protocol. This group required consistent exogenous insulin dosing to maintain whole blood glucose levels below 15 mmol/L.

As expected, and throughout the protocol, the ad-libitum fed group consumed more total grams of food over the 21-day period than both the Sema and CR groups (means 242±13 g, 158±10 g, 157±5 g, respectively) (Figure 3, Table 1). Also as expected, rats in the control group weighed more (ANOVA $p<0.001$), on average, than in the other two groups (392± 22.5 g),

while the mass of the other two groups were similar (337± 8.7 g vs. 348± 15.6 g in the Sema and caloric restricted groups respectively; p=0.31 Tukey's HST) (Figure 4).

As noted above, the OGTT was performed after a partial overnight fast during week 2 of treatment. On average, the control group had a higher baseline blood glucose level compared to the two other treatment groups, and glycemia was elevated more after gavage in controls relative to the other two groups (Two-way ANOVA p=0.0367 main group effect) but not different between the two treatment groups (Figure 5).

The insulin tolerance test was similarly performed after an overnight fast during week three of treatment (Figure 6). The controls started at a higher blood glucose level (~15 mmol/L) relative to the other groups (Two-way ANOVA p=0.014 main group effect), with the two treatment groups at similar near euglycemic levels (~6-9 mmol/L). Nonetheless, as time progressed after the injection of the insulin bolus, all groups had a fall in glucose concentrations and all reached a similar lowest point of ~3-4 mmol/L by 60 minutes. Of note, 60 minutes after insulin administration and at the end of the ITT, all rats received a standardized dose of 1ml of 25% dextrose solution to recover them from hypoglycemia (see Supplementary Figure S2).

5.2. POST TISSUE HARVEST ANALYSIS

5.2.1. IMPACT OF SEMAGLUTIDE VS CALORIC RESTRICTION INDUCED WEIGHT LOSS ON SKELETAL MUSCLE MASS

As expected, all representative muscles of the control group individuals all weighed significantly more than the same muscle in either the CR or Sema groups (Figure 7, Table 2). There were also slight differences in some of the representative muscles between the two treatment groups. For example, the Sema group showed overall slightly lower absolute muscle weights compared to the CR group. Comparing the weight of the individual muscles between the two groups, GAS was significantly smaller by about 8% in the Sema group (mean: 3539±242) mg as compared to the CR group (mean: 3935±266 p=0.0135). The SOL also weighed less (about 10%) in the Sema group (mean: 137±13 mg) than the CR weight-loss group (mean: 157±7 mg) (T-test, p=0.009). However, the TA mass was not significantly different between groups, however (T-test, p=0.78), but also was numerically less in the Sema group (mean: 713±29 mg) than the CR group (mean:782±59 mg). The EDL also trended lower in the Sema group than in the CR group but the difference was not significant with respective mean of 132±8 and 145±15 for the Sema and CR group (T-test, p=0.29).

The differences in relative muscle masses (muscle mass divided by body weight) were less notable (Figure 8, Table 3). Here, we saw no statistical differences between the CR weight-loss group and the control group (p=0.49; 0.92; 0.99; 0.21, for GAS, TA, SOL and EDL respectively). There were, however, small but statistical differences between the CR weight-loss

group and the Sema group for the relative mass of the GAS (T-test, p=0.042) and SOL (T-test, p=0.027), where the Sema rats appeared to have lost about 5-10% more relative muscle mass. A similar but non-significant trend was noted for TA (T-test, p=0.445) but not for the EDL (T-test, p=0.99).

5.2.2. IMPACT OF SEMAGLUTIDE VS CALORIE RESTRICTION INDUCED WEIGHT LOSS ON SKELETAL MUSCLE CROSS SECTION AND FIBRE TYPES

Average muscle fiber cross-sectional area (CSA) of the GAS is shown in Figure 9 and Table 4. The fibers within the GAS of the control group tended to have larger CSA, by ~10%, than both experiment groups, however these differences were not statistically significant (ANOVA p=0.0743). Within the two treatment groups, there were very small apparent differences (≈3-4 percent) that also did not reach statistical significant (p=0.81 Tukey's HST).

Fiber type distributions within the TA muscle are shown in Figure 10. Across all groups (i.e., Controls, Sema and CR), type I fibers made up 17.4, 19.2 and 18 percent of the total cross section area, while the type IIa fibers made up 45.9, 42.6 and 45.3 percent of the total cross section area and the type IIb fibers accounted for 36.6, 39.1 and 36.7 percent of the total cross-sectional areas in the controls, SEMA and CR groups, respectably, and these ratios were not significantly different among the groups for any of the three fiber types.

5.2.3. IMPACT OF SEMAGLUTIDE VS CALORIE RESTRICTION INDUCED WEIGHT LOSS ON SKELETAL MUSCLE PROTEIN EXPRESSION

Analysis of proteins expression through western blots revealed differential phosphorylation in some key proteins of the PI3K pathway. Samples from the TA and GAS muscle were used in the analysis. Slightly lower phosphorylation (activation) of AKT and Ribosomal Protein S6 was observed in both of the treatment groups compared to the control, but all but one did not reach statistical significance (Figures 11 and 12). No significant difference was found between the two treatment groups.

Analysis of the degradation marker MuRF-1, revealed significant and noticeable differences between the control group, and both the CR and the Sema groups (Figure 13). However, there were no significant differences observed between the Sema and CR groups.

5.3 TABLES

Conditions	N	Average Blood Glucose (mmol/L) Day 21 (Final day)	Average Total Food Consumed (Grams)	Average Final Body Weight (Grams)
Control	11	19.6 ± 2.4	241.9 ± 6.7	392.0 ± 22.2
Sema	8	10.6 ± 2.1	158.0 ± 4	338.0 ± 8.27
Calorie restriction	8	10.3 ± 2.7	157.1 ± 2	347.0 ± 16.2

TABLE 1. SUMMARY OF METABOLIC STATISTICS OF THE THREE GROUPS. Values displayed as mean ± SD.

Conditions	Gastrocnemius	Tibialis Anterior	Soleus	Extensor Digitorum Longus
Control	4648 ± 182	948 ± 31	183 ± 5.6	213 ± 6.9
Sema	3539 ± 242	713 ± 31	137 ± 6.6	133 ± 11.7
Calorie restriction	3935 ± 266	782 ± 59	157 ± 7.2	141 ± 11.6

Table 2: Summary of absolute muscle masses of the different muscle groups across the conditions. Values reported in mg ± standard deviation.

Conditions	Gastrocnemius/ body weight	Tibialis Anterior/ body weight	Soleus/ body weight	Extensor Digitorum Longus/ body weight
Control	11.3 ± 1.06	2.11 ± 3.4	0.46 ± 0.046	0.47 ± 0.1
Sema	10.3 ± 1.68	1.94 ± 0.07	0.41 ± 0.03	0.42 ± 0.047
Calorie Restriction	11.28 ± 0.85	2.08 ± 0.12	0.46 ± 0.013	0.41 ± 0.025

Table 3: Summary of relative muscle masses of the different muscle groups divided by body weight across the conditions. Values reported in mg/g ± standard deviation.

Conditions	Average CSA of GAS fibers
Control	429 ± 239
Sema	376 ± 456
Calorie Restriction	391 ± 287

Table 4: Cross section area of the gastrocnemius muscle of the three groups. Values represented as mean μm ± standard deviation.

6.0 DISCUSSION

6.1. PRINCIPLE FINDINGS

In this study, we have found some evidence of muscle mass loss with prolonged semaglutide treatment over two-to-three weeks in T2D rats that is not fully explained by drug-induced reductions in caloric intake. We also note that while CR appeared to be as effective as Sema for weight loss, improved OGT and enhanced insulin sensitivity, CR tended to be less detrimental for absolute muscle mass loss. While both our Sema treated and CR rats lost muscle mass compared to the no weight-loss control, the Sema group had about 5% more absolute and relative muscle mass loss in most of hind limb (i.e., weight baring) muscles than the CR group. Nonetheless, this study did not observe any major differences in activation of the skeletal muscle PI3K pathway between the two weight-loss groups, but did note noticeable reductions in activation of this pathway compared the no-treatment control group. To summarize, we discovered evidence of marginally increased muscle mass loss with semaglutide treatment when compared to caloric restriction, but were unable to discover any significant differences in protein synthesis pathways between the two conditions

Pharmacologically assisted weight loss with GLP-1RAs is approaching the ~20% desired weight loss magnitude that is typically seen with bariatric surgery[7]. Thanks to this effectiveness, as well as widespread advertisement and attention, GLP-1RAs have been extremely popular and in demand even by those who have not been diagnosed with clinical obesity, or diabetes, that is unresponsive to lifestyle intervention[74]. While the efficacy of GLP-1RA is impressive[27], the long-term efficacy and safety of drug use remains of some concern, particularly now that

the drug is being tested in aging clinical populations who do not have obesity, such as age-related cognitive decline, dementia and Alzheimer's disease[105]. One such aspect, is the relatively underexplored effects of long-term GLP-1RA use on receptor agonists on the skeletal muscle mass in clinical populations of overweight and obesity and T2D where the maintenance of muscle mass and health is deemed important [19].

There is a complex relationship between excess body weight and muscle mass in patients living with obesity or T2D. In fact, in some studies, those who weigh more than what might be considered “healthy” from a BMI perspective can have more muscle mass than those with normal weight[30]. Subsequently, loss of skeletal muscle is an inseparable part of weight loss[30], and is a large contributor to reduced energy expenditure of the body, which leads to most patients regaining most of the weight lost after 5 years[30]. Skeletal muscle can be described by both quantity (size and number of fibers) and quality (the composition of the fibers, the ability of the muscle to generate force and or produce and/or consume ATP), which is influenced by obesity and diabetes[47,52]. For example, individuals living with obesity have higher muscle mass, but are also relatively weaker with reduced function and mobility (as evidence by lower specific force and normalized power of the muscle fibers), which could partly be explained by lower muscle quality[30]. Skeletal muscle is also the main contributor to insulin-stimulated disposal of glucose, and impaired glucose utilization is often seen in obesity and is the main characteristic of diabetes. Furthermore, studies indicate that weight loss correlates with increased insulin sensitivity[106] despite the aforementioned loss of skeletal muscle. Insulin is also an anabolic hormone, which contributes to reduced proteolysis and building more muscle[53], but again, weight loss which correlates to increased insulin action causes muscle loss

and not gain. That is all to say, the hormonal and metabolic pathways involved in diabetes, obesity and the maintenance of skeletal muscle in general are extremely complex and interconnected.

Due to this complexity, separating natural loss of muscle mass from excessive loss is difficult. As the pharmacologic treatment with GLP-1RAs interact with many homeostatic pathways in the body governing nutrition and weight, separating natural and excessive loss of muscle from GLP-1 treatment also proves to be extremely complicated [23,24,70,72]. Currently in the literature, there does exists research investigating the body composition of various clinical sub populations treated with GLP-1RAs [16,29,83,72]. While this information is very valuable, these studies do not include information on proportion of muscle mass loss out of total amount of lean mass (which includes bones, organs, liquid, etc.). Because of the variability of lean mass composition, interpreting how changes of lean mass correspond with changes in skeletal muscle mass is very difficult during weight reduction[86]. Gold-standard techniques for assessment of muscle composition include MRI (magnetic resonance imaging) or CT (computed tomography)[107], but they are uncommon due to cost and availability. Therefore, these factors have led to the absence of a clear standard in assessing changes in skeletal muscle and body composition. Overall, there haven't been sufficient studies directly measuring skeletal muscle mass (or function), and there has not been much comparison between pharmacological GLP-1-induced weight loss and traditional diet/ calorie restriction weight-loss. Therefore, in the present study we aimed to directly compare skeletal muscle between diabetic rats losing weight through CR vs. GLP-1RA, to get a better idea of the direct and indirect effects of GLP-1 in

an attempt to discover some of the possible mechanisms and skeletal muscle off target effects of this pharmacotherapy.

From the results obtained, we observed similar weight loss and subsequent glycemic improvements between both Sema and CR groups when compared with the non-weight-loss control group. This is consistent with previous literature, as both short term caloric restriction[108] and GLP-1 treatment[80] are associated with reduced hyperglycemia in type 2 diabetes. Lower glucose levels could be expected in this rat model of insulin-requiring T2D at baseline from the GLP-1 treated group due to the fact that GLP-1 induces insulin secretion[61], but as both groups achieved close to healthy blood glucose levels this effect could have been masked. Nevertheless, the animals undergoing CR alone or GLP-1RA-induced weight reduction all improved their glycemic control throughout the protocol, and by the end of the treatment period none of the rats in either treatment group required daily insulin dosing to maintain glucose levels below 15 mmol/L. In fact, by week 3, the glycemia in both groups of rats had largely normalized to what would no longer be considered as “diabetic”. This was not observed in the control ad-libitum fed group, as they required continuous and close to daily insulin injections to maintain a healthy blood sugar level and prevent ketosis. At the end of the treatment period, the control animals also weighed significantly more than both weight-loss groups. While the weight and glucose level differences were expected, it not only speaks to the efficacy of semaglutide in improving glycemic control and helping with weight loss but also shows that traditional CR can also be extremely effective in these aspects. While much of the popularity of GLP-1RA treatment lies in helping patients with satiety and controlling their diet in the first place, and it is important to keep in mind that pharmaceutical intervention might not

always be needed, and that traditional dietary control could be an effective alternative to pharmaceuticals.

During the OGTT, we observed higher starting blood glucose levels from the control animals, as well as higher peak and lower rate of recovery. On top of this, the reduced glucose disposal resulting in the higher peak and longer recovery times compared to the treatment groups, does suggest improvements in both treatment group's ability to deal with glucose in the blood, although whether that is due to decreased insulin resistance or increased insulin production is unclear.

During the ITT conducted on week 3 of treatment where much of the weight loss had occurred and had stabilized in the two treatment groups, all three groups, including the controls had similar drops in blood sugar, but the control group had a much more dramatic rise in blood sugar after the end of the ITT where they received oral glucose to recover from the hypoglycemic range. Though the difference in results between the control group and the other groups were to be expected, the similarity between the results of the GLP-1 treated group and the calorie restricted weight-loss group was surprising, As one of GLP-1's main effects is known to be treatment of diabetes and improving insulin sensitivity[76,83], it was surprising to see the group only undergoing CR had similar blood glucose levels in response to ITT and oral glucose. In fact, at some time-points the semaglutide-treated group had higher, and at other points lower blood glucose levels compared to the CR group, although the differences were very small and insignificant, and can therefore most likely be attributed to statistical chance. Although improvements in glucose tolerance and insulin action due to caloric restriction is already an

established fact[108–110], GLP-1RA treatment is known to have a significant effect on increasing insulin sensitivity and improving glucose disposal[62,80,111].

In terms of our hypothesized differential effect of GLP-1 induced weight loss on specific fiber types, we found no significant differences between the fiber type specific cross section area between the CR and Sema weight loss groups. While this is by no means confirmation that GLP-1RA does not differentially affect skeletal muscle fiber types, it does suggest that at least there is no obvious differential reduction in the area of specific fiber types in diabetic rodents treated with Sema, at least under the conditions of this study and in this span of time. Although no significant differences in fiber specific cross-sectional areas were found between the groups, many things are not reflected in simply the area of the cross section that may be more important for function and/or health of the muscle or organism. As an example, conducting functional analysis or investigating the effect of GLP-1RA on the metabolic profiles of the various skeletal muscle fiber types, which is an essential part of what differentiates them[48,112], was not part of the scope of this project. Another important factor that was not included in the scope of this project is recruitment of microvasculature and capillary to fiber ratio, which could have profound effects on the viability of the muscle[113,114]. Other factors such as fat infiltration[115] or skeletal muscle satellite cell activation could also be important factors that require their own investigation. Due to the extremely complicated nature of possible effects that these conditions can have on skeletal muscle, much further probing and studying is required in order to draw better supported conclusions.

In a similar vein, our partial analysis of the PI3K pathway as a potential inducer of the differential muscle loss, resulted in statistically non-significant results. For this we analyzed the

phosphorylation of Protein Kinase B (also known as AKT), alongside the Ribosomal Protein S6 (RPS6, or simply S6), which are both highly regulated and important enzymes in the PI3K pathway. The PI3K pathway is highly associated with insulin action[42], skeletal muscle proliferation[44] and exercise induced muscular hypertrophy[41]. Some of these pathways have already been shown to be affected by GLP-1 in other tissues and in other models of health and disease [45–47]. Activation of AKT and S6 are both key steps in the PI3K/mTOR pathway leading to increased protein synthesis, by activating downstream transcription factors as well as suppressing activity of factors promoting muscular atrophy. Our results, however, showed the differences between the treatment groups were small and/or not statistically significant, although there were significant reductions in the phosphorylation status of AKT and RPS6 when compared to the control group. Moreover, our analysis of muscle degradation marker MuRF-1, which is also a part of the PI3K pathway as an enzyme, led to non-significant results between the two treatment groups as well. MuRF-1 is also an enzyme downstream of AKT which is inhibited by the activation of AKT, so the non-significant results following AKT could be expected. Future studies may wish to further probe these and other markers of skeletal muscle protein turnover when GLP-1RA are taken.

This study has numerous limitations that should be acknowledged (see also section 6.2 below). Reflecting on this study, one important point to consider is the differences between the conditions tested and day-to-day life. As pointed out previously, one of the main advantages of GLP-1RA treatment is the reduction in appetite that helps patients maintain their calorie deficit[62]. Therefore, in a real-life scenario with an uncontrolled diet it would be quite difficult to expect or enforce a similar caloric restriction condition that the rats were placed in, on oneself

or human subjects. Moreover, only male rats were used in this study and the number of rats used might not have been enough for some of the comparisons made, especially due to the reallocation of the groups we had to do due to baseline batch differences in body weights. As noted in our methods section, we needed to change rat suppliers and that change may have confounded some of our results. Finally, the duration of study may have been too short (or too long) to observe differences in muscle protein turnover markers which could have occurred either before the time points selected for muscle collection or at some later date after treatment was initiated. In addition, we did not have any markers of muscle function, nor muscle mitochondrial content or vasculature, which is also worth exploring.

In conclusion, even after these studies have been performed, it remains difficult to conclude with certainty if GLP-1RA therapy affects skeletal muscle more than the drug-induced reduction in caloric intake in this rat model of T2D. Nonetheless, both CR and GLP-1RA cause large reductions in absolute skeletal muscle mass, without changing fiber type distributions, while similarly improving whole body glycemia and likely insulin sensitivity. GLP-RA did seem to result in marginally lower relative muscle mass, but no differences in activation of protein synthesis pathways were found. It could either be argued that slight reduction in muscle mass might not be all that detrimental, since improvements to overall insulin sensitivity and glycemia in those with T2D have been observed.

6.2. STUDY STRENGTHS AND LIMITATIONS

This study aimed to directly compare muscle weight and histological parameters between calorie restriction weight loss and semaglutide treatment, which is different from the commonly seen body composition measures performed in GLP-1 trials and clinical trials. This study presents several strengths that help to highlight its novelty. Firstly, as mentioned before, we directly measured multiple aspects of skeletal muscle mass and metabolism in a male rat model of T2D, rather than using surrogate measures of body composition. What makes this thesis more compelling is the diet-matching of the CR weight loss group to the Sema group, in order to limit effects due to nutritional differences, compared to self regulated diet in humans or pre-decided diets in animal studies. One main point of consideration in this study was induction of T2D in the rodents, as T2D is the main clinical population for GLP-1 medications, with high overlap with even the population only seeking to use GLP-1RAs as a means of weight loss. We also believe skeletal muscle to be extremely important in T2D for overall metabolic health and function, and therefore important to be studied in the context of T2D and incretin therapies, as well.

There were also a number of limitations in this research that should be acknowledged and addressed in further research in this topic. Primarily, the sample size of rats assigned to each group was rather small, even smaller than what we originally intended. This is because due to a supplying issue, our main batch of animals had to be ordered from a different supplier than our pilot batch (which was from our usual supplier), and despite our best efforts to match their parameters, our main batch ended up with rats that were overall smaller and more resistant to gaining weight or diabetes. This not only meant most of the data from the first

batch could not be used, but our main batch was also rats that we were not as accustomed to handling. That brings us to the second point. Due the main batch exhibiting different weight and glucose parameters than our pilot, our experience with keeping our usual rats in the desirable glucose range and rescuing them at the perfect time was mostly not applicable. As an example, the insulin tolerance test that usually yields us 4 blood draw timepoints was only able to be continued until the 3rd, and our rescues had to be more generous with sucrose solution and sugary feed for the next day. This might have caused unequal eating, which usually would have been avoided with our experience in the exact amount of food needed to rescue an animal from hypoglycemia.

6.3. THESIS SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

Our findings overall suggest that there is legitimacy to the concerns of skeletal muscle mass loss due to semaglutide weight loss intervention, although an investigation of muscle quality or functional strength was not part of this study. These results highlight the importance of investigating the possible off target effects of medication, particularly one with as wide a breadth of effects as GLP-1 receptor antagonists. The mechanisms and particular aspects of this effect remain unclear, but nonetheless these results provide a reason or motivation to conduct more rigorous testing on this topic.

Possible future directions for this study include investigating these effects with larger sample sizes and investigating animals with different metabolic profiles (in different phases of growth, high fat fed for longer, etc.). A worthwhile avenue of research would be investigating differential expression and activation of other proteins and markers associated with muscle

atrophy and hypertrophy (IGF-1R levels, PI3K levels, mTOR activation, FoxO levels, Myostatin, MyoD, etc.), as this study was quite limited in its scope. Yet another possible avenue would be investigating the effects of resistance training or activity on these parameters, which we did not have the resources to perform. Finally investigating not only muscular weight and area, but in fact muscle quality through functional tests and similar methods would also be of great value, as such markers were outside the scope of this study.

In conclusion, this study serves as a confirmatory pilot to indicate that further research in this topic is required and is valuable. The results support the concern of differential skeletal muscle mass loss between GLP-1 receptor antagonist treatments but were unable to draw conclusions about possible pathways or differential effect on muscle fibre types.

7.0 REFERENCES

1. Global, regional, and national burden of diabetes from 1990 to 2021, with projections of prevalence to 2050: a systematic analysis for the Global Burden of Disease Study 2021. *Lancet Lond. Engl.* **402**, 203–234 (2023).
2. Purnell, J. Q. Definitions, Classification, and Epidemiology of Obesity. in *Endotext* (eds. Feingold, K. R. et al.) (MDText.com, Inc., South Dartmouth (MA), 2000).
3. Gómez-Ambrosi, J., Catalán, V. & Frühbeck, G. The evolution of the understanding of obesity over the last 100 years. *Int. J. Obes.* **49**, 168–176 (2025).
4. Grandl, G., Novikoff, A., Liu, X. & Müller, T. D. Recent achievements and future directions of anti-obesity medications. *Lancet Reg. Health Eur.* **47**, 101100 (2024).
5. Drucker, D. J., Habener, J. F. & Holst, J. J. Discovery, characterization, and clinical development of the glucagon-like peptides. *J. Clin. Invest.* **127**, 4217–4227 (2017).
6. Chou, J.-J. *et al.* Dietary Intake and Weight Changes 5 Years After Laparoscopic Sleeve Gastrectomy. *Obes. Surg.* **27**, 3240–3246 (2017).
7. Wilding, J. P. H. *et al.* Once-Weekly Semaglutide in Adults with Overweight or Obesity. *N. Engl. J. Med.* **384**, 989–1002 (2021).
8. Drucker, D. J. Discovery of GLP-1–Based Drugs for the Treatment of Obesity. *N. Engl. J. Med.* **392**, 612–615 (2025).
9. Market Intelligence Report: Antidiabetic Drugs, 2012-2021. https://www.canada.ca/en/patented-medicine-prices-review/services/npduis/analytical-studies/mir-antidiabetic-drugs-2012-2021.html (2023).
10. American Diabetes Association Professional Practice Committee. 8. Obesity and Weight Management for the Prevention and Treatment of Type 2 Diabetes: Standards of Care in Diabetes–2025. *Diabetes Care* **48**, S167–S180 (2024).
11. Wang, N. *et al.* Liraglutide reduces bone marrow adipogenesis by miR-150-5p/ GDF11 axis in diabetic rats. *Eur. J. Pharmacol.* **978**, 176793 (2024).
12. Tonon Firmino, F. *et al.* High Dose of Liraglutide Impairs Renal Function in Female Hypertensive Rats. *J. Cardiovasc. Pharmacol.* **85**, 120–128 (2025).
13. Thomsen, R. W., Mailhac, A., Løhde, J. B. & Pottegård, A. Real-world evidence on the utilization, clinical and comparative effectiveness, and adverse effects of newer GLP-1RA-based weight-loss therapies. *Diabetes Obes. Metab.* **27 Suppl 2**, 66–88 (2025).
14. Weiss, E. P., Jordan, R. C., Frese, E. M., Albert, S. G. & Villareal, D. T. Effects of Weight Loss on Lean Mass, Strength, Bone, and Aerobic Capacity. *Med. Sci. Sports Exerc.* **49**, 206–217 (2017).
15. Armamento-Villareal, R. *et al.* Changes in thigh muscle volume predict bone mineral density response to lifestyle therapy in frail, obese older adults. *Osteoporos. Int. J. Establ. Result Coop. Eur. Found. Osteoporos. Natl. Osteoporos. Found. USA* **25**, 551–558 (2014).
16. Jiao, R. *et al.* Characterizing body composition modifying effects of a glucagon-like peptide 1 receptor-based agonist: A meta-analysis. *Diabetes Obes. Metab.* **27**, 259–267 (2025).
17. Volpe, S. *et al.* Once-Weekly Semaglutide Induces an Early Improvement in Body Composition in Patients with Type 2 Diabetes: A 26-Week Prospective Real-Life Study. *Nutrients* **14**, 2414 (2022).

18. Karakasis, P., Patoulias, D., Fragakis, N. & Mantzoros, C. S. Effect of glucagon-like peptide-1 receptor agonists and co-agonists on body composition: Systematic review and network meta-analysis. *Metabolism.* **164**, 156113 (2025).
19. Linge, J., Birkenfeld, A. L. & Neeland, I. J. Muscle Mass and Glucagon-Like Peptide-1 Receptor Agonists: Adaptive or Maladaptive Response to Weight Loss? *Circulation* **150**, 1288–1298 (2024).
20. Neeland, I. J., Linge, J. & Birkenfeld, A. L. Changes in lean body mass with glucagon-like peptide-1-based therapies and mitigation strategies. *Diabetes Obes. Metab.* **26**, 16–27 (2024).
21. Heymsfield, S. B. *et al.* Body-size dependence of resting energy expenditure can be attributed to nonenergetic homogeneity of fat-free mass. *Am. J. Physiol. Endocrinol. Metab.* **282**, E132-138 (2002).
22. Fujimoto, B. A. *et al.* Disrupted glucose homeostasis and skeletal-muscle-specific glucose uptake in an exocyst knockout mouse model. *J. Biol. Chem.* **296**, 100482 (2021).
23. Zurlo, F., Larson, K., Bogardus, C. & Ravussin, E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J. Clin. Invest.* **86**, 1423–1427 (1990).
24. Srikanthan, P., & Karlamangla, A. S. (2011). Relative muscle mass is inversely associated with insulin resistance and prediabetes - Google Search. https://www.google.com/search?q=Srikanthan%2C+P.%2C+%26+Karlamangla%2C+A.+S.+(2011).+Relative+muscle+mass+is+inversely+associated+with+insulin+resistance+and+prediabetes&rlz=1C1RXQR_enCA1147CA1147&oq=Srikanthan%2C+P.%2C+%26+Karlamangla%2C+A.+S.+(2011).+Relative+muscle+mass+is+inversely+associated+with+insulin+resistance+and+prediabetes&gs_lcrp=EgZjaHJvbWUyBggAEEUYOdIBBzg0M2owajeoAgCwAgA&sourceid=chrome&ie=UTF-8.
25. Sargeant, J. A. *et al.* A Review of the Effects of Glucagon-Like Peptide-1 Receptor Agonists and Sodium-Glucose Cotransporter 2 Inhibitors on Lean Body Mass in Humans. *Endocrinol. Metab.* **34**, 247–262 (2019).
26. Pyke, C. *et al.* GLP-1 receptor localization in monkey and human tissue: novel distribution revealed with extensively validated monoclonal antibody. *Endocrinology* **155**, 1280–1290 (2014).
27. White, G. E. *et al.* Real-world weight loss effectiveness of GLP-1 agonists among patients with type 2 diabetes: a retrospective cohort study. *Obes. Silver Spring Md* **31**, 537–544 (2023).
28. Old, V. J., Davies, M. J., Papamargaritis, D., Choudhary, P. & Watson, E. L. The Effects of Glucagon-Like Peptide-1 Receptor Agonists on Mitochondrial Function Within Skeletal Muscle: A Systematic Review. *J. Cachexia Sarcopenia Muscle* **16**, e13677 (2025).
29. Jiao, R. *et al.* Characterizing body composition modifying effects of a glucagon-like peptide 1 receptor-based agonist: A meta-analysis. *Diabetes Obes. Metab.* **27**, 259–267 (2025).
30. Neeland, I. J., Linge, J. & Birkenfeld, A. L. Changes in lean body mass with glucagon-like peptide-1-based therapies and mitigation strategies. *Diabetes Obes. Metab.* **26**, 16–27 (2024).
31. Evans, W. J. Lean body mass should not be used as a surrogate measurement of muscle mass in malnourished men and women: Comment on Compher et al. *JPEN J. Parenter. Enteral Nutr.* **46**, 1497–1499 (2022).
32. Abdulla, H. *et al.* Glucagon-like peptide 1 infusions overcome anabolic resistance to feeding in older human muscle. *Aging Cell* **19**, e13202 (2020).

33. Dong, Z. *et al.* Protein kinase A mediates glucagon-like peptide 1-induced nitric oxide production and muscle microvascular recruitment. *Am. J. Physiol.-Endocrinol. Metab.* **304**, E222–E228 (2013).
34. SUBARAN, S. C. *et al.* GLP-1 at physiological concentrations recruits skeletal and cardiac muscle microvasculature in healthy humans. *Clin. Sci. Lond. Engl. 1979* **127**, 163–170 (2014).
35. Keller, A. C. *et al.* Saxagliptin Restores Vascular Mitochondrial Exercise Response in the Goto-Kakizaki Rat. *J. Cardiovasc. Pharmacol.* **65**, 137 (2015).
36. Takada, S. *et al.* Dipeptidyl peptidase-4 inhibitor improved exercise capacity and mitochondrial biogenesis in mice with heart failure via activation of glucagon-like peptide-1 receptor signalling. *Cardiovasc. Res.* **111**, 338–347 (2016).
37. Old, V. J., Davies, M. J., Papamargaritis, D., Choudhary, P. & Watson, E. L. The Effects of Glucagon-Like Peptide-1 Receptor Agonists on Mitochondrial Function Within Skeletal Muscle: A Systematic Review. *J. Cachexia Sarcopenia Muscle* **16**, e13677 (2025).
38. Wu, L. *et al.* GLP-1 regulates exercise endurance and skeletal muscle remodeling via GLP-1R/AMPK pathway. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1869**, 119300 (2022).
39. Yoon, M.-S. The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. *Nutrients* **9**, 1176 (2017).
40. Glass, D. J. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. *Curr. Top. Microbiol. Immunol.* **346**, 267–278 (2010).
41. Wu, C., Jiang, F., Wei, K. & Jiang, Z. Exercise activates the PI3K-AKT signal pathway by decreasing the expression of 5α-reductase type 1 in PCOS rats. *Sci. Rep.* **8**, 7982 (2018).
42. Kang, C.-W., Park, M. & Lee, H.-J. Mulberry (Morus alba L.) Leaf Extract and 1-Deoxynojirimycin Improve Skeletal Muscle Insulin Resistance via the Activation of IRS-1/PI3K/Akt Pathway in db/db Mice. *Life* **12**, 1630 (2022).
43. Kuramoto, N. *et al.* Role of PDK1 in skeletal muscle hypertrophy induced by mechanical load. *Sci. Rep.* **11**, 3447 (2021).
44. Tong, G. *et al.* Effects of GLP-1 Receptor Agonists on Biological Behavior of Colorectal Cancer Cells by Regulating PI3K/AKT/mTOR Signaling Pathway. *Front. Pharmacol.* **13**, (2022).
45. Shi, X. *et al.* Central GLP-2 Enhances Hepatic Insulin Sensitivity via Activating PI3K Signaling in POMC Neurons. *Cell Metab.* **18**, 86–98 (2013).
46. Yang, J.-L., Chen, W.-Y., Chen, Y.-P., Kuo, C.-Y. & Chen, S.-D. Activation of GLP-1 Receptor Enhances Neuronal Base Excision Repair via PI3K-AKT-Induced Expression of Apurinic/Apyrimidinic Endonuclease 1. *Theranostics* **6**, 2015–2027 (2016).
47. Zhang, H. *et al.* Glucagon-like peptide-1 protects cardiomyocytes from advanced oxidation protein product-induced apoptosis via the PI3K/Akt/Bad signaling pathway. *Mol. Med. Rep.* **13**, 1593–1601 (2016).
48. Lillioja, S. *et al.* Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. *J. Clin. Invest.* **80**, 415–424 (1987).
49. Stuart, C. A. *et al.* Slow-Twitch Fiber Proportion in Skeletal Muscle Correlates With Insulin Responsiveness. *J. Clin. Endocrinol. Metab.* **98**, 2027–2036 (2013).
50. Wang, L., Guo, F., Wei, S. & Zhao, R. Neonatal Intramuscular Injection of Plasmid DNA Encoding GLP-1Reduces Serum Insulin Level and Modifies Skeletal Muscle MyosinHeavy Chain Composition in Adult Rats. *Physiol. Res.* 571–579 (2010) doi:10.33549/physiolres.931815.

51. Rubino, F. *et al.* Definition and diagnostic criteria of clinical obesity. *Lancet Diabetes Endocrinol.* **13**, 221–262 (2025).
52. Caroline M. Apovian, M. D. Obesity: Definition, Comorbidities, Causes, and Burden. **22**, (2016).
53. Wu, H. & Ballantyne, C. M. Skeletal muscle inflammation and insulin resistance in obesity. *J. Clin. Invest.* **127**, 43–54.
54. Li, Y. *et al.* The Change of Skeletal Muscle Caused by Inflammation in Obesity as the Key Path to Fibrosis: Thoughts on Mechanisms and Intervention Strategies. *Biomolecules* **15**, 20 (2025).
55. ROLLAND, Y. *et al.* SARCOPENIA: ITS ASSESSMENT, ETIOLOGY, PATHOGENESIS, CONSEQUENCES AND FUTURE PERSPECTIVES. *J. Nutr. Health Aging* **12**, 433–450 (2008).
56. Galicia-Garcia, U. *et al.* Pathophysiology of Type 2 Diabetes Mellitus. *Int. J. Mol. Sci.* **21**, 6275 (2020).
57. Khan, M. A. B. *et al.* Epidemiology of Type 2 Diabetes – Global Burden of Disease and Forecasted Trends. *J. Epidemiol. Glob. Health* **10**, 107–111 (2020).
58. Fonseca, V. A. Defining and Characterizing the Progression of Type 2 Diabetes. *Diabetes Care* **32**, S151–S156 (2009).
59. DeFronzo, R. A. & Tripathy, D. Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. *Diabetes Care* **32**, S157–S163 (2009).
60. Sylow, L., Kleinert, M., Richter, E. A. & Jensen, T. E. Exercise-stimulated glucose uptake - regulation and implications for glycaemic control. *Nat. Rev. Endocrinol.* **13**, 133–148 (2017).
61. Holst, J. J. The Physiology of Glucagon-like Peptide 1. *Physiol. Rev.* **87**, 1409–1439 (2007).
62. Drucker, D. J. Mechanisms of Action and Therapeutic Application of Glucagon-like Peptide-1. *Cell Metab.* **27**, 740–756 (2018).
63. Baggio, L. L. *et al.* GLP-1 Receptor Expression Within the Human Heart. *Endocrinology* **159**, 1570–1584 (2018).
64. Drucker, D. J. GLP-1 physiology informs the pharmacotherapy of obesity. *Mol. Metab.* **57**, 101351 (2021).
65. Sabbagh, M. *et al.* Safety considerations of semaglutide in the potential treatment of Alzheimer's disease: A pooled analysis of semaglutide in adults aged ≥ 65 years. *Alzheimers Dement. Transl. Res. Clin. Interv.* **11**, e70076 (2025).
66. Kim, M. *et al.* GLP-1 receptor activation and Epac2 link atrial natriuretic peptide secretion to control of blood pressure. *Nat. Med.* **19**, 567–575 (2013).
67. Badve, S. V. *et al.* Effects of GLP-1 receptor agonists on kidney and cardiovascular disease outcomes: a meta-analysis of randomised controlled trials. *Lancet Diabetes Endocrinol.* **13**, 15–28 (2025).
68. Grunddal, K. V. *et al.* Expression Profile of the GLP-1 Receptor in the Gastrointestinal Tract and Pancreas in Adult Female Mice. *Endocrinology* **163**, bqab216 (2022).
69. Gribble, F. M. & Reimann, F. Metabolic Messengers: glucagon-like peptide 1. *Nat. Metab.* **3**, 142–148 (2021).
70. Gurjar, A. A. *et al.* Long acting GLP-1 analog liraglutide ameliorates skeletal muscle atrophy in rodents. *Metabolism* **103**, 154044 (2020).
71. Ogden, C. L., Carroll, M. D., Fryar, C. D. & Flegal, K. M. Prevalence of Obesity Among Adults and Youth: United States, 2011-2014. *NCHS Data Brief* 1–8 (2015).

72. Lemstra, M., Bird, Y., Nwankwo, C., Rogers, M. & Moraros, J. Weight loss intervention adherence and factors promoting adherence: a meta-analysis. *Patient Prefer. Adherence* **10**, 1547–1559 (2016).
73. Prescription Medications to Treat Overweight & Obesity - NIDDK. *National Institute of Diabetes and Digestive and Kidney Diseases* https://www.niddk.nih.gov/health-information/weight-management/prescription-medications-treat-overweight-obesity.
74. Watanabe, J. H., Kwon, J., Nan, B. & Reikes, A. Trends in glucagon-like peptide 1 receptor agonist use, 2014 to 2022. *J. Am. Pharm. Assoc.* **64**, 133–138 (2024).
75. What Are My Options for Type 2 Diabetes Medications? | ADA. https://diabetes.org/health-wellness/medication/oral-other-injectable-diabetes-medications.
76. American Diabetes Association Professional Practice Committee. 9. Pharmacologic Approaches to Glycemic Treatment: Standards of Care in Diabetes—2025. *Diabetes Care* **48**, S181–S206 (2024).
77. American Diabetes Association Professional Practice Committee. 10. Cardiovascular Disease and Risk Management: Standards of Care in Diabetes—2025. *Diabetes Care* **48**, S207–S238 (2024).
78. Sagastume, D. *et al.* The effectiveness of lifestyle interventions on type 2 diabetes and gestational diabetes incidence and cardiometabolic outcomes: A systematic review and meta-analysis of evidence from low- and middle-income countries. *eClinicalMedicine* **53**, 101650 (2022).
79. Lebovitz, H. E. Thiazolidinediones: the Forgotten Diabetes Medications. *Curr. Diab. Rep.* **19**, 151 (2019).
80. Nadkarni, P., Chepurny, O. G. & Holz, G. G. Regulation of glucose homeostasis by GLP-1. *Prog. Mol. Biol. Transl. Sci.* **121**, 23–65 (2014).
81. Drucker, D. J. Efficacy and Safety of GLP-1 Medicines for Type 2 Diabetes and Obesity. *Diabetes Care* **47**, 1873–1888 (2024).
82. Chaston, T. B., Dixon, J. B. & O'Brien, P. E. Changes in fat-free mass during significant weight loss: a systematic review. *Int. J. Obes.* **31**, 743–750 (2007).
83. McCrimmon, R. J. *et al.* Effects of once-weekly semaglutide vs once-daily canagliflozin on body composition in type 2 diabetes: a substudy of the SUSTAIN 8 randomised controlled clinical trial. *Diabetologia* **63**, 473–485 (2020).
84. Heymsfield, S. B. *et al.* Response to 'Lean body mass should not be used as a surrogate measurement of muscle mass in malnourished men and women: Comment on Compher et al'. *JPEN J. Parenter. Enteral Nutr.* **46**, 1500–1501 (2022).
85. Oliver, C. J. *et al.* Fat-Free Mass: Friend or Foe to Metabolic Health? *J. Cachexia Sarcopenia Muscle* **16**, e13714 (2025).
86. Jastreboff, A. M. *et al.* Tirzepatide Once Weekly for the Treatment of Obesity. *N. Engl. J. Med.* **387**, 205–216 (2022).
87. Zammit, P. S. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin. Cell Dev. Biol.* **72**, 19–32 (2017).
88. Singh, A. K. *et al.* Small molecule adiponectin receptor agonist GTDF protects against skeletal muscle atrophy. *Mol. Cell. Endocrinol.* **439**, 273–285 (2017).
89. Hong, Y., Lee, J. H., Jeong, K. W., Choi, C. S. & Jun, H. Amelioration of muscle wasting by glucagon-like peptide-1 receptor agonist in muscle atrophy. *J. Cachexia Sarcopenia Muscle* **10**, 903–918 (2019).

90. Huang, Y., Ling, L. & Xiong, Z. Establishment of rat model of sarcopenia by ovariectomy and estrogen replacement therapy. *J. Army Med. Univ.* **45**, 1937–1946 (2023).
91. Finol, H. J., Lewis, D. M. & Owens, R. The effects of denervation on contractile properties or rat skeletal muscle. *J. Physiol.* **319**, 81–92 (1981).
92. Haines, R. W. *et al.* Elevated urea-to-creatinine ratio provides a biochemical signature of muscle catabolism and persistent critical illness after major trauma. *Intensive Care Med.* **45**, 1718–1731 (2019).
93. Cheng, T.-C., Huang, S.-H., Kao, C.-L. & Hsu, P.-C. Muscle Wasting in Chronic Kidney Disease: Mechanism and Clinical Implications—A Narrative Review. *Int. J. Mol. Sci.* **23**, 6047 (2022).
94. Muskiet, M. H. A. *et al.* GLP-1 and the kidney: from physiology to pharmacology and outcomes in diabetes. *Nat. Rev. Nephrol.* **13**, 605–628 (2017).
95. Hosten, A. O. BUN and Creatinine. in *Clinical Methods: The History, Physical, and Laboratory Examinations* (eds. Walker, H. K., Hall, W. D. & Hurst, J. W.) (Butterworths, Boston, 1990).
96. Evans, W. J. *et al.* Reductions in functional muscle mass and ability to ambulate in Duchenne muscular dystrophy from ages 4 to 24 years. *J. Physiol.* **602**, 4929–4939 (2024).
97. Bonilla, E. *et al.* Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. *Cell* **54**, 447–452 (1988).
98. Sato, R. *et al.* Decreased Appendicular Skeletal Muscle Mass is Associated with Poor Outcomes after ST-Segment Elevation Myocardial Infarction. *J. Atheroscler. Thromb.* **27**, 1278–1287 (2020).
99. Liu, H. *et al.* Regulation of Contractile Proteins and Protein Translational Signaling in Disused Muscle. *Cell. Physiol. Biochem.* **30**, 1202–1214 (2012).
100. Karagounis, L. G. *et al.* Contraction-induced changes in TNFα and Akt-mediated signalling are associated with increased myofibrillar protein in rat skeletal muscle. *Eur. J. Appl. Physiol.* **109**, 839–848 (2010).
101. Gao, H. *et al.* GLP-1 amplifies insulin signaling by up-regulation of IRβ, IRS-1 and Glut4 in 3T3-L1 adipocytes. *Endocrine* **32**, 90–95 (2007).
102. Wang, Y., Song, X., Wang, Y. & Wang, N. Specific interaction of insulin receptor and GLP-1 receptor mediates crosstalk between their signaling. *Biochem. Biophys. Res. Commun.* **636**, 31–39 (2022).
103. Bose, A. K., Mocanu, M. M., Carr, R. D., Brand, C. L. & Yellon, D. M. Glucagon-like Peptide 1 Can Directly Protect the Heart Against Ischemia/Reperfusion Injury. *Diabetes* **54**, 146–151 (2005).
104. D'Souza, N. C. *et al.* Evaluating the effectiveness of a novel somatostatin receptor 2 antagonist, ZT-01, for hypoglycemia prevention in a rodent model of type 2 diabetes. *Front. Pharmacol.* **15**, 1302015 (2024).
105. Chuansangeam, M., Phadungsaksawasdi, P., Park, H. J. & Yang, Y.-H. Exploring the link between GLP-1 receptor agonists and dementia: A comprehensive review. *J. Alzheimers Dis. Rep.* **9**, 25424823251342182 (2025).
106. Guilherme, A., Virbasius, J. V., Puri, V. & Czech, M. P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **9**, 367–377 (2008).
107. Tirzepatide and muscle composition changes in people with type 2 diabetes (SURPASS-3 MRI): a post-hoc analysis of a randomised, open-label, parallel-group, phase 3 trial - The

Lancet Diabetes & Endocrinology. https://www.thelancet.com/journals/landia/article/PIIS2213-8587(25)00027-0/fulltext.
108. Weiss, E. P. *et al.* Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial2. *Am. J. Clin. Nutr.* **84**, 1033–1042 (2006).
109. Atkinson, R. L. & and Kaiser, D. L. Effects of calorie restriction and weight loss on glucose and insulin levels in obese humans. *J. Am. Coll. Nutr.* **4**, 411–419 (1985).
110. Mezhnina, V. & Kondratov, R. Regulation of glucose homeostasis by calorie restriction and periodic fasting. *Aging* **12**, 23422–23424 (2020).
111. Drucker, D. J. GLP-1-based therapies for diabetes, obesity and beyond. *Nat. Rev. Drug Discov.* (2025) doi:10.1038/s41573-025-01183-8.
112. Essén, B., Jansson, E., Henriksson, J., Taylor, A. W. & Saltin, B. Metabolic Characteristics of Fibre Types in Human Skeletal Muscle. *Acta Physiol. Scand.* **95**, 153–165 (1975).
113. Tan, R. *et al.* Skeletal muscle fiber-type-specific changes in markers of capillary and mitochondrial content after low-volume interval training in overweight women. *Physiol. Rep.* **6**, e13597 (2018).
114. Barnouin, Y. *et al.* Coupling between skeletal muscle fiber size and capillarization is maintained during healthy aging. *J. Cachexia Sarcopenia Muscle* **8**, 647–659 (2017).
115. Wang, L., Valencak, T. G. & Shan, T. Fat infiltration in skeletal muscle: Influential triggers and regulatory mechanism. *iScience* **27**, 109221 (2024).

8.0 FIGURES

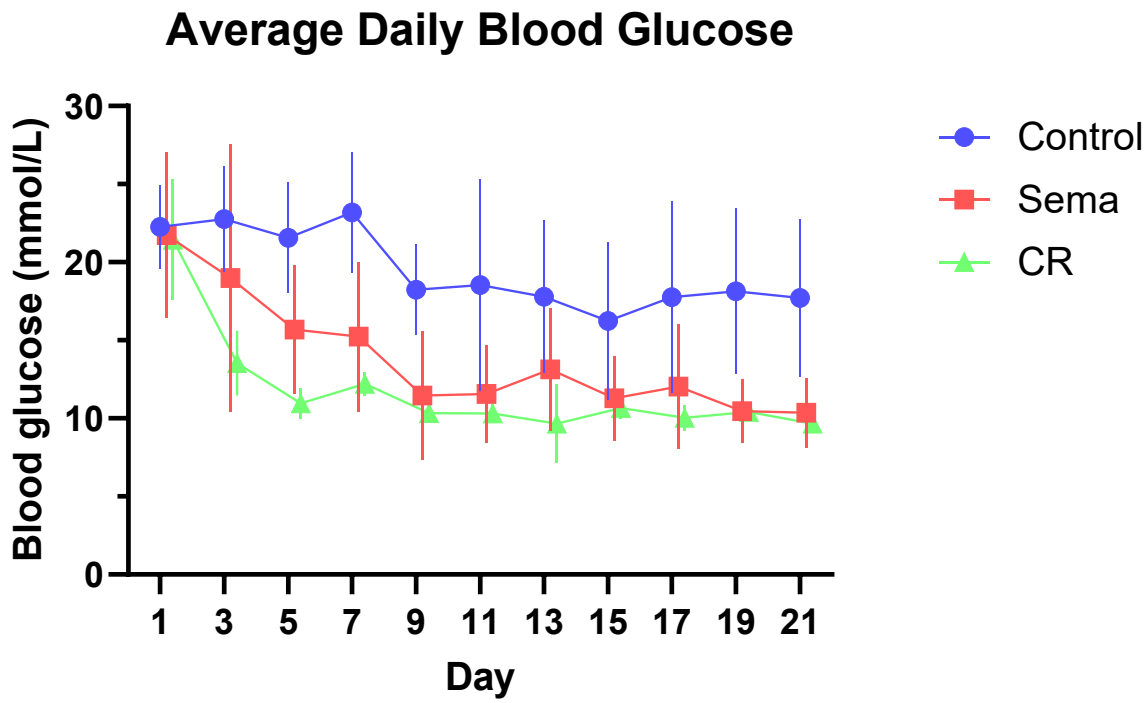

FIGURE 1. AVERAGE DAILY BLOOD GLUCOSE LEVELS OF THE SEMA, CR and CONTROL GROUPS DURING THE 21-DAY STUDY PROTOCOL: Control rats (ad-libitum fed, blue) exhibited significantly higher blood glucose levels than the two treatment groups (Sema: semaglutide treated, red, CR: calorie restriction, green) throughout the protocol (ANOVA group effect p=0.0001). The two treatment groups had similar reductions in daily blood glucose levels over time.

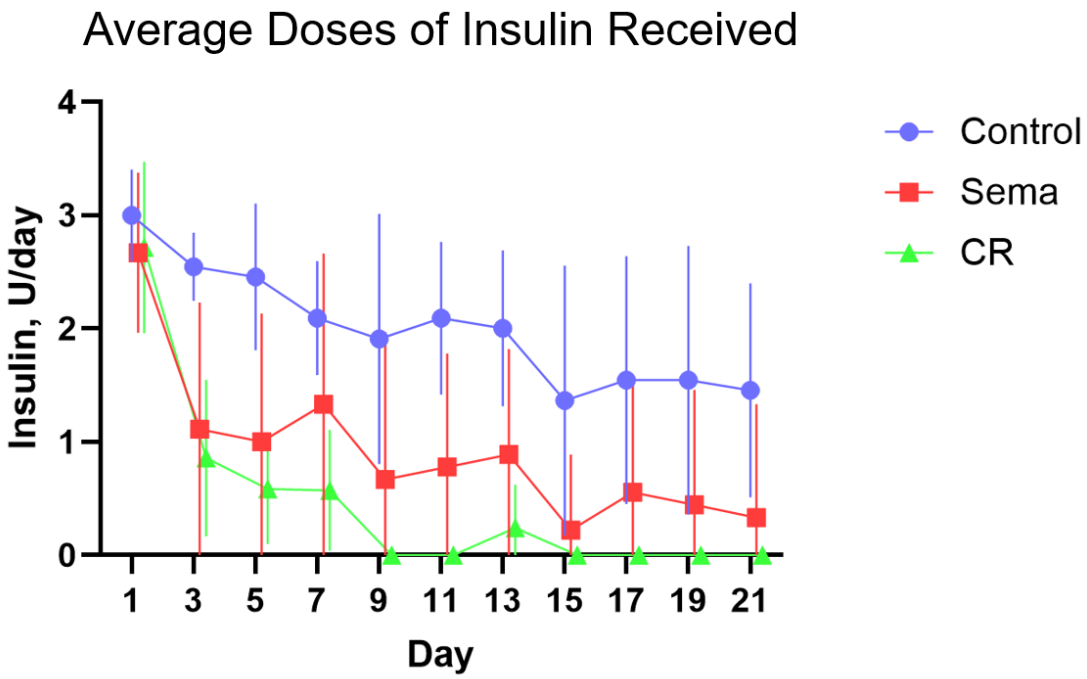

FIGURE 2. TOTAL DAILY INSULIN DOSE REQUIREMENTS FOR THE SEMA, CR and CONTROL GROUPS DURING THE 21-DAY STUDY PROTOCOL. Rats were given exogenous doses of insulin daily based on their daily blood glucose levels to help maintain their blood glucose levels in a moderate hyperglycemic state (see methods). Control (ad-libitum fed, blue) rats required more daily insulin relative to the two other treatment (Sema: semaglutide treated, red, CR: calorie restriction, green) groups (P=<0.001 Two Way ANOVA group effect).

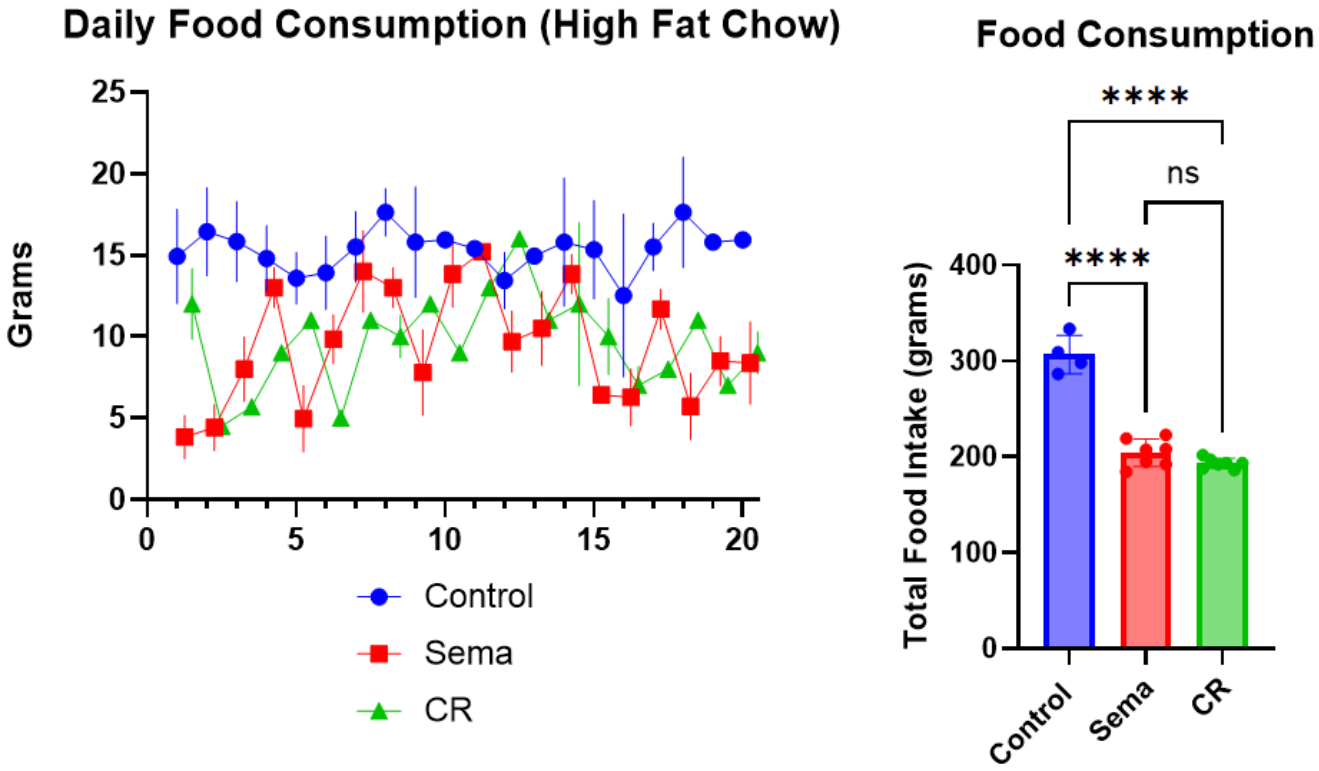

Figure 3A/3B: AVERAGE DAILY FOOD INTAKE PER GROUP OVER THE 21 DAYS/ AVERAGE TOTAL FOOD CONSUMED PERS GROUP OVER THE 21 DAYS. A) Control (ad-libitum fed, blue) animals had a mostly stable food consumption, which was higher than that of the two experiment groups (Sema: semaglutide treated, red, CR: calorie restriction, green). Food intake was sharply reduced following semaglutide injections, and would show gradual recovery to baseline until the next injection. Due to the diet-matched nature of the protocol, the CR (calorie restricted, green) group roughly had the same daily food consumption as the Sema group, only offset by one day. B) Both treatment groups (Sema and CR) consumed significantly less food compared to the control group (ANOVA $P<0.001$). Due to the diet-matching nature of the protocol, the two treatment groups had similar total food intake over the 21 day study period.

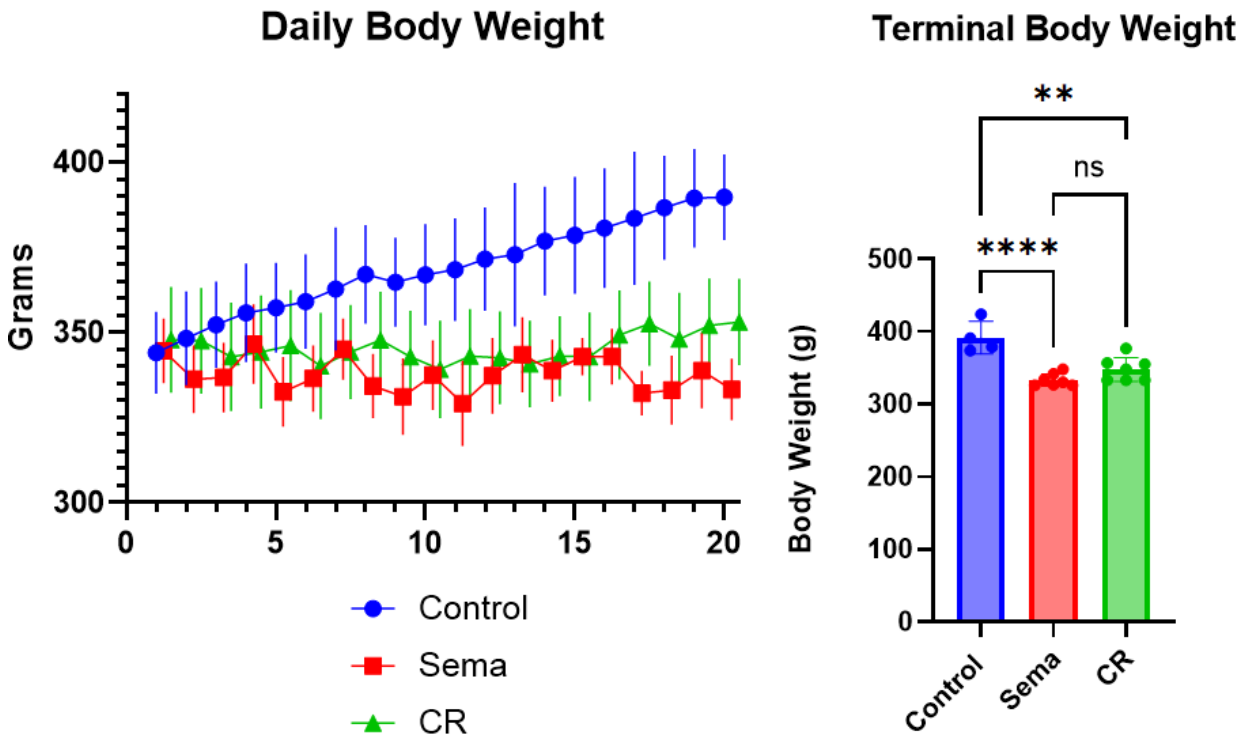

FIGURE 4A/B: AVERAGE DAILY BODY WEIGHT OF THE RATS PER GROUP/ AVERAGE TERMINAL BODY WEIGHT OF THE RATS PER GROUP. A) Control (ad-libitum fed, blue) animals continued to gain weight throughout the protocol, while the two treatment groups (Sema: semaglutide treated, red, CR: calorie restriction, green) did not. The two treatment groups followed a similar body weight patter, only offset by one day, similar to their food consumption. B) The two treatment groups had a significantly lower terminal body weight (ANOVA $P<0.001$) compared to the control group. The difference in the average terminal body weight of the two treatment groups was not statistically significant.

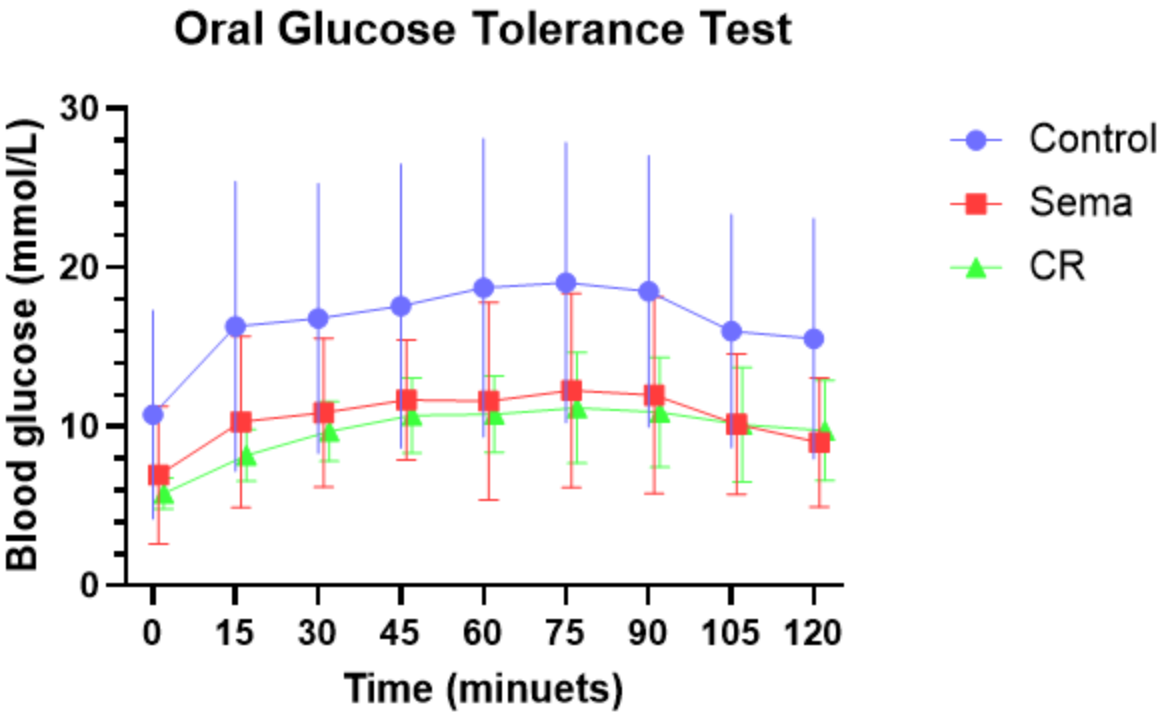

FIGURE 5. BLOOD GLUCOSE CONCENTRATIONS THROUGHOUT THE ORAL GLUCOSE TOLERANCE TEST. Although no significant differences interaction effect was found (P=0.4961), the two treatment groups (Sema: semaglutide treated, red, CR: calorie restriction, green) followed the same trend as the control group (ad-libitum fed, blue) although at a significantly lower blood sugar (Main group effect P<0.0001).

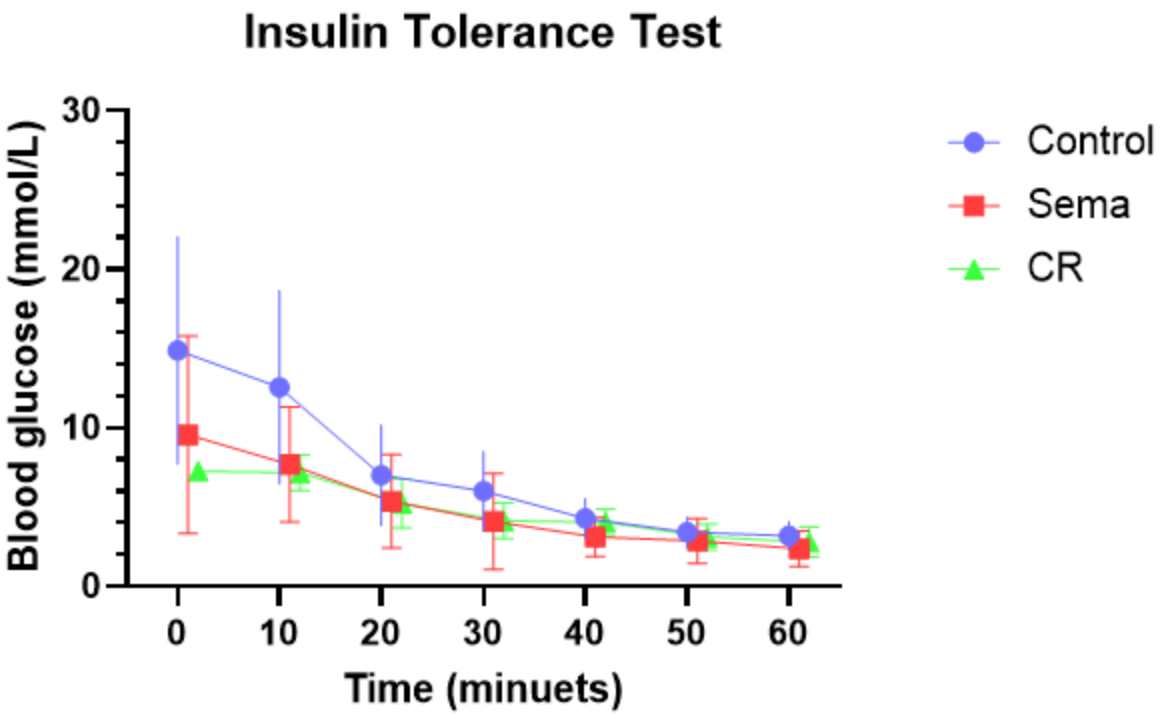

FIGURE 6. BLOOD GLUCOSE CONCENTRATIONS THROUGHOUT THE INSULIN TOLERANCE TEST Rats were given an 8u/kg bolus dose of insulin (NovoRapid, Novo Nordisk, Mississauga, Canada) and monitored for 60 min. The Sema (semaglutide treated, red) and CR (calorie restricted, green) groups had a lower baseline blood glucose level compared to the control (ad-libitum fed, blue), but all groups dropped to the same level within the hour-long protocol, before being rescued with a dextrose gavage (data not shown).

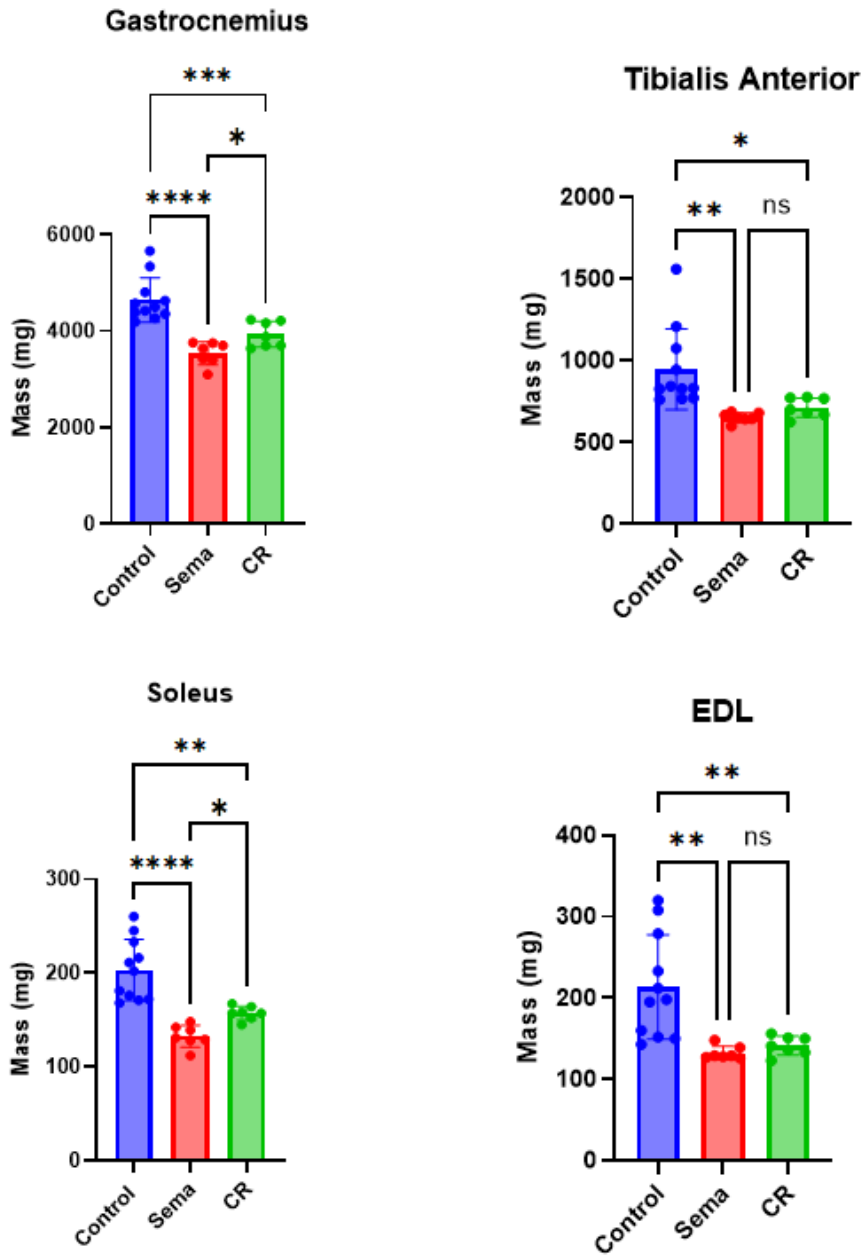

FIGURE 7: AVERAGE MUSCLE WEIGHTS AT THE TIME OF EUTHANIZATION IN THE THREE TREATMENT GROUPS. Muscles groups were separated and weighed immediately after the sacrifice of the animal on Day 21. The two treatment groups (Sema: semaglutide treated, red, CR: calorie restriction, green) exhibited significantly lower muscle weight compared to the control (ad-libitum fed, blue) group (* = P<0.05, ** = P<0.01, ***=P<0.001 denoted by 3 stars). Comparing the two treatment groups, the Sema group had smaller GAS and SOL muscle masses relative to the CR rats, by about 8% (p=0.0135; p=0.001, respectively), with the TA and EDL no differing significantly between the two groups.

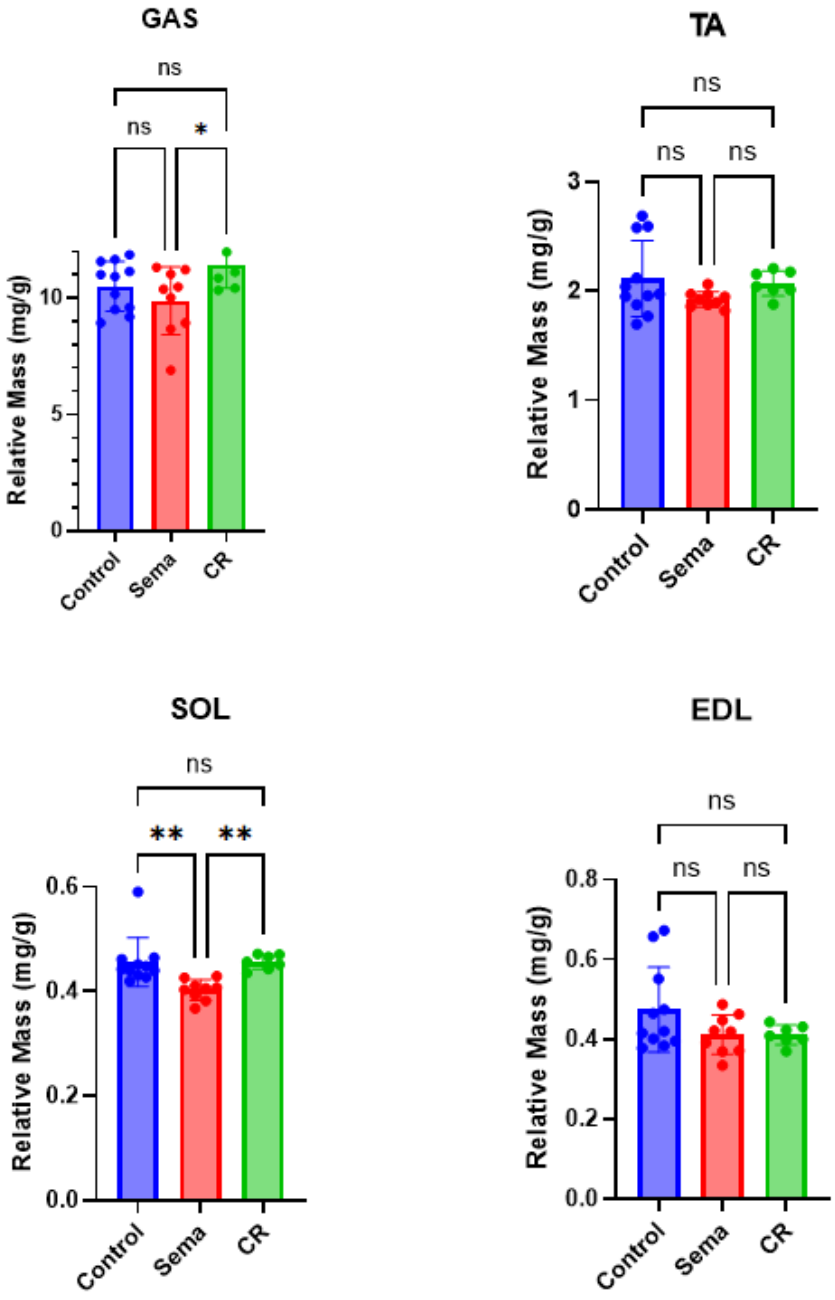

FIGURE 8: RELATIVE MUSCLE MASS OF THE GAS, TA, SOL AND EDL MUSCLE, NORMALIZED TO BODY WEIGHT IN THE THREE TREATMENT GROUPS. Muscles groups were separated and weighed immediately after the sacrifice of the animal (Day 21). Values are obtained by dividing the mass of the muscle measured (mg) by the weight of the rat at the time of euthanization (g). Comparing the CR (calorie restricted, green) group with the control group (ad-libitum fed, blue), no significant differences were found in relative muscle mass. The Sema (semaglutide treated, red) group, however, exhibited reduced SOL relative weight compared to both groups, and reduced GAS relative weight compared to the CR group.

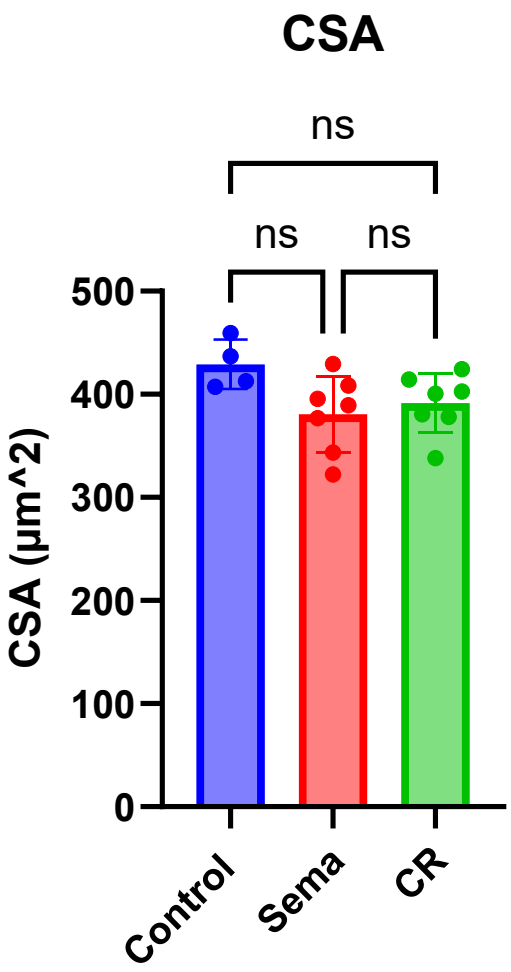

FIGURE 9: AVERAGE FIBER CROSS SECTIONAL AREA OF THE GASTRACNEMIUS MUSCLE OF THE THREE TREATMENT GROUPS. Frozen muscle samples were sectioned using a cryostat and imaged under a microscope, measuring average fiber CSA. No statistically significant difference was found, although the CR (calorie restricted, green) group tended to have larger CSA relative to the control (ad-libitum fed, blue) and Semag (semaglutide treated, red) group (ONE WAY ANOVA: p=0.07).

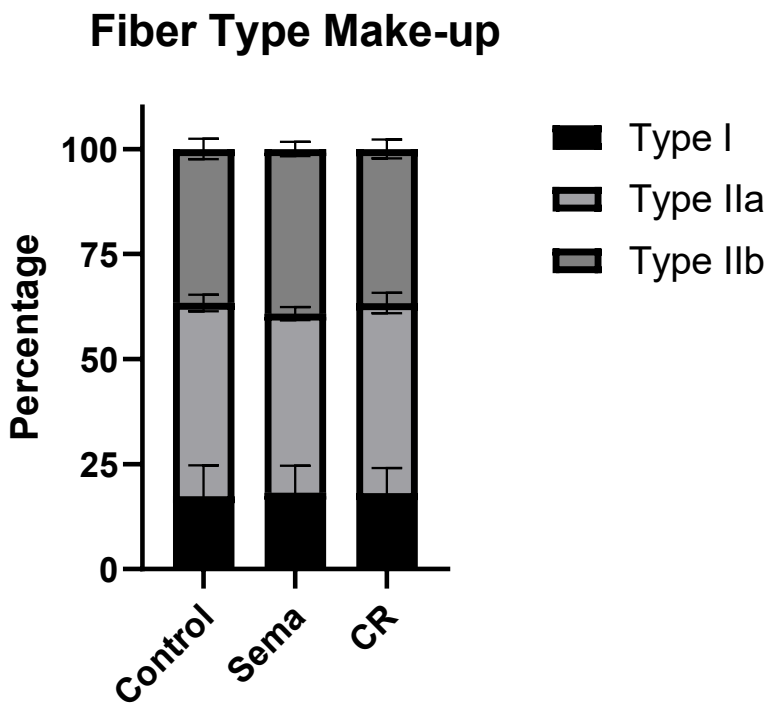

FIGURE 10: DISTRIBUTION OF THE DIFFERENT FIBRE TYPES IN THE TA MUSCLE IN THE THREE TREATMENT GROUPS. Control on the graph denotes ad-libitum fed control animals, Sema denotes semaglutide treated group and CR denotes the calorie restricted group. Muscle section slides were stained for the different fiber types and imaged using a fluorescent microscope. No significant differences were found in fiber type makeup of the three groups.

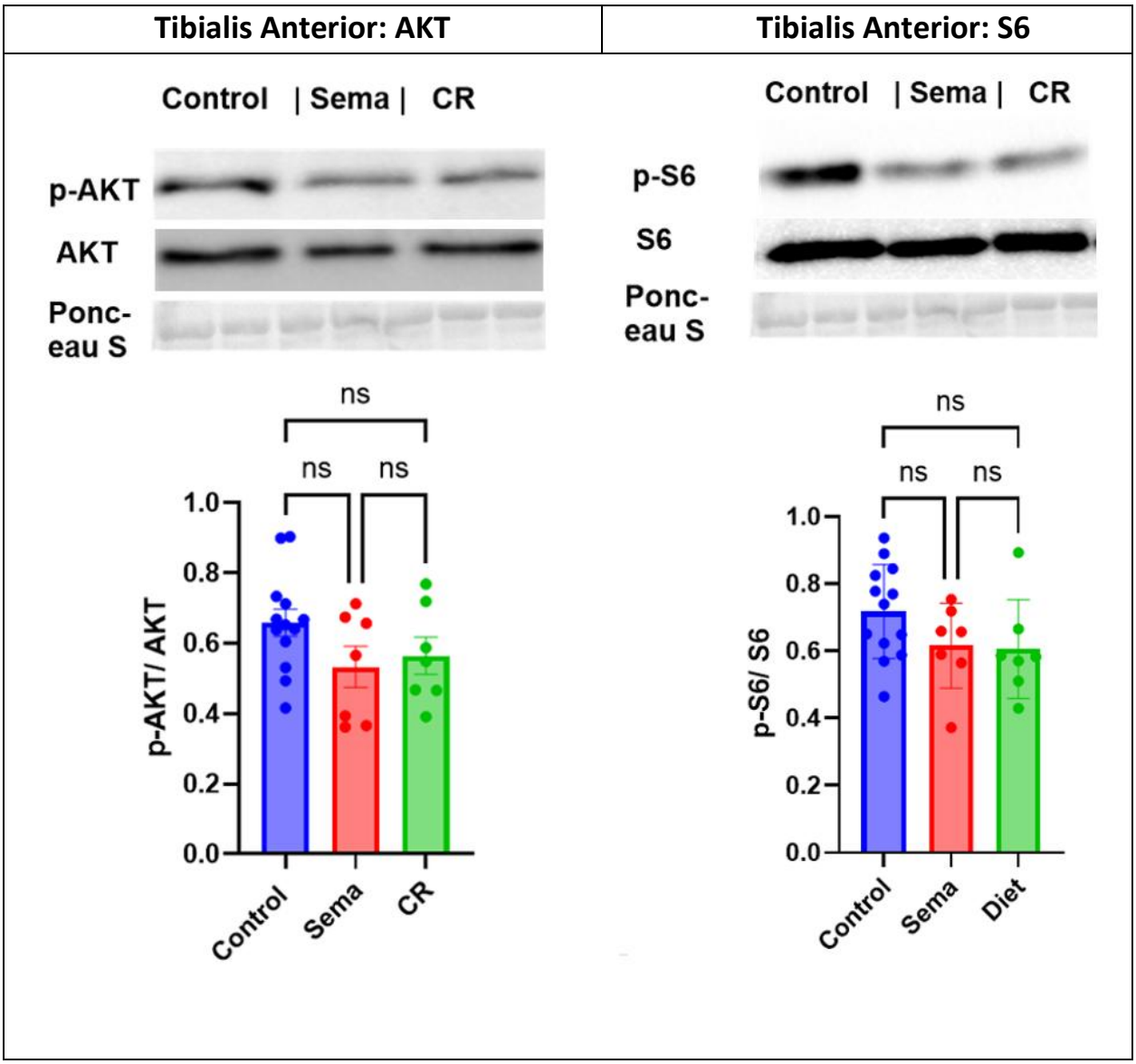

FIGURE 11: ACTIVATION OF PI3K PATHWAY IN THE TIBIALIS ANTERIOR COMPARED BETWEEN THT THREE GROUPS. pAKT/AKT (56kDa) and pS6/S6 (30kDa) ratio was analyzed as quotient of pAKT versus AKT and pS6 versus S6 expression. Representative blots for both AKT and pAKT are displayed above. Difference between the control group (ad-libitum fed, blue) and both Sema (semaglutide treated, red) group and the CR (calorie restricted, green) group in terms of AKT activation was non-significant (P=0.14 ANOVA). Furthermore, no significant differences in S6 phosphorylation (P=0.15 ANOVA) between the control group and two treatment groups was observed (P=0.28 Sema and P=0.21 CR respectively, Tukey's HST).

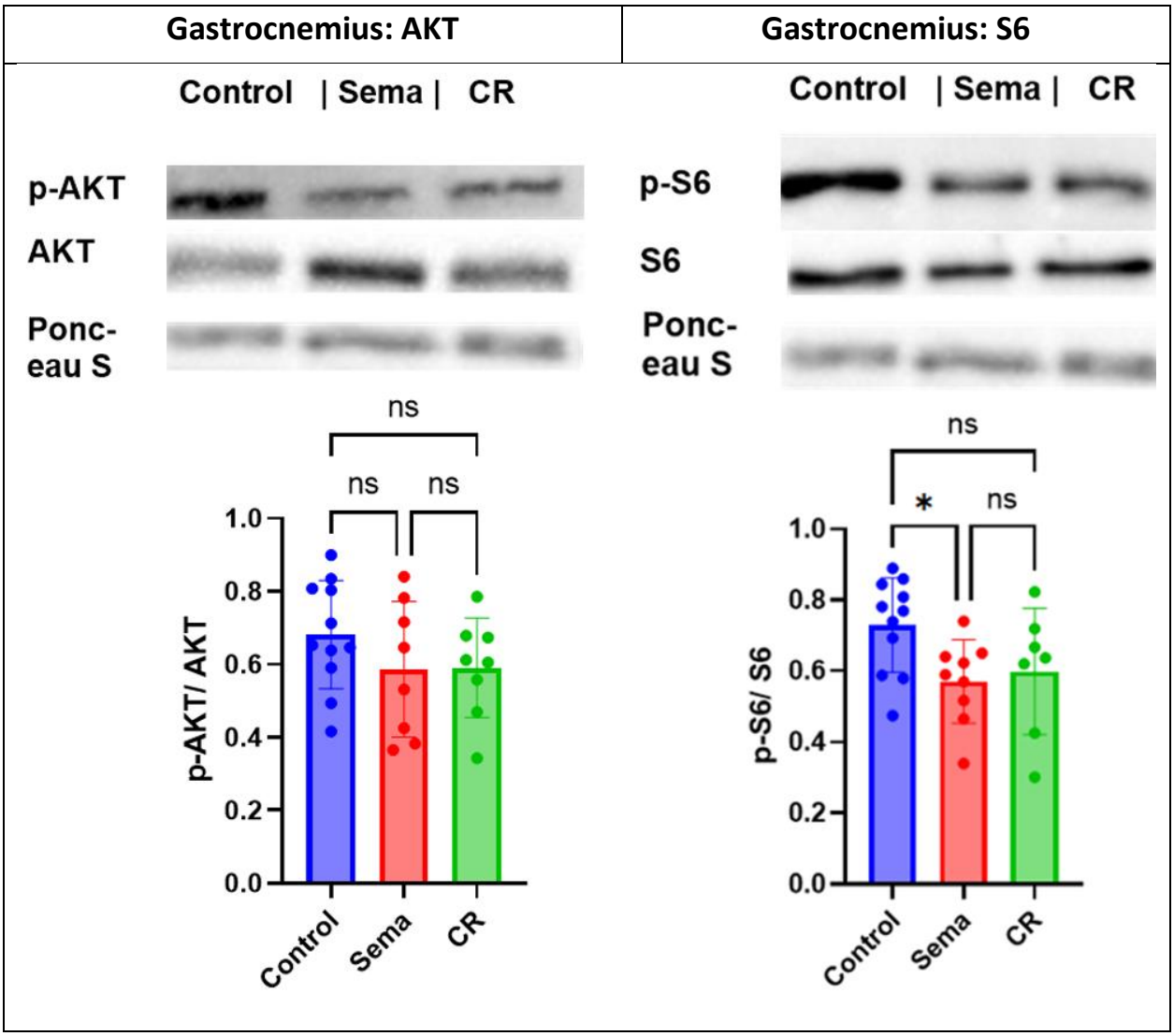

FIGURE 12: ACTIVATION OF THE PI3K PATHWAY IN THE GASTROCNEMIUS MUSCLE IN THE THREE TREATMENT GROUPS. pAKT/AKT (56kDa) and pS6/S6 (30kDa) ratio was analyzed as quotient of pAKT versus AKT and pS6 versus S6 expression. Representative blots for both AKT and pAKT are displayed above. Difference between the control group (ad-libitum fed, blue) and both Sema (semaglutide treated, red) group and the CR (calorie restricted, green) group in terms of AKT activation was non-significant (P=0.33 ANOVA). Control group did have significantly higher S6 phosphorylation when compared to the Sema group (p=0.042 ANOVA, 0.0481 Tukey's HST) but was non-significantly different from the CR group (P=0.15 Tukey's HST).

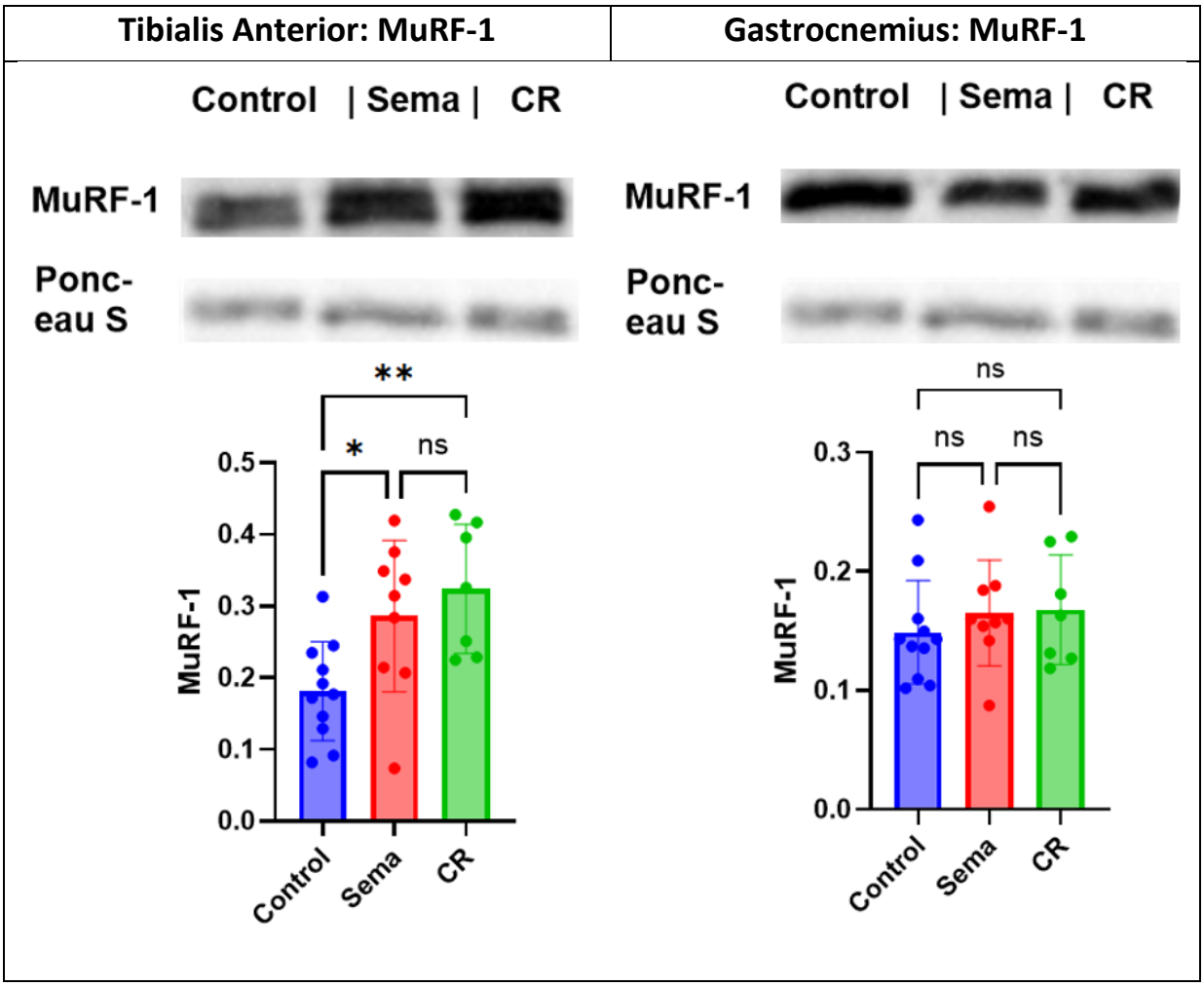

FIGURE 13: MURF-1 LEVELS IN THE TIBIALIS ANTERIOR AND GASTROCNEMIUS MUSCLEIN THE THREE TREATMENT GROUPS. Values are reported as reading for MuRF-1 levels divided by reading of control (Ponceau S). Control (ad-libitum fed, blue) group had significantly less MuRF-1 levels in the Tibialis Anterior muscle compared to the Sema (semaglutide treated, red) and CR (calorie restricted, green) group (P=0.005 ANOVA, P=0.036 and P=0.007 respectively, Tukey's HST), but the two treatment groups did not defer significantly (p=0.6 Tukey's HST). No groups were significantly different in terms of MuRF-1 expression in the Gastrocnemius muscle (P=0.6 ANOVA).

9.0 SUPPLEMENTARY FIGURES AND TABLES

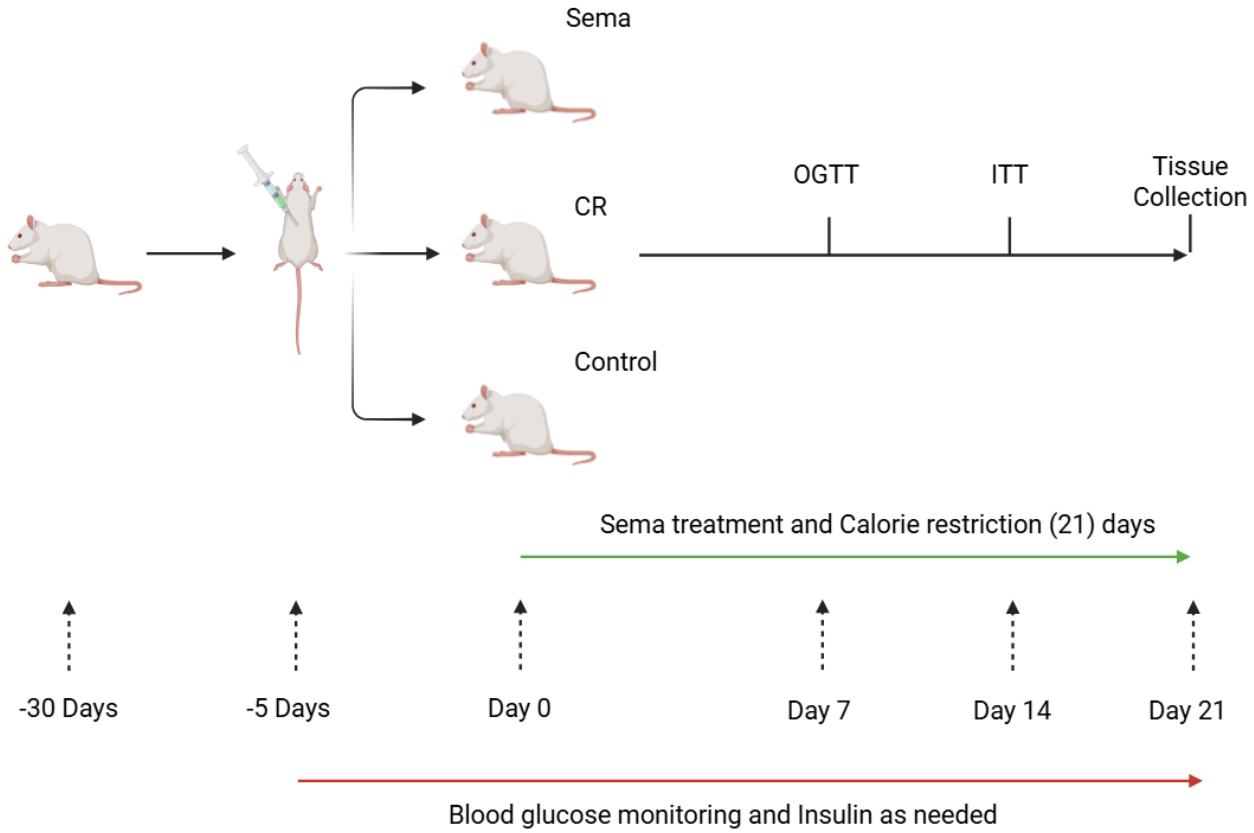

Figure S1: Visual representation of the protocol.

Target	Supplier	Catalogue #
p-AKT	**Cell Signaling Technology**	**9271S**
AKT	Cell Signaling Technology	9272
p-S6	Cell Signaling Technology	2211S
S6	Thermo Fisher	MA5-15164
MuRF-1	Thermo Fisher	PA5-76695

Table S1: Antibodies Used for Imaging

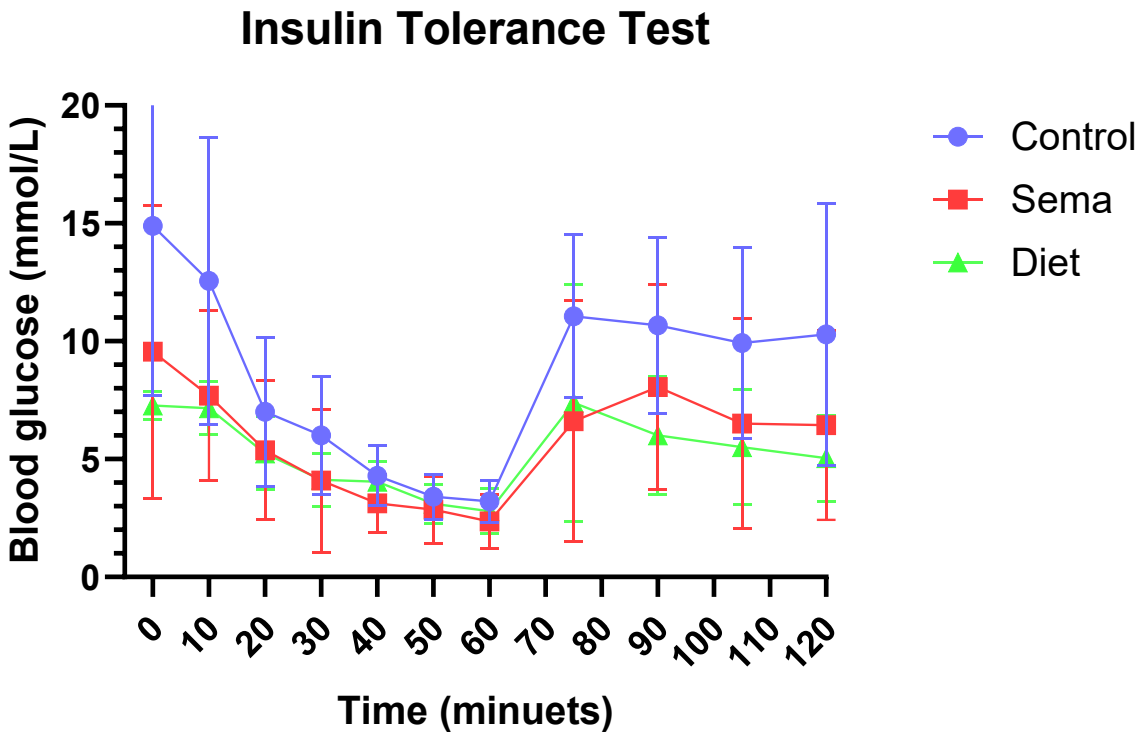

Figure S2: Blood glucose readings throughout the insulin tolerance test including the response to the recovery dose of gavage dextrose used to recover the rats from hypoglycemia.