# Impacts of Empagliflozin (EMPA) Treatment on Skeletal Muscle Microvasculature in Type 1 Diabetic Rats

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ABSTRACT

In individuals with type 1 diabetes (T1D) and models of the disease, hyperglycemia is associated with microvascular complications, including reduced capillary ratio in skeletal muscle (SM). We investigated whether a blood glucose lowering drug, empagliflozin (EMPA), has any impact on the microvasculature in SM. T1D was induced in eight-week-old male rats using streptozotocin (65 mg/kg, n=21). Twenty-one rats were given daily insulin and 11 of 21 rats also consumed EMPA (30 mg/kg/day) for 14 days. Twelve healthy non-diabetic rats served as controls. Capillary content and angiogenic factors were assessed in three separate leg muscles. EMPA+insulin treated rats exhibited better glycemic control, but capillary to fibre ratio and VEGFa mRNA was not impacted by EMPA+insulin treatment in the tibialis anterior. PECAM mRNA and VEGFa protein expression was also not impacted by EMPA+insulin treatment. EMPA has no obvious impact on the vasculature of SM in type 1 diabetic rodents.

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LIST OF ABBREVIATIONS

**BG:** blood glucose

**C:F:** capillary to fibre ratio

**CVD:** cardiovascular disease

**DEPICT-1:** Dapagliflozin Evaluation in Patients with Inadequately Controlled Type 1 Diabetes

**DKA:** diabetic ketoacidosis

**EMPA:** empagliflozin

**EMPA-REG OUTCOME:** Empagliflozin, Cardiovascular Outcome Event Trial in T2D Mellitus Patents

**GASTROC:** gastrocnemius

**HbA1c:** Hemoglobin A1c

**HHF:** hospitalization for heart failure

**HLA:** human leukocyte antigen complex

**PECAM:** platelet endothelial cell adhesion molecule-1

**SM:** skeletal muscle

**SGLT2:** sodium glucose co-transporter 2

**STZ:** streptozotocin

**T1D:** type 1 diabetes

**T2D:** type 2 diabetes

**TA:** tibialis anterior

**TSP-1:** thrombospondin-1

**VEGF-A:** vascular endothelial growth factor-A

## 1. INTRODUCTION

Individuals with type 1 diabetes (T1D) continue to face a challenge in maintaining their blood glucose levels despite the advancements in insulin therapy[1]. Hyperglycemia is associated with microvascular complications in both rodent models[2–5] and individuals with T1D[6,7]. Skeletal muscle plays a large role in maintaining blood glucose levels[8] and whole-body insulin sensitivity[8,9], however the examination of skeletal muscle microvascular health in T1D is not well studied relative to other tissues examined in diabetes, such as the kidney or retina. Failing to properly regulate blood glucose levels and deliver insulin to the skeletal muscle can exacerbate the challenge in maintaining blood glucose levels[10]. Consequently, an adjunct therapy to insulin that further promotes a lowering of blood glucose levels in T1D, without promoting hypoglycemia, could potentially promote maintenance of the microvasculature in the skeletal muscle.

One possible therapy to improve glycemic control and overall cardiometabolic health in T1D is sodium glucose co-transporter 2 (SGLT2) inhibitors, a class of drugs widely prescribed to manage hyperglycemia in patients living with type 2 diabetes[11]. Clinical trials of SGLT2 inhibitors have been shown to be beneficial for individuals with T1D with the main outcomes being reduced hemoglobin A1c (HbA1C) levels, body weight and blood pressure[12], and improvements in renal and cardiovascular function[11]. SGLT2 inhibitors have been shown to delay the development of diabetic nephropathy[13] in diabetic rodents and promote the expression of pro-angiogenic factors in the skeletal muscle of diabetic rodents with ischemia[14]. Clinical studies also suggest that SGLT2 inhibitors can increase the risk of diabetic ketoacidosis, a potentially life threatening condition[12,15].

The aim of this study is to evaluate the impact of empagliflozin (EMPA), a commonly prescribed SGLT2 inhibitor for type 2 diabetes in humans[15], on microvasculature in skeletal muscle of a juvenile rat model of T1D. To our knowledge, no other studies have investigated the impact of EMPA, on microvasculature in the skeletal muscle of rodent models and/or humans with T1D.

## 2. LITERATURE REVIEW

### 2.1 Type 1 Diabetes

#### 2.1.1 Pathology and Etiology

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin-secreting β-cells in the pancreas. Due to the pancreas's inability to produce insulin, individuals with T1D rely on exogenous insulin to help maintain their blood glucose levels. Modern blood glucose management includes intensive insulin therapy, including basal and prandial insulin delivery using an insulin pump or multiple daily injections. Blood glucose management also includes frequent glucose monitoring using subcutaneously implanted glucose sensors and or self-monitoring of capillary blood glucose levels, along with the assessment of hemoglobin A1c (HbA1c) levels to help regulate blood glucose levels. There has been a 3% to 5% increase per year in the incidence of T1D worldwide since the 1960s[16]. Canada is estimated to hold the sixth highest incident rate of T1D, with an estimated age standardized rate of 20-30 per 100,000 per year in children under 14 years of age[17].

Despite the increase in the incidence of T1D, the cause for T1D remains unknown. It is currently understood that T1D is caused by a combination of genetic predisposition and environmental factors that lead to alterations in gene expressions[18]. Currently, over 50 genetic loci have been associated with T1D, with the strongest association being with the human leukocyte antigen complex (HLA)[18]. Briefly, the HLA is responsible for marking cells of the human body to identify them as their own cells to prevent their destruction by the immune system[19]. Two regions on two specific chromosomes (chromosome 6 and 11) that code for the HLA have been identified to have a strong association with T1D[19]. However, the alleles on these chromosomes associated with T1D vary among different ethnic groups[18]. Additionally, clinical

studies have shown differences in DNA methylation between monozygotic twins[20,21], with a 40% concordance rate[18], suggesting a role of epigenetics contributing to the development of T1D. The proposed environmental factors that lead to epigenetics are a combination of drugs[22–24], pollutants[25–27], foods[16], infectious agents[28,29], and differences in gut microflora[30–33].

### 2.1.2 Hypoglycemia and Hyperglycemia in T1D

Individuals with T1D continue to face a challenge with managing their blood glucose levels, despite the advancements in blood glucose monitoring devices and insulin treatment[34]. People with T1D need to be wary of hypoglycemia and hyperglycemia since they contribute to their own set of challenges with the management of T1D and this population is at risk of having both.

#### 2.1.2i Hypoglycemia in T1D

Hypoglycemia is clinically defined to be when blood glucose levels fall below 4 mmol/L and is associated with symptoms such as nausea, tingling, trembling, hunger and other symptoms[35]. Hypoglycemia can become severe and lethal in individuals with T1D if symptoms are not treated in time. The risk for severe hypoglycemia, defined as blood glucose levels below 2.8 mmol/L[35], increases if individuals with T1D had a prior episode of severe hypoglycemia[36–38], have low HbA1C levels (<6.0%)[37,39–41], are unaware of hypoglycemia[42], have neuropathy[43], and are in the adolescent and pre-school age group[44]. Severe hypoglycemia episodes are estimated to have a 1.0-1.7 incidence rate per patient per year (30-40% annual prevalence)[45]. The reason for such high prevalence in this population is due to the failed blood glucose counter regulatory system in people with T1D[45].

Briefly, in healthy individuals, the response to hypoglycemia begins by decreasing the secretion of insulin and increasing the secretion of glucagon when blood glucose concentrations reach 4.4-4.7mmol/L[46] followed by decreased glucose catabolism in the body except in the brain[46]. If blood glucose levels further decrease beyond the 3.6 mmol/L, then glucagon secretion is further increased and epinephrine is released which stimulates hepatic glycogenolysis and promotes gluconeogenesis[46]. However, T1D is associated with a failed counterregulatory system which leads to an increased risk of hypoglycemia in this population. The mechanisms behind the failed counterregulatory system have been investigated in both humans[47,48] and rodent models[49,50] with T1D, but remain unclear. In short, insulin cannot be decreased in secretion due to the loss of pancreatic β-cells and uptake of exogenous insulin in individuals with T1D. Therefore, hypoglycemia is more likely to occur due to the sustained and unregulated levels of hyperinsulinemia[45]. In addition, the majority of people with T1D are no longer able to produce glucagon after 5 years of diagnosis[51]. This defect is thought to be due to intra- and extra-pancreatic factors. One theory is that an elevated secretion of pancreatic somatostatin[52], a hormone that inhibits glucagon release, and a failure in local α and β-cell signaling in the pancreas during hypoglycemia leads to the reduction of glucagon production and tracks to impaired production of glucagon in T1D [53]. Additionally, reduced stimulation of the autonomic nervous system[45] and reduced secretions of catecholamines[45,54] are thought to play a role in the failed counterregulatory system in T1D[45]. Thus, a high risk of insulin and exercise-induced hypoglycemia[45]. As such, the glycemic management in T1D remains a major challenge and most with the disease develop diabetes-related micro and macrovascular disease with early mortality[55].

2.1.2ii Hyperglycemia in T1D

Hyperglycemia can be defined as random blood glucose levels greater than 11.1 mmol/L[56] or an HbA1c% >7%[1]. Chronic hyperglycemia, in both T1D and type 2 diabetes (T2D), is typically associated with mild to moderate symptoms at its early stages, such as, thirst, frequent urination, weight loss despite usual food intake, and sometimes nausea[57]. At the later stages it can be associated with diabetic ketoacidosis in those living with undiagnosed or untreated T1D. A large portion of individuals with T1D fail to meet the clinical target of HbA1C% (7%), with adolescents being the age group furthest away from this clinical target[1]. With increasing blood glucose levels in healthy individuals, insulin secretion increases which promotes (a) increased glucose uptake at the skeletal muscle, (b) increased hepatic glycogenesis, (c) and reduced hepatic gluconeogenesis[58]. Once the blood glucose concentration surpasses 10-11mmol/L, glucose is excreted renally via the sodium glucose co-transporters 1 and 2 (SGLT1 & SGLT2)[59]. However, people with T1D are unable to produce insulin, thus they are unable to downregulate elevated blood glucose and the threshold for glucose "spillover" rises and ketosis develops. Even with exogenous insulin, individuals with T1D still face challenges in downregulating high blood glucose levels[60]. It is thought that this may be due to insulin resistance that occurs in the peripheral tissues, such as, the skeletal muscle[60]. Briefly, insulin resistance leads to reduced promotion of blood glucose uptake and utilization thus promoting an increase in blood glucose levels[61]. In addition, it is though that dysregulated glucagon secretion and a positive feedback loop where glucose is reabsorbed at the kidneys can also further promote hyperglycemia[62–65]. Indeed, patients with T1D have an observed increase in maximal capacity of renal glucose reabsorption (~20% increase from 10-11mmol/L threshold), with the highest increase recorded tracking up to 14mmol/L[66,67].

Hyperglycemia also increases the risk of diabetic ketoacidosis (DKA) in individuals with T1D. DKA is a lethal condition, where the liver produces abnormally high amounts of ketone bodies (hyperketonemia), resulting in a severe acidification of the bloodstream and interstitial fluids, due to multiple hormonal abnormalities[68]. Individuals with DKA usually present symptoms such as dehydration, polyuria, polydipsia (extreme thirst), and lethargy[68]. The prevalence of DKA is higher at onset of diabetes in children with an estimated 26% of cases occurring at onset[69], which increased during the COVID-19 pandemic by 22.6%[70]. Ketone body production during prolonged exercise and fasting is a normal physiologic process associated with high periods of lipolysis, where ketone bodies are used by tissues for energy[68]. However, in DKA, hyperketonemia, caused by reduced insulin administration or illness in T1D can cause the blood to become dangerously acidic leading to cerebral oedema (fluid build up around brain) and other lethal complications[71]. Briefly, DKA occurs when insulin deficiency leads to the loss of the cells' ability to uptake glucose[68] (thereby promoting hyperglycemia) and excessive rates of hepatic ketone production with high levels of adipose tissue and liver lipolysis[68]. This is often associated with increased glucagon secretion, elevations in hepatic glycogenolysis, hepatic gluconeogenesis, and hepatic ketogenesis[68]. Stress hormones, such as, cortisol, growth hormone, and epinephrine also do contribute to DKA by increasing lipolysis, glycogenolysis, and hepatic gluconeogenesis, which further promote hyperglycemia[68]. DKA can lead to an increase in insulin resistance[72], this because of the hyperketonemia and high free fatty acids in circulation from the increased lipolysis. Thus, there is less of a response to insulin as there is an increased reliance on lipolysis for metabolism. These can also further promote hyperglycemia creating a vicious cycle. Although causes for DKA are variable, majority of DKA cases occur following lapse in insulin uptake or treatment, infection, or increased alcohol uptake[73].

Recurrent exposure to hyperglycemia in T1D is a major risk factor for macrovascular complications like cardiovascular disease (CVD) and microvascular complications like nephropathy and retinopathy[55]. In macrovascular diseases, the main underlying mechanism for CVD is atherosclerosis which leads to narrowing of vessels[74,75]. In T1D, the risk for CVD is increased and although the underlying mechanism for the increased risk is unclear[75], dysfunction in the endothelium plays an important role[74]. Human and *in vitro* studies have shown that this dysfunction is attributed to (a) dysfunction of endothelial cells[76,77] due to the hyperglycemia and metabolic stress and (b) failure to repair vasculature[78]. Epidemiological studies have also shown that patients with T1D have a higher ischemic heart disease mortality rate compared to healthy individuals[79] and a higher cerebrovascular mortality rate at all ages[80]. Studies have also shown that intensive treatment of T1D and lower HbA1C% is associated with a 42% reduction in all cardiovascular events[81]. These studies[74,76–81] show that although the underlying mechanism is unclear, hyperglycemia does play a large risk factor in contributing to macrovascular complications.

Individuals with T1D have an elevated risk for a number of microvascular related diseases including nephropathy, retinopathy, and limb amputation[55]. Nephropathy, a major cause of end-stage renal failure in Western countries[82], clinically presents itself by developing proteinuria (high levels of protein in urine) and a decline in the glomerular filtration rate[74]. The development of nephropathy is complex as multiple factors play into the progression of the disease[74]; thus, the role hyperglycemia plays will be the main focus discussed herein. Hyperglycemia promotes endothelial dysfunction[74] through oxidative stress and accumulation of advanced glycation end products[83]. Additionally, an *in vivo* study[84] has shown that hyperglycemia promotes cellular senescence through SGLT2. Hyperglycemia is also promoted

through hypertrophy of the kidney, in particular the proximal tubule[74,85],which leads to an increase in the uptake of the filtrate, thereby causing an increase in the glomerular filtration rate[86]. Likewise, retinopathy is the leading cause of blindness in adults[74,87], where hyperglycemia leads to pathological changes[74] that causes a reduction in capillary density and degeneration of capillaries[74]. These microvascular complications are well documented and understood due to the vast studies that have investigated their mechanisms and prevalence. However the same cannot be said for diabetic myopathy which may also be linked to microvascular dysregulation within skeletal muscle in poorly controlled T1D[88].

Myopathy clinically presents itself in diabetes with decreased muscle mass, muscle weakness, and reduced myofiber size all strongly associated with poor glycemic control and underinsulinization[89]. Due to the limited studies investigating this complication the exact mechanism of action is unknown. However, it is proposed that due to repeated insulin uptake there are chronic periods of hyperglycemia in the intracellular space[90]. This leads to oxidative stress[91], upregulation of certain gene expressions related to collagen expression, increased basement membrane thickening, reduced satellite cell number, and decreased myofiber size[92]. Signs of skeletal muscle myopathy seem to appear before the onset of the other vascular complications as seen in a group of recently diagnosed patients (1-28 weeks with T1D)[93]. This indicates that healthy skeletal muscle is important in delaying the onset of the severe complications and managing this disease. In order to understand why diabetic myopathy is concerning especially in people with T1D, one needs to appreciate the important role skeletal muscle plays in managing T1D.

## 2.2 Skeletal Muscle in T1D

Skeletal muscle is (a) the largest site for fatty acid oxidation[94], (b) the largest organ for glucose uptake to maintain homeostatic blood glucose levels[8], and (c) the most critical organ for the maintenance of whole body insulin sensitivity[9]. Glucose can be taken up through various ways into the skeletal muscle, depending on if the organism is in the basal (resting, fasted), fed or exercising state. In the basal state, when insulin levels are low, a relatively small amount of glucose is transported into muscle via GLUT-1 facilitated diffusion[95]. In the fed state, when insulin levels rise, glucose is taken up in large amounts into skeletal muscle via insulin-mediated translocation of the insulin-sensitive GLUT-4 transporters[95]. During exercise, when insulin levels typically drop, glucose uptake and oxidation is mediated by contraction-mediated GLUT-4 translocation to the plasma membrane that dramatically increases glucose uptake[96]. Therefore, skeletal muscle plays an important role in mediating blood glucose levels and maintaining insulin sensitivity; by taking up glucose through non-insulin dependent ways.

The literature has shown a reduced regeneration capacity in skeletal muscle in young adults with T1D[97], increased intramyocellular lipid content in adults with T1D[98–100], mitochondrial deficiencies[90,101] even in physically active young adults[102] compared to healthy controls. These myopathies are concerning as they can interfere with the ability of the skeletal muscle to regulate blood glucose well and maintain insulin sensitivity, thus, further exacerbating the challenge in managing T1D. Research has also observed, in both rodents and humans with T1D, negative impacts on skeletal muscle capillarization and dysregulated angiogenesis associated with hyperglycemia, which will be further discussed herein.

### 2.2.1 Skeletal Muscle Capillaries in T1D

Capillary density, capillary number, and the tortuosity of capillaries in skeletal muscle are all important determinants of overall skeletal muscle health[103–105]. Capillary density provides an indication of the extent of muscle perfusion and is determined by counting the total number of capillaries relative to the muscle area. Capillary number when compared to the relative number of myofibers in the muscle provides an indication of angiogenesis, this measurement is referred to as capillary to fibre ratio (C:F). Capillary tortuosity is referring to the 'twists' of the capillaries and provides an indication of how well the muscle tissue is perfused[106]. Higher amounts of these measurements usually indicate better muscle health[103–105]. The impacts of T1D on the capillaries in skeletal muscle remains an understudied area with mixed results. In type 1 diabetic rodents, studies have shown reduced C:F compared to healthy counterparts[3,107]. However, in human studies no difference was found in the C:F when compared between individuals with T1D, who were undergoing intensive insulin therapy, and healthy individuals[108,109]. Nevertheless, Wallberg-Henriksson and colleagues[110] showed that when adults with T1D are placed on an exercise training regimen, lasting 2-3 months, the increase seen in the C:F ratio that is normally observed in the healthy controls, was absent. Moreover, in type 1 diabetic rodents, capillaries in skeletal muscle were observed to be smaller in length and smaller in diameter, which indicate negative impacts in skeletal muscle health[107,111]. Considering the importance of skeletal muscle in managing T1D, the reduced skeletal muscle regeneration capacity, repressed exercise adaption in the skeletal muscle of individuals with T1D, and decreased C:F in type 1 diabetic rodents, it is then vital to understand the underlying molecular processes of capillary growth and how that may change in T1D.

2.2.2 Skeletal Muscle Angiogenesis and T1D

Angiogenesis is the process that describes the growth of capillaries from pre-existing ones[112]. It is essential for repair, healing from wounds, and for exercise training adaptations[112–114]. There are two types of angiogenesis that occur in the body, termed (1) sprouting and (2) intussusceptive. Briefly, sprouting angiogenesis is when the capillary basement membrane gets degraded followed by endothelial cell proliferation and migration where then a new vessel is formed 'sprouting' off a pre-existing vessel[115,116]. Alternatively, intussusceptive angiogenesis involves splitting the lumen of a pre-existing vessel to create a new vessel[117]. Briefly, this occurs when the pre-existing endothelial cells re-organize themselves within the lumen to split the original lumen into new vessels[117,118]. Both can occur in skeletal muscle however it is more commonly seen that sprouting angiogenesis occurs in skeletal muscle[119]. Sprouting angiogenesis is considered to be less energy efficient than intussusceptive angiogenesis due to requiring more endothelial cell proliferation and basement membrane degeneration[112,120]. Decreasing angiogenesis in diseases like cancer, ophthalmic conditions, and other diseases have shown to be therapeutic while, promoting angiogenesis in diseases such as, ischemic heart disease, peripheral arterial disease, and others have also shown to be therapeutic[112]. Therefore, achieving an appropriate dynamic balance in angiogenesis is essential for maintaining the function of tissues in the body[121].

There are a multitude of factors that can impact the process of angiogenesis to either promote or impede angiogenesis which are both important to achieve the balance needed by the body to maintain function[121]. Vascular endothelial growth factor-A(VEGF-A) and thrombospondin-1 (TSP-1) have been identified as two critical factors for promotion and inhibition, respectively. VEGF-A and TSP-1 are produced by multiple cells in the body[122].

VEGF-A tends to be upregulated in response to hypoxia, metabolic stress, and exercise[123–125]. VEGF-A in the skeletal muscle are stored in vesicles[125] and are relocated to the skeletal muscle's subsarcolemma in response to hypoxia or metabolic stress which are then released into the interstitial space[126]. TSP-1's production and secretion are variable and depend on cell type and tissue type. TSP-1 has been observed to increase in mRNA expression in the skeletal muscle of healthy organisms following a one hour bout of treadmill running similar to VEGF-A[127]. Furthermore, capillary regression was found to be associated with an increase in TSP-1 expression and no change in VEGF-A levels following detraining that was preceded by an exercise training period[128]. This suggests that the balance between both VEGF-A and TSP-1 play an important role in the capillarization in skeletal muscle. The current limited literature points to T1D impacting VEGF-A and TSP-1 levels at both the mRNA and protein level. Kivela and colleagues[3] have showed decreased VEGF-A mRNA and increased TSP-1 mRNA levels in type 1 diabetic mice and reduced C:F compared to heathy controls. Mixed results with regards to protein levels of VEGF-A in skeletal muscle of type 1 diabetic rodents have been reported. Kivela and colleagues[3] reported a decrease in VEGF-A protein levels and Aiken and colleagues[4] reported an increase and no change in VEGF-A protein levels in two different muscles compared to healthy controls. The differences in these protein expressions may be due to the different muscle fibre types that were used for analysis and the different type 1 diabetic rodent models used in the various studies. Indeed, Birot and colleagues[123], showed that VEGF-A expression is fibre type specific, with VEGF-A being more abundantly produced by fast-twitch glycolytic fibre. In fact, when looking at the study by Kivela et al[3],T1D was induced via streptozotocin and all calf muscles were used (soleus, gastrocnemius, and plantaris) which together are mixed fibre twitch types. On the other hand, Aiken et al[4], used a model where T1D developed due to genetic

modifications and used soleus where no change was observed and the plantaris, a mixed fibre muscle with greater fast-twitch fibre morphology, observed an increase in VEGF-A compared to healthy controls. Aiken et al[4] further assessed TSP-1 protein levels and found them to be elevated and found reduced C:F in both muscles compared to healthy controls. This coincides with the elevated TSP-1 mRNA and reduced C:F findings by Kivela et al. Aiken et al then further analyzed the ratio between VEGF-A and TSP-1 which was reported to be higher in the type 1 diabetic rodents compared to healthy controls. Considering these results, it appears that both VEGF-A and TSP-1 play an important role in the capillary health of skeletal muscle in T1D with the increase in TSP-1 appearing to have a larger impact on capillarization in skeletal muscle. Future studies should assess both factors to gain a better understanding of the balance of angiogenesis occurring in skeletal muscle.

## 2.3 SGLT2 Inhibitors

### 2.3.1 Mechanism of Action

SGLT2 is a transport protein located in the proximal tubule of the nephron in the kidney[129]. In healthy individuals, both SGLT2 and SGLT1 reabsorb all glucose from the filtrate back into the body[130], where SGLT2 is responsible for ~97% of the glucose reuptake[131]. Renal glucose reabsorption at these transport proteins requires the coupling of sodium molecules, where the ratio of sodium to glucose is 2:1 in SGLT1 and 1:1 in SGLT2[132]. SGLT2 inhibitors reduce renal glucose reabsorption by competitively inhibiting SGLT2 in the presence of sodium thereby blocking glucose transport[133], resulting in glucosuria (high glucose levels in urine). In this way, SGLT2 inhibitors are excellent drug candidates for managing hyperglycemia since they are insulin independent in their mechanism of action. Clinical trials in people with T2D have shown this to be true as shown by reduced HbA1C % and SGLT2 inhibitors having a dose-dependent effect on lowering glucose levels[134]. Due to this, there are currently many variations

of SGLT2 inhibitors being prescribed for people with T2D as an oral medication to help manage their hyperglycemia[11], with the three currently approved SGLT2 inhibitors by the Food and Drug Administration being: dapagliflozin, canagliflozin, and empagliflozin[135]. Inhibitors of SGLT2 have also been shown to have a wide range of beneficial effects in T2D in addition to managing hyperglycemia which will be briefly discussed herein.

### 2.3.2 Beneficial Effects of SGLT2 Inhibitors in T2D

Studies in people with T2D have shown SGLT2 inhibitors to have beneficial effects on metabolism[11], renal function[11], blood pressure[11], and cardiovascular function[136]. Due to the increased renal clearance of glucose due to SGLT2 inhibitors, compensatory pathways are promoted to meet the demand to replenish the lost glucose[11]. Studies have demonstrated this as an observed increase in glycogenolysis and gluconeogenesis in the liver in patients with T2D[137] and an increase in renal gluconeogenesis in type 2 diabetic rodents[138] after SGLT2 inhibition. Following consistent use of SGLT2 inhibitors the glycogen stores become depleted as shown in diet induced obese mice[139,140], thus this lead to a reduction of utilization of glucose by peripheral tissues to preserve glucose for brain utilization[11]. Studies in patients with T2D have also shown an increase in ketogenesis in the liver as an alternative and more energy efficient metabolite[141]. This is due to the promotion of lipolysis[142]as observed in individuals with and without T2D receiving SGLT2 inhibitor treatment[142], in order to meet the demands of energy since glucose is being underutilized by peripheral tissues[11]. Due to this shift in metabolite usage and glucosuria, weight loss is observed in people with T2D as glucosuria can promote a negative energy balance[11]. This has been demonstrated in clinical studies in adults with T2D[134,143] receiving SGLT2 inhibitor treatment, where there is association between overall weight loss and fat mass loss and the dose of SGLT inhibitors[144] to meet the demand of lipolysis. This weight loss does

reach a nadir after ~6 months of SGLT2 inhibitor use, even though glucosuria is still observed[11]. It is suggested that this is because with time the amount of glucosuria declines (due to the improved blood glucose levels) thus the energy loss's magnitude decreases[11]. Therefore, the smaller energy loss can be offset by food intake leading to a new energy balance and plateau in weight[11].

With regards to renal function, the Empagliflozin, Cardiovascular Outcome Event Trial in T2D Mellitus Patents (EMPA-REG OUTCOME)[145]showed that incidence of acute kidney injury, common in those with diabetes, was lower with SGLT2 inhibitor treatment vs. placebo. This may be due to reducing the burden of sodium-reabsorption at the proximal tubule[11]. This reduced reabsorption of solutes at the proximal tubule can also lead to a reduction in the glomerular filtration rate, which as mentioned previously is usually increased in diabetes. This reduced glomerular filtration rate has been associated with improved kidney function in patients with T2D[145,146] . Indeed, in adults with T2D who have received SGLT2 inhibitor treatment for a long period of time, had a slower decline in renal function compared to placebo[145–147] and had reduced their risk of developing end-stage renal disease[11]. These improvements appear to be independent of improvements in glucose levels and weight[11].

In terms of blood pressure, although the mechanism is not well understood, SGLT2 inhibitors are observed to cause acute reduction in plasma volume in patients with T2D[11]. It is believed that the glucose osmotic effect and increase in sodium flow back past the proximal convoluted tubule are interpreted by the nephron as increased filtration[11]. This is despite the fact that plasma volume and sodium amount are reduced in individuals with T2D after receiving SGTL2 inhibitor treatment for 6 weeks[148]. The increased flow back of sodium and lower sodium levels in the body is believed to then signal changes to promote a 'new' homeostatic steady state

where plasma volume is reduced[11]. This is because the literature has shown that SGLT2 inhibition's association with natriuresis is modest[149] and the osmotic diuretic effects of glucosuria that are observed with SGLT2 inhibition are also observed in persons without diabetes[150].

With reference to cardiovascular function, SGLT2 inhibitors have shown improvements in function in large clinical trials in people with T2D who have CVD and/or risk factors of CVD. In the EMPA-REG OUTCOME trial, the risk for the three major adverse cardiovascular events (MACE, include non- fatal myocardial infraction, cardiovascular death, and nonfatal stroke) was reduced by 14%, the risk of cardio vascular death was reduced by 38%, the risk of hospitalization for heart failure (HHF) was reduced by 35%, and all cause morality was reduced by 32%[151]. The reduction in HHF was also similar in CANVAS, a large-scale trial involving patients with T2D and risks of CVD (33% and 14% respectively) that took canagaloflizin[151]. Similarly, in another large clinical trial, patients with T2D and underlying kidney disease had a 20% reduction in MACE[151]. These improvements in cardiovascular function are important considerations for clinical practice, however, the mechanism of action is unclear. It is proposed that SGLT2 inhibitors work on a multitude of pathways; (1) they could improve ventricular loading condition[152,153], (2) they can improve cardiac energy metabolism[154,155], and (3) they have antifibrotic effects as seen in rat models of post myocardial infraction[156].

All these studies demonstrate how SGLT2 inhibitors are viable treatments for T2D. However, it is important to consider some potential adverse effects that can occur with SGLT2 inhibitors. Although few in number, some clinical trials in patients with T2D have reported increasing the risk of urinary infections[147,157,158] where the events are mild in severity[15]. Another side effect is the potentially life-threatening development of DKA, although more common in

T1D, occurred in a small number of participants in the CANVAS trial[15]. It is important to note that DKA appears to occur more so when the SGLT2 inhibitor is cangaloflizn[15]. The CANVAS trial also showed that participants were at an increased risk of lower extremity amputation and bone fractures[15]. These side effects have not been observed in any other SGLT2 inhibitor[15]. With all these impacts in mind, research is emerging to understand if SGLT2 inhibitors can be beneficial in T1D, which will be further discussed.

2.3.3 SGLT2 Inhibitors in T1D

As already discussed above in this thesis, chronic hyperglycemia in T1D is a major risk factor for macro and microvascular diseases and while intensive insulin therapy may minimize the complications[159], it is associated with weight gain, increased glucose variability, and hypoglycemia[160–162]. Therefore, the majority of this population does not meet glycaemic targets[1]. Thus, a therapy that coincides with insulin therapy that promotes better glycemic control without increasing the risk of hypoglycemia is needed as it can minimize the risk of developing microvascular diseases.

Previous studies of SGLT2 inhibitors in rodent models of T1D have already shown that the inhibitors can improve glycemic control alongside insulin therapy[163,164] . This has also been shown in human clinical trials with T1D as measured by a reduced HbA1c levels by~0.3-1.0%[165–169]. These clinical studies also showed that the total amount of exogenous insulin that was taken was decreased in these participants[165–169] and reduced body weight[165–168]. In the same human studies, a few DKA events were reported in each study (Perkins et al[165]: 5%, Henry et al[168]:14.5%, Sands et al[169]:6.06%). While alone these numbers are not concerning, the repeated and similar prevalence in three of the human studies encouraged researchers to educate participants about DKA and provide blood ketone monitoring devices to help reduce DKA

events. Indeed this was confirmed by two large clinical trials, one being Tandem3[170] and the second being Dapagliflozin Evaluation in Patients with Inadequately Controlled Type 1 Diabetes(DEPICT-1)[171]. In the Tandem-3 trial[170], participants were educated about DKA and given blood ketone meters before beginning the 24-week blind randomized control trial. Participants with T1D were randomized into a group receiving SGLT2 inhibitor or placebo. After the 24 weeks, participants in the SGLT2 inhibitor group had a reduced HbA1C%, decreased body weight, decreased total insulin uptake compared to placebo and no DKA or hypoglycemic events occurred. Similarly in the DEPICT-1 trial[171], participants were again educated on DKA and provided blood ketone meters before beginning the double blind randomized control trial. Following a similar study design to Tandem-3, the participants in the SGLT2 inhibitor group had reduced HbA1C%, reduced body weight, reduced total insulin uptake, and no difference in DKA prevalence when compared to the placebo group. However, more urinary tract infections occurred in the participants in the SGLT2 inhibitor group compared to the placebo group, a common non-severe side effect. These results show how important it is to educate this population about DKA and provide tools to help monitor their blood ketone levels as it played a larger role in preventing DKA. Indeed, this is supported by a study by Patel et al[172] that suggested that the failure of patents with T1D to recognize early signs of DKA largely increases the risk factor for developing DKA rather than due to an increase in ketogenesis. In the study by Patel et al[172], participants with T1D, underwent an overnight suspension of insulin before and after treatment of SGLT2 inhibitor or placebo. Ketone bodies, free fatty acids, plasma insulin, and glucagon were measured continuously after suspension of insulin for the entire 6-hour duration of the insulin suspension. The study found that the SGLT2 inhibitor treatment did not significantly impact the levels of free fatty acids, ketone bodies, and glucagon levels during

the suspension. Therefore, these results highlight the importance of educating this population about DKA as it appears that failure to recognize early symptoms of DKA is what largely increases the risk for developing DKA.

In addition to these large clinical trials of drug efficacy on glycemic control, a few clinical studies have also shown improvements in renal and cardiovascular function in patients with T1D. Cherney et al found that subjects with T1D had reduced renal hyperfiltration after empagliflozin treatment for 8 weeks in euglycemic and hyperglycemic clamp conditions via a decreased glomerular filtration rate [173]. Additionally, individuals with uncomplicated T1D who received SGLT2 inhibitor treatment for 8 weeks improved their arterial stiffness in euglycemic and in hyperglycemic clamp conditions[174]. Similarly in a study conducted by Lunder et al, adults with T1D improved arterial stiffness after 12 weeks of treatment with a SGLT2 inhibitor[175]. The participants in this study were randomized to receive (a) SGLT2 inhibitor only, (b) SGLT2 inhibitor and metformin (another glucose lowering drug prescribed for people with T2D) (c) metformin only, or (d) placebo. The study found that endothelial function improved after 12 weeks in all treatment conditions except placebo as measured via flow mediated dilation and reactive hyperaemia index, with the greatest improvement being in the SGLT2 inhibitor and metformin group. The study also found that arterial stiffness improved after 12 weeks of treatment in all treatments except placebo as measured by pulse wave velocity, with the highest benefit being the SGLT2 inhibitor and metformin treatment. Based on these results and the multitude of benefits SGLT2 inhibitors have, the literature has investigated the possible mechanisms of action SGLT2 inhibitors play on vasculature.

2.3.4 The Impact of SGLT2 Inhibitors on Vasculature at the Molecular and Cellular Level

The literature has shown that SGLT2 inhibitors can have a positive impact on the health of vasculature which in turn can decrease the risk of macro and microvasculature complications from occurring[176]. *In vitro* work has shown that SGLT2 inhibitors can reduce endothelial inflammation by reducing[177] and or inhibiting[178] reactive oxidation species production, increasing nitric oxide availability[177] (important for vasodilation), reducing production of inflammatory markers[178,179] even in the presence of hyperglycemia[180], which contributes to reduced endothelial dysfunction [181]. *In vitro* work has also shown that SGLT2 inhibitors can reduce proliferation[176], prevent migration[176], and arrest the growth of vascular smooth muscle cells[182] (VSMC). VSMCs play an important role in maintaining vascular tone[176]. They can undergo phenotypic conversions and increase in their proliferation which can both lead to negative impacts on the function of the vasculature such as remodeling that occurs in atherosclerosis, an example of macrovasculature disease[183,184]. Thus, the results observed in the *in vitro* work points to SGLT2 inhibitors reducing the risk of negative vascular remodeling[176]. These results were also confirmed *in vivo* in a type 2 diabetic rodent model, where VSMCs function was restored to the level of their healthy counterparts after 8 weeks of SGLT2 inhibitor treatment[185]. *In vivo* work in type 1 and type 2 diabetic animal models have also shown SGLT2 inhibitors to be associated with reduced endothelial dysfunction[178,180,186,187]via reduced glucose toxicity[186], reduced oxidative stress[178,186,187], and reduced inflammation[178,180,186]. Other *in vivo* work in diabetic rodents showed that SGLT2 inhibitors delay diabetic nephropathy development by reducing the oxidative stress induced by hyperglycemia[13] and by inhibiting oxidative stress, inflammation, and fibrosis[188]. Reduced fibrosis via reduced oxidative stress was also seen in a non-diabetic rodent model where renal ischemia was induced followed by SGLT2 inhibitor treatment where it was postulated that perhaps SGLT2 inhibitors were possibly playing a role

through a VEGF dependent pathway[189]. However, research investigating the impact of SGLT2 inhibitors on the vasculature in skeletal muscle is extremely limited. In a study by Sugiyama et al, patients with T2D who were treated with SGLT2 inhibitors for 6 months showed improved peripheral microvascular endothelial function[190]. Another *in vivo* study in a type 2 diabetic rodent model with hind limb ischemia received SGLT2 inhibitor treatment found that the skeletal muscle cells viability and proliferation improved compare to the counterpart control non-ischemic limb where the SGLT2 inhibitors were given via an intramuscular injection into the limb that had ischemia[14]. The same study also exposed cell lines to hyperglycemic conditions and showed that protein levels of VEGF-A increased in response to the SGLT2 inhibitor. These results are intriguing and if proven to be true in type 1 diabetic rodent models then SGLT2 inhibitors might be a beneficial therapeutic to help reduce and minimize complications of diabatic myopathy, particularly with regards to the reduced capillarization and impairments in angiogenesis.

## 3. RATIONALE AND OBJECTIVES

### Rationale

Individuals with T1D continue to face a challenge with regulating their blood glucose levels, with a large portion of this population having hyperglycemic levels (>7% HbA1c)[1]. This is despite the advancements in blood glucose monitoring technology and the intensive insulin therapy that is taken up exogenously which includes basal and prandial insulin delivery via an insulin pump or daily insulin injections. Chronic hyperglycemia is a major risk factor for microvascular complications that tend to occur in people with T1D[191,192], with the most understudied complication being diabetic myopathy. From the limited literature that has explored the health of capillaries in the skeletal muscle in type 1 diabetes, hyperglycemia has been associated with reduced capillarization in the skeletal muscle in rodent models of T1D[3,4] and reduced adaptations to exercise, such as the increased capillary to fibre ratio in skeletal muscle that is typically observed after exercise, which have been shown to be diminished in people with T1D[110]. These results are concerning for this population as skeletal muscle is a critical organ for managing this disease since it is the largest organ for blood glucose regulation[8] and a mediator of whole body insulin sensitivity[9]. Indeed, a loss in the number of capillaries in skeletal muscle can further exacerbate the challenge in maintaining blood glucose levels due to a diminished ability to uptake blood glucose and failing to transport insulin into the skeletal muscle. Therefore, additional therapies that coincide with insulin therapy to promote better blood glucose level regulation without risking hypoglycemia in this population is needed.

One possible therapeutic are SGLT2 inhibitors, a drug currently used to treat hyperglycemia in people with type 2 diabetes. Clinical trials of this drug coinciding with insulin therapy in individuals with T1D have shown to be safe and effective in promoting healthy

glycemic levels[170,171]. Research in rodent models and humans with T1D have also shown improvements in renal function, cardiovascular function, and reduced endothelial dysfunction[173,174,187,193]. SGLT2 inhibitors have also been shown to promote the expression of important pro-angiogenic factors of diabetic rodents with ischemia[14]. Accordingly, SGLT2 inhibitors have the potential to promote capillary maintenance in skeletal muscle by reducing the stress of hyperglycemia on the capillaries in the skeletal muscle.

For the first time the impacts of SGLT2 inhibitors on capillary maintenance in the skeletal muscle in a juvenile type 1 diabetic rodent model are being investigated. This study will provide insight on whether the improved glycemia that occurs in SGLT2 inhibitor treatment coinciding with insulin therapy can promote maintenance of capillary health in the skeletal muscle within 2 weeks of treatment.

**Objectives**

The primary objective of this thesis was to determine the impacts of SGLT2 inhibition on capillary to fibre ratio in the skeletal muscle of a juvenile type 1 diabetic rodent model as a way to evaluate its possible impacts on capillary maintenance in skeletal muscle in T1D. To further examine the impacts of SGLT2 inhibitors on capillary maintenance in the skeletal muscle in T1D, protein expression of VEGF-A, mRNA expression of VEGF-A and platelet endothelial cell adhesion molecule-1 (PECAM) was assessed as secondary outcomes. In order to assess the impacts of SGLT2 inhibition on blood glucose levels, the risk of diabetic ketoacidosis, and the risk of a urinary tract infection blood glucose levels, HbA1C%, blood ketone levels, and leukocyte levels in urine were assessed.

## Hypothesis

We hypothesized that SGLT2 inhibition coinciding with insulin therapy will promote the maintenance of capillaries in the skeletal muscle of type 1 diabetic juvenile rodents via indirect effects of improved glycemia. Therefore, we expected a normalization of the VEGF-A protein, VEGF-A mRNA, and PECAM mRNA expression in type 1 diabetic rats as compared to the healthy counterparts. We did not expect to see an increase in the risk of DKA or urinary tract infection occurring in the SGLT2 inhibition and insulin treatment.

4. MANUSCRIPT

# EMPA DOES NOT IMPACT CAPILARY CONTENT IN SKELETAL MUSCLE IN A JUVENILE MALE RODENT MODEL OF TYPE 1 DIABETES

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## Contribution by Authors

The protocol for this study was designed through a collaborative effort from Dr. Tara Haas, Dr. Michael Riddell, and myself. Dorsa Shakeri, Ninoschka D'Souza, Sara Atherley, Emily Hoffman, and I contributed to the daily animal care. HbA1c % ELISA assay was performed by myself, Dorsa Shakeri, and Ninoshcka D'Souza. C-peptide ELISA assay was performed by Sara Atherly. Westerns were performed by myself, Stephen Mora, and Gagandepp Mann. I performed RT-PCR and qPCR analysis. Capillary counting for capillary to fibre ratio was performed by Dorsa Shakeri. George Nader taught me how to extract RNA from skeletal muscle for the purposes of RT-PCR and qPCR analysis. I analyzed all data concerning blood glucose concentration, HbA1C%, c-peptide, capillary to fibre ratio, correlation analysis, westerns, and qPCR. Dr. Riddell is the principal investigator and supervisor of this project. The manuscript found below was written by me with edits from my supervisory committee after my thesis defence (Dr, Michael Riddell, Dr. Tara Haas, and Dr. Jean-Paul Paluzzi).

## Introduction

Type 1 Diabetes (T1D) is an autoimmune disease characterized by complete or near complete autoimmune destruction of the insulin-secreting pancreatic β-cells, thereby causing hyperglycemia[194]. It is estimated that over 300,000 Canadians have T1D with a growing average incidence rate[17]. Modern blood glucose management includes intensive insulin therapy, including basal and prandial insulin delivery using an insulin pump or multiple daily injections, and frequent glucose monitoring using subcutaneously implanted glucose sensors and or self-monitoring of capillary blood glucose levels, along with the assessment of hemoglobin A1c (HbA1c) levels to help regulate blood glucose levels. Nevertheless, blood glucose control is often suboptimal in T1D, particularly in childhood and young adulthood, with most individuals exposed to frequent episodes of both hypoglycemia and hyperglycemia[1]. Chronic hyperglycemia, as defined by a HbA1c level of >7.0% or a mean glucose of >11.1mmol/L, is a major risk factor for several complications such as nephropathy and retinopathy due to negative impacts on the microvasculature[191,192]. Associations between hyperglycemia and endothelial cell dysfunction have been well documented in the literature both in animal models[195] and human patients with this disease[196]. The endothelial dysfunction seems to occur even during early development of the disease[196–198] with strong negative correlations observed between HbA1c levels and endothelial function, in adolescents and young adults with T1D[88,199–201].

Researchers have demonstrated a loss in the number of capillaries in the skeletal muscle of rodents with T1D [2–5], dysregulated angiogenesis[3], and defective peripheral skeletal muscle perfusion in adults with T1D when compared to healthy controls[202]. These changes in the skeletal muscle microvasculature with T1D[4,202] accompany other myopathic changes including alterations in muscle morphology[203], impaired muscle recovery from injury[204], and reduced

mitochondrial function[205], all likely resulting in imperfect glycemic control and frequent exposure to hyperglycemia.

Skeletal muscle plays a vital role in maintaining blood glucose levels by (a) transporting glucose across the capillaries to be metabolized by the skeletal muscle and (b) absorbing insulin across endothelial cells[206]. Therefore, a loss in the number of capillaries in the skeletal muscle in T1D is concerning as it can further exacerbate the challenge in maintaining blood glucose homeostasis. Another likely reason for the challenge in maintaining blood glucose levels within a healthy range in those with T1D[10] and for a possible deterioration in overall muscle health and capillary density, even in pediatric patients with the disease[207]; is failing to properly deliver sustained and physiologic insulin levels to the skeletal muscle. Accordingly, the maintenance of capillaries and endothelial cell health in skeletal muscle in people with T1D is important in managing normal blood glucose levels since skeletal muscle is the largest organ for glucose disposal[206]. Consequently, any adjuvant therapy to insulin that further lowers blood glucose levels, but without promoting hypoglycemia, could potentially promote the maintenance of the capillaries in the skeletal muscle.

One emerging adjuvant therapy for improving glucose control in T1D is sodium-glucose cotransporter-2 (SGLT-2) inhibitors, where the SGLT-2 in the proximal tubule of the kidney is inhibited to promote renal clearance of glucose. SGLT-2 inhibitors are currently used to treat type 2 diabetes[11] and have been shown to be beneficial for individuals with T1D since they lower glucose levels by mimicking the blood glucose reducing effects of insulin via increased renal clearance of glucose[11]. In adults with T1D, SGLT-2 inhibitors typically lower mean HbA1c levels by 0.4-0.5%, reduce body weight by 2.5-4 kg, reduce total daily insulin needs by 10-20%, and significantly lower glucose excursions around mealtimes[208].

The clinical benefits of SGLT2 therapy may go well beyond these well described improvements to metabolic control in T1D. For example, SGLT-2 inhibitors have also been shown to improve endothelial function *in vivo*[185,209] and *in vitro*[177,178] by attenuating endothelial inflammation[177,178,180] and oxidative stress[176,178]. In diabetic rodents, SGLT2 inhibitors delay the development of diabetic nephropathy[13], a micro-vascular complication in those with T1D[176], through many mechanisms of actions, one postulated to be by preventing renal fibrosis[13]. Indeed, it was demonstrated that in a SGLT2 inhibitor treated non-diabetic rodent model with renal ischemia, renal fibrosis was observed to be reduced through a vascular endothelial growth factor (VEGF) dependent pathway[189], a protein important in regulating angiogenesis of vasculature[210]. Moreover, SGLT-2 inhibitors have been shown to promote the expression of important pro-angiogenic factors in the skeletal muscle of diabetic rodents with ischemia, another complication that occurs in T1D[14] . Therefore, SGLT2 inhibitors have the potential to promote maintenance of the capillaries in the skeletal muscle of those with T1D. However, it remains unclear if SGLT2 inhibitors can maintain capillaries in the skeletal muscle, by way of improving glycemic control.

The aim of this study is to evaluate the impact of empagliflozin (EMPA), a commonly prescribed SGLT-2 inhibitor for type 2 diabetes in humans, on capillary maintenance in a juvenile rat model of T1D, with capillary-to- fibre (C:F) as the primary outcome and mRNA and protein levels of angiogenic factors as secondary outcomes. We hypothesized that EMPA will promote maintenance of the capillaries in the skeletal muscle of young rats with T1D via indirect effects through improved glycemia as measured by frequent whole blood glucose monitoring and HbA1c levels. To our knowledge, this research will for the first time examine the effects of SGLT-2 inhibitors on capillary maintenance in the skeletal muscle of T1D rodents.

## Materials and Methods

### *Animal Model*

Thirty-three, healthy, six-week-old male Sprague Dawley rats (SD, Charles River) were separately housed under temperature-controlled conditions in a twelve-hour light–dark cycle and randomized into a healthy control group (CON, n=12), an EMPA and insulin treated T1D group (SGLT2i, n=11), and an insulin treated T1D group (Vehicle, n=11). This study is approved by the York University Animal Care Committee and was conducted in accordance with the Canadian Council for Animal Care Guidelines.

### *Training Rats to Voluntarily Consume SGLT2i using Flavoured Jellies*

The rats were habituated to vivarium conditions for seven days with access to regular chow and water. On the eighth day, the rats were fasted overnight followed by presenting the vehicle "jellies" (see preparation of jellies below for contents in jellies) in the houses of the rats at the same time as when the jellies would be presented during the treatment (9am, see drug dosing strategy below). Regular chow (10-15g) was then added back to the cage once the jelly was consumed. The vehicle jelly was then presented simultaneously with regular chow (20-25g) in the evening at the same time as when jellies were presented during treatment (4pm). The simultaneous introduction of the vehicle jellies and regular chow lasted for eight days.

### *Induction of T1D using Streptozotocin*

Following the training of the rats on jelly consumption, T1D was induced (Figure 1), through an intraperitoneal injection of streptozotocin (STZ, 65 mg/kg of body weight, Sigma, #S0130) followed by the consumption of 20% sugar water for the next twenty-four hours. STZ enters the insulin producing β-cells through a GLUT2 transporter and causes DNA damage

through a series of molecular reactions which then induces necrosis of the β-cells [211]. In order to confirm the onset of T1D, blood glucose (BG) levels were measured using a blood glucose meter and tail poke bleed 24 hours after the STZ injection, where fed a BG ≥ 15 mmol/L is considered to be diabetic, as per standard guidelines[56]. Regular water was then provided following the confirmation of T1D onset. Rats that did not become diabetic within two days after the initial STZ injection were given a second injection (65 mg/kg of body weight). Any rats that did not become diabetic following the second injection were excluded from this study.

*Treatment and Daily Monitoring*

Following the onset of T1D in the vehicle and SGLT2i groups, rats in the CON and vehicle groups were presented with vehicle jellies while rats in the SGLT2i group were presented with drug jelly in the morning and evening for two weeks (Figure 1). Each drug jelly had 15 mg/kg body weight of EMPA to meet the desired drug dosage per day (30 mg/kg of body weight). All rats had access to forty grams of regular chow during the day. Rats were weighed daily in the morning followed by a blood glucose (BG) check before presenting the jelly. In the evening, all rats had their BG checked before presenting the jelly. The SGLT2i rats had their BG checked after consuming their jelly to determine the appropriate amount of insulin that will be given via a subcutaneous injection of Humulin R. Whereas the vehicle rats received their subcutaneous injection of Humulin R based on their evening BG at the same time the SGLT2i rats received theirs. Jellies were weighed before presenting them and again before the next round of jelly presentation to account for any drug not consumed. All blood glucose checks were made using Contour Next glucose meter from a sterile tail poke. The amount of insulin that was given to each rat was dependent on their body weight and their BG levels (Appendix 2). No insulin was given if a rat had BG ≤ 14 mmol/L, to mimic poorly controlled T1D. If a rat's BG levels were ≥

30 mmol/L in the morning, one unit of basal insulin (insulin glargine, Lantus, Sanofi Canada) was given subcutaneously. SGLT2i rats were treated to achieve a target of BG 15-20 mmol/L to simulate the blood control generally observed in youth living with type 1 diabetes[212]. In order to achieve this target, the two different human derived insulins were used as the duration of action and time it takes for the insulins to begin their mechanism of action differs (Humilin R: reaches the blood stream ~15-30 mins after injection and is effective for 3-6 hours, Lantus: reaches the blood stream ~1.5 hours after injection and is effective for 12-24 hours[213]). Although the insulin is human derived with slight differences in the amino acid sequences compared to rat insulin, the human derived insulin is still effective in lowering BG in rats[214].

*Blood Ketone Measurements*

Blood ketone levels were measured in a fasted state (~16 hour fast) before the onset of T1D, 72 hours after the onset of T1D, and after the two weeks of drug/vehicle treatment. Random (i.e., fed) blood ketone levels were also measured towards the end of the two-week treatment (Figure 1). All ketone checks were made using the Freestyle Precision ketone meter from a sterile tail poke using a fine gauge needle to collect ~5ul of capillary blood.

*Preparation of Jellies and EMPA drug solution*

Jellies were made as a mode to orally administer EMPA or saline to the rats based on a protocol by Zhang 2021[215] with modifications to mask the bitter the flavour of the EMPA/SGLT2i treatment, as described below. Specifically, the vehicle jellies were made using strawberry flavouring, sucralose sweetener, unflavoured gelatin powder, 0.9% saline, and anti-bite nail varnish to stimulate the bitter flavour found in the EMPA jellies. The EMPA jellies contained the same ingredients as the vehicle jellies with the exception of the anti-bite nail

varnish and saline, where the latter was substituted with EMPA drug solution. A 20% (weight/volume) sucralose sweetener was made to use as the solvent for the gelatine powder and powdered EMPA. EMPA (Jardiance tablets, 10 mg/tablet, DIN 02443937) were powdered and dissolved in 20% sucralose sweetener water (2.2 ml, final concentration: 4.5 mg/ml). Twelve grams of gelatine powder were then dissolved in the sucralose sweetener water to create a 24% (weight/volume) gelatine solution by heating it to 55-60˚C, loosely covering it, and constantly stirring until the solution became clear. Strawberry flavouring (10ml) was then added, followed by the addition of anti-bite nail varnish (0.5ul) in the vehicle stock solution only. The mixture was constantly stirred at the same temperature for ten minutes. Using silicone-based molding trays, the saline or EMPA drug solution was pipetted into a mold followed by pipetting the appropriate hot jelly stock solution into the mold to achieve the required concentration (15 mg/kg of body weight/jelly). The mixture was then mixed thoroughly with a toothpick. New jellies were made every three to four days with the remaining gelatine stock solutions stored at 4˚C

*Skeletal Muscle Tissue Isolation and Urine Analysis*

After the two-week treatment (Figure 1), all rats were anaesthetized with inhaled isoflurane. Tibialis anterior (TA), gastrocnemius (GASTROC), and soleus muscles were removed, weighted, and immediately frozen in liquid nitrogen (mRNA and protein measurements) or frozen in liquid nitrogen-cooled isopentane for histology. All samples were stored (at -80˚C) until analysis. Urine was collected from the rats' bladder through a syringe and deposited on urinary strips (Chemstrip 9, 10398411119) to validate the effectiveness of the drug (i.e: glucose concentrations in the urine sample) and to note if there was any urinary tract infection (i.e: leukocytes detected in the urine sample).

*Muscle capillary:fibre analysis*

A cryostat set to -20˚C was used to obtain 10 $\mu$m thick transverse cryosections from the mid-belly of the TA as previously described[4]. Cryosections were briefly fixed in paraformaldehyde (4%) and stained with Rhodamine Wheat Germ Agglutinin (1:200; #RL10225, VectorLabs) to detect capillaries and to outline muscle fibers. Images of sections were captured using a digital colour charge coupled device camera after being viewed with an Olympus microscope (x20 objective). Capillary and muscle fiber counts were averaged from four to nine independent fields of view per muscle per rat.

*RNA Extraction and Real-Time Quantitative PCR (qPCR) Analysis*

Total RNA was isolated from TA, GASTROC, and soleus of all rats as previously described[216]. The primers that were used in the qPCR analysis are TaqMan Assay-on-Demand Hprt1 (Thermofisher, #Rn01527840_m1), PECAM (Thermofisher, #Rn01467262_m1), and VEGF-A (Thermofisher, #Rn00582935_m1), with the target gene being PECAM and VEGF-A. Expression of PECAM was calculated relative to Hprt1 in all muscles while VEGF-A was calculated relative to Hprt1 only in the TA and all results were expressed as $2^{-\Delta Ct}$.

*Western blotting*

Immunoblotting on protein extracts from the TA, GASTROC, and soleus were obtained from all rats as previously described[4]. The blots were probed overnight as previously described[4] with an antibody against VEGF-A (Novus Bio, clone VG1, # NB100-664). Blots were also incubated with Ponceau-S for 15 minutes prior to antibody incubation. Images of the blots were obtained using ChemiDoc XRS+ System (BIORAD, # 1708265). Blots were analyzed with Image Lab Software (BIORAD, #1709690) quantifying all bands that appeared for both Ponceau S and VEGF-A.

*Elisa Kits*

Elisa kits were performed to determine HbA1c levels (Crystal Chem, #80300) and c-peptide concentrations (Crystal Chem, #90055) to provide information on glycemic control and endogenous insulin secretion. Higher HbA1c levels, expressed as a % glycosylation of the red cell, appear to increase the risk of complications in the capillaries[1] while the c-peptide measurement allows us to monitor how effective the STZ injection was in destroying the insulin producing β-cells.

*Statistical Analysis*

Statistical analyses of data were performed using Prism 8 software except for the Cochran-Mantel-Haenszel Test where R was used. One-way ANOVA was performed for capillary:fibre, western blot analysis, Elisa kits, mRNA from qPCR, random and blood ketone levels, followed by a Tukey's post hoc where appropriate. A two-way repeated measure ANOVA was performed for blood glucose levels, weight, and food consumption followed by a Tukey's post hoc for all except for blood glucose levels where a Bonferroni's post hoc was performed in addition to the Tukey's to compare the difference between morning and evening blood glucose levels. A two-way ANOVA was also performed for fasted ketone blood levels. Correlation analyses between variables were performed with nonparametric two-tailed Pearson correlation. A Cochran-Mantel-Haenszel Test was performed for leukocyte levels in urine. A $p$ value of $\leq 0.05$ was considered statistically significant. All values are presented as the mean ± standard deviation.

**Results**

*STZ was Effective in causing T1D in rats*

C-peptide levels were significantly higher in the CON group (1.150 ng/ml ± 1.525) compared to both diabetic groups ($p<0.05$) and there was no difference between the diabetic groups (vehicle: 0.1014 ng/ml ±0.1150, SGLT2i: 0.1529±0.2656 ng/ml | $p>0.05$, Figure 2).

*Diabetes was associated with hyperphagia and weight loss*

The animal weights and the amount of food consumed by the diabetic animals were not significantly different between the two diabetic groups during the two-week treatment period ($p>0.05$, Figure 3a, $p>0.05$, Figure 3b, respectively). However, the body weights of the healthy control rats were significantly higher than both diabetic treatment groups ($p<0.05$), and the daily food consumption by the healthy controls was significantly less than both diabetic treatment groups ($p<0.05$).

*EMPA is Effective in Lowering HbA1c levels and Morning and Evening Blood Glucose Levels*

HbA1c levels were compared between the SGLT2i, Vehicle, and CON group using a one-way ANOVA (Figure 3b). The test showed that the SGLT2i group (10.65% ± 0.74) had significantly lower HbA1c levels compared to the vehicle group (12.56% ± 1.66, $p<0.05$) with the CON being significantly less than both diabetic groups (5.64% ± 1.02). Average blood glucose levels were significantly different all three groups, where the CON (6.12 mmol/L ± 0.34) was significantly lower compared to both diabetic groups ($p<0.05$) and the SGLT2i group (18.32 mmol/L ± 4.36) was significantly lower than the vehicle group (29.03 mmol/L ± 2.45 | $p<0.05$, Figure 4a) as indicated by the Tukey's post hoc following the one-way ANOVA. A Bonferroni's multiple comparison test was also conducted to test if differences existed within groups between the morning and the evening blood glucose sampling time periods (Figure 4a). The test showed that both diabetic groups' blood glucose levels were significantly higher in the evening

compared to their respective morning blood glucose measurements (vehicle; $p<0.05$ & SGLT2i; $p<0.05$). These results show that the SGLT2i treatment promoted lower blood glycemic levels, indicating that EMPA was effective in lowering hyperglycemia in these rats.

*EMPA Treatment did not appear to increase the risk of DKA or urinary tract infection*

A two-way ANOVA of fasted blood ketone levels showed no differences among all three groups at the three different measured time points ($p>0.05$, Figure 4a). The test also showed no difference among the three time points within each respective group ($p>0.05$, Figure 5a). A one-way ANOVA of random blood ketone levels, followed by a Tukey's post hoc test, showed the CON (0.7 mmol/L ± 0.2) to be significantly lower than both diabetic groups ($p<0.05$) with no difference between the vehicle (1.6 mmol/L ± 0.7) and the SGLT2i (1.7 mmol/L ± 1.1 | $p>0.05$, Figure 5b) groups. A Cochran-Mantel-Haenszel test showed that there was no difference between the observed and expected data for all three groups, indicating that EMPA did not increase the risk of a urinary tract infection in these animals as measured via leukocytes/uL of urine. (CON: 2.75±2.36, Vehicle: 2.50±2.08, SGLT2i: 2.75±1.89 Leuk/uL | $p>0.05$, Figure 6).

*Hyperglycemia in T1D is associated with lower C:F*

Mean TA C:F was similar among the three groups ($p>0.05$, Figure 7a). However, the individual C:F appeared to correlate modestly with individual HbA1c levels when combining all animals including the controls (Figure 7b, $p>0.05$, $r=-0.38$)..

*T1D appears to increase the production of VEGF-A at the protein level in the T A*

A one-way ANOVA followed by a Tukey's post-hoc conducted on VEGF-A intensity relative to Ponceaus S, showed that the CON (0.0042 ± 0.0016 intensity) has significantly lower VEGF-A protein expression compared to the vehicle group in the TA (0.0118 ± 0.0082 intensity

| $p<0.05$, Figure 8a). There was no difference between the SGLT2i group ($0.0099 \pm 0.0076$) and the CON group ($p>0.05$) nor a difference between the SGLT2i and the vehicle group ($p>0.05$). In the soleus and the GASTROC, no differences were observed after conducting a one-way ANOVA on the VEGF-A intensity relative to Ponceaus S (Figure 8b, c, respectively).

*No difference in PECAM mRNA levels between all three groups in the TA, soleus, GASTROC or in VEGF-A mRNA levels in the TA*

PECAM mRNA levels relative to HRPT1 mRNA in the TA, soleus, and Gastroc were not significantly different between the groups when using a one-way ANOVA ($p>0.05$, Figure 9). Similarly, no significant difference was found when looking at VEGF-A mRNA levels relative to HRPT in the TA($p>0.05$, Figure 10). PECAM mRNA levels were assessed as a proxy for endothelial cell number[217].

**Discussion**

The literature has well documented how hyperglycemia in T1D is associated with microvascular complications including nephropathy and retinopathy in both rodents and humans with T1D [6,74,75,218]. However, microvasculature impairments in skeletal muscle in rodents and humans with T1D remains to be a heavily understudied area. Impairments in the ability of skeletal muscle to regulate blood glucose levels and deliver insulin can further exacerbate hyperglycemia in T1D[10], where hyperglycemia is observed to be highly prevalent in individuals with T1D, and in particular youth with the disease at a time when skeletal muscle and muscle capillaries are expected to grow[1]. This study aimed to assess if SGLT2 inhibitors could impact the maintenance of capillaries in skeletal muscle in a juvenile rat model of T1D, via indirect effects of improved glycemia.

In support of previous literature and the hypothesis of this study, SGLT2 inhibitors promoted improved glycemia as measured via blood glucose levels and HbA1 levels without promoting hypoglycemia[165–169]. Our study also showed, in support of our hypothesis and other literature[3,4]that hyperglycemia is associated with reduced C:F in skeletal muscle. This is despite no differences observed in C:F between the diabetic and healthy rats which is in contrast to our hypothesis and other literature[3,4]. Indeed, this study showed that the VEGF-A protein levels were higher in the TA in the diabetic groups and unchanged in the soleus and GASTROC compared to the CON group, which is in accordance with the findings of Aiken and colleagues[4]. This study also reported no differences in PECAM mRNA levels between all groups (CON, Vehicle, and SGLT2i). On the other hand, VEGF-A mRNA levels trended to be lower in the diabetic animals compared to the healthy animals, with no difference between the diabetic animals. These results support the notion that EMPA can promote better glycemic control in T1D, but may not have an impact on the capillaries in the skeletal muscle under the conditions of this study (i.e., sedentary young STZ-treated T1D rats undergoing insulin therapy that improves but does not normalize glycemic control).

In line with rodent models of T1D[162,163] and clinical trials of SGLT2 inhibitors in individuals with T1D[170,171], we have shown that EMPA, can improve glycemic control, as measured by HbA1c levels and average blood glucose levels, without promoting hypoglycemia nor increasing the risk of DKA (as measured by blood ketone levels), when taken as an adjuvant to insulin therapy. Indeed, the SGLT2i group's mean HbA1c levels was closer to the clinical target of 7% as compared to the vehicle group which was closer to 13%. Similar to the clinical trials of SGLT2 inhibitors in humans with T1D, the amount of insulin given to the animals in the SGLT2i group during treatment was 15% (Appendix 3) of the amount of insulin given to the

vehicle group[159]. Additionally, no differences were found in leukocyte levels in urine between the groups, indicating that EMPA did not increase the risk of a urinary tract infection in this particular model of T1D undergoing SGLT2 inhibitor therapy. This is contrary to the results of the DEPICT-1 clinical trial[171], where individuals with T1D received SGLT2 inhibitor treatment for 24-weeks were found to have more urinary tract infections and higher risk for DKA as compared to the individuals with T1D that received a placebo. The difference in the reported results is likely due to the length of time the SGLT2 inhibitor treatment was provided and the larger number of human study participants, where this study lasted for two weeks while the DEPICT-1 trial lasted for 24 weeks. Urinary tract infections are a relatively rare event in human clinical trials of SGLT2 inhibitor therapy and T1D[15], with an average of 8.67% of genital infection occurring with the lowest incidence being ~6% and the highest being 12%[159], which may not be observed in a small rodent study such as ours.

In accordance with the literature[4], this study reported capillary regression associated with hyperglycemia, despite no differences reported in mean C:F among all groups which is in contrast to previous literature. A few possible reasons as to why the mean difference between the C:F was not observed in our study are; (1) the length of diabetes, (2) the insulin treatment that the animals were receiving, (3) and how T1D was induced. Sexton et al[107] and Kivela et al[3], induced diabetes using STZ like this study, however, their animals were not treated with insulin and remained diabetic for a minimum of 5 weeks, unlike our animal model, where insulin was given daily and the duration of diabetes was only ~2.5 weeks. Similarly, Aiken and colleagues[4], who also found a difference in C:F between healthy and diabetic rats like Sexton et al. and Kivel et al., used a biobreeding model that spontaneously develops T1D between 50-90 days and once T1D was confirmed insulin treatment lasted for two weeks. However, our model induced T1D

via STZ at eight weeks of age in animals that are not genetically prone to develop diabetes and were treated immediately for only two weeks. In fact, studies in humans with insulinized T1D have shown no differences in C:F compared to healthy controls[108,109], instead human studies have demonstrated that the typical increase in C:F observed after several weeks of exercise training in healthy individuals is not observed in individuals with T1D who are also exercise training[110]. These results then point to a subtle defect in baseline angiogenesis in T1D, perhaps only observed in those under very poor glycemic control who may fail to fully adapt to exercise stimuli.

The current limited literature has shown alterations in expression of VEGF-A at both the mRNA[3] and protein level[3,4] and alterations in PECAM expression at the protein level in T1D[219]. This indicates that hyperglycemia in T1D can negatively impact the health of the vasculature which can exacerbate the diabetic myopathy that clinically presents itself in this population as reduced muscle mass, myofiber size, and poor metabolic control[89]. Accordingly, we investigated VEGF-A protein levels and PECAM mRNA in all the skeletal muscles harvested and VEGF-A mRNA in the TA. Herein we report an increase in VEGF-A protein levels in the TA in the vehicle group compared to the CON group, in line with the findings by Aiken et al[4], with no difference between with the CON group and the SGLT2i group. We also reported no difference in the amount of VEGF-A protein amount across all treatment groups in the soleus and GASTROC, like the findings in Aiken et al[4] where no difference in VEGF-A protein expression in the soleus between the diabetic and non-diabetic animals were found. The differences between protein expression of VEGF-A in the different muscles may be attributed to the fiber type composition, where VEGF-A appears to be more transcribed in fast-twitched glycolytic fibers[123]. Indeed, Aiken and colleagues[4] reported the increased VEGF-A protein in the plantaris, a mixed

fiber type composition with more predominant fast-glycolytic fibers like the TA as reported in our study. It is estimated that the fast glycolytic fibers composition is observed to be ~97% in the TA of rats[220]. Whereas the fiber type composition of the soleus is majorly slow oxidative fibers with an estimated 80% of the muscle having this fiber type[220]. The GASTROC, although predominantly is fast twitched, the deeper portion of the GASTROC, which was the portion used for the western analysis herein, is more hybrid with only ~70% of the fibers being fast twitched[220].

Interestingly, the VEGF-A mRNA expression trended to be lower in the diabetic groups compared to the CON group, similar to what was observed by Kivela et al[3], except that study found statistical significance. A possible explanation as to why the VEGF-A mRNA levels trended to be lower in our study but were not significantly lower in the diabetic groups compared to the CON group, may be due to the length of time the animals were diabetic and the insulin treatment the animals were receiving. Kivela and colleagues[3], induced diabetes in mice with STZ for 5 weeks with no insulin treatment, unlike our study where the STZ induced diabetes was treated immediately after induction with daily insulin, with or without EMPA, for two weeks. A possible explanation for the inverse relationship in the amounts of VEGF-A mRNA and VEGF-A protein reported in all three groups is due to how VEGF-A secretion is regulated in the skeletal muscle. Briefly, VEGF-A protein is stored in vesicles throughout the skeletal muscle[125] and are then relocated to the skeletal muscle' subsarcolemma in response to a hypoxia or a metabolic stressor and then released into the interstitial space[126]. It is thought that once these vesicle stores release VEGF-A into the interstitial fluid, VEGFA mRNA's transcription is elevated to replenish the VEGF-A stores in the skeletal muscles. This is due to how VEGF-A has been observed to respond to exercise, where VEGF-A mRNA is elevated during rest after exercise in muscle[125,221–

[223], while VEGF-A protein is elevated at the early onset of exercise in the interstitial fluid[126,224]. Thus, it is possible that the skeletal muscle mRNA levels are lower in comparison to the elevated protein levels in the diabetic groups due to the low demand to replenish the high VEGF-A protein amounts and vice versa in the CON group. Altogether, these results indicate that the improved glycemia by EMPA treatment may not impact the protein or mRNA amounts of VEGF-A and T1D may increase VEGF-A protein expression.

Additionally, PECAM mRNA levels were found to not be different between all groups in all the muscles analyzed, indicating that EMPA may not impact the endothelium's health in skeletal muscle which contrasts with previous literature[3,4]. This is because PECAM is a transmembrane glycoprotein that is abundantly expressed on endothelial cells[225], thus, serving as a proxy for endothelial cell number in the skeletal muscle. Therefore, a reduced expression of PECAM may indicate that there is a reduced endothelial cell number. Roudier and colleagues[226], demonstrated impairments in angiogenesis in rodent models with type 2 diabetes, where PECAM protein levels were observed to be lower in the skeletal muscle compared to their healthy counterparts. Similarly, Jin and colleagues[219] showed a reduction in PECAM protein levels in the skeletal muscle of type 1 diabetic rodents compared to healthy counterparts. A possible explanation for the different results as reported by Jin et al[219] and this study is due to the type of treatment the rodents received and the length of diabetes. Jin et al[219] provided no treatment for their diabetic rodents and kept them diabetic for eight weeks, whereas our study provided insulin treatment immediately after inducing T1D and the protocol lasted for two weeks. The alterations observed in Jin et al[219] that we did not observe may simply be due to the fact that our animals were not diabetic for a long period of time and we provided insulin treatment to maintain their blood glucose levels in a euglycemic range. In fact, this highlights the novelty of our work, as for

the first time we have shown the integral role insulin treatment plays in T1D, as previous literature has assessed similar markers without insulin treatment.

Furthermore, Nugrahaningrum and colleagues [14], showed that in type 2 diabetic rodents with hind limb ischemia, an intramuscular injection of SGLT2 inhibitors into the ischemic limb increased PECAM protein level expression in comparison to a type 2 diabetic rodent with hind limb ischemia and a placebo intramuscular injection. However, our results indicate no impact by EMPA on PECAM mRNA levels. The difference for these results is most likely explained by the difference in the methodology as; (1) Nugrahaningrum et al[14] used a type 2 diabetic model, (2) induced hind limb ischemia which is associated with necrosis and other negative impacts, (3) SGLT2 inhibitor treatment was provided via an intramuscular injection which does not match how SGLT2 inhibitor treatments are administered clinically, as they are taken orally. It is also possible that because Nugrahaningrum et al[14] provided the SGLT2 inhibitor treatment via an intramuscular injection the possible effects of the SGLT2 inhibitor are more pronounced. These mixed results are important to note as it indicates that more research is required to elucidate these differences and better understand how T1D may impact PECAM expression.

This study has potential limitations and perhaps the most noticeable is the variability in the mRNA data, particularly in the T1D rats. Variability observed in the diabetic groups are common, perhaps because glycemic control varies among rodents, and humans, with the condition. Similarly, the variability in the CON group is substantial and this may be due to which piece of the muscle was used for analysis since there may be a difference in the expression of the markers as previously discussed based on the myofiber type and the variability is more pronounced due to the small sample size. Certainly, this large degree of variability can be minimized by a larger sample size, but this limitation does not invalidate the results observed in

this study as all results provide a similar narrative, which is that EMPA and insulin treatment in this study did not impact capillary content under the conditions of this study. Future research should increase the sample size, while minimizing glycemic variability, to better elucidate if there are any differences between mRNA levels between type 1 diabetic rats treated with or without SGLT2 inhibition.

In conclusion, we demonstrated that SGLT2 inhibition can significantly improve glycemia when administered alongside insulin therapy, without increasing the risk of DKA or hypoglycemia. However, SGLT2 inhibition may not impact skeletal muscle capillarization in a juvenile rat model of T1D in a two-week treatment. Future research could include assessing the impacts of SGLT2 inhibition on a prolonged model of this disease in the presence and absence of insulin. This is to further our understanding of the impact of SGLT2 inhibition on skeletal muscle in T1D at different points of T1D progression.

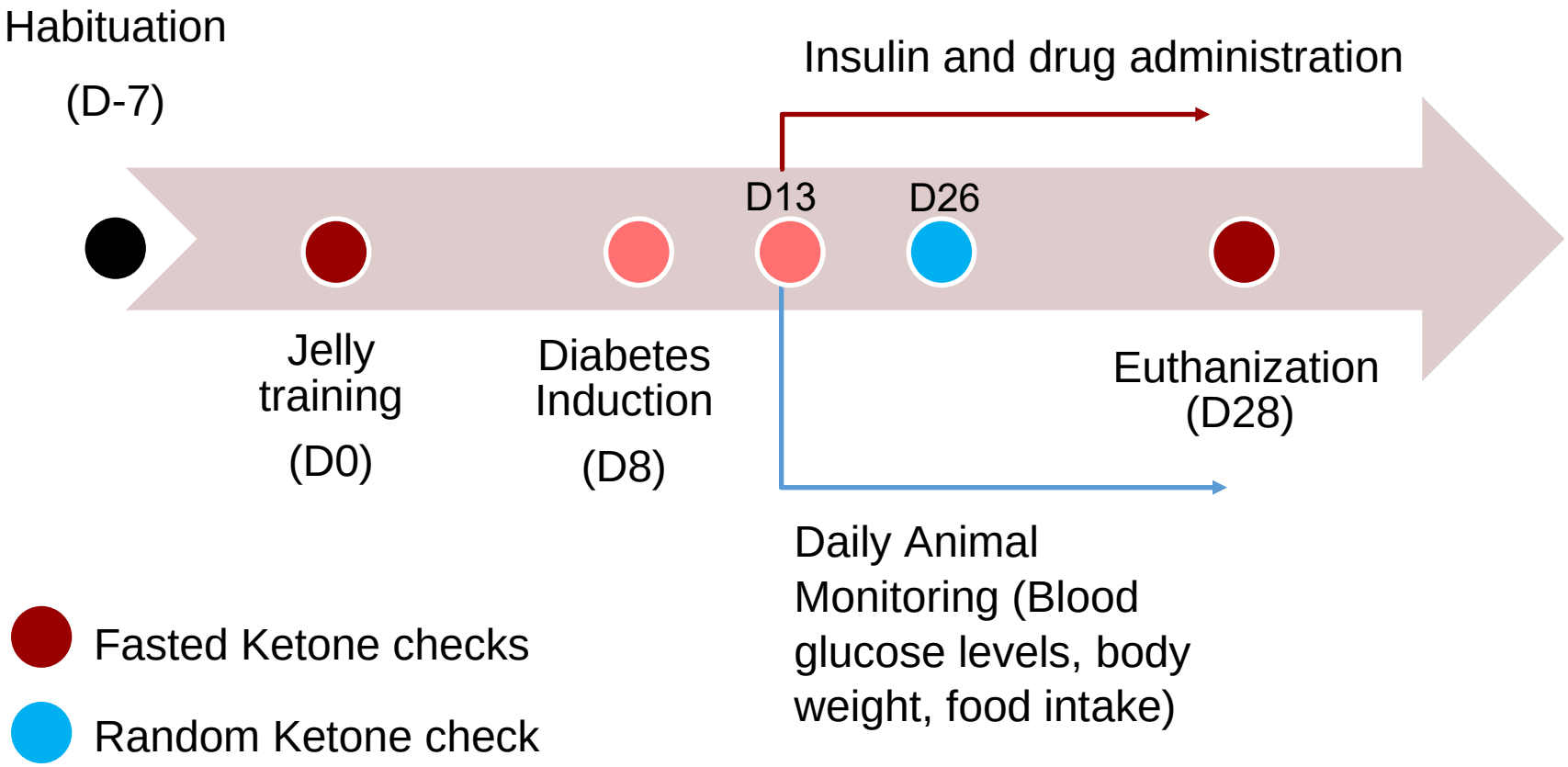



**Figure 1. Study Timeline.** The timeline of the study.

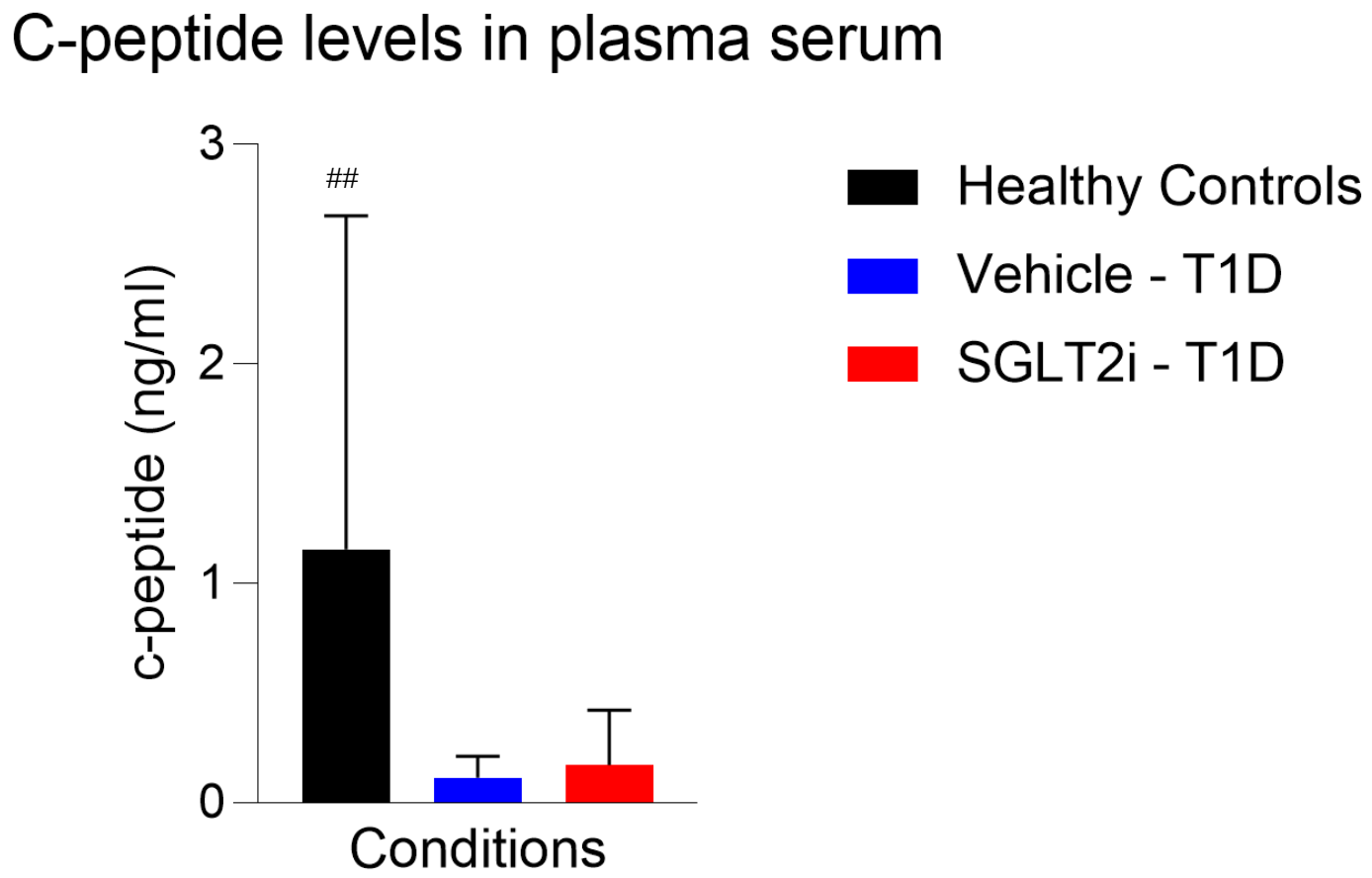



**Figure 2. C-peptide levels in plasma serum.** STZ was effective in making the animals diabetic. The CON group was significantly greater than both diabetic groups ( ##p<0.05) and there was no difference between the diabetic groups. The data plotted is the mean with the standard deviation of the plasma serum measured after the 14-day treatment. (CON: n=5, Vehicle: n=10, SGLT2i: n=11).

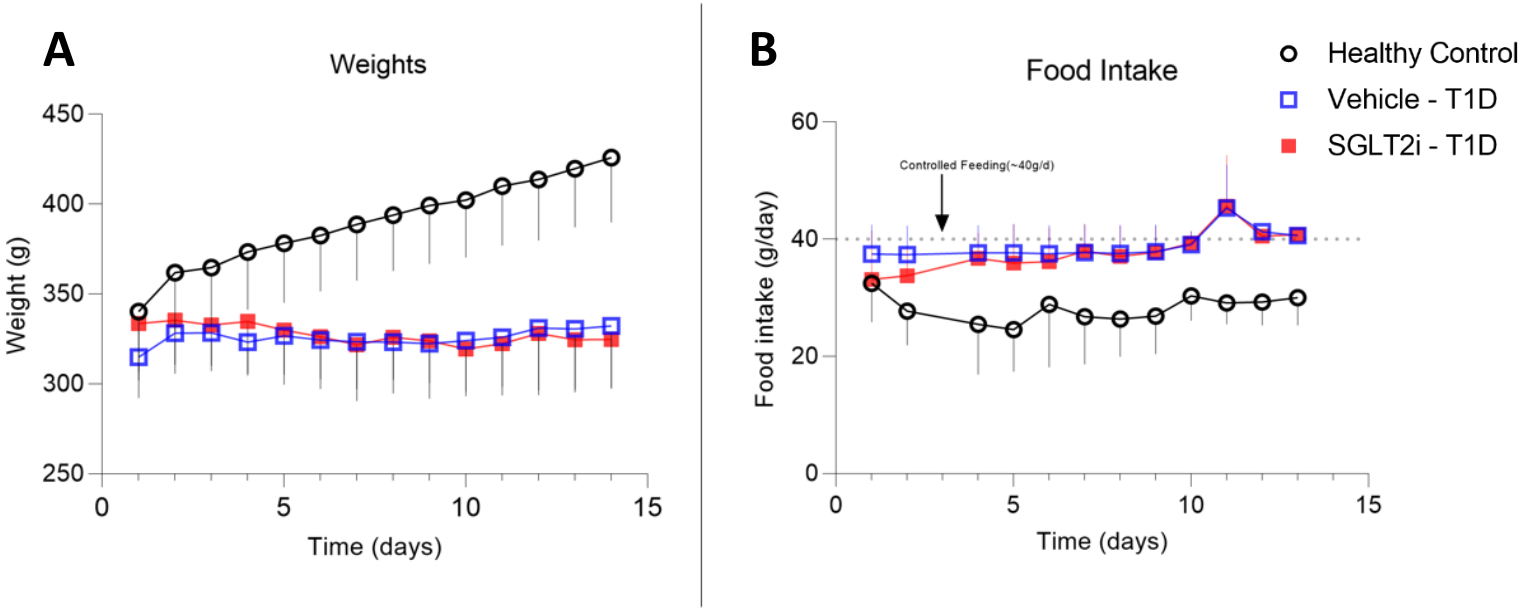



**Figure 3. Body weight and food intake over the two-week treatment.** Diabetes was associated with hyperphagia and weight loss, SGLT2i (EMPA) had no effect in a 2-week treatment period. Figure 1b, controlled feeding began on the third day of treatment. Figure 1b, the spike on the 11$^{th}$ day in the diabetic food intake amount was due to providing more food to the animals due to concerns about weight loss. The data plotted represents the mean and standard deviation for daily body weight and food intake per group (CON: n=12, Vehicle: n=10, SGLT2i: n=11).

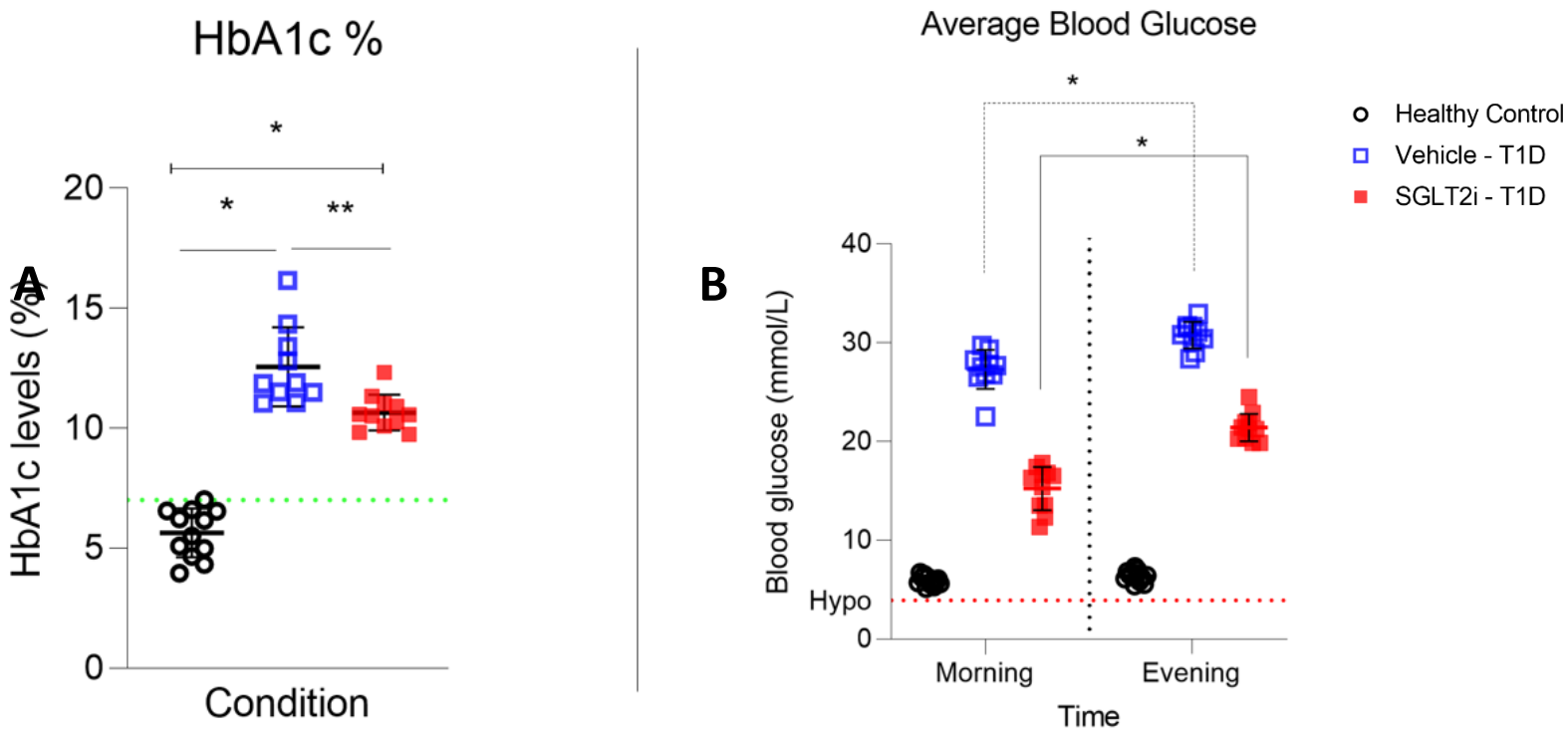



**Figure 4. Glycemic control after the two-week treatment period.** SGLT2i group had significantly better glycemic control when compared to the vehicle group. 2b, CON was significantly lower than both diabetic groups, with no difference between the morning and the evening. 2b, the diabetic groups were significantly higher compared to their respective group in the evening compared to the morning. The green dashed line on 2a is representative of the 7% HbA1c clinic target. The red dashed line in 2b, is representative of hypoglycemia (3.9 mmol/L). No hypoglycemia events occurred as shown in 2b. The data plotted represents the mean and standard deviation for the average blood glucose over the 14-day treatment period in 2b and the HbA1c% after the 14 day treatment period in 2a. (CON: n=12, Vehicle: n=10, SGLT2i: n=11). *,**$p<0.05$

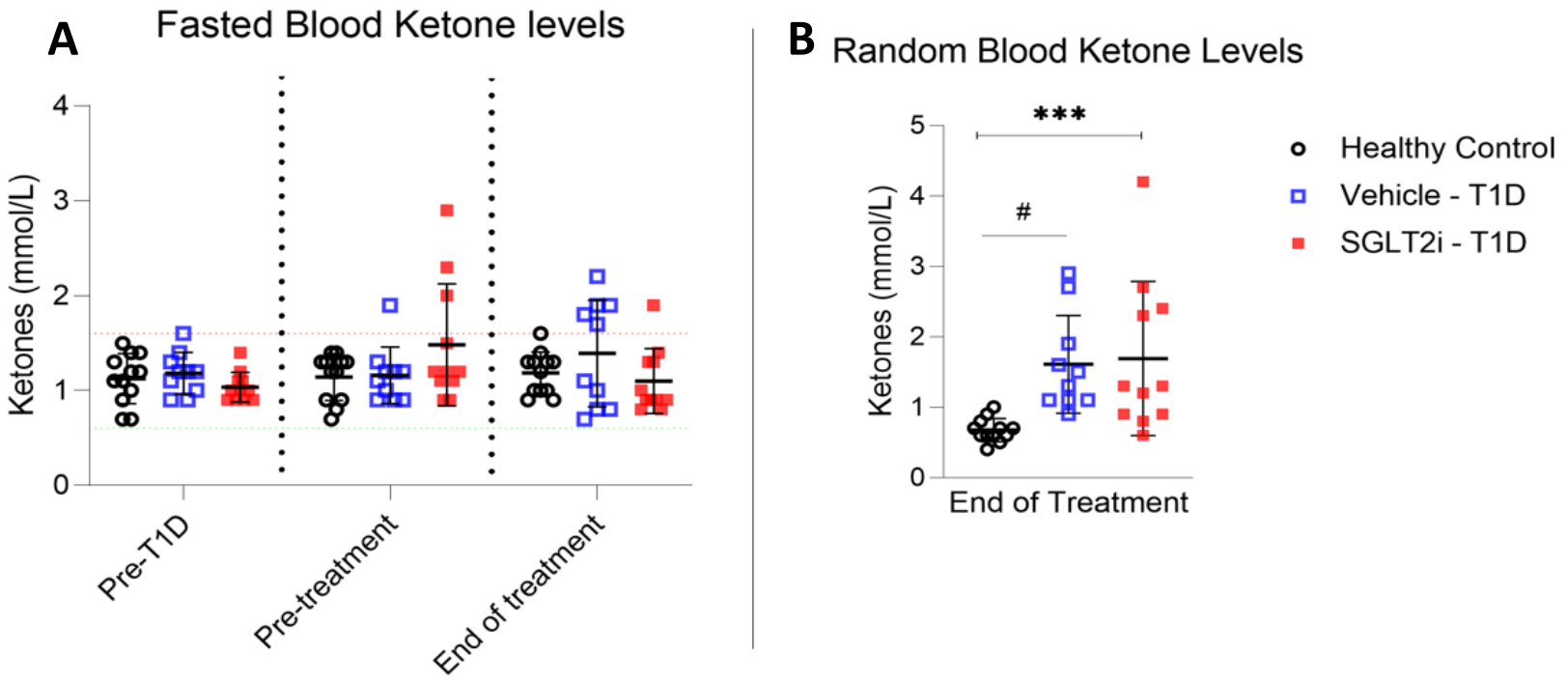



**Figure 5. Blood ketone levels during the duration of the study.** EMPA treatment did not increase the risk of DKA as measured by blood ketone levels. 3b, CON was significantly lower than both diabetic groups (#,***$p<0.05$) with no difference between the diabatic groups. 3a, there was no difference between all groups across all time points. The red dashed line in 3a, is representative of the levels of blood ketones that indicates an increased risk of DKA (1.5 mmol/L), while the green line is an indication of normal levels of blood ketones typically observed (0.5 mmol/L). The data plotted represents the mean and standard deviation for the fasted blood ketone levels before inducing T1D, before treatment, and after the 14-day treatment period in 3a and the non fasted blood ketone levels on the last day of treatment period in 3b. (CON: n=12, Vehicle: n=10, SGLT2i: n=11).

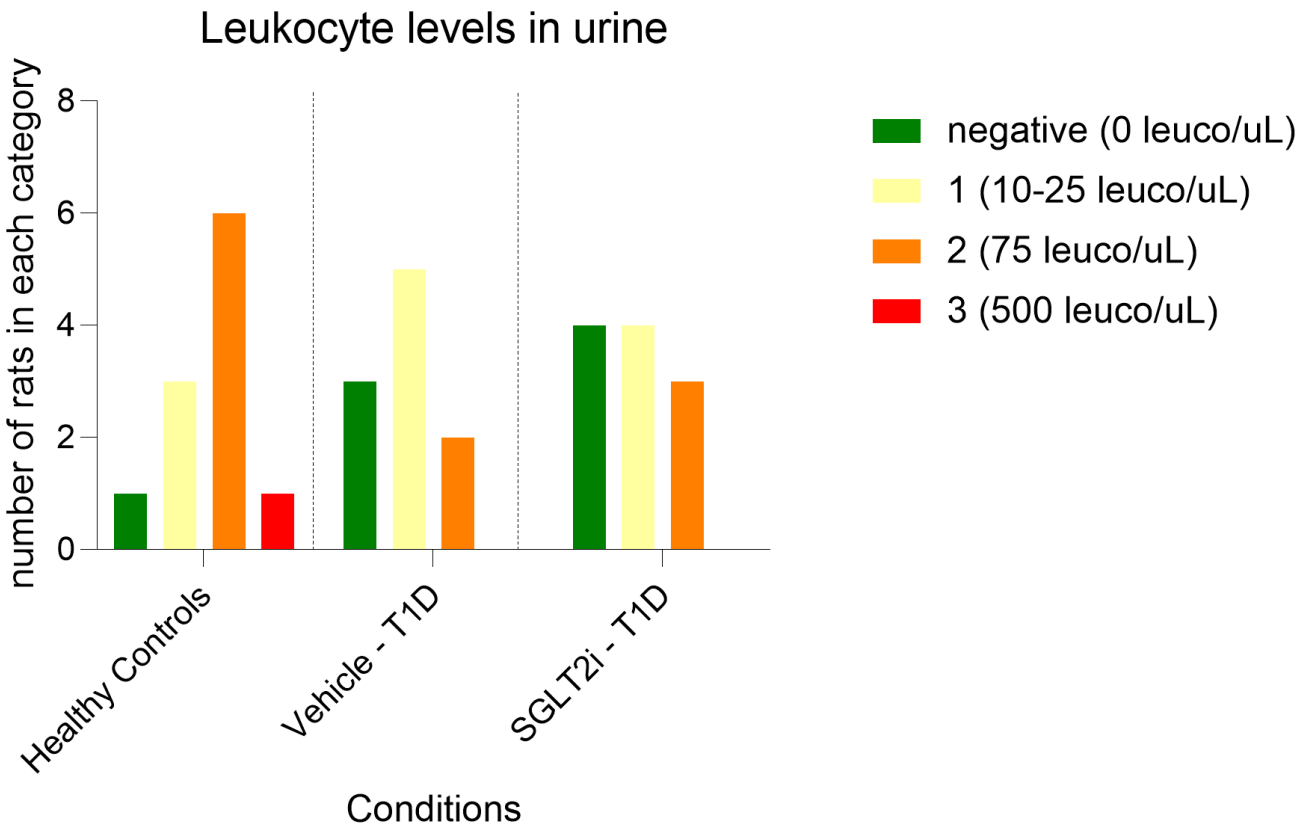



**Figure 6. Leukocyte levels in urine.** EMPA treatment did not increase the risk of urinary tract infection as measured by urine leukocyte levels. The data plotted is a representation of the number of rats that fit into each category that the urine strip detected. None of the diabetic rats fell into category 3. (CON: n=11, Vehicle: n=10, SGLT2i: n=11).

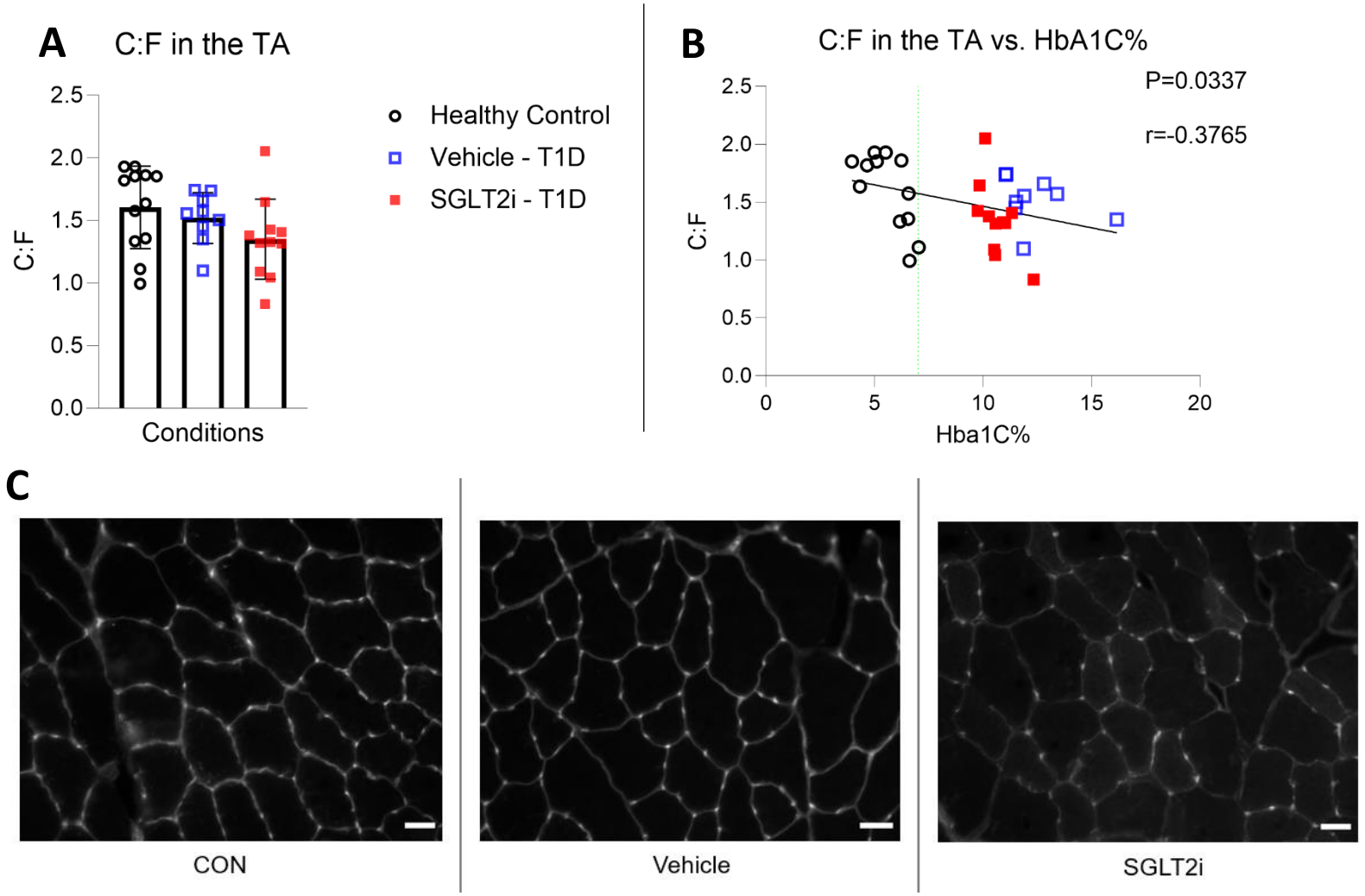



**Figure 7. C:F in the TA muscle and its association with HbA1c levels.** EMPA treatment did not maintain capillaries in the skeletal muscle in the as assessed by C:F. Hyperglycemia is associated with reduced C:F as shown in 5a. The green line in 5b is representation of the 7% HbA1c% clinical target. 5c Representative images of TA taken at 20x(scale bar=30µm). The data plotted in 5a is the mean with the standard deviation of the C:F measured after the 14-day treatment. (CON: n=12, Vehicle: n=9, SGLT2i: n=11).

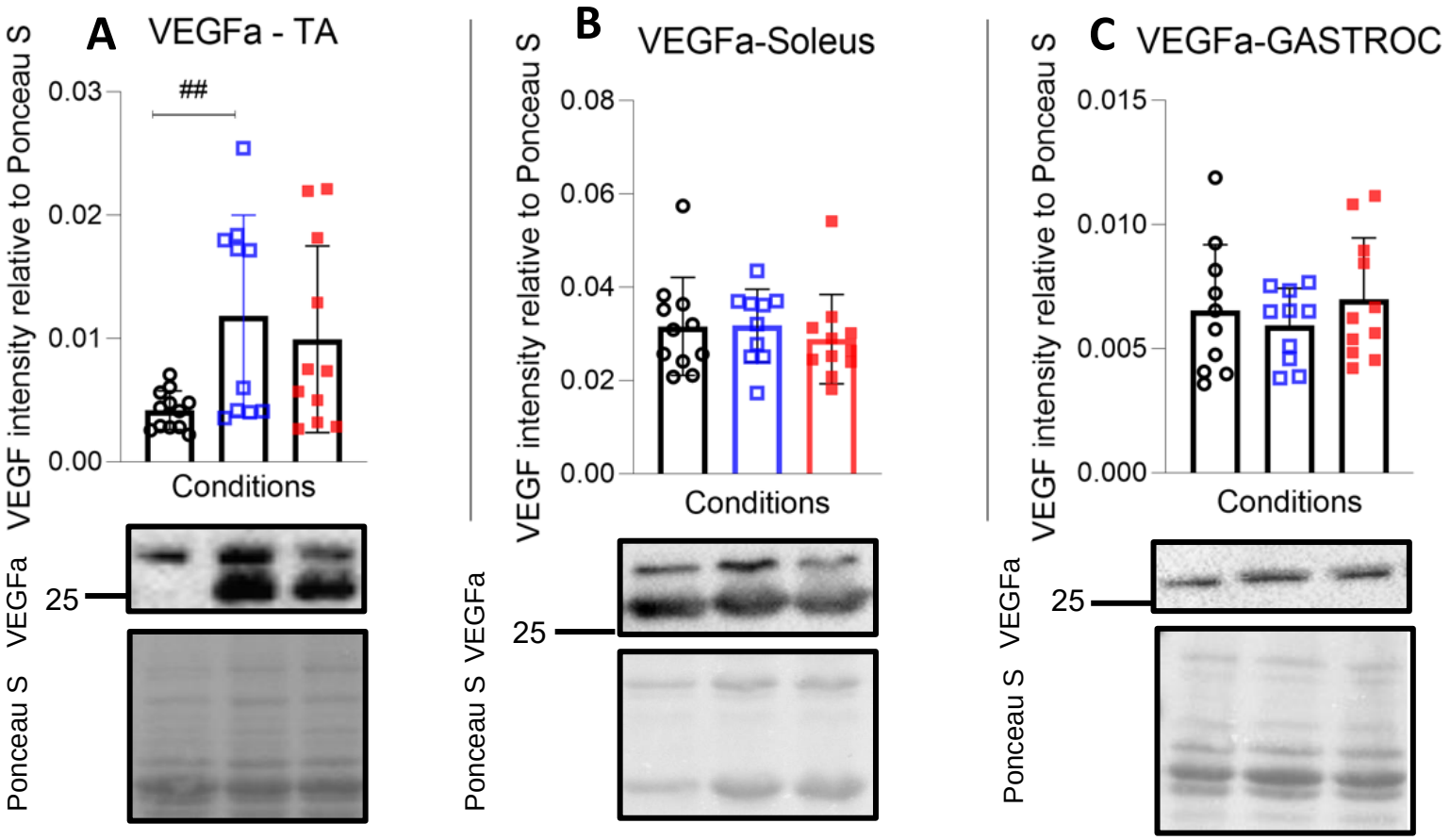



**Figure 8. VEGFa protein expression in the TA, soleus, GASTROC muscles.** EMPA treatment did not impact VEGFa expression in the skeletal muscle. 6a, the CON group was significantly higher when compared only to the vehicle group (##p<0.05) in the TA only. The data plotted is the mean with the standard deviation of the VEGFa expression measured after the 14-day treatment. (CON: n=12, Vehicle: n=10, SGLT2i: n=11).

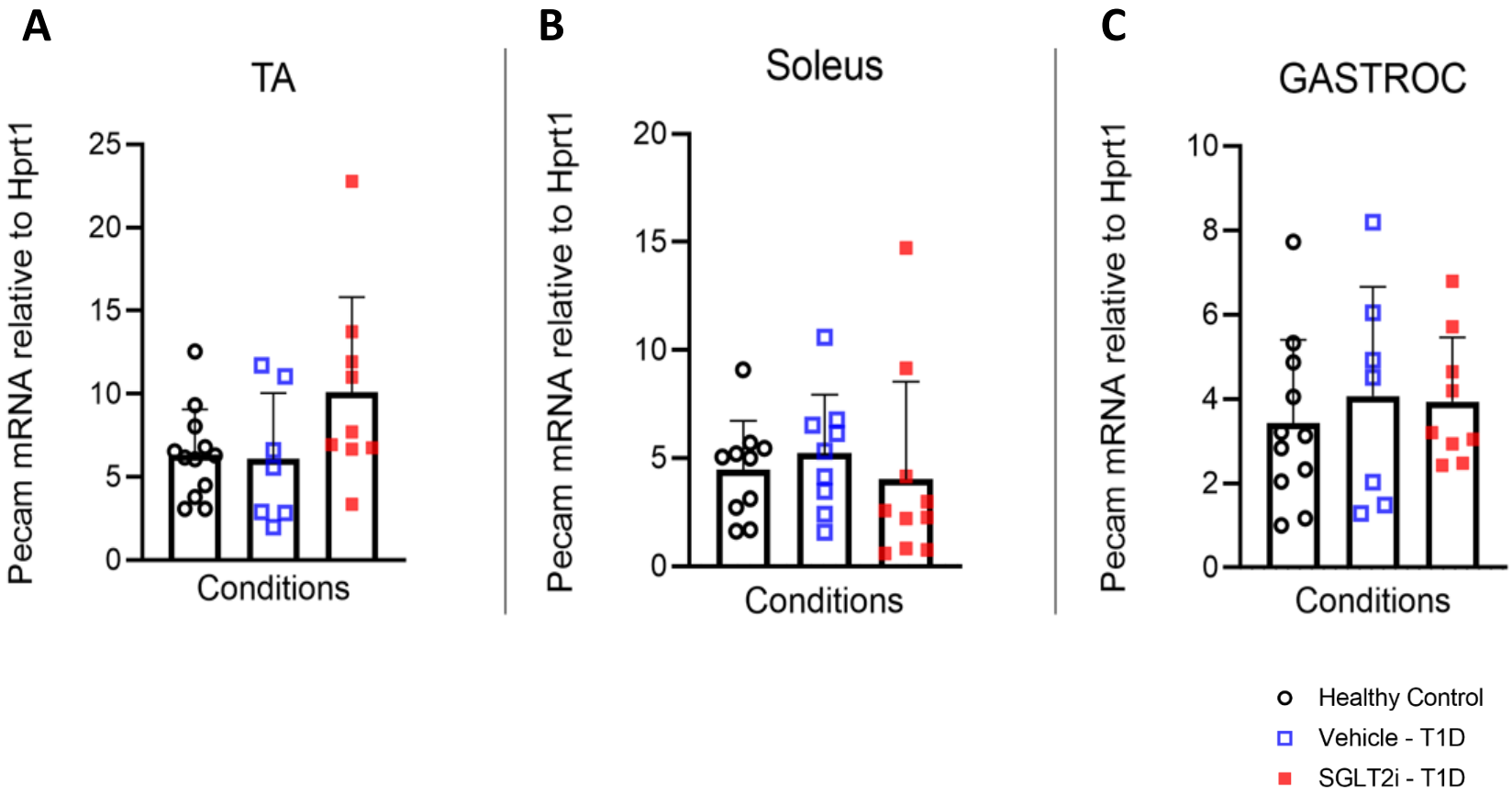



**Figure 9. PECAM mRNA expression relative to Hprt1 in TA, soleus, GASTROC muscles.** EMPA treatment did not impact PECAM expression in the skeletal muscle. The data plotted is the mean with the standard deviation of the PECAM expression measured after the 14-day treatment, with outliers not shown. (CON: n=12, Vehicle: n=10, SGLT2i: n=11).

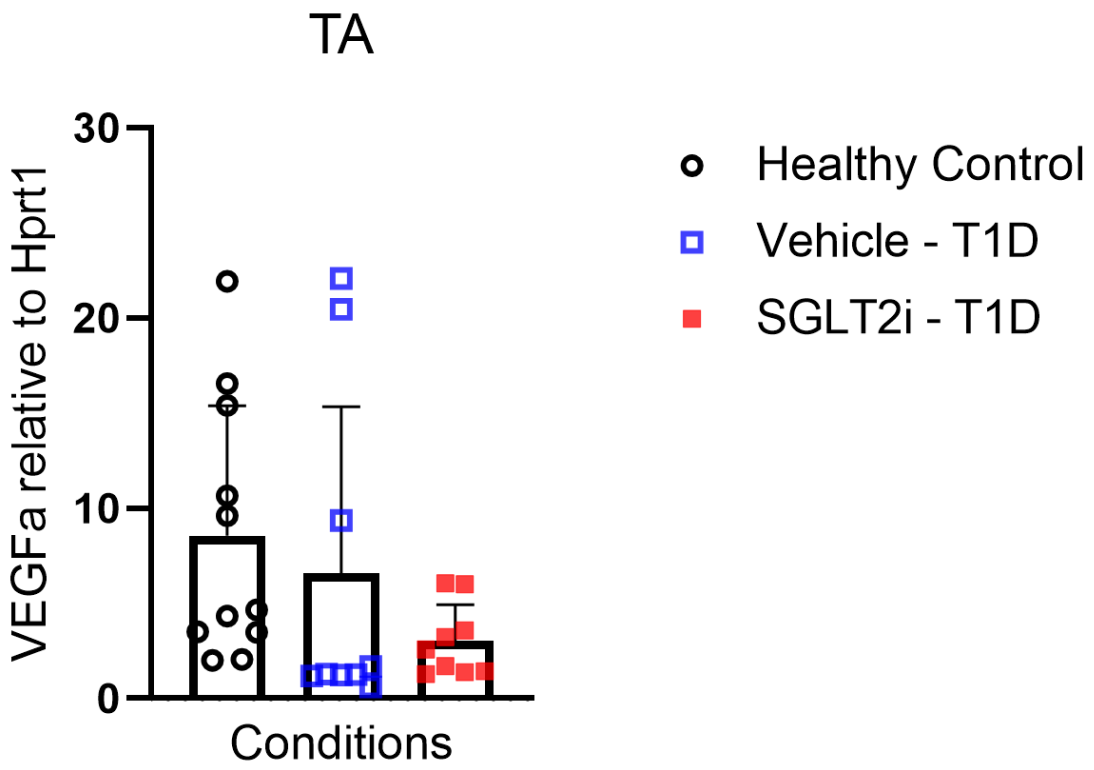



**Figure 10. VEGFa mRNA expression realative to Hprt1 in TA muscle**. EMPA treatment did not impact VEGFa mRNA expression in the skeletal muscle. However, the data trended to be lower in both diabetic groups compared to the CON group. The data plotted is the mean with the standard deviation of the VEGFa expression measured after the 14-day treatment, with outliers not shown. (CON: n=12, Vehicle: n=10, SGLT2i: n=11).

# 6. SUMMARY OF FINDNGS AND FUTURE DIRECTIONS

For the first time, the impacts of SGLT2 inhibition on the health of capillaries in skeletal muscle in a juvenile type 1 diabetic rodent model have been investigated. We demonstrated that SGLT2 inhibition and insulin treatment promote better glycemia without increasing the risk of DKA nor the risk of a urinary tract infection and does not appear to impact the capillaries in skeletal muscle in 2 weeks. We also demonstrated that capillarization in skeletal muscle of type 1 diabetic rodents receiving insulin therapy is not negatively impacted in 2 weeks. This is in contrast to other studies that demonstrated a reduction in the capillarization of skeletal muscle in diabetic rodents with the key difference being that the animals in previous literature did not receive insulin treatment and were diabetic for a longer period of time. To test if length and treatment of diabetes plays a role in the negative impacts on C:F another group of animals that have diabetes with no treatment provided for 2 weeks would be required. It would also be ideal that this study is repeated with treatment lasting for a longer period of time to assess if the length of the treatment was the limitation in the lack of differences observed in the C:F.

We also showed that VEGF-A protein expression appears to increase in T1D in the TA and this was supported by the mRNA VEGF-A expression. Future research should also assess the mRNA VEGF-A expression in the soleus and GASTROC to support the findings observed in the protein expression. Furthermore, future research should also assess PECAM protein expression as previous literature has shown negative impacts on protein expression in diabetic rodents and we showed that PECAM mRNA expression appeared to be unchanged. Additionally, it would be ideal to test if there are any changes in the proteins that impact the anti-angiogenic processes as studies[121] have outlined that the anti-angiogenic proteins may have a larger impact on capillarization in the skeletal muscle than the pro-angiogenic proteins. This could also give us

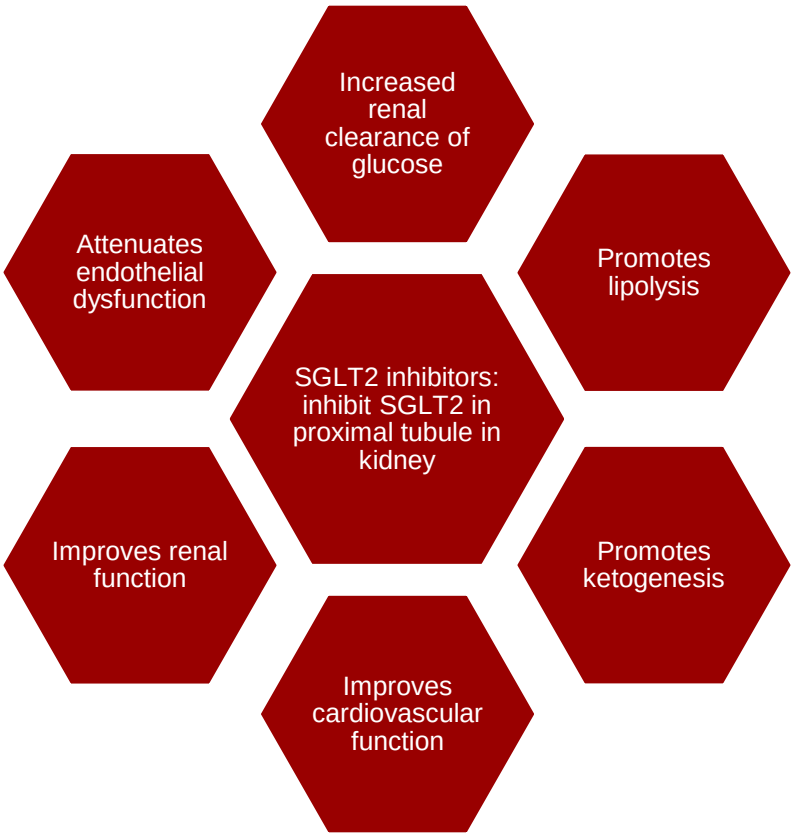
a better insight as to why there were no alterations to the C:F in the TA in the diabetic rodents. Future directions should include assessing TSP-1 a major anti-angiogenic factor which has also shown to be elevated in diabetic rodents[3,4].

Overall, our findings showcased that SGLT2 inhibitor treatment coinciding with insulin therapy is effective in promoting improved glycemic levels (as compared to insulin only treatment) without promoting hypoglycemia, increasing the risk of DKA, and the risk of a urinary tract infection. Additional studies with larger sample sizes, longer treatment periods, and a group of untreated diabetic rodents would provide a clearer understanding of our findings and possible application.

## 7. APPENDIX

| Blood glucose | U/BW |
|---|---|
| 1.0-14.9 | 0 |
| 15.0-16.9 | 0.25 |
| 17.0-20.0 | 0.25 |
| 20.1-22.9 | 0.5 |
| 23.0-25.0 | 1 |
| 25.1-28.0 | 1.5 |
| 28.1->33.3 | 2 + 1U Lantus |

**Appendix 1:** Insulin given to rats based on blood glucose levels and body weight in units




**Appendix 2:** SGLT2 inhibitors' mechanism of action and off target effects

| Condition | Average insulin taken in the morning (U) | Average insulin taken in the evening (U) | Total of average insulin taken (U) |
|---|---|---|---|
| Vehicle | 0.71 | 1.18 | 1.89 |
| SGLT2i | 0.10 | 0.19 | 0.29 |

**Appendix 3:** Average amount of insulin each group received. SGLT2i group received 15% of what vehicle group received.

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