The Effects of Short-Term Low-Carbohydrate and Ketogenic Interventions on Cardiometabolic Health.

by

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Abstract

Type 2 diabetes (T2D) is currently one of the most common chronic diseases worldwide and is characterized by impaired insulin secretion and action, elevated blood glucose and chronic inflammation. Lifestyle interventions including exercise and nutritional manipulations are considered first-line non-pharmacological treatments for this metabolic disease. While most health care providers agree that diet is of extreme importance for the prevention and management of T2D, the type of diet that should be consumed is highly debated. One dietary intervention, the low-carbohydrate high-fat (LCHF) diet, has been shown to be particularly effective at improving diabetes symptoms by reducing glucose excursions and lowering hyperinsulinemia. The first study of this thesis was to determine if changes in insulin levels in saliva were reflective of those observed in plasma following high and low-carbohydrate meals in 20 individuals (10 normal weight; BMI 20.0–24.9 kg/m2 and 10 with overweight/obesity; BMI > 28.0 kg/m2). The findings of this study indicate that saliva could potentially be used to delineate between low and high insulin levels following mixed meals, providing a potential avenue for personalizing dietary choices based on non-invasive saliva insulin measurement. In a second study, the effect of three 4-day diet interventions: i) Low-fat low-glycemic index (GL); ii) LCHF, and iii) LCHF with 15-min post-meal walks (LC+Ex) on the inflammatory and glycemic profiles of 11 individuals with T2D were compared. The main findings were that: a) LCHF and LCHF+EX improved glycemic control and proinsulin levels to a greater extent than GL and b) all diets improved some markers of inflammation. For the third and fourth study, we investigated the isolated effect of a new exogenous ketone monoester (KM) drink taken before a 2-hour oral glucose tolerance test in 20 healthy young individuals (BMI < 25 kg/m2) and 15 adults with overweight/obesity (BMI ≥ 28 kg/m2). The main findings are that the KM improved glucose control and decreased non-esterified fatty acids levels without a concomitant increase in circulating insulin. Overall, low-carbohydrate diets and ketone supplements seem to be effective
at controlling glucose levels and could be considered as preventive and management therapies for metabolic diseases.
Lay Summary

Type 2 diabetes is a chronic disease characterized by elevated blood sugar and altered function of the hormone insulin. In this thesis, we investigated if changes in insulin levels in saliva were reflective of those observed in blood following different meals. We also determined the effects of a 4-day low-carbohydrate high-fat (LC) diet and newly developed ketone drinks on blood sugar control, metabolism, and inflammation in adults classified as healthy, obese or with type 2 diabetes. The main findings of these studies were: 1) saliva could potentially be used to non-invasively track insulin levels following a meal, 2) A LC diet is better for reducing blood sugar levels when compared to a low-fat diet but both diets improve some markers of inflammation 3) An oral ketone drink can lower blood sugar and lipid levels.
Preface
I declare that this thesis is the result of my own work and that I have acknowledged all sources in the case of co-authored work with my contribution to each study outlined below.

Chapter 1: Introduction. For figures and tables that were originally published elsewhere, permission has been granted by the respective journals for use in this thesis.

Chapter 2: A version of Chapter 2 has been published. Myette-Côté, É., Baba, K., Brar, R., & Little, J. P. (2017). Detection of Salivary Insulin Following Low versus High Carbohydrate Meals in Humans. *Nutrients*, 9 (11), 1204. The permission to include this work in the present thesis has been granted by the publisher. The protocol of this study was approved by the Ethics Committee of the UBC Clinical Research Ethics Board (H1502638) and registered at ClinicalTrials.gov (NCT02699203).

Candidate Contribution
Planning: The candidate was responsible for writing the study design and the ethics application along with Dr. Jonathan Little and was involved in all recruitment, organization and testing of the participants.

Data Collection and Analysis: The candidate supervised all human participant testing done within the lab and performed tasks including recruitment, screening, manual blood pressure, anthropometric measurements, and meals organization for participants. The candidate performed both the saliva and blood samples collection and did perform the glucose analyses in the biological laboratory while the insulin analyses were performed by co-authors Baba, K., and Brar, R.

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Manuscript: The candidate prepared all the table and figures except Figure 4, which was prepared by Botezelli, J.D. and Johnson, J.D. and Figure 5, which was prepared by Bammert, T.D. and DeSouza C.A. The candidate prepared the first version of the manuscript, and coordinated edits from co-authors. The candidate submitted the manuscript, prepared the response to the reviewer comments, and revised the manuscript accordingly.

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Candidate Contribution

Planning: The candidate was responsible for writing the study design and the ethics application along with co-author Little J.P and Clarke K. and was involved in all recruitment, organization and testing of the participants.
Data Collection: The candidate supervised all human participant testing done within the lab and performed tasks including recruitment, screening, manual blood pressure, anthropometric measurements, study drinks preparation for participants. The candidate performed the blood samples collection and the glucose and non-esterified fatty acids analyses. The insulin, c-peptide and GLP-1 were analyzed by the candidate along with Little J.P. Neudorf. H. processed all blood samples for storage and subsequent analyses as part of her Honours thesis. Adiponectin was analyzed by co-author Rafiei, H.

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Data Collection: The candidate supervised all human participant testing done within the lab and performed tasks including recruitment, screening, manual blood pressure, anthropometric measurements, study drinks preparation for participants. The candidate performed blood samples collection and analyzed glucose, non-esterified fatty acids, lactate and triglycerides. The insulin, c-peptide, GLP-1, glucagon and leptin were also analyzed by the candidate along with the help of undergraduate students. Hemodynamic functions were performed and analyzed by co-author Caldwell, H.G with guidance from co-author Ainslie, P.

Manuscript: The candidate prepared all figures and tables except Table 2 that was prepared by co-author Caldwell, H.G. and Ainslie P. The candidate prepared the first version of the manuscript, and coordinated edits from co-authors. The candidate submitted the manuscript, which was under peer review at the time of final thesis submission.
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List of Abbreviations

AcAc: Acetoacetate
AUC: Area under the curve
BHB: Beta-hydroxybutyrate
BMI: Body mass index
CBF: Cerebral blood flow
CCA: Common carotid artery
CD: Cluster of differentiation
CGM: Continuous glucose monitor
CONGA: continuous overall net glycemic action
CVD: Cardiovascular disease
Ctrl: Control drink
DAG: Diacylglycerol
EDTA: Ethylenediaminetetraacetic acid
GPCR: G-protein-coupled receptors
GI: Glycemic index
GL: 4-day low-fat low-glycemic index diet
GLP-1: Glucagon-like peptide-1
HbA1c: Glycated haemoglobin
HCM: High-carbohydrate breakfast meal
HCFL: High-carbohydrate low fat
HDAC: Histone deacetylases
HR: Heart rate
iAUC: incremental area under the curve
IFG: Impaired fasting glucose
IGT: Impaired glucose tolerance
IL: Interleukin
IR: Insulin resistance
IRS: Insulin receptor substrate protein
JNK: c-Jun N-terminal kinase
KB: Ketone body
KD: Ketogenic diet:
KE: Ketone ester
KM: Ketone monoester
KS: Ketone salts
LC: 4-day low-carbohydrate high-fat diet
LCM: Low-carbohydrate breakfast meal
LC+Ex: 4-day low-carbohydrate high-fat diets with 15-min post meal walks
LCHF: Low-carbohydrate high-fat
LDL-C: Low density lipoprotein-cholesterol
LMPs: leukocyte-derived microparticles
MAGE: mean amplitude of glycemic excursion
MAP: Mean arterial pressure
MCP-1: monocyte chemoattractant protein-1
METs: Metabolic equivalents
MMPs: Monocyte-derived microparticles
MPs: Microparticles
NEFA: Non-esterified fatty-acids
NF-k B: nuclear factor-kappa B
NLRP3: Nod-like receptor protein 3
NW: normal weight
OGIS: Oral glucose insulin sensitivity
OGTT: Oral glucose tolerance test
OO: Overweight/obese
p-JNK: Phosphorylated c-Jun N-terminal kinase
PBMCs: peripheral blood mononuclear cells
TG: Triglycerides
TLR2: Toll-like receptor 2
TLR4: Toll-like receptor 4
TNF-a: Tumor necrosis factor alpha
PKC: Protein kinase C
T1D: Type 1 diabetes
T2D: Type 2 diabetes
VAS: Visual analogue scales
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Gilles and Suzanne, I realized a long time ago already that no words or ways would ever be enough to express how thankful I am for everything you have done for us. You two are my greatest source of inspiration and I know for sure that only because of all the sacrifices you made for me, can I be where I am today. Through your constant care, love and precious guidance, I found the strength to continue in the hope of making you proud.
Dedication

À mes parents Gilles et Suzanne et à la mémoire de mon bon ami Robert Jolicoeur.
Chapter 1: Introduction

1.1 The development of obesity

The epidemic of obesity is, by its nature, a complex issue where lifestyle, environmental and individual genetic factors are in constant interaction [1]. Over the last several decades, human lifestyle behaviors have progressively shifted to favor sedentary activities while time spent performing physical activities has been declining [2, 3]. The resulting decrease in daily energy expenditure was paired with an increase in the consumption of cheap and easily available processed foods leading to higher energy intake [4, 5], the latter being mainly attributable to increased intake of carbohydrates (CHO) [6]. Under such circumstances, the prevalence of obesity and related chronic diseases have sharply risen putting an enormous burden on our health and economic systems [7, 8]. Over the last two decades in Canada, the prevalence of adults classified as overweight has increased by from 28% to 34% while the prevalence of obesity has more than doubled from 6% to 18% of the population [9]. In parallel, there has been a large increase in cases of type 2 diabetes (T2D), which is now one of the most prevalent chronic diseases in Canada with an estimated 9.3% of the population affected (~3.4 million people) [10]. Type 2 diabetes is characterized by a progressive inability of the body tissues to adequately respond to the hormone insulin, a state called insulin resistance (IR). When the pancreatic beta-cells are no longer able to overcome this resistance with an appropriate compensatory increase in insulin secretion, chronic hyperglycemia (the main symptom and diagnostic criteria of T2D) ensues [10].

Obesity is one of the main risk factors for developing T2D; individuals with obesity are 2-4 times more likely to develop the disease than normal weight individuals [11]. Among individuals with T2D, up to 90% are considered overweight or obese while 41% are considered inactive [12]. The obese state is characterized by adipocytes that release abnormally high amounts of non-esterified fatty-acids (NEFA), glycerol and pro-inflammatory cytokines that all contribute to the worsening of metabolic health and IR [13]. Lifestyle interventions using
nutritional approaches and/or exercise have been shown to prevent obesity and T2D [14, 15] and to improve cardiovascular risk factors including insulin sensitivity and body weight status in T2D [16, 17]. Cardiovascular diseases are the main cause of death in individuals with T2D and develop between 2-4 times more frequently in this population as compared to individuals free of diabetes [10].

Despite important financial investments and the World Health Organization making one of their main targets to halt the rise in obesity and T2D, the prevalence of these conditions worldwide shows no sign of slowing [18]. The etiology of obesity, IR, and T2D is a long-standing debate that has attracted substantial research attention over the years. Currently, there are two main schools of thought debating on the underlying causes of obesity and IR. Given the importance and the massive implications for future public health policies and clinical interventions, it is essential to pursue this quest in order to better understand and treat these metabolic conditions.

1.1.1 The “calories in, calories out” classical model
The calorie concept stipulates that body mass will be increased or decreased following a positive or negative total energy balance, respectively. In this model, an increase in energy intake combined with reduced energy expenditure is hypothesized to lead to elevated levels of circulating metabolites and insulin, which would favor fat storage (Figure 1A) [19]. This theory is based on the first law of thermodynamics that states that energy cannot be created or destroyed, thus all calories must be accounted for by simple balancing of energy intake and expenditure [20]. Once assumed as accurate, this model quickly became the main framework for obesity prevention and management and individuals were encouraged to “eat less and move more” in order to create a negative energy balance [21]. Despite its ongoing use [22], the “static calories in, calories out” theory has been suggested to be inaccurate and followed by new theories such as the “settling point” and the “set point models” of body weight regulation which
include long-term feedback control for energy intake and expenditure [23]. The main limitation of these theories is that they assume that all calories are created equal and do not take into account the potential hormonal and thermic effects of different macronutrients on energy balance, fuel partitioning, or body weight/fat gain.

1.1.2 The carbohydrate-insulin model

The carbohydrate-insulin model suggests that weight gain develops not primarily because of a positive energy balance, but because of the fat partitioning effect of high-carbohydrate diets related to excess insulin secretion (Figure 1B). CHO are known to be the most potent insulin secretagogue in the human diet [24]. Once in circulation, insulin has potent effects to inhibit adipose tissue lipolysis, favour the storage of free fatty acids as triglyceride in adipocytes, and reduce fat oxidation by active tissues such as muscles, liver and heart [25]. The proponents of this model have theorized that in such “internal starvation” state, fat oxidation is diminished, leading to decreased energy expenditure, increased food intake and body fat accumulation [26, 27]. Other factors such as sleep, stress, protein amount and type, and fatty acids profile can also play a role in modulating insulin secretion [19].

To date, only a few controlled feeding studies have been conducted with the objective to tests the superiority of low-carbohydrate high-fat (LCHF) diets in terms of energy expenditure and body weight management. In 2012, Ebbeling et al. showed that following a 10-15% weight loss over 12 weeks, an isocaloric LCHF diet led to a significantly smaller (compensatory) decrease in resting and total energy expenditure as compared to a low-glycemic (40% CHO) and a low-fat diet (60% CHO) [28]. A few years later, Hall et al. evaluated the effect of two 6-day isocaloric diets low in fat and moderate in CHO (140g/day) with participants confined to a metabolic ward [29]. In this study, the post-intervention 24-hour energy expenditure was not different between conditions. Fat oxidation was higher and insulin levels were significantly lower in the carbohydrate-restricted group but a higher fat loss occurred in the low-fat group. A year
later, this same group conducted a 4-week study comparing a ketogenic diet (KD) to a low-fat diet [30]. Surprisingly, this study was not randomized so all the participants started the ketogenic diet after 4-weeks on a low-fat diet and an average weight-loss of 0.8 kg. The daily energy expenditure was slightly but significantly higher following the ketogenic diet while the total fat oxidation increased as compared to low-fat diet. The authors concluded a slowing in body fat loss during the KD phase which is likely attributable to the non-randomized nature of the study. The validity and conclusions of this study have been criticized [31] and it is clear that randomized, longer-term studies allowing metabolic adaptations to the LCHF diet and trials with larger sample sizes are still required to elucidate the potential metabolic advantage of LCHF diets.

1.2 Classification and diagnosis of glucose intolerance

Prediabetes and T2D diagnoses are made when any of the following 3 variables reach levels that are considered elevated as compared to normal; fasting plasma glucose, 2-hour oral glucose tolerance tests (OGTT) plasma glucose and glycated haemoglobin A1c (HbA1c) (see
Table 1) [32, 33]. FPG represents the levels of glucose in circulation following an overnight fast of at least 8-hours while the 2-hour OGTT represents the plasma glucose value at the 2-hour time point following the consumption of a standardized 75-g D-glucose drink. HbA1c can be measured at any moment of the day using a venous blood sample and reflects the average plasma glucose over the previous 2 to 3 months [34]. The diagnosis criteria found in Table 1 are based on thresholds that are associated with the development of micro-vascular complications over time [35-37]. However, as noted above, IR, insulin secretion demand, and beta-cell compensation/dysfunction are associated with obesity and are thought to occur many years before the diagnoses of prediabetes or T2D. Thus, earlier diagnoses of IR, insulin hypersecretion, and/or beta-cell dysfunction, along with novel intervention strategies to reduce these pathologies, are attractive targets for reducing the burden of T2D.

Table 1. Diagnostic criteria for prediabetes and type 2 diabetes.

<table>
<thead>
<tr>
<th>Organizations</th>
<th>Normal</th>
<th>Prediabetes</th>
<th>Type 2 diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FPG (mmol/L)</strong></td>
<td>DC</td>
<td>≤ 6.0</td>
<td>6.1-6.9</td>
</tr>
<tr>
<td></td>
<td>ADA</td>
<td>≤ 5.5</td>
<td>5.6-6.9</td>
</tr>
<tr>
<td><strong>2-h OGTT (mmol/L)</strong></td>
<td>DC &amp; ADA</td>
<td>≤ 7.7</td>
<td>7.8-11.0</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>DC &amp; ADA</td>
<td>≤ 5.6</td>
<td>5.7-6.4</td>
</tr>
</tbody>
</table>

DC; Diabetes Canada, ADA; American Diabetes Association, FPG; fasting plasma glucose, HbA1c; glycated haemoglobin.

1.3 Prediabetes

In 2015, the estimated prevalence of prediabetes in adults in Canada was 5.7 million, or 22.1% of the population [10]. Prediabetes is an intermediate state of fasting and/or postprandial hyperglycemia that sits between normoglycemia and T2D levels. At this stage, though glucose
is only slightly more elevated than normal, insulin resistance and beta-cell dysfunction have already been established (Figure 2) [38]. The British Whitehall II study has previously confirmed this by showing that in people who developed diabetes, glucose levels, insulin sensitivity and beta-cell secretion capacity were already impaired 13 years before diabetes diagnosis with the greatest deterioration in these metabolic parameters being observed between 2-6 years before diagnosis [39]. Pathologies within individuals with prediabetes can differ with some depicting isolated impaired fasting glucose (IFG), isolated impaired glucose tolerance (IGT), or both. As shown in Figure 3, individuals with different IR and beta-cell pathologies will respond differently to an OGTT [40]. IFG seems to be predominantly associated with hepatic insulin resistance while IGT is more associated with skeletal muscle IR [41].

While Diabetes Canada consider individuals with prediabetes not to be at increased risks for microvascular diseases [10], other reports indicate there are already increased risks [42, 43]. Nevertheless, prediabetes is associated with an increased risk of cardiovascular diseases and all-cause mortality [44-46]. Prediabetes is also considered an important risk factor for diabetes with a 5%-10% annual conversion rate to diabetes; with a similar proportion converting back to normoglycaemia [47]. According to different sources, between 70-90% of individuals who have prediabetes will eventually develop diabetes at some point in their life [15, 47]. In spite of these numbers, the Diabetes Prevention Program successfully showed a reduced conversion from prediabetes to diabetes by 58% with intensive lifestyle intervention compared with 31% for metformin [48]. With data supporting the fact that the progression from prediabetes to diabetes is preventable and even reversible, investment in diabetes lifestyle intervention programs should be augmented [49].
Figure 2. Natural history of type 2 diabetes. The relationship between beta-cell function and increasing plasma glucose levels before and after diabetes diagnosis. Key points include (1) insulin resistance precedes onset of diabetes mellitus, (2) type 2 diabetes requires both insulin resistance and loss of insulin secretion, and (3) over time, most type 2 diabetes patients lose insulin secretory capacity. Used with permission from publisher Taylor and Francis [38].
1.4 Type 2 diabetes pathophysiology

1.4.1 Insulin resistance

As discussed previously, obesity is associated with IR. However, not all individuals with obesity are insulin resistant and some individuals with IR and/or T2D are normal weight [50]. One explanation for this discrepancy is that IR is not directly caused by excessive total fat mass storage but more specifically by the accumulation of intracellular lipids in insulin-sensitive tissues such as skeletal muscles and liver [51, 52]. In skeletal muscle, insulin first binds to its insulin receptor at the surface of the muscle leading to phosphorylation of the intracellular insulin receptor substrate protein (IRS). IRS can then activate two main signaling pathways; i) the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway that is responsible for glucose metabolism, glycogen, lipid and protein synthesis; and ii) the Ras–mitogen-activated protein kinase (MAPK) pathways that is responsible for general gene expression and cooperates with the PI3K pathway to control cell growth and differentiation [53].
When lipid metabolites such as diacylglycerols (DAG) accumulate intracellularly, novel protein kinase C (PKC) gets activated and serine phosphorylation of IRS-1 is increased while tyrosine phosphorylation is decreased. As a result, IRS-1 is prevented from interacting with the insulin receptor, leading to impaired insulin-stimulated glucose transport activity. Similarly, in the liver, DAG accumulation leads to PKC activation and decreased IRS-2 tyrosine phosphorylation, resulting in impaired glucose and lipid metabolism [51, 54]. Intracellular DAG accumulation occurs when the rate of NEFA delivery to tissues exceeds the rates of intracellular fat oxidation and/or conversion to neutral lipid. The main causes of DAG accumulation are overnutrition, defects in adipocyte metabolism and/or mitochondrial NEFA oxidation and some specific gene variations affecting lipid metabolism [54]. Despite the DAG-mediated insulin resistance theory being the most prevailing hypothesis at this time, alternative hypotheses and pathways are likely involved in the development of insulin resistance, including roles for inflammation, endoplasmic-reticulum stress and/or mitochondrial dysfunction [55]. Regardless of the exact mechanisms, the development of IR, over time, in multiple tissues including skeletal muscle, liver, adipose tissue, and brain will lead to increased insulin demand, prevailing hyperinsulinemia, and pancreatic beta-cell stress [56].

1.4.1 Muscle insulin resistance

Skeletal muscles are known to be the main site of glucose disposal, accounting for 80% of the glucose removal during an euglycemic hyperinsulinemic clamp [57, 58] and around 30-40% during an oral glucose challenge [59, 60]. When insulin binds to its receptor at the surface of the muscle, the ensuing signaling transduction cascade leads to the translocation of glucose transporter 4 vesicles to the plasma membrane [61]. In the postprandial state, the glucose in circulation can then enter the cells where ~45-75% of it will be used for glycogen synthesis, while ~25-55% will be oxidized for energy [62-64]. Under conditions of muscle insulin resistance, insulin-stimulated glucose disposal is markedly decreased (up to 50%) [65, 66].
Several factors such as impaired insulin signaling, impaired glucose transport, altered glucose phosphorylation, and reduced glycogen synthesis all contribute to this impairment [67-70]. While glucose oxidation has been reported to be reduced by ~30% in T2D, non-oxidative glucose disposal including glycogen synthesis seems to show the biggest impairments with a 50% decrease as compared to healthy individuals [70]. Within the muscle, mitochondrial capacity is also impaired with insulin resistance, which leads to decreased fat and glucose oxidation, impaired ATP synthesis and increased propensity for intramuscular fat deposition [71-74]. As shown by several longitudinal studies, muscle insulin resistance occurs early in the development of T2D and can be detected up to a decade before beta-cell failure or overt hyperglycemia [75, 76]. Skeletal muscle IR is usually accompanied by IR in other tissues such as the liver, possibly because of their limited capacity to convert glucose to glycogen, which is then diverted to the liver for triglycerides (TG) conversion [77, 78]. The nature of the relationship between obesity and skeletal muscle IR seems to be causative, since losing and gaining weight correlate with insulin sensitivity improvement and worsening, respectively [79, 80].

1.4.1.2 Liver insulin resistance

The liver is of primary importance for glucose homeostasis since, contrary to skeletal muscle, it is involved in both the removal and release of glucose into the circulation. While muscle IR is mostly associated with postprandial hyperglycemia, hepatic IR contributes to elevated glucose in both the fasting and postprandial state [81]. Indeed, once the liver becomes IR, insulin loses its ability to inhibit hepatic glucose production, which leads to excessive glucose release and subsequent hyperglycemia [82]. A study by Firth et al. clearly showed that individuals with T2D had elevated hepatic glucose production before and up to 6 hours following a meal [83]. Furthermore, another study observed that individuals with T2D had excessive rates of systemic glucose entry which was due to the failure of insulin to adequately suppress endogenous glucose release after meal ingestion (45% higher vs. control) [84]. Lack of suppression of
postprandial glucagon release in this population has also been reported to contribute to excessive hepatic glucose output by accelerating glycogenolysis [85]. Possibly due to a defect in glucokinase activity, hepatic glucose uptake is also impaired and can contribute to ~30% of the decrease in total body glucose disappearance observed in T2D [86].

Lastly, hepatic IR is associated with the accumulation of TG and fatty acids metabolites such fatty acyl-CoA, DAG, ceramide, and glyco-sphingolipid [87]. This can be attributable to a combination of factors observed in IR states including increased de novo lipogenesis, increased free fatty acids flux from adipocytes and impaired mitochondrial fat oxidation that all contribute to the development of non-alcoholic fatty liver disease [87, 88].

1.4.1.3 Adipose tissue insulin resistance

White adipose tissue serves as the main site of energy storage in the form of triglycerides. Hormones such as insulin stimulate lipogenesis and excess energy storage while preventing the release and oxidation of free fatty acids through diminished protein kinase A-mediated lipid droplet phosphorylation and increased malonyl-CoA activity, respectively [89]. In opposition, catecholamines and natriuretic peptides stimulate lipolysis through hepatic and lipoprotein lipases, which result in the release of NEFA into the circulation for delivery and subsequent oxidation in mitochondria of other tissues (e.g., skeletal muscle) [89]. In insulin resistant adipocytes, insulin-mediated regulation of lipolysis is impaired resulting in TG being hydrolyzed and fatty acids released at a highly abnormal rate [90]. As a consequence, individuals with T2D have elevated exposure to circulating NEFA, which when accompanied by decreased energy expenditure (e.g., physical inactivity) and mitochondrial dysfunction, results in deposition of NEFA in ectopic tissues such as skeletal muscle and liver [91]. Elevated levels of NEFA are strongly associated with IR [92] and decreasing circulating NEFA using an antilipolytic agent such as acipimox improves glucose metabolism [93, 94]. It is possible that NEFA competes with glucose for substrate oxidation (glucose-NEFA cycle) leading to downregulation of the main
glycolytic enzymes and subsequent impairment in glucose uptake and oxidation [95]. Infusing NEFA has also been shown to impair insulin sensitivity through an increase in intracellular lipid metabolites, causing the insulin-receptor signaling cascade to diminish [96]. Hypertrophied insulin resistant adipose tissues are also known to display infiltration of immune cells (primarily lymphocytes and monocytes/macrophages), elevated expression and release of cytokines (primarily tumor necrosis factor alpha (TNF-a) and interleukin (IL)-6), and altered adipokines (increased leptin and resistin, with reduced adiponectin) [97]. Increased release of inflammatory cytokines and reduced release of adiponectin from inflamed, obese adipose tissue can lead to inter-organ cross-talk that impairs insulin sensitivity in distant tissues. Thus, the loss of insulin sensitivity in adipocytes causes dysregulation of lipid partitioning and altered metabolism of both lipid and glucose in non-adipose tissues, including liver, skeletal muscle, and pancreas [98].

1.4.2 Beta cell compensation, decompensation and failure

Sustained insulin secretion demand on beta-cells in IR states leads, over time, to the inability of the pancreas to further compensate by increasing insulin release [99]. Though IR and beta-cell dysfunction both contribute to chronic hyperglycemia, beta-cell dysfunction supersedes IR in inducing T2D [100]. Hyperglycemia is a well-known consequence of IR and beta-cell dysfunction but can also play a role in their development [101]. Impairments in beta-cell mass and function manifest in many ways including decreases in the insulin response to intravenous glucose [102, 103], a decline in the ability of glucose to potentiate the insulin response to non-glucose secretagogues [104] [71], alterations in pulsatile insulin release [105, 106] [74, 75] and ultradian oscillatory insulin secretion [107] [76], inefficient proinsulin processing to insulin [108] and more.

Through a feedback loop where IR produces increased glucose levels, beta-cell secretion and growth are stimulated and evolve through different stages throughout the progression to diabetes [100, 109]. First, beta-cells are thought to compensate/adapt to the
increasing demand posed by insulin resistant tissues by increasing their mass. This increased in beta-cell mass seems to be due to increased beta-cell numbers over hypertrophy [109]. When fasting glucose reaches the prediabetic range of ~5.6-7.0 mmol/L, a mild beta-cell decompensation occurs characterized by a loss of first-phase glucose-stimulated insulin secretion [99]. It is hypothesized that a few years later, a more severe decompensation where a loss of acute glucose-stimulated insulin secretion to non-glucose secretagogues occurs along with loss of beta-cell mass, ultimately causing a more rapid rise in glucose to the diabetic levels. The last stage of evolution is more often found in type 1 diabetes (T1D) and rarely in T2D and is characterized by severe beta-cell mass reduction and failure, islet structural damage and blood glucose levels above 20 mmol/L [110].

Very-low calorie diets have previously been shown to be effective at reducing body weight, insulin resistance and restoring beta-cell function in obese and T2D individuals [111, 112]. It is currently unknown if a low-carbohydrate or KD, by reducing insulin secretion demand, could also restore beta-cell function in these populations.

1.4.3 Chronic low-grade inflammation

Most complications where insulin resistance is present possess an inflammatory component, which makes the underlying mechanisms linking these conditions together a popular and important research area [113]. Cross-sectional and prospective studies previously concluded that T2D and obesity are low-grade systemic inflammatory states as shown by elevated levels of acute-phase proteins, cytokines and chemokines [114-116]. Increased levels of interleukin-1β, IL-6, C-reactive protein, TNF-a and total leukocyte count are all predictive of T2D development [115-118]. The innate immune system of individuals with T2D has also been shown to be over activated and implicated in diabetes-related pathogenesis [119, 120]. Indeed, the expression of cell surface toll-like receptors -2 (TLR2) and -4 (TLR4) and their ligands are
increased in individuals with T2D and contribute to a sustained proinflammatory environment [121].

In T2D and obesity, increased circulating levels of glucose and NEFA contribute to reactive oxygen species production and the recruitment of immune cells within tissues such as adipocytes [122, 123]. As a result, pathways such as c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-k B) are activated and inflammatory proteins are expressed and released in circulation, potentially contributing to systemic inflammation, IR and beta-cell dysfunction [124, 125]. Hyperglycemia, by increasing reactive oxygen species production [126] and activating toll-like receptors [127] has also been shown to directly induce inflammation, creating a vicious cycle [128, 129].

Microparticles (MPs; sometimes called microvesicles) are small membrane-bound vesicles that are released from almost all cell types (e.g., platelets, leukocytes, monocytes, erythrocytes, and endothelial cells) following cell activation or apoptosis [130-133]. In individuals with T2D, MPs levels are increased as compared to age-matched control [134]. MPs are thought to be mediators and markers of inflammation [135] and to be involved in several processes including coagulation and vascular function [136-139]. MPs are related to pathogenesis of various cardiovascular diseases and T2D through their pro-inflammatory and pro-coagulant properties that can promote vascular complications [135]. Nutritional interventions including ketogenic diets [140], weight-loss [141] and exercise [142] have been shown to improve inflammatory profiles in individuals with IR but given the novelty and limited research on MPs there is little information on how lifestyle interventions impact these emerging mediators of inflammation.

1.5 Type 2 diabetes complications

T2D is classified as a chronic disease with hyperglycemia as its main characteristic. Standards in medical care for diabetes have long been recommending pharmacological and lifestyle
interventions to prevent and manage the deleterious effects of elevated glucose on the vasculature and organs. The deleterious effects of prolonged hyperglycemia are usually separated into microvascular complications and macrovascular complications [143]. T2D diagnosis (fasting, 2h post-OGTT and HbA1C) are based on thresholds for developing microvascular complications but it is cardiovascular diseases that are the leading cause of morbidity and mortality in this population and contribute the most to the direct and indirect costs of diabetes [10]. Over the past few decades, several groups have investigated the effects of aggressive glucose control on the risk of developing macrovascular complications [144-148]. A recent review suggests that only targeting glycemic control without the underlying causes (e.g., IR, beta-cell dysfunction, inflammation) might not be sufficient to prevent CVD in T2D [149].

1.5.1 Microvascular complications
Microvascular complications are comprised of diabetic nephropathy, neuropathy, and retinopathy with the risk of developing such complications being proportional to the magnitude and duration of hyperglycemia [10]. While nephropathy can lead to chronic kidney disease, neuropathy is characterized by damage to nerves that can lead in extreme cases to limb amputations. Diabetes-related retinopathy is the most frequent cause of new blindness among adults [10].

The main mechanism by which elevated glucose seems to damage capillary endothelial cells is through excess oxidative stress. Based on Pitocco et al., this seems to be caused by an imbalance between the activity of endogenous pro-oxidative enzymes (such as NADPH oxidase, xanthine oxidase, and mitochondrial respiratory chain) and antioxidative enzymes (such as superoxide dismutase, glutathione peroxidase, heme oxygenase, and catalase) resulting in the production of reactive oxygen species that exceeds the available antioxidant defense systems [150]. Long-term studies in T1D (Diabetes Control and Complications Trial) [151], and T2D [152] (Kumamoto trial) and the United Kingdom Prospective Diabetes Study)
[147] have shown that improving glycemic control significantly reduces the risk of microvascular complications. Noteworthy, a longitudinal study in T2D has shown that between 1/3 to 1/4 of individuals with marked hyperglycemia did not develop microvascular complications, suggesting that other factors such as duration of diabetes, age, blood pressure, insulin, cholesterol, inflammation, and others might also play an important role in microvascular complications development [153].

1.5.2 Macrovascular complications

It is well known that individuals with diabetes are at increased risk of premature mortality and morbidity due to complications of atherosclerosis [154]. Cardiovascular diseases (CVD) such as coronary artery disease, stroke, and peripheral vascular disease are the leading cause of mortality among Caucasian individuals with T2D who have a three- to four-fold increased risk of CVD-related mortality when compared to non-T2D counterparts [155].

In T2D, hyperinsulinaemia and IR stimulates sympathetic nerve activity which increases blood pressure [156]. IR and hyperglycemia are also strongly linked to endothelial dysfunction, which is an early step in the development of atherosclerosis [157]. By potentially impairing endothelial function and blood pressure regulation, IR, the associated hyperinsulinemia, and hyperglycemia have all been suggested to contribute to CVD [158].

Nutrition therapy such as a Mediterranean diet have been shown to decrease major cardiovascular events by ~30% as compared to a low-fat control diet [159]. For exercise interventions, though several randomized trials have shown improvements in traditional cardiovascular factors, no clinical trials have demonstrated a reduction in major cardiovascular endpoints or mortality [160]. Contrary to microvascular complications, the Diabetes Control and Complications Trial, the Kumamoto trial, and the United Kingdom Prospective Diabetes Study failed to show significant improvements in cardiovascular outcomes following intensive pharmacological glucose control interventions [151]. Similar conclusions were drawn from the
following three major randomized controlled trials; the Action to Control Cardiovascular Risk in Diabetes [144], Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation [161, 162], and Veterans Affairs Diabetes Trial [146, 163]. In contrast to lowering HbA1C, improving CVD risk factors such as blood pressure and cholesterol, greatly reduces CVD risk and mortality in patients with T2D [149].

Anti-diabetes medications such as glucagon-like peptide-1 receptor agonists, sodium glucose co-transporter 2 inhibitors and thiazolidinediones have been shown to be effective at decreasing CVD risk and macrovascular events by 14–26% [164-168]. However, other drugs such as dipeptidyl peptidase 4 inhibitors, sulfonylureas and insulin do not appear to reduce CVD events or mortality [146, 161, 169, 170]. Abdul-Ghani et al. suggested that the drugs that offer CVD protection act through mechanisms that are independent of the reduction in plasma glucose concentration and traditional CVD risk factors [149].

1.6 Nutritional therapies for type 2 diabetes

Lifestyle interventions including nutrition therapy and exercise are non-pharmacological first-line treatments for prevention and management of T2D [10]. Previously, lifestyle interventions have been shown to be more effective than metformin in preventing the onset of T2D in adults with elevated plasma glucose [171]. As stated by Diabetes Canada; “The goals of nutrition therapy are to maintain or improve quality of life and nutritional and physiological health; and to prevent and treat acute- and long-term complications of diabetes, associated comorbid conditions and concomitant disorders” [10]. Over the last few decades, the steadily increasing rates of obesity and T2D have made scientists, clinicians, journalists, and the general public question the efficacy of the “gold standard” national dietary recommendations [172]. Metabolic syndrome, obesity and T2D are all insulin resistant states characterized by glucose intolerance. Despite this, the current low-fat dietary guidelines for T2D necessitate the consumption of the majority of calories as CHO, a recommendation that is now being hotly debated [173].
1.6.1 Current dietary guidelines

Governments and advisory bodies around the world have slightly different specifics in their dietary guidelines but, in general, guidelines for healthy individuals and those with T2D are relatively similar [10, 174, 175]. According to Diabetes Canada, the daily recommended acceptable macronutrient distribution ranges for macronutrients as a percentage of total energy includes 45% to 60% energy from CHO, 15% to 20% energy from protein and 20% to 35% energy from fat, with 5% to 10% energy derived from linoleic acid and 0.6% to 1.2% energy derived from alpha linolenic acid (CDA). For CHO, low glycemic index (GI) and high-fibre foods are to be prioritized while fructose and sucrose should be kept below 10% of total daily energy. Importantly, Diabetes Canada endorsed the dietary reference intakes recommending the consumption of no less than 130 g/day of available CHO for adults to provide glucose to the brain [176, 177]. The DRIs also specify a recommended dietary allowance for protein of 0.8 g per kg body mass for adults. Saturated fat should be ≤10% of energy and cholesterol intake need dietary cholesterol ≤200 mg/day. Essential polyunsaturated fatty acids n-3 alphalinolenic acid daily consumption should be 1.1 g/day for women and 1.6 g/day for men. Replacement of saturated fatty acids or CHO with high quality sources of monounsaturated fatty acids is also encouraged. In terms of food to be consumed, the typical diet should comprise a wide variety of legumes, fruits, starchy and non-starchy vegetables, nuts, whole grains, low-fat dairy and lean animal protein.

1.6.2 Low-carbohydrate high-fat diets

Diabetes Canada and the American Diabetes Association both define low-CHO diet as one containing less than 130g of CHO per day [10, 175]. Although definitions can vary, a diet with less than 26% of total energy provided by CHO is generally defined as a low-CHO diet, while diets with less than 10% (20-50g/day) of total energy provided by CHO falling into the very low-CHO KD category (Table 2) [173]. When CHO is restricted, the contribution of fat to total energy
intake is increased, resulting in a LCHF diet. Within an LCHF diet, fat comprises the majority of energy in the diet (65-80% of total calories; with generally no limit on saturated fat intake) while protein intake is kept moderate (10-25%, 1.2-2.0 g/kg body weight) to prevent the stimulation of insulin secretion by certain amino acids [178]. In terms of food, an LCHF diet will favor meat, fish, poultry, eggs, full-fat dairy, nuts, seeds, and non-starchy vegetables. To optimize fluid and electrolyte balance, daily sodium supplementation of 1-5g/day is recommended. Noteworthy, a well-designed LCHF diet needs to be individualized based on the patient’s metabolic state (glucose tolerance, insulin resistance, blood lipids, body mass index, past experience on diets, etc.) and may need to be modified over time going from induction to maintenance phases.

Table 2. Suggested definitions for different forms of LCHF diets. Used with permission from publisher Elsevier [173].

<table>
<thead>
<tr>
<th>Type of LCHF Diet</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Very low-carbohydrate ketogenic diet (VLCKD)</strong></td>
<td>Carbohydrate, 20-50 g/d or &lt;10% of the 2000 kcal/d diet, whether or not ketosis occurs. Derived from levels of carbohydrate required to induce ketosis in most people. Recommended early phase (“induction”) of popular diets such as Atkins Diet or Protein Power.</td>
</tr>
<tr>
<td><strong>Low-carbohydrate diet:</strong></td>
<td>&lt;130 g/d or &lt;20% total energy. The ADA definition of 150 g/d as its recommended minimum.</td>
</tr>
<tr>
<td><strong>Moderate-Carbohydrate Diet:</strong></td>
<td>200–400 g/d. Upper limit, approximate carbohydrate intake before the obesity epidemic (41%).</td>
</tr>
<tr>
<td><strong>High-Carbohydrate Diet:</strong></td>
<td>&gt;450 g/d. Recommended target on ADA websites. The 2010 Dietary Guidelines for Americans recommends 45–65% carbohydrate. The average American diet is estimated to be ~40% carbohydrate.</td>
</tr>
</tbody>
</table>

**Carbohydrate Consumption (NHANES):**
- **Men**
  - 1991–1994: 42% (~250 g for 2450 kcal/d)
  - 1999–2000: 43% (~310 g for 2600 kcal/d)
- **Women**
  - 1971–1974: 45% (~150 g for 1550 kcal/d)
  - 1999–2000: 52% (~210 g for 1900 kcal/d)

1.6.2.1 Low-carbohydrate high-fat diets and weight loss

Current dietary recommendations for weight management and obesity treatment advocate the use of a high-carbohydrate low-fat (HCFL), moderate energy-restricted diet [179]. National dietary authorities currently do not advise LCHF diets, presumably because of the perceived risks posed by their high content in saturated fats and cholesterol. Reviews on the safety of such diets have been written, however, and do not highlight any major concerns [180, 181].
Over the last few decades, the efficacy of LCHF diets for weight loss has been the focus of several investigations. In studies of ≥6 months duration using ketogenic diets (< 50g/day) in individuals with obesity, weight loss has usually been recorded to be superior to HCLF diets [182-185]. Systematic reviews and meta-analysis on the subject have also concluded that LCHF diets were superior at 3 and 6 months and superior to or as good as HCLF at ≥1-year for weight loss interventions [186-189]. In individuals with T2D, a 3-month LCHF diet has been shown to induce greater weight loss (6.9 vs. 2.1 kg, \( P = 0.003 \) as compared to a diet following the Diabetes UK nutritional recommendations [190]. Adherence to LCHF diets measured in clinical trials have been reported to be similar to other diets with equal calories [191]. Taken together, these results strongly suggest that LCHF diets should be considered as an alternative tool against obesity.

1.6.2.2 Low-carbohydrate high-fat diets and cardiometabolic risk factors

The literature on the effects of LCHF diets on cardiometabolic risk factors has sometimes shown inconsistent results. One reason to explain this is that the inclusion criteria used by some authors to define LCHF vary widely and can include diets with up to 45% of CHO. Systematic reviews and meta-analysis have consistently shown that LCHF diets are effective at reducing HbA1C, insulin, TG, systolic and/or diastolic blood pressure and at increasing high density lipoprotein-cholesterol in individuals with obesity [186-188] and T2D [192, 193]. Total cholesterol and low density lipoprotein-cholesterol (LDL-C) have shown inconsistent results with most [186-188] but not all studies or reviews [193, 194] showing increased levels following LCHF diets. Given the parallel improvement in other cardiometabolic risk factors and that the distribution of LDL-C subclasses and ApoB tend to improve or remain unchanged under CHO restriction [195], the long-term implications of isolated increases in total or LDL-cholesterol remains to be determined. However, an increase in LDL cholesterol after a LCHF diet is an
issue of potential concern and likely needs to be considered when following this dietary approach in the long-term.

1.6.2.3 Low-carbohydrate high-fat diets and inflammation

Obesity and diabetes are known as low-grade inflammatory conditions characterized by heightened inflammation in circulating white blood cells [196] and infiltration of immune cells into adipose tissue [197, 198]. In these populations, various nutritional weight-loss interventions have been shown to improve inflammatory profile [199-201]. In a cross-over design study using LCHF diets in overweight men, TNF-α, IL-6, C-reactive protein and intercellular adhesion molecule-1 all decreased following 6 weeks of both LCHF and HCLF diets. Because participants lost weight in both dietary interventions, the authors concluded that “in the short-term, weight loss is primarily the driving force underlying the reductions in most of the inflammatory biomarkers” [202]. In two other studies in overweight men and women with atherogenic dyslipidemia and T2D, both LCHF and HCLF diets significantly decreased the concentration of several serum inflammatory markers, but the overall anti-inflammatory effect of the LCHF diet was superior at reducing TNF-α, IL-6, interleukin-8, monocyte chemoattractant protein-1 (MCP-1), E-selectin, intercellular adhesion molecule 1, plasminogen activator inhibitor-1, C-reactive protein and white blood cell count [140]. Since nutritional interventions in participants with obesity and T2D are usually accompanied by weight-loss, it makes it difficult to isolate the direct effect of the LCHF diet on inflammation. Acute studies assessing the impact of a single meal on inflammatory markers have suggested that consumption of high-fat meals are pro-inflammatory, leading to increased activation of pro-inflammatory signaling pathways in circulating white blood cells, along with production and release of pro-inflammatory cytokines [203]. Such pro-inflammatory effects of a high-fat meal are even more pronounced in individuals with T2D [204]. However, several of these “high-fat meal” studies were actually performed using high-carbohydrate high-fat foods, highly processed fast food, or whipped cream as the food source.
The direct effect of a well-designed LCHF diet composed of whole food, without the confounding variable of weight-loss, on inflammation remains to be elucidated.

1.7 Nutritional Ketosis

Normal postprandial ketone body (KB) concentrations in humans consuming a western diet are about ~0.1 mmol/L and will typically rise to about ~0.2-0.3 mmol/L following an overnight fast. Ketosis is a metabolic state that is often defined as circulating KB levels >0.3-0.5 mmol/L. In humans, various interventions ranging from exhaustive exercise to extended fasting can lead to physiological ketosis, the level of which differs widely depending on the intervention used (Figure 4) [205]. Ketosis is not to be confused with life-threatening diabetic ketoacidosis, which along with elevated KB (often >10 mmol/L), is accompanied by elevated glucose and decreased blood pH, bicarbonate and electrolytes [206]. As compared to glycogen depleting exercise and prolonged fasting, approaches such as ketone supplements and ketogenic diets constitute more sustainable alternatives to induce and maintain ketosis over time.
Figure 4. Changes in BHB under various physiological states. Used with permission from publisher John Wiley and Sons [205].

1.8 Ketone bodies

KB refer to carbon containing compounds that incorporate a carbonyl group with two hydrocarbon groups attached to it \((R-C(=O)-R')\). Acetoacetate (AcAc), BHB and acetone comprise the three KB, (though BHB is technically not a KB body as the ketone moiety is reduced to a hydroxyl group; Figure 5). While AcAc and BHB are short-chain, four carbon organic acids that provide energy to extra-hepatic tissues, acetone, a three-carbon organic acid does not contribute to energy production.
Figure 5. Circulating ketone bodies

1.9 Hepatic ketogenesis

The primary source for ketogenesis are NEFA coming from white adipose tissues, with ketogenic amino acids such as leucine and lysine contributing to a small extent (~5%) [207]. Under conditions of low circulating insulin and intracellular glucose, adipose tissue lipolysis will be stimulated and NEFA will reach the liver to be converted into acetyl coenzyme A through beta-oxidation. Under normal conditions, acetyl coenzyme A enters the tricarboxylic acid cycle after pairing with oxaloacetate, which accumulates from pyruvate produced during glycolysis [208]. However, in conditions of cellular glucose starvation, a large proportion of oxaloacetate is shifted to the gluconeogenesis pathway. When combined with overproduction of acetyl coenzyme A from NEFA, acetyl coenzyme A is diverted to form Aceto-Acetyl CoA then 3-hydroxy-3-methylglutaryl-CoA through the fate-committing enzyme 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2. From there, 3-hydroxy-3-methylglutaryl-CoA will release an acetyl coenzyme A and become AcAc, which can further be reduced by beta-hydroxybutyrate dehydrogenase-1 to BHB, or spontaneously decarboxylated to acetone (Figure 6) [209]. While acetone is exhaled through the lungs, BHB transport is still not completely understood but appears to be released into the circulation through monocarboxylate transporter SLC16A6 [210]. Although the liver is the main site of production for KB, other sites such as astrocytes, kidneys, pancreatic beta-cells and others have also been proposed to possess ketogenesis properties, however their
contribution to systemic hyperketonemia is limited to non-existent [211]. The rate of ketogenesis is regulated by hormone-sensitive lipase, acetyl CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA synthase. Through its direct actions on these enzymes, a high ratio of hormones glucagon:insulin will stimulate ketogenesis while a low one will inhibit it. Other counter regulatory hormones as well as low glycogen stores also contribute to the stimulation of ketogenesis while elevated concentrations of BHB will act as a feedback mechanism to control its own production through inhibition of adipocyte lipolysis [212]. It has been estimated that under starvation conditions, a human can synthesize as much as 150 grams of KB over a 24-h period [213].

1.10 Extra-hepatic ketolysis
KB concentrations results from the balance of ketogenesis and ketolysis at any given moment. BHB is the main KB found in circulation and enters extra-hepatic cells through monocarboxylate transporter-1 and 2 following a concentration gradient [209]. Once inside the cells, BHB will be oxidized by beta-hydroxybutyrate dehydrogenase-1 to AcAc, which will then be converted into AcAc-CoA by the enzyme succinyl-CoA:3-oxoacid CoA transferase before yielding two molecules of acetyl coenzyme A that will enter the tricarboxylic acid cycle for ATP production. Succinyl-CoA:3-oxoacid CoA transferase ketolytic activity is highly present in the in heart and kidneys, followed by skeletal muscles and then the brain. In the liver, the ketolytic activity of these enzymes are low and ketone oxidation is insignificant [214]. Though the main fate of KB is oxidation in extra-hepatic tissues, a small fraction will also contribute to cholesterol- and lipogenesis in hepatocytes and adipocytes (Figure 6) [209].
1.11 Physiological adaptations to ketogenic diets

Several decades ago, pioneering work by Cahill paved the way to a better understanding of fasting keto-adaptation [213, 215]. Among other things, Cahill’s work clearly showed that under prolonged fasting conditions, many tissues and organs such as the liver, kidneys, muscles and brain can adapt and ensure survival by producing and using ketone bodies. A somewhat similar but more relevant and applicable keto-adaptation occurs when one follows a well-formulated LCHF diet over several weeks leading to important metabolic and enzymatic adaptations in both the muscle and the liver (Figure 7 and Table 3) [216]. Among them, hepatic metabolic regulation undergoes numerous changes that are associated with: i) lower glycogen storage, ii) reduced circulating glucose, insulin and triglycerides; and iii) a concomitant increase in circulating non-
esterified fatty acids and KB [216]. These changes, when sustained over time, lead to a specific metabolic state that has been termed "keto-adapted" where most cells shift from using glucose to NEFA and KB as the main fuel source to meet energy needs [217, 218]. Although the rate of fat oxidation changes quite rapidly (within days) of switching to a LCHF diet [29], the time necessary to reach this so-called keto-adapted state is currently unknown but it has been suggested that several weeks may be necessary for complete switch to optimal fat and ketone utilization [216].

Figure 7. Schematic of hepatic metabolic regulation induced by a low-carbohydrate diet. Used with permission from publisher Elsevier [216].
1.12 Ketogenic diets vs. exogenous ketone supplements

The physiological adaptations characteristics of the “keto-adapted” state are likely exclusive to low-carbohydrate intake conditions and are not believed to be present when ketosis is achieved acutely using exogenous ketones (either orally or by infusion). Despite similarly increasing blood BHB concentrations, several important differences exist between these two states of ketosis. First, AcAc breakdown occurs spontaneously, which makes stability difficult. Thus, the majority of exogenous ketone supplements are made exclusively of BHB and do not mimic the physiological liver synthesis ratio of AcAc:BHB. Moreover, many BHB supplements are often composed of a racemic mixture of D- and L-isomer, the latter not being produced by the liver and potentially not used as efficiently by the tissues once in circulation [219, 220]. Finally, when compared to a KD, exogenous supplements elevate ketones on a background of replenished glycogen stores and unchanged carbohydrate intake, insulin secretion and circulating NEFA. For all these reasons, these two methods of achieving ketosis should not be considered to have equivalent effects and more studies are required to compare their respective effects on metabolism. Nonetheless, the recent discovery and commercialization of exogenous oral ketone
supplements is intriguing as both an intervention and research tool to substantially raise circulating KB in humans without having to force a prolonged period of fasting or follow a longer-term carbohydrate-restricted diet. Exogenous oral ketones therefore allow for a more direct test of the impact of raising BHB when compared to fasting or an LCHF diet where a myriad of systemic metabolic changes accompany the increase in circulating KB.

1.13 Exogenous ketones
For decades, raising circulating ketone levels was only achievable by severely restricting CHO, performing prolonged exercise sessions, consuming medium-chain triglycerides or by infusing ketones. Nowadays, new oral supplements have emerged and drastically changed the time course and the way by which ketosis can be attained. There are currently two main oral supplement formulations available; ketone salts (KS) and ketone esters (KE). Ketone infusion and oral ketone supplements both can acutely (within 15-60 minutes) raise KB to levels equivalent to weeks on a KD or even several days of fasting [221, 222].

1.13.1 Ketone infusion
As early as the 1940’s, KB infusions were being performed in humans to better understand the relationships between ketone and glucose metabolism [223]. Ketone infusion methods are typically done using racemic mixtures of sodium D- and L- AcAc or BHB that are infused intravenously at a constant rate over a period of several hours. This method is effective at raising KB concentrations, but requires medical expertise and is by nature, invasive.

1.13.2 Oral ketone salts
Ketone salts are the most affordable and palatable form of oral ketone supplements on the market. As their name suggests, a ketone salt is produced through an acid-base reaction where the free acid AcAc or BHB is buffered by a cation (sodium, calcium, potassium, magnesium). In
humans, a 50% D- and L-isoforms mixture of KS has been shown to effectively raise D-BHB and L-BHB to levels up to ~1 and ~2 mmol/L respectively. The implication of raising L-BHB is currently unknown but its high concentrations in circulation 4-hour after ingestion, combined with previous mechanistic studies, suggests that it undergoes less oxidative metabolism [219, 220, 222].

Given the large amount of ketones (~50 grams) needed to reach nutritional ketosis, the current KS supplement formulations are, for the most part, impractical as they provide high level of minerals (e.g., sodium) that might cause gastrointestinal distress and potential alkalinizing effect over time [224]. New KS formulations with improved cation balance are currently under development.

1.13.3 Oral ketone esters

As an alternative to KS, two KE; the R,S-1,3-butanediol acetoacetate diester [225] and the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate ketone monoester (KM) [221] have been studied in humans. The ketone diester is synthesized by transesterification of t-butylacetoacetate with R,S-1,3-butanediol while the KM is synthesized by transesterification of ethyl-(R)-3-hydroxybutyrate and (R)-1,3-butanediol. The AcAc diester delivers two AcAc molecules and one racemic BHB molecule while the KM delivers two BHB molecules. Both KE have been shown to raise BHB levels anywhere between 1-6 mmol/L between 15 minutes to 3 hours following ingestion [221, 226-228]. While the only study in humans using the ketone diester reported severe gastrointestinal distress at 2 daily doses of 250 mg/kg of body mass [228], doses of the KM between 140-714 mg/kg body mass taken 1-3 times daily appear well tolerated [221, 222].

The KM is currently the most appropriate way for delivering high doses of BHB. Studies directly comparing ketone esters to KS show that the KM is more effective at raising blood D-BHB [222]. However, their bitter and unpleasant taste constitutes an important barrier for longer-term use where repeated doses would be necessary over time.
1.14 The metabolic and signaling effects of ketone bodies

To assure its survival, the human body possesses a variety of adaptive mechanisms to environmental stresses such as nutrient deprivation. For a long time, KB were perceived as detrimental and dangerous to human health mostly because of their connection with the life-threatening metabolic disease state of diabetic ketoacidosis. However, a multitude of studies, including classic studies from the groups of Cahill and Robinson have provided a better understanding of the potential roles of KB as important carriers of energy and regulators of cellular functions [214, 217]. More recently, changes in the activation of nutrient-responsive pathways in situations of dietary restriction have been linked to reduced aging and prolonged lifespan [211]. Mechanistic studies demonstrate that BHB possesses direct and indirect signaling capabilities that can affect gene expression, lipid metabolism, inflammation, central nervous system function and more (Figure 8) [229]. Thus, it is now believed that KB, particularly BHB, may act not only as an alternative fuel source but as key signaling molecules that orchestrate widespread adaptation linking nutritional status to tissue function.
Figure 8. Schematic of direct and indirect signaling functions of the ketone body BHB. Indirect signaling functions require catabolism to other molecules, whereas direct signaling functions are actions of BHB itself. The major downstream effects of signaling functions are noted. Abbreviations: BHB, B-hydroxybutyrate; CoA, coenzyme A; FFAR3, free fatty acids receptor 3; GABA, y-amino-butyric acid; HDAC, histone deacetylase; HCAR2, hydroxycarboxylic acid receptor 2; NAD, nicotinamide adenine dinucleotide; VGLUT, vesicular glutamate transporter. Used with permission from publisher Annual Reviews [229].

1.14.1 Ketone bodies as metabolic fuel

KB are the most efficient substrate in terms of oxygen consumption for ATP production and act as extra-hepatic energy source for active tissues such as the heart, kidneys, skeletal muscles and brain [224, 230]. In conditions of nutrient deprivation, providing these tissues with an alternative energy source can greatly reduce hepatic gluconeogenesis from amino acids, which limits the associated protein catabolism. Through these means, the generation of KB is essential for long-term survival in starvation [213]. In normal conditions, KB concentrations are low (0.1 mmol/l) and do not significantly contribute to energy provision [226]. Despite the main driver of KB oxidation being blood KB concentration, their relationship in muscle is curvilinear
and KB oxidation seems to saturate at levels around 1-2 mmol/L [231, 232]. Skeletal muscles make up the majority of the human tissues (~40%) and constitutes a major site for KB utilization. Based on previous studies, it appears that in muscles, KB are providing ~5% of the energy in the post-absorptive state, ~10% following an overnight fast [233, 234] and ~20-50% after 72 h of fasting [234, 235]. However, this declines to 15% after 24 days of starvation [234]. Contrary to long-chain NEFA, KB have the capacity to effectively cross the blood brain barrier and become a major source of energy for brain cells [236]. The KB concentration:utilization relationship in the brain seems to be more linear [232]. Indeed, KB can provide ~33% of cerebral energy requirements following 3-4 days of fasting [237] and increased to ~67% following several weeks of starvation [215]. The heart and kidneys are also known to be sites of KB uptake and oxidation in both animals and humans [213, 214]. Contrary to glucose, KB utilization in the brain and the heart appears to be unaffected in individuals with metabolic diseases such as diabetes or Alzheimer’s [238, 239].

1.14.2 The anti-lipolytic effect of BHB

G-protein-coupled receptors (GPCR) have important roles in metabolism and metabolic diseases. More specifically, GPCR109A is a cell-surface receptor found in adipose tissue [240] and immune cells [241] that can be activated by D- and L-BHB (EC50 of 0.7mM), nicotinic acid, and butyrate but not AcAc [240, 242]. The activation of GPCR109A by BHB leads to a decrease in adipose tissue lipolysis, which is thought to be a negative feedback mechanism to regulate ketogenesis by limiting NEFA availability [242]. The inhibition of adenylyl cyclase and subsequent decrease in cyclic adenosine monophosphate, which in turn inhibits hormone-sensitive TG lipase [240, 241] is responsible for the anti-lipolytic effect observed. Studies using ketone infusion and exogenous ketone supplements have consistently shown reduced level of NEFA [226, 243, 244]. The potential clinical application of these findings are intriguing give that
pharmacological agonists of GPCR109A (e.g., nicotinic acid) have been successfully used to control dyslipidemias and glucose in humans [245, 246].

1.14.3 The glucose-lowering effect of ketone bodies

To date, most studies exploring the direct effects of elevated plasma ketones on markers of metabolic control have employed ketone infusion methods. The hypoglycemic action of KB has been shown in BHB infusion studies in both animals and humans [232, 244, 247-249]. Among them, Miles et al. and Mikkelsen et al. showed that BHB, infused to levels of ~2.0 mM, significantly decreased circulating glucose [232, 247]. Other studies including obese children and adults have also reported similar observations [243, 250]. More recently, an oral KE supplement ingested following the consumption of a standard meal decreased glucose levels from an average of 5.5 to 4.7 mM (~15%) over a 4-hour period [222].

The mechanisms of action for the glucose-lowering action of ketones are not well understood but may be due to an inhibitory effect of hepatic glucose production, which has been shown to be suppressed by ~20% during BHB infusion [232, 247]. A similar fall in blood glucose during ketone infusion can occur in the absence of changes in insulin or glucagon [243, 244, 250], suggesting that a change in glucoregulatory hormones are not the primary driver of this effect. However, the potential for KB infusion to stimulate beta-cell insulin secretion has been previously demonstrated [251-253]. Studies using oral KE supplement have also observed a two-fold increase in insulin levels during a post-exercise hyperglycemic clamp [254] and a small increase in the fasting state [222] whereas no change was seen when KE were combined with a post-exercise high-dose protein-carbohydrate drink [255].

Lowering glucose and free fatty acids could be of potential value for individuals with glucose intolerance and insulin resistance. So far, the only study in individuals with type 2 diabetes has used AcAc infusion and did not demonstrate a glucose-lowering effect [256].
1.14.4 The potential antioxidant and anti-inflammatory effects of BHB

BHB is known as an endogenous inhibitor of class I and IIa histone deacetylases (HDACs), a family of proteins that regulate gene expression by deacetylating histone and non-histone proteins. As a generality, histone deacetylation is associated with reduced gene expression, so inhibition of HDACs by BHB should lead to increased gene expression. Through the inhibition of HDACs, BHB has been shown to activate the transcription of forkhead box O3 and downstream antioxidant genes, which is associated with protection from oxidative damage [257].

Like other GPR109A ligand such as niacin, BHB may also exert anti-inflammatory effects in tumor-necrosis factor alpha and lipopolysaccharide-induced inflammation by decreasing pro-inflammatory proteins and cytokines levels through inhibition of NF-kB translocation to the nucleus [258, 259]. BHB has also been shown to decrease activation of the Nod-like receptor protein 3 (NLRP3) inflammasome and subsequent activation of caspase-1 and interleukin IL-1B and IL-18 secretion [260, 261]. The NLRP3 inflammasome is known as a mediator of the inflammatory response to nutrient excess and mitochondrial dysfunction [262, 263]. Though BHB is generally considered anti-inflammatory, some studies have reported pro-inflammatory effects of AcAc when levels are > 5 mmol/L (reviewed here: [209]). Overall, these antioxidant and anti-inflammatory effects of BHB are intriguing and suggest that raising BHB, either through a ketogenic diet or BHB supplementation, could have widespread benefits in chronic diseases that have an inflammatory component. However, limited evidence exists in humans that directly assesses the anti-inflammatory effects of BHB due to potential confounding factors with ketogenic diets (e.g., weight loss, other metabolic adaptations) and lack of studies on exogenous ketone supplements.

1.15 Aim and hypotheses

Given the important role of hyperinsulinemia in pathogenesis of obesity and T2D, the non-invasive measurement of insulin is of great interest and could be advantageous for identifying
individuals with elevated basal and postprandial insulin. As a first step toward a non-invasive insulin monitor, we conducted the 1st study of this dissertation with the industry partner CoreHealth Technologies Inc. to determine if insulin levels could be detected non-invasively in saliva. We hypothesized that saliva insulin responses in lean vs. obese individuals would reflect plasma insulin responses and that saliva insulin could be used to delineate between low and high insulin responses following the consumption of meals with different carbohydrate contents.

Despite the clear promises of LCHF diets for individuals with T2D, several unanswered questions remain. Specifically, whether eating 70% energy from fat impacts inflammation, in the absence of the confounding effects of weight loss, is unknown. Furthermore, whether the LCHF dietary approach can reduce beta-cell stress and potentially promote beta-cell rest has not been adequately addressed. In order to test these hypotheses, our second study used a 4-day LCHF dietary intervention in T2D participants to determine the short-term impacts of consuming a whole-food LCHF diet, independent of substantial weight loss, on cellular markers of inflammation, glucose control and beta-cell stress.

The emerging roles of BHB as a signaling molecule and alternative fuel source have led to a resurgence in ketone research. The development of exogenous ketone ester drinks now provide the opportunity to rapidly raise blood ketones to study their direct effects without the confounding influence of prolonged fasting or a LCHF diet. However, due to their novelty there is very limited research on the metabolic effects of exogenous ketone supplements. The aims of Studies 3 (in lean healthy volunteers) and 4 (in individuals with overweight and obesity) were to determine the metabolic effect of an exogenous ketone ester drink on glucose tolerance and to explore the potential underlying mechanisms involving glucoregulatory hormones and circulating metabolites.
Chapter 2: Detection of salivary insulin following low versus high carbohydrate meals in humans.

BACKGROUND

Compelling data show that elevated insulin levels are associated with the development of pathological conditions such as obesity and T2D [264]. For the past few decades hyperinsulinemia has been mainly considered as a consequence of obesity but this concept has recently been revisited [265]. A revised model of obesity and T2D suggests a more central and causal role of hyperinsulinemia, which is thought to precede and drive metabolic abnormalities [266, 267]. Insulin hypersecretion and IR are detectable up to several years prior to abnormalities in glucose tolerance [268-272]. Over time, persistent elevated insulin secretion can no longer be maintained by pancreatic beta-cells, leading to chronic hyperglycemia and the associated diagnoses of prediabetes or T2D [273], which significantly increases the risk for CVD and mortality [274]. Thus, elevations in basal and stimulated insulin levels may constitute an important early marker of metabolic dysfunction that could be monitored in both apparently healthy and at-risk individuals.

The theoretical and practical importance of postprandial insulin levels have previously been emphasized by proposing the use of a food insulin index that ranks foods based on their ability to elevate postprandial insulin [275, 276]. Although potentially useful, a general food insulin index based on the postprandial blood insulin responses to isolated foods in healthy volunteers [275] would not take into account the inter-individual variability in insulin secretion and/or responses to mixed meals nor would it be able to account for expected differences in postprandial insulin levels between individuals with different levels of insulin resistance. From a clinical and monitoring perspective, measuring postprandial insulin levels would be highly valuable but presents several challenges including the requirement for repeated blood sampling.
Developing non-invasive, user-friendly alternatives to monitor insulin levels in humans therefore holds potential value.

Saliva as a body fluid contains several of the same components as blood but can be sampled non-invasively. In clinical research settings, saliva is already being used as a diagnostic and/or monitoring tool to reflect the content of circulating hormones, proteins, adipokines and inflammatory biomarkers [277]. Although it has been known for several years that insulin can be measured in saliva, most of the studies conducted measured salivary insulin levels in the fasted state [278] and following an oral glucose tolerance test [279, 280]. Results are generally supportive that saliva insulin may serve as an adequate surrogate to blood insulin levels, although the absolute concentration is lower and there may be a lag in salivary insulin changes when compared to blood in response to glucose ingestion.

To date, it is known that salivary insulin increases following the consumption of a glucose load or a mixed meal high in CHO [281, 282]. Pasic et al. 1988 also assessed salivary insulin levels following mixed-meals but only in individuals with T1D who were on exogenous insulin, which makes the conclusions difficult to apply to other populations [283]. These studies support the notion that saliva insulin may reflect blood insulin levels but do not provide insight into whether changes in saliva insulin are sensitive enough to delineate between meals with different insulin responses or whether subtle differences in postprandial insulin, which might reflect systemic IR or increased metabolic risk, are detectable via saliva insulin measures. Such information is needed to determine if saliva could be a non-invasive means to accurately measure postprandial insulin and potentially identify individuals at risk for T2D due to hyperinsulinemia.

Accordingly, the primary aim of this study was to determine if saliva insulin could be used to delineate between postprandial insulin levels following the ingestion of low- and high-carbohydrate mixed meals designed to elicit low and high insulin responses, respectively. A
secondary aim was to compare young lean participants to young overweight/obese participants to determine if subtle differences in postprandial hyperinsulinemia could be detected in saliva.

METHODS

Participants
Sixteen individuals were recruited through poster advertisement and word of mouth across the University campus. Based on the World Health Organization guidelines [284] eight individuals were classified as normal weight (NW) (BMI 20.0-24.9 kg/m² with a waist to hip ratio <0.90 male or <0.85 female) and eight classified as overweight/obese (OO) (BMI ≥28.0 kg/m² with a waist to hip ratio ≥0.90 male or ≥0.85 female). One female participant classified as obese was excluded from the analysis because of abnormally elevated insulin levels (values were >2.2 times the interquartile range above the median resulting in severe skewness with her data included). All participants met the following eligibility criteria: 1) being between 20 and 39 years of age; 2) not diagnosed with any medical conditions; 3) not taking any medications known to impact metabolism (on stable oral contraceptive pills for at least 3 months was accepted); 4) not following a vegetarian or LCHF diet; 5) not a competitive athlete or participating in structured endurance training. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the UBC Clinical Research Ethics Board (H1502638) and registered at ClinicalTrials.gov (NCT02699203).

Study design
The study followed a randomized crossover design. Randomization was performed using the online research randomizer program accessible at: https://www.randomizer.org. Eligible participants completed two isocaloric meal conditions separated by at least 72 hours: 1) low-carbohydrate breakfast meal (LCM); 2) high-carbohydrate breakfast meal (HCM)
Study protocol

Visit 1: After the eligibility criteria were confirmed and informed consent obtained, anthropometrics and blood pressure measurements were collected. Participants were given a dietary journal to record all the food and drinks consumed for the 24-hour prior to their first experimental condition. During this 24-hour period, participants were instructed not to exercise and to follow their typical eating patterns such that replication would be easily accomplished on the day preceding their second experimental condition. Visit 1 occurred 2-10 days prior to Visit 2.

Visit 2 and 3: After an overnight (>10 h) fast, participants arrived at the laboratory where the research coordinator reviewed the dietary journal and confirmed that no exercise was performed in the previous 24 hours. If there were no irregularities an indwelling venous catheter was inserted by a certified phlebotomist in the antecubital space of the arm. Fasting blood and saliva samples were then collected followed by the consumption of the meal, which was consumed within 10 minutes. Five minutes before the second sampling time point participants were asked to rinse their mouth with water to remove any food remnants. Blood and saliva samples were then collected at 15, 30, 60, 90 and 120 minutes following meal completion. Following visit 2 participants were provided with their 24-hour diet record and given instructions to follow their meal plan exactly prior to the next visit. On the morning of visit 3 (3-10 days following visit 2) the research coordinator reviewed the 24-hour dietary journal for compliance, and confirmed that no exercise had been performed on the day before. Participants then went through the same procedures as visit 2 but consumed the alternate meal.

Meals

The LCM (10% CHO, 65% fat, 25% protein) was composed of whole eggs, egg whites, avocado, red peppers and onions while the HCM (55% carbohydrate, 20% fat, 25% protein, GI: 48) [285] was composed of plain rolled oats, mixed berries (blueberries, raspberries,
strawberries) and stevia sweetened whey protein isolate. Both mixed meals were isocaloric (500 kcal) and were designed to reflect food typically eaten at breakfast that would elicit a low and high insulin response respectively.

**Blood and saliva sample collection and processing**

Repeated blood samples were collected in 4 ml Ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer, Franklin Lakes, New Jersey, USA) using an intravenous catheter (BD Nexiva, Sandy, Utah, USA). Saliva samples were collected using a passive drool collection device for a period of 60 seconds (Salimetrics LLC, State College, Pennsylvania, USA). Both samples at each corresponding time point were kept on ice and then centrifuged together within 20 minutes (1550 g, 15 minutes, 4° Celsius). Plasma was immediately stored with the centrifuged saliva samples at -20° Celsius prior to analyses. For saliva analyses, samples were first thawed and then centrifuged again (1550 g, 15 minutes, 4° Celsius) and the clarified supernatant used for insulin analysis.

**Biochemical analyses**

Plasma glucose was measured by the hexokinase method on a clinical chemistry analyzer (Chemwell 2910, Awareness Technologies). Plasma and salivary insulin were measured in duplicate by ELISA following the manufacturer’s protocol (Mercodia Ultrasensitive Insulin ELISA) with absorbance read on a microplate reader (iMark, Bio-Rad). The coefficient of variation for duplicate samples was 10.7% for plasma insulin and 6.0% for salivary insulin. Although a previous study has used ELISA to assess fasting saliva insulin [278] we first performed validation experiments and preliminary testing revealed that there was interference in the assay with neat saliva. Spike and recovery tests showed 80+/−7% recovery (N=6) when saliva was diluted 1:2 with the zero standard provided in the kit. Diluting more than this did not appreciably increase recovery (80-85%) and tended to result in samples with low insulin
concentration (e.g., lean fasting) to be below the detection limit. Therefore, 1:2 diluted saliva was used in the ELISA.

**Statistics**

Data was analyzed using SPSS v22. Normality was assessed using Q-Q plots and Shapiro-Wilk test within each group. Appropriate transformation (natural log or 1/square root) on non-normally distributed variables resulted in normal distribution. Baseline differences were assessed using an unpaired Student t-test. Baseline fasting glucose, plasma insulin and salivary insulin level were computed using the average of both the LCM and HC conditions for each participant. Area under the curve (AUC) and incremental AUC (iAUC) were calculated using GraphPad Prism v6.0. A two factor (group X meal) mixed ANOVA with repeated measures on the second factor was used to analyze AUC and iAUC for plasma glucose, plasma insulin, and saliva insulin. Significant interactions were followed up with pre-planned contrasts comparing NW to OO within meal and LCM to HCM meals within groups using Bonferroni corrections for multiple comparisons. Cohen's $d$ effect size was calculated for all of the pre-planned comparisons. Potential relationships between salivary and plasma AUC following both meals were assessed using separate Pearson correlations. The relationship between fasting saliva and plasma insulin levels was assessed using Spearman rank-order correlation for non-normal data. Significance was set at $P<0.05$.

**RESULTS**

All participants complied with replication of their diet and refrained from exercising for the 24-hour period preceding each experimental condition. Baseline characteristics are presented in Table 4. As expected, the OO group had a higher body mass index (BMI) and waist to hip ratio (WHR) (both $p<0.001$). The OO group also had a significantly higher systolic blood pressure, fasting blood glucose, fasting plasma insulin and fasting salivary insulin (all $p<0.05$). There were
no differences between the two groups in terms of age, resting heart rate and diastolic blood pressure.

**Table 4. Baseline participants' characteristics**

<table>
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<th></th>
<th>Normal weight</th>
<th>Overweight/Obese</th>
<th>P value</th>
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<tr>
<td>Number of participants (M/F)</td>
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<td>7 (6/1)</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.1 (4.1)</td>
<td>30.6 (4.3)</td>
<td>0.133</td>
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<td>Body mass index (kg/m²)</td>
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<td>Waist to Hip ratio (cm)</td>
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<td>0.93 (0.06)</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>129 (12)</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
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<td>83 (7)</td>
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<td>Fasting saliva insulin (mU/L)</td>
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<td>2.5 (2.0)</td>
<td>0.039</td>
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</tbody>
</table>

**Saliva and Plasma Insulin**

The saliva and plasma insulin responses to the meals for both groups are presented in Figure 9. Total and incremental AUC are shown in Figure 10. Significant Meal X Group interactions were observed for salivary insulin AUC (p=0.025), plasma insulin AUC (p=0.014), salivary insulin iAUC (p=0.036) plasma insulin iAUC (p=0.015).

Comparison of LCM to HCM within groups: In the lean group, salivary insulin AUC (by ~89%; p=0.005, d=1.7), plasma insulin AUC (by ~205%; p<0.001, d=3.7), salivary insulin iAUC
(by ~307%; p=0.002, d=2.0) and plasma insulin iAUC (by ~519%; p<0.001, d=3.4) were higher after HCM as compared to LCM. In the OO group, salivary insulin AUC (by ~90%; p=0.001, d=2.7), plasma insulin AUC (by ~230%, p=0.002, d=3.2) salivary insulin iAUC (by ~340%; p=0.003, d=2.5) and plasma insulin iAUC (by ~582%; p=0.002, d=2.5) were also higher after HCM as compared to LCM.

Comparison of OO to NW groups within meals: Salivary insulin AUC (by ~100%, p=0.003, d=1.9), plasma insulin AUC (by ~98%, p=0.014, d=2.0) salivary insulin iAUC (by ~106%, p=0.022, d=1.4) and plasma insulin iAUC (by ~111%, p=0.020, d=1.8) were significantly higher in the OO group as compared to the NW group after the HCM. After the LCM, salivary insulin AUC (p=0.010, d=1.6) and plasma insulin AUC (p=0.007, d=2.2) were significantly higher by ~100% and ~83%, respectively, in the OO group as compared to the NW group. Plasma insulin iAUC and salivary insulin iAUC, despite being higher in the OO group compared to the NW group after LCM, did not reach statistical significance (respectively p=0.067, d=1.2 and p=0.119, d=0.9).
Figure 9. Two-hour plasma and saliva insulin responses to low-carbohydrate (LCM) and high-carbohydrate (HCM) breakfast meals in normal weight (NW) and overweight/obese (OO) participants. A) Plasma insulin levels in NW participants. B) Plasma insulin levels in OO participants. C) Saliva insulin levels in NW participants; and (D) saliva insulin in OO participants. Statistical analyses were performed on the areas under the curve shown in Figure 10.
Figure 10. Plasma and saliva insulin area under the curve (AUC) in normal weight (NW) and overweight/obese (OO) following low-carbohydrate (LCM) and high-carbohydrate (HCM) breakfast meals. A) Plasma AUC in NW and OO participants. B) Plasma incremental AUC (iAUC) in NW and OO participants. C) Saliva AUC in NW and OO participants; and (D) Saliva iAUC in NW and OO participants. *P≤0.005 vs. LCM meal within group. #P<0.05 vs. NW group within meal.

**Plasma glucose**

The plasma glucose responses to the meals for both groups are shown in Figure 11. No Meal X Group interactions were found for plasma glucose AUC (p=0.436) (NW; HCM: 618±189 vs LCM: 586±94, OO; HCM: 751±156 vs LCM: 678±94) or plasma glucose iAUC (p=0.261) (NW; HCM: 64±108 vs LCM: 21±32, OO; HCM: 117±118 vs LCM: 33±42). The main effect of meal for plasma glucose AUC approached statistical significance (HCM: 680±182 vs LCM: 629±102, p=0.057). However, there was a significant main effect of meal for plasma glucose iAUC (HCM;
89±112 vs LCM; 27±36, p=0.012) indicating higher values after the HCM compared to the LCM, as expected. There were no significant differences between the NW or OO groups for plasma glucose AUC (p=0.296) and iAUC (p=0.122).

Figure 11. Two-hour plasma glucose responses to low-carbohydrate (LCM) and high-carbohydrate (HCM) breakfast meals in normal weight (NW) and overweight/obese (OO) participants. A) Plasma glucose in NW participants. B) Plasma glucose in OO participants.

Relationships between plasma and saliva insulin

The fasting saliva:plasma insulin ratio was 1:3.6 in NW and 1:2.8 in OO. Fasting plasma insulin and fasting saliva insulin showed a significant positive correlation (\(\rho = 0.602, p=0.017\)) (Fig.12A). Plasma insulin AUC was significantly correlated with saliva insulin AUC after the LCM (\(r = 0.821; p<0.001\)) and the HCM (\(r = 0.882; p<0.001\)) (Fig.12B, 12C). Saliva flow rate over 60
seconds was not significantly different across each time points or between meals and groups (data not shown).

Figure 12. Relationships between plasma and saliva insulin concentrations. A) Fasting plasma and saliva insulin. B) Two-hour plasma and saliva insulin total area under the curve (AUC) following the high-carbohydrate breakfast meal (HCM). C) Two-hour plasma and saliva AUC following the low-carbohydrate breakfast meal (HCM).

Peak insulin values

The peak salivary and plasma insulin values are presented in Table 5. Significant Meal X Group interactions were observed for peak salivary insulin (p=0.012) and peak plasma insulin (p=0.008).

Comparison of LCM to HCM meals within groups: In the lean group, peak salivary insulin (p=0.008) as well as peak plasma insulin (p=0.001) were higher after HCM as compared to LCM. In the OO group, peak salivary insulin (p=0.001) and peak plasma insulin (p<0.001) were also higher after HCM as compared to LCM.

Comparison of OO to NW groups within meals: Peak salivary insulin (p=0.028) and peak plasma insulin (p<0.001) were significantly higher in the OO group as compared to the NW group after the LCM. After the HCM, peak salivary insulin (p=0.004) and peak plasma insulin (p=0.002) were also significantly higher in the OO group as compared to the NW group.
Table 5. Insulin peak values following the ingestion of mixed meal breakfasts in normal weight and overweight/obese participants.

<table>
<thead>
<tr>
<th></th>
<th>Normal weight</th>
<th>Overweight/Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td><strong>LCM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak saliva insulin (mU/L)</td>
<td>1.0-3.9</td>
<td>2.5 (1.1)</td>
</tr>
<tr>
<td>Peak plasma insulin (mU/L)</td>
<td>7.6-13.3</td>
<td>9.7 (2.1)</td>
</tr>
<tr>
<td><strong>HCM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak saliva insulin (mU/L)</td>
<td>2.5-8.2</td>
<td>4.8 (2.2)#</td>
</tr>
<tr>
<td>Peak plasma insulin (mU/L)</td>
<td>22.1-61.8</td>
<td>39.9 (15.8)#</td>
</tr>
</tbody>
</table>

*Meal X Group interactions were significant for all insulin outcomes (P<0.05)*

*significantly different from normal weight within meal

**significantly different from low-carbohydrate meal within group

**DISCUSSION**

The objective of this study was to verify if salivary insulin could be used as a tool to delineate between high and low insulin levels following the ingestion of a high- or low-CHO mixed meal. Results consistently demonstrated that expected differences in saliva insulin responses to meals were evident; the HCM led to larger postprandial insulin responses compared to the LCM and OO participants had higher responses than NW. Fasting saliva insulin was also higher in OO versus NW participants and significant correlations between saliva and plasma insulin were found for fasting insulin and the insulin AUC after both the HCM and LCM meal.

Obesity is generally accompanied by metabolic impairments including insulin resistance and hyperinsulinemia [286]. In accordance with Marchetti et al [279], the present study showed that fasting insulin differences between groups were detectable in both plasma and saliva samples with higher levels observed in the OO as compared to NW (See Table 5). Fasting saliva insulin concentration was ~30% of the plasma concentration with absolute mU/L values.
slightly higher, but in the same general range, to those previously recorded in adults without diabetes [279, 281, 282]. Small differences in fasting saliva insulin levels could be due to the fact that these earlier studies used a radioimmunoassay technique whereas we used ultrasensitive ELISA. We also discovered interference in the insulin ELISA with undiluted saliva, a phenomenon that did not appear to be tested in these previous studies. Nonetheless, fasting saliva and plasma insulin were positively correlated ($\rho = 0.60$, $p = 0.017$) indicating individuals with higher basal plasma insulin levels also had higher fasting concentration of saliva insulin. A correlation of $r = 0.92$ was previously observed in the fasting state by Fabre et al. 2012 using a larger sample of overweight adolescents [278]. The stronger correlation observed in this study might be due to the difference in sample size ($n = 277$ vs $n = 15$) and/or the population (children vs adults). The differences between OO and NW groups in fasting saliva (and plasma) insulin were seen despite both groups having glucose levels in the normoglycemic range, highlighting the potential utility of saliva insulin for detecting underlying IR.

Significant Meal x Group interactions were observed for 2-hour plasma and salivary insulin AUC. Since differences in baseline (fasting) insulin levels were detected, we also computed the respective iAUC, which also revealed similar statistically significant meal X group interactions. **Cohen’s $d$** effect sizes for pairwise comparisons were large to very-large (0.9 to 3.7) and confirmed the magnitude of the differences observed between LCM and HCM as well as the NW and OO groups. CHO are known as the most potent stimulator of insulin secretion [24] with increased secretion observed when combined to insulinoitropic amino acids [287]. In our study, the HCM increased the total and incremental insulin AUC over the 2-hour postprandial period as compared to LCM in both groups with a greater increase in the OO as compared to NW. Importantly, these differences were similarly observed in both plasma and saliva samples suggesting that expected differences in insulin levels following the consumption of distinct meals can potentially be tracked in the saliva. Previously Fekete et al. 1993 and Messenger et al. 2003 showed that salivary insulin was increased following a mixed meal tolerance test in lean and
overweight individuals (body mass index 21-28 kg/m²) [281, 282]. However, the effects of body mass index on salivary and plasma insulin levels were not assessed in these studies nor were different meals compared. Thus, our observations add to the limited literature on saliva insulin monitoring by showing that differences in salivary insulin can be tracked between meals with different insulinogenic effects and between NW and OO participants.

It is of interest to note the significant correlations between plasma and salivary insulin total AUC (LC: \( r = 0.821 \) and HC \( r = 0.882 \)). Positive linear correlations varying from 0.50 to 0.91 have previously been observed after both an oral glucose tolerance or single meal tolerance tests in healthy adults, obese individuals and people with both T1D and T2D [279, 283, 288]. Generally speaking, the peak insulin response in saliva was delayed by 30-45 minutes relative to blood, although without traceable insulin and more frequent sampling we were not able to quantify the exact time lag for peak insulin from blood to saliva. In line with our findings, a 15 to 60 minute delay in the peak concentration of salivary insulin has consistently been reported by other groups following a mixed meal [281, 282] and an oral glucose load [279, 283].

No interactions or group effect were observed in terms of glucose AUC and \( \text{iAUC} \). However, we observed a MEAL effect for the postprandial glucose \( \text{iAUC} \) (\( p = 0.012 \)) while the AUC approached significance level (\( p = 0.057 \)). The absence of glucose differences between groups combined with the higher insulin levels in OO as compared NW suggests that the OO group was able to successfully compensate for a higher degree of insulin resistance by increasing insulin secretion. Moreover, these results suggest that some metabolic impairments and underlying insulin resistance are present, and can be detected with both plasma and saliva insulin measurements, in overweight to obese but otherwise healthy, young and normoglycemic participants.

This study has some limitations that should be acknowledged. First, the relatively small sample size limit our ability to fully appreciate the range of achievable postprandial saliva insulin levels in healthy adults. Second, our results are limited to the insulin and glucose responses in
the fasting state and following a morning mixed meal, which might differ from meals consumed at other times of the day. Finally, more frequent samples along with a longer postprandial period (e.g., 3-4 hours) would have been useful in order to better compute the insulin curves and determine the timing of when saliva insulin returns to basal levels. Despite the above limitations, our study is strengthened by the fact that we used a randomized crossover design for the meal intervention and assessed metabolic responses to “real-life” mixed-meals.

In conclusion, this study provides evidence that saliva could potentially be used to delineate between high and low insulin levels in both the fasting state and following mixed-meals. Additional studies using larger sample sizes are warranted to better explore the kinetics and consistency of salivary insulin responses following mixed-meals to further validate this non-invasive assessment of metabolic health.
Chapter 3: Short-term low-carbohydrate high-fat diet with or without post-meal walks on glycemic control and inflammation in type 2 diabetes: A randomized trial.

BACKGROUND

Inflammation is associated with the pathogenesis of insulin resistance, T2D and related complications. Immune cells become activated and infiltrate various tissues contributing to a state of chronic low-grade inflammation [289]. High glucose promotes pro-inflammatory cytokine release, elevates surface protein expression of the key innate immune cell activator TLR4, potentiates pro-inflammatory signaling pathways (e.g., JNK), and causes release of characteristic pro-inflammatory MPs in monocytes/macrophages [290-292]. In T2D, elevated levels of circulating pro-inflammatory cytokines [293], increased monocyte TLR4 expression [294], over-activation of the JNK pathway in peripheral blood mononuclear cells (PBMCs) [295], and elevated circulating monocyte-derived microparticles (MMPs) [134] have been repeatedly demonstrated, linking mechanistic work with clinical features of T2D. Thus, high glucose appears to trigger activation of innate immune cells, suggesting that hyperglycemia itself drives a vicious cycle of inflammation and IR in T2D.

Lifestyle therapy is a frontline treatment for improving glucose control in people with T2D. Most current dietary guidelines advocate a “healthy” diet low in saturated fat and sugar, with emphasis on low GI foods. However, accumulating evidence shows that consuming a LCHF diet is more effective for lowering glucose [296]. In a companion paper, we demonstrated that a short-term LCHF diet quickly stabilizes glucose in T2D patients [297]. However, whether the rapid improvement in glucose control is accompanied by lowered inflammation has, to our knowledge, not been tested. It is possible that factors related to T2D pathophysiology, other than hyperglycemia, are more important in driving chronic inflammation. It is therefore unknown whether lowering glucose by following a LCHF diet can reduce innate immune cell activation and inflammation in T2D.
Consuming a LCHF diet with less than 130 grams of CHO is contraindicated by the American Diabetes Association, partly due to the perceived negative impacts of consuming high amounts of fat [298]. Acute feeding studies indicate that an isolated “high-fat meal” increases inflammation and triggers pro-inflammatory activation of immune cells, particularly in individuals with insulin resistance or T2D [145, 299]. Thus, attempting to lower hyperglycemia using a LCHF diet may have unintended consequences. Strategically-timed exercise may be one way to counteract this. Padilla et al. have shown that exercising in the postprandial period reversed the detrimental impacts of a high-fat meal on endothelial function [300]. Additionally, several recent studies have highlighted the benefits of post-meal walking exercise on glycemic control [301]. Combining a LCHF diet with post-meal exercise may be an optimal combination for improving glucose control and reducing inflammation.

The objective of the present study was to determine whether reducing hyperglycemia with a LCHF diet could lower markers of innate immune cell activation and systemic inflammation in people with T2D. A secondary objective was to examine if the combination of a LCHF diet with strategically-timed post-meal walking was superior to a LCHF diet alone. In order to reduce the confounding influence of both weight loss and long-term adaptation to a LCHF diet [302] we employed a 4-day controlled feeding protocol in attempts to isolate the direct effects of lowering glucose using a whole food LCHF diet, with or without exercise. A control condition involving low GI, low-fat whole foods was employed to directly compare the LCHF conditions to “gold-standard” dietary guidelines [177]. The primary outcome was glucose control, defined as average glucose assessed by continuous glucose monitor (CGM). Secondary outcomes included a comprehensive array of systemic, cellular, and molecular indices of inflammation from plasma and PBMCs, along with standard markers of metabolic control.
METHODS

Ethical approval
The study was approved by the University of British Columbia Clinical Research Ethics Board (ID H15-01952) and was registered on clinicaltrials.gov (NCT02683135). The study conformed to the standards set by the Declaration of Helsinki. Participants provided written informed consent prior to study commencement during the initial screening visit.

Overview
Individuals with physician-diagnosed T2D (HbA1c >6.5%, FPG >7.0 mmol/L, or 2-h glucose OGTT >11.1 mmol/L (32)) were recruited to complete three, short-term controlled intervention periods in a randomized crossover design: i) Low-fat low-glycemic index diet, (GL); ii) low-carbohydrate high-fat diet (LC); and LC with 15-min post-meal walks (LC+Ex). The randomization sequence was generated by a computer random number generator and was revealed to study staff from a master list after participant provided informed consent. Neither participants nor staff were blinded. All food was provided to participants using a local service (https://mealprepforyou.ca/) and diets were matched for energy and protein content. Fasting blood samples following an overnight fast were collected before and after each experimental intervention. Accelerometers (Actigraph wGTX3+) were worn to monitor activity for all conditions and confirm the completion of post-meal walks.

Participants
Sixteen (8 males and 8 females) participants aged between 48-72 y, not on exogenous insulin and without diagnosed cardiovascular, kidney or any other diabetes complications were recruited from the local community in Kelowna, BC, Canada from November 2015 to March 2017. Individuals currently involved in a regular exercise routine (>3 days of structured exercise per week), following a LCHF diet, or unwilling to consume the provided meat-containing diets
were excluded. Five of the sixteen participants did not complete all three conditions, due to family reasons (n=1), inability or unwillingness to follow study diets (n=3) and change of medications (n=1, addition of SGLT2 inhibitor after completing one condition) (Figure 13). Participants were instructed to take their medications as usual during the three experimental conditions and their compliance was assessed with a medication log.

This study was designed as a pilot trial in order to generate effect sizes to guide a larger randomized controlled trial. Nygaard et al. (2009) previously demonstrated a ~15% reduction in postprandial glucose area under the curve with post-meal walking [303]. Using means and SD for CGM from the literature and our own T2D studies [304], a sample size of 11 would be needed to detect a 15% difference in mean glucose with 80% power at an alpha of 0.05 assuming a correlation among repeated measures of r = 0.7 (G*Power v3.1.9.3). To account for drop-outs and/or missing data, we aimed to recruit 16 patients with T2D.

**Experimental Protocol**

During the initial screening visit, anthropometric measurements (height, body weight, body mass index and waist circumference) were taken and both a physical activity readiness and a Godin leisure time exercise questionnaires were filled, followed by the three randomized 4-day diet interventions. The 24-hour period preceding each diet interventions was standardized for physical activity and included a standardized mixed meal on the evening followed by a ≥10-hour fast. A washout period of 9-14 days was introduced between each intervention where participants were asked to return to their regular diet and physical activity habits. Fasting blood samples were collected at the same time in the morning before and after each 4-day intervention for future analysis. During each intervention, a CGM (iPro² professional CGM, Medtronic, Northridge, CA, USA and Enlite™ sensor) was worn to measure glycemic control. Average blood glucose across four days was the primary outcome. Area under the curves were calculated using the trapezoid method [305] as the mean amplitude of glycemic excursion
(MAGE) and continuous overall net glycemic action (CONGA) were computed using EasyGV (Version 9.0.R2., University of Oxford). All intervention diets were isoenergetic, with calories estimated using the Harris Benedict equation [306] and habitual intake (i.e., matched from first trial). An example meal plan for one-day of each diet is provided in Table 6.

**4-day low-fat low-glycemic index diet (GL)**

GL diet followed the current dietary guidelines for adults with T2D comprising low-fat, low glycemic index, whole foods [307]. Each meal comprised ~55% of total energy from carbohydrate (predominately from low glycemic index and high fiber carbohydrate sources), 20% energy from fat (aiming for <7% saturated fatty acids); and 25% protein (primarily from lean meats).

**4-day low-carbohydrate high-fat diet (LC)**

LC diet provided the same energy content as the GL diet but with CHO limited to ~10% of total energy. The percent protein was matched at ~25%, with the remainder of the energy coming from fat (~65% of total kcal).

**4-day low-carbohydrate high-fat diet with 15-min post-meal walks (LC+Ex)**

Participants performed 15 minutes of walking beginning ~30 minutes after breakfast, lunch and dinner. The exercise intensity of the post-meal walking was light-to-moderate, which was confirmed on day-one of the intervention by having participants walk on a horizontal treadmill in the laboratory at a comfortable pace that elicited a rating of perceived exertion (CR-10 scale) of 3 ± 1 (equating to ~60% of maximal heart rate, 93 ± 13 beats per minute). Participants were instructed to replicate this pace at home for each post-meal walk. Accelerometers were worn to confirm compliance and intensity. Participants consumed the same diet as the LC intervention but with the addition of the estimated individualized calories expended over the three daily 15
minutes post-meal walks ((2.5 metabolic equivalents (METs) x 3.5 x Weight (kg) x 45 (minutes)) / 200) [308].

**Hormones and metabolites**

Morning fasting blood samples were collected by venipuncture using a 21-gauge needle into EDTA tubes and were centrifuged at 1,550g for 15 min at 4°C. Following centrifugation, plasma samples were stored at −80°C for batch analyses. Plasma TG (Pointe Scientific INC, MI, USA) and glucose (Pointe Scientific INC, MI, USA) were analyzed on a Chemwell 2910 automated analyzer (Awareness Technologies, Palm City, USA). Plasma insulin (Human Insulin ELISA, Crystal Chem, IL, USA) was analyzed on an iMark™ Microplate Absorbance Reader (Bio Rad, CA, USA). Active proinsulin (ALPCO, STELLUX Chemi Human Total Proinsulin ELISA, NH, USA) was analyzed on an POLARstar Omega plate reader (BMG Labtech, Durham, NC, USA). Plasma C-peptide was measured by Meso Scale Discovery C-peptide Singleplex (MD, USA). All assays were run in duplicate. The coefficient of variation (CV) for duplicate samples was 2.9% (TG), 3.0% (glucose), 5.0% (insulin), 4.5% (proinsulin) and 6.6% (C-peptide).

**Inflammatory markers**

Fasting plasma concentrations of TNF-a, MCP1, IL-6, IL-10 and IL-18 were analyzed by multiplex immunoassay (U-PLEX Human Panel, Meso Scale Discovery, MD, USA) and read on a Meso Quickplex SQ 120. Plasma was centrifuged at 1,500g for 15 min at 4°C to remove debris and analyzed in duplicate. The CV was 7.6% (TNF-a 4.6% (MCP1), 5.7% (IL-6), 7.9% (IL-10) and 4.1% (IL-18).

**Flow cytometry**

Fc Receptor blocking reagent (130-059-901; Miltenyi Biotec, Bergisch Gladbach, Germany) was added to 90 µl of whole blood and incubated for 10 min at 4°C in the dark. Conjugated
antibodies (all Miltenyi Biotec) for human cluster of differentiation (CD) 14 (Vioblue, 130-094-364), TLR2 (PE, 130-099-016), and TLR4 (APC, 130-096-236) were added followed by a 10 min incubation at 4°C in the dark. Next, 1 ml of red blood cell lysis buffer (120-001-339, Miltenyi Biotec) was added followed by a 15 min incubation at room temperature in the dark. 2 µl of propidium iodide (130-093-233; Miltenyi Biotec) was added for dead cell exclusion and samples were analyzed on a MACSQuant Analyzer 10 flow cytometer. Ten thousand monocytes were counted in each sample and data were analyzed with MACSQuantify version 2.6 (Miltenyi Biotec). CD14+ monocytes were identified via a hierarchical gating strategy. Specifically, cells that stained positive for PI were excluded, and then cells were characterized as CD14+ and confirmed to be monocytes via characteristic scatter profile. TLR2 and TLR4 median fluorescence intensity were then determined on CD14+ monocytes with fluorescence minus one controls used to determine positive and negative populations. Total granulocyte, monocyte, and lymphocyte numbers were determined based on characteristic scatter profiles.

**Monocyte and leukocyte-derived microparticles**

MMPs and leukocyte-derived microparticles (LMPs) were characterized using flow cytometry, as previously described [309, 310]. Plasma samples were centrifuged at 13,000g for 2 minutes and 200µL of platelet-free plasma was transferred to TruCount tubes (BD Biosciences, New Jersey, USA). MP size threshold was established using Megamix-Plus SSC calibrator beads (Biocytex, Marseille, France) and only events <1 µm in size were counted. MPs were defined as events falling within the established size ranges (0.16, 0.20, 0.24 and 0.5 µm). Monocyte CD14+ and leukocyte CD45+ specific antibodies (BioLegend, San Diego, California) identified MMPs and LMPs for events falling within the respective MP size range. Samples were incubated with antibodies for 20 minutes in the dark at room temperature, fixed with 2% paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, California), diluted with PBS, and analyzed using BD Biosciences FACSAria I High Speed Cell sorter and flow cytometer (University of Colorado
Anschutz Medical Campus Allergy and Clinical Immunology/Infectious Disease Flow Core). The concentration of MMPs and LMPs were determined using the formula: ([number of events in region containing MPs / number of events in absolute count bead region] x [total number of beads per test / total volume of sample]). MP analyses were performed on n=8.

**Western Blotting Analysis**

PBMCs were extracted from ~8 ml whole blood using Leucoep® tubes (163288, Greiner Bio-One International GmbH, Kremsmunster, Austria) according to the manufacturer’s instructions and stored at -80°C for batch analyses. Samples were lysed by sonication in ice-cold RIPA buffer (50mM Tris-HCL, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl) containing protease (#04693159001; complete™, Protease Inhibitor Cocktail, Roche) and phosphatase (100mM NaVO₃, 20mM NaF, EDTA 10mM) inhibitors and centrifuged (14,000g for 20 minutes). Protein was quantified in supernatants with the bicinchoninic acid assay (#23225, Pierce™ BCA Protein Assay Kit) and 20µg samples were prepared in Laemmli Buffer (#7722, Blue Loading Buffer, 1.25M DTT), heated at 95°C for 5 minutes and separated using 12% SDS-polyacrylamide gel electrophoresis (#170-3930, Mini Trans-Blot®, Bio-Rad). Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes using Towbin Buffer (25mM Tris-HCL, 192mM glycine, 20% methanol, pH 8.3) for 3h at 70V. Membranes were stained with Ponceau S Solution (P7170 Sigma-Aldrich®) to verify efficiency of protein transfer. Membranes were blocked in TBS-T+iBlock solution (20mM Tris-HCL, 140mM NaCl, 0.1% Tween-20, 0.2% iBlock™) for 1h at RT and then incubated in primary Phospho-SAPK/JNK (Thr183/Tyr185; phosphorylated-JNK (p-JNK)) antibody (#4668, 1:1000, Cell Signaling Technology®) in TBST+iBlock solution overnight at 4°C followed by secondary anti-rabit IgG HRP-Linked antibody (#7074, 1:5000, Cell Signaling Technology®) in TBST+iBlock solution for two hours at RT. Proteins were detected using Luminata Forte HRP Substrate (#WBLUF0100, Millipore Sigma®) and the images were acquired using Amersham Hyperfilm ECL (#28906835, GE Life
Membranes were stripped (#21059, ThermoFisher Scientific) for 20 minutes at RT and blocked again in TBST+iBlock. The same procedure was adopted to quantify total SAPK/JNK (#9252, 1:1000, Cell Signaling Technology®). Results were quantified using Adobe Photoshop CC 2017 and relativized to the protein content using the Ponceau Staining as described by Gilda et al. [311]. Samples from each participant were run on the same gel and bands were expressed relative to an internal standard containing pooled human PMBC lysate included in each blot. PBMC analyses were performed on n=6 due to sample availability.

Statistical analysis
Data were analyzed using SPSS v.21 (SPSS Inc., Chicago, IL, USA). Normality was assessed using Q-Q plots and Shapiro-Wilk tests within each experimental condition. Four-day CGM data were analyzed using a one-way repeated-measures ANOVA with Fisher’s LSD post-hoc testing. A linear mixed-effects model (Condition and Time as fixed factors, Subject as random factor) was used to determine the treatment effects for all inflammatory and metabolic markers. Significant interactions and time effects were followed up with pre-planned contrasts comparing pre vs. post-intervention within each condition. Cohen’s $d$ effect size was calculated for these pre-planned comparisons within each condition. Significance was set at $P < 0.05$. Data in Tables 8-10 and Figures 14-17 are presented as mean (SD).
Figure 13. Consolidated standards of reporting trials flow diagram. CVD, cardiovascular disease.
### Table 6. Composition of study diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>GL</th>
<th>LC</th>
<th>LC+Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein: Fat %</td>
<td>5: 5: 10</td>
<td>15: 11: 39</td>
<td>15: 11: 39</td>
</tr>
<tr>
<td>Sat.:</td>
<td>40</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Poly.:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mono.:</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

| Mean glycemic index | 40 | N/A | N/A |

| Diet | CHO: Carbohydrates, GL: Low-glycemic index guidelines diet, LC: Low-carbohydrate high-fat diet, LC+Ex: Low-carbohydrate high-fat diet and exercise, Sat.; Saturated fat, Poly.; Polyunsaturated fat, Mono.; Monounsaturated fat. Menu shown is an individual example based on a daily energy intake of ~1700 kcal including 3 meals of 500 kcal each and 200 kcal snacks for a 68-yr old female participant with an estimated energy expenditure of 1700 kcal per day (body mass of 68.2 kg, height 1.51 m, physical activity level of 1.3). In the LC+Ex condition, 135 kcal was added to compensate for the daily post-meal walks. |
|------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Breakfast | 95g-Oats | 25g-Whey | 20g-Blueberries | 30g-Raspberries | 150g-Whole Egg | 110g-Egg Whites | 55g-Avocado | 30g-Peppers | 40g-Onions | 40g-Carrots | 10g-Almonds |
| Lunch | 105g-Chicken Breast | 230g-Yams | 40g-Green Beans | 17g-Cashew | 105g-Ground Turkey | 38g-Cashew | 15g-Olive oil | 35g-Spinach | 30g-Carrots | 30g-Cucumber |
| Dinner | 100g-Turkey | 85g-Brown Rice | 14g-Cashew | 40g-Broccoli | 100g-Steak (Rib eye) | 30g-Cashew | 13g-Olive Oil | 30g-Apple | 30g-Spinach | 50g-Cucumber |
| Snack | 1 x Solo Bar (Solo GI Nutrition) | 50g-Cheddar cheese | 20g-Almonds |

RESULTS

Baseline characteristics and body weight changes

Characteristics of the participants who completed the study are shown in Table 7. Leisure-time exercise per week was 0.1 ± 0.3 for strenuous exercise, 1.7 ± 0.9 for moderate exercise and 2.3 ± 0.7 for light exercise. The average total score (arbitrary units calculation; (9 x strenuous) + (5 x moderate) + (3 x lights)) at baseline was 16.3 ± 4.5. A significant overall time effect was observed for body weight, which decreased by (-2.0 ± 1.0 kg, -2.0 ± 0.8 kg and -1.9 ± 1.1 kg for LC, LC+Ex and GL respectively, \( P < 0.001 \)) with no condition by time interaction (\( P = 0.969 \)).
Table 7. Characteristics of participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants (M/F)</td>
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</tr>
<tr>
<td>Age (year)</td>
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<tr>
<td>Body mass index (kg/m²)</td>
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<tr>
<td>Waist circumference (cm)</td>
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</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>124 (9)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77 (5)</td>
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<tr>
<td>Glycated haemoglobin (%)</td>
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</tr>
<tr>
<td>Time since diagnosis (year)</td>
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</tr>
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</table>

**Medications**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET (n)</td>
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</tr>
<tr>
<td>MET + SU (n)</td>
<td>2</td>
</tr>
<tr>
<td>MET + GLP-1 (n)</td>
<td>1</td>
</tr>
<tr>
<td>MET + SU + DPP4 (n)</td>
<td>1</td>
</tr>
<tr>
<td>Statin (n)</td>
<td>3</td>
</tr>
<tr>
<td>Anti-hypertensive (n)</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD) except for number of participants (count; male/postmenopausal female) and medications (count). MET; Metformin, SU; Sulfonylurea, GLP-1; Glucagon-like-peptide-1, DPP4; dipeptidyl peptidase-4.
**Accelerometer**

One-way repeated measures ANOVA showed a significant effect for length of time at moderate intensity over the 4-day interventions (235 ± 136) in LC+Ex compared to LC (75 ± 92) and GL (117 ± 137) (P<0.001). As expected, total moderate intensity minutes in LC+Ex were higher when compared to LC (+161 ± 107 minutes, P = 0.01, d = 4.5) and GL (+119 ± 88 minutes, P = 0.02, d = 3.8) with no significant difference between LC and GL. Time spent in sedentary, light and vigorous intensity activity was similar between all conditions (data not shown).

**Continuous glucose monitoring**

Four-day continuous glucose monitoring data are presented in Table 8. One-way repeated measures ANOVA showed a significant effect for 4-day mean, standard deviation, MAGE, CONGA and time over 10 mmol/L (all P < 0.02). Four-day mean glucose was significantly lower in the LC+Ex as compared to LC (-5%, P < 0.05, d = 0.8) while both LC+Ex and LC conditions were lower than GL (-16%, P < 0.001, d = 1.9 and -12%, P < 0.001, d = 2.3, respectively) (Figure 14A). CONGA was also significantly lower in the LC+Ex as compared to LC (-5%, P < 0.05, d = 0.9) while both LC+Ex and LC conditions were lower than GL (-11%, P < 0.01, d = 1.2 and -6%, P < 0.01, d = 1.2 respectively). LC+Ex and LC were not significantly different but were both respectively lower than GL for standard deviation (both -53%, P < 0.01, respectively d = 1.6 and d = 2.4), MAGE (-61%, P ≤ 0.001, d = 2.1 and -54%, P < 0.001, d = 2.8, respectively) (Figure 14B) and time over 10 mmol/L (-90%, P < 0.05, d = 2.0 and -83%, P < 0.05, d = 2.5). No significant difference between conditions was observed for time under 4 mmol/L.
Figure 14. Mean glucose and mean amplitude of glycemic excursions (MAGEs) from continuous glucose monitoring during each 4-day diet intervention. Mean glucose (A) and MAGE (B) calculated from continuous glucose monitoring throughout each intervention. Fisher least-significant difference post hoc tests following significant one-way repeated-measures ANOVA; GL, low-fat lowglycemic index guidelines diet; LC, low-carbohydrate high-fat diet; LC+Ex, low-carbohydrate high-fat diet and exercise. †P ≤ 0.001 vs. CON. ‡P < 0.05 vs. LC.
Figure 15. Changes in fasting glucose, C-peptide, insulin and proinsulin following each four-day diet intervention. A) Fasting glucose, (B) fasting C-peptide, (C) fasting insulin, and (D) fasting proinsulin presented as change scores (Post minus Pre within each condition). GL: Low-fat low-glycemic index guidelines diet, LC; Low-carbohydrate high-fat diet, LC+Ex; Low-carbohydrate high-fat diet and exercise. Linear mixed models revealed a significant main effect of time for fasting glucose ($P < 0.001$) and a significant condition X time interaction for proinsulin ($P < 0.001$). † $P \leq 0.01$, ‡ $P = 0.001$, for pre-planned contrast of Post vs. Pre within condition.
Table 8. Four-day continuous glucose monitoring

<table>
<thead>
<tr>
<th></th>
<th>GL</th>
<th>LC</th>
<th>LC+Ex</th>
<th>ANOVA</th>
<th>GL vs LC</th>
<th>GL vs LC+Ex</th>
<th>LC vs LC+Ex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (mmol/L)</td>
<td>7.4 (1.6)</td>
<td>6.5 (1.2)</td>
<td>6.2 (1.1)</td>
<td>0.001</td>
<td>0.000</td>
<td>0.001</td>
<td>0.049</td>
</tr>
<tr>
<td>SD (mmol/L)</td>
<td>1.7 (0.9)</td>
<td>0.8 (0.3)</td>
<td>0.8 (0.4)</td>
<td>0.001</td>
<td>0.002</td>
<td>0.003</td>
<td>0.476</td>
</tr>
<tr>
<td>MAGE (mmol/L)</td>
<td>4.3 (2.2)</td>
<td>2.0 (1.0)</td>
<td>1.7 (0.9)</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
<td>0.109</td>
</tr>
<tr>
<td>CONGA (mmol/L)</td>
<td>6.6 (1.4)</td>
<td>6.2 (1.1)</td>
<td>5.9 (1.0)</td>
<td>0.004</td>
<td>0.005</td>
<td>0.005</td>
<td>0.037</td>
</tr>
<tr>
<td>Time &gt;10 mmol/L (%)</td>
<td>13.5 (18.1)</td>
<td>2.3 (5.5)</td>
<td>1.3 (3.9)</td>
<td>0.020</td>
<td>0.018</td>
<td>0.022</td>
<td>0.218</td>
</tr>
<tr>
<td>Time &lt;4 mmol/L (%)</td>
<td>0.3 (0.7)</td>
<td>0.5 (1.1)</td>
<td>1.1 (2.4)</td>
<td>0.332</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD). P-values are shown for the overall one-way repeated-measures ANOVA and for the Fisher LSD post-hoc tests. Please see text for effect sizes. SD; Standard deviation, MAGE; Mean amplitude of glycemic excursion, CONGA; Continuous overall net glycemic action, GL; Low-fat low-glycemic index guidelines diet, LC; Low-carbohydrate high-fat diet, LC+Ex; Low-carbohydrate high-fat diet and exercise.
**Metabolites and hormones**

Metabolites and hormone data are presented in Table 9 and the change from baseline for fasting glucose, c-peptide, insulin and proinsulin are presented in Figure 15. A significant condition by time interaction was found for proinsulin ($P < 0.001$), with a significant decrease in LC (-27%, $P = 0.001$, $d = 1.5$) and LC+Ex (-35%, $P = 0.005$, $d = 1.5$) but not for GL (-8%, $P = 0.065$, $d = 0.7$). A significant main effect of time was observed for fasting glucose and proinsulin:C-peptide ratio (both $P < 0.001$). In pre-planned contrasts, only LC+Ex and LC decreased fasting glucose (-10%, $P = 0.007$, $d = 1.3$, and -10%, $P = 0.011$, $d = 1.0$ respectively) and proinsulin:C-peptide ratio (-31%, $P = 0.045$, $d = 0.7$ and -17%, $P = 0.004$, $d = 1.2$ respectively).

**Inflammatory markers**

Inflammatory markers are presented in Table 10. Western blot images of p-JNK and total JNK in PBMCs as well as change from baseline for p-JNK are shown in Figure 16 and MMPs and LMPs are presented in Figure 17. A significant overall time effect was observed for MCP1 ($P = 0.045$) and p-JNK ($P < 0.001$). In pre-planned contrasts, only LC+Ex decreased MCP1 (-12%, $P = 0.003$, $d = 1.3$), whereas p-JNK decreased in all three conditions (All $P < 0.05$, GL; -32%, $d = 1.6$, LC; -45%, $d = 1.7$, LC+Ex; -44%, $d = 1.4$). Despite the randomized crossover design, a main effect of condition was observed for p-JNK and TLR4 (both $P \leq 0.01$). Levels of p-JNK were overall lower in LC+Ex as compared to GL and LC while overall TLR4 was lower in GL as compared to LC and LC+Ex. There were no differences in total granulocyte, monocyte, or lymphocyte cell counts with any of the interventions (Table 10). A significant condition by time interaction was observed for MMPs ($P = 0.040$) with a significant decrease in GL (-76%, $P = 0.035$, $d = 1.8$) and a tendency for a reduction in LC (-70%, $P = 0.064$, $d = 0.9$) whereas there was no significant
change in LC+Ex (+0.5%, $P = 0.990$, $d = 0.0$). For LMPs, there was a tendency for a significant main effect of time ($P = 0.067$), with exploratory pairwise comparisons within conditions showing tendencies for reductions in GL (-63%, $P = 0.055$, $d = 1.2$) and LC (-16%, $P = 0.057$, $d = 0.9$) but not LC+Ex (-9%, $P = 0.516$, $d = 0.3$).

Figure 16. Changes in PBMCs p-JNK following each four-day diet intervention.  
A) Representative western blot image showing total and phosphorylated JNK (p-JNK) before (pre) and after (post) each intervention. Band intensities were expressed relative to a pooled sample of human PBMCs included in every blot (pool). B) Data for p-JNK/total JNK presented as change scores (Post minus Pre within each condition). GL: Low-fat low-glycemic index guidelines diet, LC; Low-carbohydrate high-fat diet, LC+Ex; Low-carbohydrate high-fat diet and exercise. PBMCs; peripheral blood mononuclear cells, p-JNK; Phosphorylated c-Jun N-terminal kinase. Linear mixed models revealed a significant main effect of time for p-JNK ($P < 0.05$). ‡ $P < 0.05$ for pre-planned contrast of Post vs. Pre within condition.
Figure 17. Changes in MMPs and LMPs following each four-day diet intervention. A) MMPs and B) LMPs presented as change scores (Post minus Pre within each condition). MMPs; Monocyte-derived microparticles, LMPs; Leukocyte-derived microparticles. Linear mixed models revealed a significant condition X time interaction for MMPs (P < 0.05). ‡ P < 0.05 for pre-planned contrast of Post vs. Pre within condition.
Table 9. Fasting hormones and metabolites before (pre) and after (post) each four-day diet intervention.

<table>
<thead>
<tr>
<th></th>
<th>GL</th>
<th>LC</th>
<th>LC+Ex</th>
<th>Linear mixed-model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre (Pre)</td>
<td>Post (Post)</td>
<td>Pre (Pre)</td>
<td>Post (Post)</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.3 (2.1)</td>
<td>8.1 (2.0)</td>
<td>8.4 (1.9)</td>
<td>7.6 (1.7)*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.0 (1.1)</td>
<td>1.9 (0.7)</td>
<td>1.9 (1.0)</td>
<td>2.1 (1.2)</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>63.9 (33.5)</td>
<td>58.0 (29.2)</td>
<td>64.8 (29.7)</td>
<td>62.1 (46.4)</td>
</tr>
<tr>
<td>Proinsulin (pmol/L)</td>
<td>33.5 (14.7)</td>
<td>30.7 (16.1)</td>
<td>35.5 (15.1)</td>
<td>26.0 (12.8)†</td>
</tr>
<tr>
<td>C-peptide (nmol/L)</td>
<td>1.11 (0.39)</td>
<td>1.18 (0.52)</td>
<td>1.18 (0.38)</td>
<td>1.07 (0.45)</td>
</tr>
<tr>
<td>Proinsulin: Insulin (ratio)</td>
<td>0.7 (0.5)</td>
<td>0.6 (0.3)</td>
<td>0.7 (0.5)</td>
<td>0.6 (0.3)</td>
</tr>
<tr>
<td>Proinsulin: C-peptide (ratio)</td>
<td>0.031 (0.013)</td>
<td>0.027 (0.012)</td>
<td>0.030 (0.013)</td>
<td>0.025 (0.008)*</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD). GL: Low-fat low-glycemic index guidelines diet, LC: Low-carbohydrate high-fat diet, LC+Ex: Low-carbohydrate high-fat diet and exercise. * P ≤ 0.01, † P = 0.001, ‡ P < 0.05 for pre-planned contrast vs. Pre within condition.
Table 10. Fasting inflammatory markers before (pre) and after (post) each four-day diet intervention.

<table>
<thead>
<tr>
<th></th>
<th>GL</th>
<th>LC</th>
<th>LC+Ex</th>
<th>Linear mixed-model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>14.6 (4.6)</td>
<td>15.7 (4.6)</td>
<td>14.1 (3.6)</td>
<td>14.8 (4.6)</td>
</tr>
<tr>
<td>MCP1 (pg/ml)</td>
<td>725 (201)</td>
<td>710 (222)</td>
<td>727 (217)</td>
<td>686 (229)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>8.1 (2.8)</td>
<td>9.1 (4.7)</td>
<td>8.2 (3.8)</td>
<td>8.8 (3.6)</td>
</tr>
<tr>
<td>IL-18 (pg/ml)</td>
<td>2810 (1217)</td>
<td>2760 (1152)</td>
<td>2838 (1199)</td>
<td>2818 (1527)</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>3.6 (2.9)</td>
<td>3.3 (2.8)</td>
<td>3.5 (3.4)</td>
<td>3.3 (2.4)</td>
</tr>
<tr>
<td>p-JNK (A.U.)</td>
<td>100 (30)</td>
<td>68 (20) †</td>
<td>105 (40)</td>
<td>58 (19) †</td>
</tr>
<tr>
<td>TLR2 (MFI)</td>
<td>7.5 (1.3)</td>
<td>6.9 (0.9)</td>
<td>7.4 (1.9)</td>
<td>6.9 (0.7)</td>
</tr>
<tr>
<td>TLR4 (MFI)</td>
<td>4.7 (0.3)</td>
<td>4.8 (0.3)</td>
<td>5.0 (0.4)</td>
<td>5.0 (0.4)</td>
</tr>
<tr>
<td>MMPs (count/ml)</td>
<td>404 (330)</td>
<td>97 (55) †</td>
<td>245 (203)</td>
<td>73 (58)</td>
</tr>
<tr>
<td>LMPs (count/ml)</td>
<td>1313 (999)</td>
<td>490 (311)</td>
<td>1055 (1317)</td>
<td>890 (1380)</td>
</tr>
<tr>
<td>Granulocytes (count/ml) x 10⁶</td>
<td>2.4 (0.6)</td>
<td>2.9 (0.6)</td>
<td>2.6 (0.5)</td>
<td>2.6 (0.4)</td>
</tr>
<tr>
<td>Lymphocytes (count/ml) x 10⁶</td>
<td>1.2 (0.2)</td>
<td>1.4 (0.4)</td>
<td>1.3 (0.3)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>Monocytes (count/ml) x 10⁵</td>
<td>2.7 (0.6)</td>
<td>3.0 (0.7)</td>
<td>2.6 (0.8)</td>
<td>2.7 (0.6)</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD). GL: Low-fat low-glycemic index guidelines diet, LC: Low-carbohydrate high-fat diet, LC+Ex: Low-carbohydrate high-fat diet and exercise. A.U.; arbitrary units, MFI; median fluorescence intensity, MMPs; Monocyte-derived microparticles, LMPs; Leukocyte-derived microparticles, p-JNK; Phosphorylated c-Jun N-terminal kinase. † P < 0.01, † P < 0.05 for pre-planned contrast vs. Pre within condition.
DISCUSSION

The main objective of this study was to determine whether reducing hyperglycemia by following a 4-day LC diet alone, or in combination with post-meal walking, could lower markers of innate immune cell activation and systemic inflammation in people with T2D. Our study showed that while LC and LC+Ex led to superior improvements in glucose control and fasting proinsulin levels as compared to GL, all three diets appeared to lower PBMC p-JNK (a marker of cellular inflammation) over the short-term.

**LCHF diets improve glucose control**

Recently, LCHF diets have been recommended as a first-line treatment for improving glucose control in T2D [173]. In studies lasting between 6-12 months, LCHF diets have been shown to reduce HbA1c and fasting glucose to a greater extent than a traditional western diet or a low-glycemic diet [312, 313]. In line with our results, the glucose-lowering effect of carbohydrate restriction can be observed quickly with no or very minimal weight-loss [314, 315]. In addition to restricting CHO, light walks performed around meal times have been shown to lower glucose excursions [316, 317]. To the best of our knowledge, our study is the first to test the combined strategy of a LC diet with post-meal walks (LC+Ex) with findings showing that this approach can improve 24-hour glucose control to a greater extent than both a LC diet alone or a GL diet. The LC diet alone was also clearly effective at lowering 24-hr glucose when compared to GL. Change in fasting glucose was not different between conditions but a significant decrease within LC and LC+Ex of similar magnitude as a previous 7-day low-carbohydrate diet study in overweight/obese individuals was observed [318]. The short duration of the current study might explain the absence of change in fasting glucose as significant weight loss or longer-term metabolic adaptations may be needed to reduce hepatic glucose output and/or insulin resistance [296, 319]. The dysglycemia of diabetes is characterized by sustained chronic hyperglycemia, postprandial glucose fluctuations, and increased glucose variability [320]. As
compared to GL, both LC and LC+Ex improved all of these glycemic outcomes including MAGE, which has been shown to be strongly and positively correlated to oxidative stress and inflammation markers [321]. Activation of oxidative stress and inflammatory pathways contributes to insulin resistance and diabetes complications [322, 323]. Therefore lowering overall hyperglycemia and glycemic variability in LC and LC+Ex may be advantageous in T2D.

**Impact of short-term dietary interventions on inflammation**

The phosphorylation of JNK is regarded as a key signaling node controlling inflammation by activating transcription factors that result in the production of pro-inflammatory cytokines and chemokines [324, 325]. Murine studies support a causal role of myeloid JNK activation in the progression of insulin resistance, adipose tissue macrophage accumulation, and systemic inflammation [326]. JNK activation is increased in T2D and can be triggered by several metabolic stressors such as elevated glucose, circulating free fatty acids, insulin, cytokines and oxidative stress [327-329]. Thus, activation of JNK in PBMCs may represent both a systemic marker of inflammation and an underlying signaling pathway involved in T2D pathophysiology. In the present study, all three short-term dietary interventions led to significant reductions in PBMCs p-JNK. It is possible that the small (~2 kg) weight-loss experienced in each intervention could contribute to the reduction in JNK activation. Since body weight went back to pre-intervention during washout periods, it is likely that participants were consuming slightly less calories during the three 4-day periods as compared to baseline. Alternatively, all three dietary conditions may have been less pro-inflammatory than the typical dietary pattern of participants given that we provided minimally processed foods with low sugar and refined carbohydrate content.

Cell-derived MPs are small (~100-1000 μm) plasma membrane vesicles that are released by most eukaryotic cells in response to activation and/or apoptosis. Depending on the cell of origin, circulating MPs exert distinct biological effects [330, 331]. MPs derived from
monocytes and leukocytes are known to play a role in inflammation [132, 332]. For example, MMPs have been shown to induce an upregulation in endothelial cell expression of intracellular cell adhesion molecule-1 and promote T-cell infiltration into the vessel wall enhancing plaque formation [333]. In addition, LMPs impact the endothelial monolayer stimulating inflammatory processes, such as the JNK signaling pathway [334], and the recruitment of chemotactic cytokines [132]. In the present study, circulating concentrations of MMPs were markedly lower in response to the GL (~75%) and LC (~70%) diets. Whilst the main effect of each diet on LMPs was not as great as MMPs, the GL and LC diet tended to reduce circulating LMP concentrations. Reductions in circulating MMPs and LMPs supports anti-inflammatory effects of each diet, potentially via reduced monocyte and leukocyte activation. Interestingly, there were no significant reductions in either MMPs or LMPs in response to combined LC diet with post-meal light-to-moderate exercise. It is plausible that exercise induced a moderate, transient increase monocyte and leukocyte activation, and in turn MP vesiculation, that counteracted the diet effects. Indeed, acute exercise has been reported to increase circulating MPs; whereas, chronic aerobic exercise is associated with reduced MP formation [335]. It is likely that a longer intervention period, resulting in a more chronic exercise stimulus, may yield different results.

Among the five cytokines assessed in our study, only MCP-1 showed a significant decrease over the 4-day interventions. In a 7-day study in overweight individuals, a LCHF diet with and without antioxidants (Vitamin C/E) led to a similar reduction in MCP-1 while, in line with our results, C-reactive protein and IL-6 levels were not affected [318]. Thus, despite the reduction in PBMC JNK activation, plasma cytokines were largely unchanged by short-term diet interventions. Longer term low-carbohydrate diets typically result in substantial reductions in body and fat mass [186], which can confound interpretation of obesity-related inflammatory parameters. In our short-term study, the lack of change in pro-inflammatory cytokines could be viewed as support for the utilization of LCHF diets in treatment of T2D. These findings suggest that previous findings of inflammatory activation after hypercaloric high-fat meals (~950 kcal),
may not translate to heightened inflammation when eucaloric/hypocaloric LCHF foods are consumed over several days [336, 337]. However, the use of plasma-borne inflammatory markers such as cytokines has recently been questioned as they may not reflect inflammatory processes occurring in cells [203]. Our findings are in agreement with this notion as changes were seen in PBMCs p-JNK and microparticles with minimal effects of the dietary interventions on key cytokines such as IL-6 and TNF-a.

**LC diets lower proinsulin**

Elevated proinsulin as well as proinsulin:insulin or proinsulin:C-peptide ratio are strong predictors of insulin resistance and are associated with beta-cell dysfunction [338, 339]. In our study, LC and LC+Ex decreased fasting proinsulin while no changes were seen following the GL diet. Although the change in proinsulin:C-peptide was not significantly different between conditions (i.e., non-significant condition X time interaction), within conditions comparisons showed that only LC and LC+Ex decreased this ratio. Here we speculate that by reducing carbohydrate intake, and thus the demand for insulin, LCHF diets can potentially provide the beta-cells with some transient rest, decrease endoplasmic reticulum stress, and improve proinsulin processing within beta cells [340]. The long-term effect of such diets on proinsulin levels and beta-cell function are unknown but since both proinsulin and glucose levels are independent cardiovascular risk factors [341, 342], a LC diet with and without post-meal walks could contribute to improving the metabolic profile and cardiovascular risk of individuals with T2D.

**Limitations and Perspectives**

Despite our efforts to maintain energy balance, a small (~1-2 kg) but significant weight loss occurred in all conditions. The short duration of each intervention did not permit adjusting the caloric intake in attempts to prevent weight loss but energy intake was matched between
conditions. Therefore, the small weight loss observed may be related to an overall increase in diet quality and/or strictly controlling food over the four days. The clinical relevance of this small reduction is unclear but we acknowledge that weight loss could have played a role in the modulation of inflammation.

All food consumed by participants was provided and consisted of healthy whole foods. Thus, it is likely that most participants improved the quality of their diet as compared to what they usually eat by removing processed foods. This might have contributed to the absence of significant differences between conditions for some outcomes since each diet seems to have provided a certain degree of improvement over habitual patterns of participants. Furthermore, despite providing fresh whole foods the strict protocol may have reduced generalizability as three participants were unable to complete all conditions citing inability to comply with study procedures.

Like most people with T2D, the participants in the current investigation were on medications. We made sure to instruct each participant to continue to take their regular medications throughout the entire study and used a daily log to confirm this but interaction between medications, diets, and inflammatory outcomes are unknown. The relatively small sample size did not allow for examination of interactions with medications or by sex but the randomized crossover design was a strength in this regard.

**TAKE-HOME MESSAGE**

In conclusion, a low-carbohydrate high-fat diet with or without daily post-meal walks improved 4-day glycemic control and fasting proinsulin levels compared to a low-glycemic index, low-fat dietary guidelines diet. The addition of post-meal walks to a LC diet further improved glycemic control. There were no consistent effects of the individual interventions on inflammatory markers, although inflammatory activation of circulating peripheral blood mononuclear cells appeared to be reduced following each intervention with no appreciable changes in
characteristic pro-inflammatory plasma cytokines. Longer-term studies using LCHF diets and exercise looking at cardiometabolic and inflammatory markers are necessary to confirm the beneficial effects of such diets in individuals with T2D.

**Perspectives and significance**

LCHF diets have recently regained popularity for the management of T2D. The macronutrient distribution characteristic of this kind of diet is in contradiction with the current dietary guidelines for diabetes. As demonstrated in this study, an LCHF diet with and without post-meal walks significantly and rapidly normalized blood glucose levels. The elevated amount of fat consumed on this diet did not negatively affect inflammation or circulating TG, which combined with the glucose-lowering effect, suggests that LCHF can be a beneficial treatment strategy for T2D. Metabolic adaptations to LCHF can take several weeks to months, and deepening our knowledge on their chronic effect on inflammatory and metabolic status will likely help us to formulate better therapeutic dietary approaches for individuals with impaired glycemic regulation.
Chapter 4: Prior Ingestion of Exogenous Ketone Monoester Attenuates the Glycemic Response to an Oral Glucose Tolerance Test in Healthy Young Individuals.

BACKGROUND

The ketone bodies, D-BHB and acetoacetate are produced by the liver under conditions of starvation, very-low carbohydrate intake and prolonged glycogen-depleting exercise [185, 214, 231]. Several studies, including classical work by Cahill et al. [213, 215], have demonstrated that BHB, the main ketone body in circulation, can act as an alternative energy substrate for metabolically active tissues such as the brain, heart, kidneys and skeletal muscles. More recently, BHB has been shown to have several cellular signaling functions including acting as an endogenous HDAC inhibitor, a ligand for cell surface receptors, and an inhibitor of the NLRP3 inflammasome [261]. These findings suggest that in addition to its well-known role as a fat-derived energy source, BHB can modify an array of physiological functions.

To date, most studies exploring the direct effects of elevated plasma ketones on markers of metabolic control in human and animal models employed ketone infusion methods [232, 244, 247-249]. A consistent finding in studies in which BHB is infused to levels ≥1.0 mmol/L is a reduction in glucose and circulating free fatty acids; potentially related to reduced hepatic glucose output and inhibition of adipose tissue lipolysis, respectively. Lowering glucose and free fatty acids could be of potential value for individuals with glucose intolerance and insulin resistance but infusing BHB is not a practical therapeutic strategy. In the past few years, the emergence of exogenous ketone supplements allows researchers to study the isolated effects of elevated ketones without the presence of metabolic keto-adaptations or the use of infusions.

Exogenous ketone supplements can be ingested in the form of KS (e.g., sodium-potassium BHB) or KE (available in monoester and diester forms). So far, a limited number of studies have tested the effect of KS and KE on circulating metabolites in rats [225, 343, 344]. Consistent with the ketone infusion studies mentioned above, exogenous ketone supplements
decrease circulating glucose and free fatty acids. The high amount of salt contained in the KS supplement along with potential gastrointestinal distress limit its therapeutic utility and can be avoided by consuming a KM supplement [224]. KM supplementation using (R)-3-hydroxybutyl (R)-3-hydroxybutyrate has been shown to provide a safe [221] and novel strategy for rapidly increasing blood BHB to approximately 3 mmol/L within ~30 minutes in healthy humans [227, 255]. Once ingested, a non-racemic KM drink will be metabolized into the D isoform of BHB, the isoform produced by endogenous ketogenesis [345, 346]. Therefore, oral consumption of KM may be an interesting alternative for increasing BHB and improving metabolic control.

Further investigation is necessary to explore the metabolic effects of ketone supplementation in humans before KM can be considered as a therapeutic option. The objectives of this study were twofold: 1) To determine whether acute ingestion of KM; (R)-3-hydroxybutyl (R)-3-hydroxybutyrate impacts plasma glucose levels during a standardized OGTT. 2) To compare changes in insulin concentrations and estimates of insulin sensitivity after acute KM supplementation. In order to do so, we conducted a randomized, placebo-controlled crossover experiment in healthy young males and females. Due to the novelty of KM and the unknown impacts on glycemic response and insulin sensitivity when these supplements are consumed prior to an OGTT, we conducted this initial study in healthy participants in order to determine the normal physiological responses. We hypothesized that, when compared to a placebo, a single dose of KM taken 30 minutes prior to a 2-hour OGTT would reduce glucose AUC and improve oral glucose insulin sensitivity (OGIS) index.

METHODS

Ethical approval

The study was approved by the University of British Columbia Clinical Research Ethics Board (ID H16-01846). The study conformed to the standards set by the Declaration of Helsinki,
except for registration in a database. Every participant provided written informed consent during the initial screening visit.

Research design

The experimental design involved an initial visit for screening and baseline testing and two experimental conditions that each required a 3-hour visit to the laboratory. On experimental visits, participants arrived at the laboratory following a ≥10-hour overnight fast. According to a randomized crossover design, participants consumed either ketone or placebo supplement followed 30 minutes later by a 75-gram glucose drink for a standard 2-hour OGTT. The 30 minutes period was chosen based on previous studies [227, 255] showing that circulating levels of BHB following the ingestion of KM supplements reached a peak concentration around this time. Participants and study personnel performing laboratory blood sample analyses were blinded to experimental conditions. Only the researcher preparing the drinks and collecting blood samples was aware of the experimental condition. At least 48 hours later, participants returned to the lab following a similar overnight fast and performed the alternate experimental condition.

Participants

Twenty healthy participants (10 males and 10 females) aged between 18-35 years were recruited to participate in this study. Exclusion criteria included 1) currently taking any medications (except for birth control for females), 2) following a low-carbohydrate diet or consuming nutritional ketone supplements, 3) considered competitive athlete engaged in competition or intensive training, 4) waist circumference >102 centimeters for men or >88 centimeters for women. Participants’ baseline characteristics are presented in Table 11.
Table 11. Characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25.4 (4.1)</td>
<td>25.9 (4.2)</td>
<td>24.8 (4.2)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.1 (2.2)</td>
<td>23.4 (2.2)</td>
<td>20.9 (1.4)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>70.2 (7.2)</td>
<td>76.1 (4.7)</td>
<td>64.4 (3.2)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>116 (8)</td>
<td>120 (8)</td>
<td>112 (7)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>76 (8)</td>
<td>77 (9)</td>
<td>74 (6)</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>4.5 (0.4)</td>
<td>4.6 (0.3)</td>
<td>4.3 (0.3)</td>
</tr>
<tr>
<td>Fasting insulin (mU/L)</td>
<td>3.2 (1.3)</td>
<td>2.8 (1.2)</td>
<td>3.6 (1.0)</td>
</tr>
<tr>
<td>Fasting NEFA (mmol/L)</td>
<td>0.43 (0.22)</td>
<td>0.35 (0.13)</td>
<td>0.52 (0.19)</td>
</tr>
</tbody>
</table>

24-hour dietary food log

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calories (kcal)</td>
<td>2030 (454)</td>
<td>2177 (404)</td>
<td>1840 (472)</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>43 (12)</td>
<td>41 (14)</td>
<td>45 (9)</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17 (5)</td>
<td>18 (3)</td>
<td>17 (6)</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>42 (9)</td>
<td>43 (12)</td>
<td>41 (5)</td>
</tr>
</tbody>
</table>

Data are presented as Mean (SD). NEFA: Non-esterified fatty acids, Glucose, insulin and FFA are reported as the average of fasting measures for both conditions.

Baseline testing

Body weight (kg), height (cm), blood pressure (mmHg) and waist circumference (cm) were measured using standardized methods. Participants were given a 24-hour food log to complete on the day prior their first experimental condition. They were also told to avoid exercising and to not consume alcohol for 24 hours prior to the experimental trials.
Experimental trials

Participants reported to the laboratory for experimental trials after an overnight fast (≥10 hours). The research coordinator confirmed with them that the 24-hour food log had been completed, that no exercise had been performed and that no alcohol had been consumed on the day before. An indwelling intravenous catheter (BD Nexiva, Becton Dickinson Infusion Therapy Systems Inc., Utah, USA) was then inserted into the antecubital vein for repeated blood sampling. At each time point, blood was drawn into 1x2ml EDTA and 1x4ml serum tubes (BD Vacutainer, Becton Dickinson Infusion Therapy Systems Inc., Utah, USA) for isolation of plasma and serum. Seven intravenous blood draws were performed for each condition. The first collection (-30 min) occurred immediately before the consumption of the ketone or placebo supplement. Participants consumed a KM supplement in the form of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (ΔG® from TΔS® Ltd, UK; 0.45 ml/kg body mass or 482 mg/kg body mass) ingested with water and vanilla-flavoured stevia (SweetLeaf) in a total volume of 100 ml. Immediately following ingestion of the ketones, participants were given 20 ml of calorie-free Gatorade G2 in attempts to remove any remaining flavour of the supplement. In the placebo condition, participants consumed 100 ml of water and vanilla-flavoured stevia (SweetLeaf) followed by the same 20 ml calorie-free Gatorade G2. Participants wore a nose clip while consuming the supplement in both conditions to further mask any flavour. Thirty minutes later, another blood sample was collected (0 min) then followed immediately by the consumption of a 75-gram oral glucose tolerance test drink (Thermo Scientific, Fisher Scientific Company, Middletown, USA). Another five blood draws occurred at 15, 30, 60, 90 and 120 minutes after ingestion of the glucose drink. At each time point, BHB was measured in whole blood (Precision Neo; Abbott Laboratories, Witney, UK). After the trial was completed, participants were asked to complete a gastrointestinal distress questionnaire and to guess whether they had the placebo or ketone supplement by answering the following; “What condition do you think you were in today?” Before leaving the laboratory, a copy of the 24-hour food log was provided to
participants who were asked to repeat the exact diet 24 hours before the following visit. Subjects were also reminded to not perform exercise or to consume alcohol 24 hours prior to the next visit.

Participants returned to the laboratory (≥10 hour fast) at least 48 hours later to complete the alternate condition. Adherence to the 24-hour diet, exercise and alcohol guidelines were confirmed upon arrival in the laboratory. If no significant deviations were observed, the protocol for the third visit was the same as the previous one, except that participants received the alternate supplement (ketone or placebo). Female participants completed both experimental conditions in the follicular phase (between 3-9 days after the beginning of their menstrual cycle).

**Blood samples**

Upon collection, 10 μL of a dipeptidyl peptidase-4 inhibitor (Cat. No. DPP4-010, Millipore, MA, USA) was added to the EDTA tube to prevent the degradation of active glucagon-like peptide-1 (GLP-1). The EDTA and serum tubes were centrifuged at 1,500 × g for 15 min at 4°C. Following centrifugation, plasma and serum samples were stored in a −80°C freezer until assays were performed in a blinded fashion. Blood metabolites were analyzed using the following commercially available kits; Serum NEFA (Wako Diagnostics HR Series, CA, USA) and serum glucose (Glucose hexokinase, Pointe Scientific INC, MI, USA) were analyzed on a Chemwell 2910 automated analyzer (Awareness Technologies, Palm City, USA). Serum C-peptide (C-peptide, Meso Scale Discovery, MD, USA) and plasma GLP-1 (M/R active GLP-1 (7-36) amide, Meso Scale Discovery, MD, USA) were analyzed on a MESO Quickplex SQ 120. Serum insulin (Human Insulin ELISA, Crystal Chem, IL, USA) and serum adiponectin (Rapid Human Adiponectin Immunoassay kit, Antibody and Immunoassay Services, Li Ka Shing Faculty of Medicine, The University of Hong Kong) were analyzed on an iMark™ Microplate Absorbance Reader (Bio Rad, CA, USA). All assays were run in duplicate. The coefficient of variation for
duplicate samples was 4.2% for serum NEFA, 2.9% for serum glucose, 7.8% for serum C-peptide, 4.1% for plasma GLP-1, 4.9% for serum insulin, and 4.0% for serum adiponectin.

**Oral glucose insulin sensitivity index**

The OGIS index was computed using the model-based method proposed by Mari et al. [347].

**Dietary food log**

The 24-hour dietary recall was analyzed using a commercial software program (Foodmate, Version 1.1, N.J., USA).

**Visual Analogue Scale**

At 120 minutes of each experimental condition, participants were asked to fill out visual analog scales (VAS) [348] for the following five symptoms; nausea, urge to vomit, bloating, belching, and cramps. Participants were asked to fill out the VAS based on their global experience and symptoms throughout the 2.5-hour experimental condition.

**Statistical Analysis**

Data were analyzed using SPSS v.21 (SPSS Inc., Chicago, IL, USA). Normality was assessed using Q-Q plots and Shapiro-Wilk tests within each experimental condition. Two-hour AUC and iAUC were calculated using GraphPad Prism v.6.0 (GraphPad Software Inc., San Diego, CA, USA) and included time points 0 to 120. AUCs and iAUCs were compared between experimental conditions using paired Student’s t-tests. VAS differences between conditions were assessed using a Wilcoxon signed-rank test. A linear mixed-effects model including time points -30 to 120 (Condition and Time as fixed factors and Subject as random factor) was used to determine the treatment effects. Significant interactions were followed up with pre-planned contrasts comparing Placebo to KM within each time point using Bonferroni corrections for
multiple comparisons. Cohen’s $d$ effect size was calculated for all of the significant pre-planned comparisons. Significance was set at $P < 0.05$. Data in Table 11 and figures are presented as mean (SD) whereas non-parametric data in Table 12 are presented as median with range.

A sample size of N=20 was calculated a priori in order to detect a 20% reduction in glucose AUC, which was the predefined primary outcome of interest. A 20% reduction in glucose was based on pilot experiments, parallels what is seen with BHB infusion [232] and acute KM ingestion [222] and was deemed clinically relevant based on similar magnitude of reduction with glucose-lowering medications [349]. Using a mean and standard deviation for glucose AUC from previous data collected in our lab (700 +/- 160 mM.120min) a 20% reduction corresponded to an effect size of 0.875 assuming a moderate correlation of r=0.5 amongst repeated measures. Using these data with an alpha level of 0.05 and 90% power a sample size of N=16 was calculated using G*Power (v3.1.9.3). In order to account for 20% dropout or missing blood samples we aimed to recruit 20 participants.

RESULTS

The BHB, glucose, insulin, and C-peptide responses over time are presented in Figure 18. A significant condition by time interaction was found for BHB ($P < 0.001$), with all time points except -30 ($P = 0.577$) being higher after KM supplementation compared to placebo (all $P < 0.001$, Figure 18A). Also, significant main effects of time were found for glucose, insulin and C-peptide (all $p < 0.001$). There was a main effect of condition observed for glucose ($P < 0.001$, Figure 18B) with glucose being lower in the KM condition. No significant effects of condition or condition X time interactions were found for insulin (respectively $P = 0.971$ and $P = 0.871$; Figure 18C) or C-peptide ($P = 0.078$, $P = 0.489$; Figure 18D).

Areas under the curve for BHB, glucose, insulin, and C-peptide are presented in Figures 19 and 20. The $K_{me}$ supplement significantly increased BHB AUC compared to placebo (1104%, $P < 0.001$, $d = 8.9$) (Figure 19A). As compared to placebo, the KM supplement significantly
decreased glucose AUC (-17%, \( P < 0.001 \), d = 1.1, Figure 19B) and glucose iAUC (-40%, \( P = 0.016 \), d = 0.7) (Figure 20A). While no differences were observed between KM and placebo for the insulin AUC: (-3%, \( P = 0.710 \), d = 0.1, Figure 19C), insulin iAUC: (-15%, \( P = 0.087 \), d = 0.4, Figure 20B) or C-peptide AUC (-9%, \( P = 0.151 \), d = 0.4, Figure 19D), C-peptide iAUC showed a significant decrease in the KM condition (-21%, \( P = 0.005 \), d = 0.9, Figure 20C). OGIS index improved by ~11% in the KM condition (\( P < 0.001 \), d = 1.0, Figure 21).

A condition x time interaction was observed for serum NEFA (\( P < 0.001 \); Figure 22A). Pre-planned contrasts comparing the two conditions within each time point revealed significant differences at time 0 (\( P < 0.001 \), d = 1.7), 15 (\( P < 0.001 \), d = 1.9), 30 (\( P < 0.001 \), d = 1.4), 60 (\( P = 0.001 \), d = 0.8), 90 (\( P = 0.004 \), d = 0.8) and 120 (\( P = 0.005 \), d = 1.0). NEFA AUC was also decreased by ~44% after KM as compared to Placebo (\( P < 0.001 \), d = 1.8, Figure 22B). A significant condition X time interaction was seen for GLP-1 (\( P = 0.002 \)), with GLP-1 being lower at time 30 in the KM condition (\( P < 0.001 \), d = 1.4; Figure 23A). There were no significant effects of condition, time, or condition X time interactions for adiponectin (all \( P > 0.239 \); Figure 23B).

Gastrointestinal symptoms were generally low to non-existent with medians of zero for all symptoms except nausea where the median was 1 (out of 100 mm) in the ketone condition (Table 12). Wilcoxon signed rank tests revealed no significant differences between conditions for symptoms of nausea, urge to vomit, bloating, belching and cramps (all \( P > 0.05 \); Table 12).
Table 12. Gastrointestinal symptoms assessed at the end of the ketone monoester (KM) supplement and placebo experimental trials.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>0-15</td>
<td>0</td>
<td>0-4</td>
<td>0</td>
<td>0-9</td>
<td>0</td>
<td>0-40</td>
<td>0</td>
<td>0-10</td>
<td>0</td>
</tr>
<tr>
<td>Urge to Vomit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bloating</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Belching</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cramps</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wilcoxon signed-rank tests

| P values | 0.06 | 0.09 | 0.60 | 0.92 | 0.89 |

Effect sizes

| Cohen’s d | 0.5  | 0.6  | 0.3  | 0.1  | 0.1  |

Results are presented in millimetres (0-100).
Figure 18. D-beta-Hydroxybutyrate (BHB), glucose, insulin and C-Peptide responses following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). (A) BHB; (B) Glucose; (C) Insulin; and (D) C-peptide. * P < 0.001 vs. placebo within time point, Bonferroni adjusted post-hoc. † P < 0.001 significant main effect of condition.
Figure 19. Two-hour area under the curve (AUC) following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). (A) Beta-Hydroxybutyrate (BHB) AUC; (B) Glucose AUC; (C) Insulin AUC; and (D) C-peptide AUC. Solid lines represent individual male participants and dashed lines represent individual female participants. *P = 0.001 vs. placebo.
Figure 20. Two-hour incremental area under the curve (iAUC) following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). (A) Glucose iAUC; (B) Insulin AUC; and (C) C-peptide iAUC. Solid lines represent individual male participants and dashed lines represent individual female participants. **P < 0.01 vs. placebo, *P < 0.05 vs. placebo.
Figure 21. Oral glucose insulin sensitivity index following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). Solid lines represent individual male participants and dashed lines represent individual female participants. *P = 0.001 vs. placebo.
Figure 22. Non-esterified fatty acids (NEFA) following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). (A) Two-hour area under the curve; (B) NEFA response over time. In panel A, solid lines represent individual male participants and dashed lines represent individual female participants. **P < 0.001 vs. placebo. * P < 0.01 vs. placebo within time point, Bonferroni adjusted post-hoc.
Figure 23. Glucagon-like peptide (GLP-1) and adiponectin responses following a single dose of ketone monoester supplement or placebo. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). (A) GLP-1; and (B) Adiponectin. *P < 0.01 vs. placebo within time point, Bonferroni adjusted post-hoc.
DISCUSSION

The objective of this study was to determine if a single dose of KM consumed 30 minutes before a 2-hour OGTT could attenuate the glycemic response in young healthy individuals. Our results demonstrate that in individuals with fasting glucose levels within the normal range (4.0-6.0 mM) [350], 2-hour total and incremental glucose AUCs are significantly lowered by a single dose of KM supplement as compared to a placebo. This reduction in glycemic response following the ingestion of KM was accompanied by a decrease in circulating NEFA levels and an improvement in the OGIS index, a marker of insulin sensitivity. The decrease in C-peptide iAUC under the KM condition also supports the notion that exogenous ketone supplementation may lower glucose via improved insulin sensitivity.

At the time of writing this manuscript, the first data in humans reporting a glucose lowering effect of KM supplements in the resting state were published [222]. This recent publication demonstrated that a KM supplement ingested following the consumption of a standard meal decreased glucose levels from 5.5 to 4.7 mmol/L over the 4-hour study. This decrease of ~15% is of similar magnitude as the 16% decrease in glucose AUC observed in our study. Our study adds to these findings and extends the glucose lowering effect to KM consumption prior to ingestion of glucose, suggesting that pre-meal supplementation with exogenous ketones could reduce the glycemic response. The hypoglycemic action of ketone bodies has been shown in BHB infusion studies in both humans and animals [232, 244, 247-249]. Among them, Miles et al. and Mikkelsen et al. showed that β-OHB, infused to levels of ~2.0 mmol/L, significantly decreased circulating glucose and suppressed endogenous glucose production by ~20% in healthy males [232, 247]. The glucose-lowering action of ketones may be due to a direct effect on hepatic glucose production, as it has been shown to occur in the absence of changes in insulin or glucagon [243, 244, 250]. However, ketone body infusion has also been shown in some studies to stimulate pancreatic beta-cell insulin secretion [251-253]. Previous studies using KM supplement have observed a two-fold increase in insulin levels.
during a post-exercise hyperglycemic clamp [254] and a small increase in the fasting state [222, 351] whereas no change was seen following a post-exercise high-dose protein-carbohydrate drink [255]. The potential insulinogenic action of ketone bodies remains a matter of debate, but our results clearly indicate that if indeed KM stimulates insulin secretion, this effect is not additive to the stimulus of a standardized 75-gram glucose drink. Contrary to a potential increase in insulin secretion following the use of exogenous ketones, we observed a decrease in C-peptide iAUC during the OGTT. Miles et al. reported an increase in C-peptide levels following the infusion of BHB in fasted humans [247]. The discrepancy between this report and our findings might come from the fact that our study involved ingestion of exogenous ketones followed by glucose instead of isolated infusion of ketones in the basal state. The decrease in C-peptide was accompanied by a decrease in incretin hormone GLP-1 in the KM condition. This observation is in agreement with Stubbs et al. who reported a decrease in GLP-1 following a KM supplement in the fasting state [351]. GLP-1 is produced by the gut in response to food (or CHO) ingestion and acts to potentiate insulin secretion [352]. Thus, a decrease in GLP-1 might be linked to the observed decrease in C-peptide iAUC following the KM supplement. More studies are needed to determine the underlying mechanisms linking KM ingestion to reduced glycemic response, including further exploration of effects on insulin sensitivity, insulin secretion and GLP-1 involvement.

As can be seen from figure 18 and 19, not every single participant responded the same way to the KM treatment in terms of glycemic or insulin responses. This is typical for human physiology experiments and our study was not designed to attempt to quantify individual responses or predictors thereof but this may be an interesting future direction for exogenous ketone research. Our study was not able to directly test the underlying mechanisms responsible for the reduced glycemic response after KM ingestion. However, based on the observations of several previous studies in both human and animal models [232, 247, 353], one could speculate that the reduced glycemic response is mainly attributable to BHB-mediated reduction in hepatic
glucose output. Studies using tracer techniques along with KM supplementation would be necessary to validate these assumptions.

While starvation and a KD increase the release and utilization of NEFA [216, 217, 354], BHB can directly inhibit lipolysis via agonism of the nicotinic acid receptor GPR109A (also known as HM74A, PUMA-G) on adipocytes [242]. In line with our results, several studies involving ketone infusion have reported a significant decrease in NEFA levels in both fasting and fed conditions [222, 232, 244, 247]. Noteworthy, the KM supplement in our study was able to immediately decrease NEFA in the fasted state (-30 vs. 0 minute time points) and further decrease NEFA levels on top of the anti-lipolytic effect of glucose ingestion (time points 15-120 minutes). Since acutely decreasing circulating NEFA is associated with improved insulin sensitivity and/or glucose tolerance, it is possible that the improvement in insulin sensitivity in the KM condition could be the result of the decrease in circulating NEFA [93, 355, 356]. The absence of change in adiponectin levels following KM is in accordance with a previous study using KS and suggests that this protein was not involved in the insulin sensitivity improvement process [343]. Adiponectin is an adipokine that is associated with improved insulin sensitivity [357] the secretion of which can be stimulated through the nicotinic acid receptor [358].

Some limitations of our study should be acknowledged. First, this study was conducted with healthy young individuals, so the results may not apply to clinical populations with metabolic impairments. Given that KM is so new, we aimed to study the response in the healthy individuals to reduce the confounding influence of insulin resistance, beta-cell dysfunction, and medications, all of which could confound interpretation of OGTT results. Along this line, more studies are needed to determine the possible impact of KM supplementation in individuals with impaired glucose tolerance. Secondly, the use of the oral glucose insulin sensitivity index limits mechanistic interpretation of our findings, compared to a more direct assessment of insulin sensitivity such as a hyperinsulinemic-euglycemic clamp or stable isotope glucose tracers. However, the OGIS correlates well with clamp-derived measures in healthy individuals and, if
KM is to be used therapeutically to lower glucose, we feel it is important to demonstrate efficacy in reducing glycemic response as ultimately individuals consume CHO, which contributes to hyperglycemia in real-life. Finally, our study could not rule out potential effects of the KM on digestion and absorption of the glucose drink, so future studies could explore gastric emptying to better understand the mechanisms behind the reduced glycemic response following the KM supplement.

In conclusion, a KM supplement that acutely increased β-OHB levels up to ~3 mmol/L attenuated the glycemic response to an OGTT in healthy humans. The reduction in glycemic response along with improved markers of insulin sensitivity was not driven by increased insulin secretion, but could be related to a BHB-mediated reduction in hepatic glucose output. These acute effects on reduced glycemic response and insulin sensitivity suggest that ketone monoester supplements could have therapeutic potential in the management and prevention of metabolic disease.
Chapter 5: A Single Ketone Monoester Drink Improves Glucose Tolerance in Individuals with Obesity: A Randomized Controlled Trial

BACKGROUND

Traditionally associated with life-threatening ketoacidosis, research on potential therapeutic effects of the ketone body, BHB, has gained substantial attention in recent years [205, 209, 229]. Hepatic ketogenesis can be achieved via dietary changes, such as fasting or carbohydrate-restriction (e.g. ketogenic diet), resulting in an increase in circulating BHB to levels of ~0.5-5.0 mmol/L, primarily to provide an alternate fuel source for the brain [359-361]. Through binding to G protein-coupled receptors and eliciting epigenetic modifications, BHB also possess several signaling functions that regulate lipolysis [362], inflammation [363], and oxidative stress [257, 364]. In addition, classic studies have observed that infusing BHB can lower circulating glucose and NEFA [244, 247]. Thus, raising circulating BHB could have potential therapeutic use in metabolic disease.

The recent development of exogenous ketones now make it possible to rapidly reach a state of ketosis without the need for severe dietary restriction or use of invasive methods [221]. Using the KM, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, we recently demonstrated that raising circulating BHB through oral supplementation was effective at reducing glucose and NEFA levels during an OGTT in young healthy individuals [226]. Compared to control drink, the glycemic response was reduced by ~16%, and NEFA AUC was reduced by ~44% with a corresponding increase in OGIS index of ~11% [226]. These observations, and other studies [222, 225], suggest that individuals with obesity or insulin resistance might benefit from strategies that elevate circulating BHB. However, BHB metabolism is altered with aging, obesity and insulin resistance, which could limit the ability of ketone supplements to exert these potentially beneficial metabolic effects [365-368]. At present, all human studies involving ketone
drinks have examined young, healthy volunteers and, therefore, results may not be applicable to older individuals with obesity or related metabolic impairments.

In addition to peripheral metabolic effects, there are also possible therapeutic applications of βHB related to the brain. When βHB is rapidly elevated by infusion there is a robust increase in cerebral blood flow (CBF) [369], suggesting that raising circulating ketones may impact cerebrovascular function. Notably, obesity-related metabolic disorders are associated with reduced CBF and abnormal vascular function [370]; therefore, elevating BHB could improve blood flow patterns in these individuals.

The main objective of this study was to determine whether the ingestion of KM prior to a 2-hour OGTT could lower glucose levels in individuals with obesity. Additional objectives were to determine how acute KM ingestion altered NEFA levels, glucoregulatory hormones and estimates of insulin sensitivity. As infusion of BHB causes a robust (39%) increase in CBF [369], our exploratory objective was to evaluate common carotid artery (CCA) hemodynamics. Based on our study using the same oral ketone supplement and protocol [19], we hypothesized that raising BHB through oral KM ingestion 30 minutes prior to an OGTT would reduce both glucose and NEFA AUCs and improve OGIS index.

**MATERIALS AND METHODS**

**Ethical approval**

The study was approved by the University of British Columbia Clinical Research Ethics Board (ID H16-01846) and was registered on clinicaltrials.gov (NCT03461068). The study conformed to the standards set by the Declaration of Helsinki and all participants provided written informed consent during the initial screening visit.
Participants

Fifteen participants (5 males and 10 females) volunteered for the study. Inclusion criteria required participants to be between the ages of 30 and 65 years and have a body mass index $\geq 28$ kg/m$^2$ with a waist circumference $>102$ cm for males and $>88$ cm for females. Exclusion criteria included: 1) taking medications affecting glucose or lipid metabolism; 2) following a LCHF diet or intermittent fasting protocol; 3) consuming ketone supplements; 4) engaged in intensive exercise training; 5) diagnosed with diabetes or heart disease; or 6) pregnant during the study. Baseline characteristics and the recruitment flowchart are presented in Table 13 and Figure 24, respectively.

Baseline testing

Body weight (kg), height (cm), blood pressure (mmHg), and waist circumference (cm) were measured using standardized methods. Participants were given a 24-hour food log to complete on the day prior to their first experimental condition. Participants refrained from exercise and alcohol consumption for 24 hours prior to the experimental trials.
Table 13. Characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>15</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>46.6 (10.0)</td>
<td>42.8 (13.9)</td>
<td>48.5 (7.6)</td>
</tr>
<tr>
<td>Body mass index (kg/m^2)</td>
<td>33.7 (5.1)</td>
<td>34.2 (3.6)</td>
<td>33.5 (5.8)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>103.4 (12.6)</td>
<td>108.8 (7.8)</td>
<td>100.5 (14.1)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 (11)</td>
<td>134 (6)</td>
<td>126 (11)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 (8)</td>
<td>84 (8)</td>
<td>86 (8)</td>
</tr>
<tr>
<td>Glycated hemoglobin (%)</td>
<td>5.5 (0.6)</td>
<td>5.3 (0.4)</td>
<td>5.6 (0.7)</td>
</tr>
<tr>
<td>Fasting glucose (mM)</td>
<td>5.6 (0.9)</td>
<td>5.3 (0.5)</td>
<td>5.7 (1.1)</td>
</tr>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>153 (76)</td>
<td>180 (95)</td>
<td>140 (64)</td>
</tr>
<tr>
<td>Fasting NEFA (mM)</td>
<td>0.46 (0.22)</td>
<td>0.36 (0.15)</td>
<td>0.51 (0.24)</td>
</tr>
</tbody>
</table>

24-hour dietary food log

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calories (kcal)</td>
<td>1913 (721)</td>
<td>2042 (956)</td>
<td>1872 (583)</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>40 (11)</td>
<td>35 (11)</td>
<td>42 (11)</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17 (4)</td>
<td>18 (3)</td>
<td>16 (4)</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>44 (11)</td>
<td>45 (9)</td>
<td>44 (12)</td>
</tr>
</tbody>
</table>

Data are presented as Mean (SD). NEFA: non-esterified fatty acids. A.U; arbitrary units. Glucose, insulin and FFA are reported as the average of fasting measures for both conditions.
**Experimental**

Data were collected at the University of British Columbia, Okanagan Campus (Kelowna, Canada) in the Exercise Metabolism and Inflammation Laboratory from April 2018 to December 2018. Participants reported to the laboratory after an overnight fast ($\geq$10 hours). Following 10 minutes of supine rest, assessment of right CCA blood flow shear patterns were performed using a 10-MHz multifrequency linear array duplex ultrasound (Terason t3200; Teratech, Burlington, MA, USA). Blood pressure and heart rate were taken in the supine position pre- and post- CCA blood flow scans (Omron Healthcare Co. Ltd. Model BP786CANN). An indwelling intravenous catheter (BD Nexiva, Becton Dickinson Infusion Therapy Systems Inc., Utah, USA) was then inserted into the antecubital vein for repeated blood sampling. Blood was drawn into 1x4ml serum tubes and 1x2ml P800 K$_2$EDTA tubes (BD Vacutainer, Becton Dickinson Infusion Therapy Systems Inc., Utah, USA) for isolation of serum and plasma. Seven intravenous blood draws were performed for each condition. The first collection (-30 min) was immediately after the fasting blood flow measurement. Participants then consumed a KE supplement ($\Delta$G from T$a$s Ltd, UK; 0.45 ml/kg body weight or 482 mg/kg body weight) ingested with water and fruit-flavoured calorie-free artificial sweetener (Mio, Kraft Foods) in a total volume of 150 ml. Immediately after, participants were given 20 ml of calorie-free sports drink (Gatorade G2) in attempts to remove any remaining flavour. In the control drink condition, participants consumed 140 ml of water combined with 10 ml of bitter flavour (Symrise, 648352) with the same sweetener followed by the calorie-free sports drink. Participants wore a nose clip while consuming the supplement in both conditions to further mask any flavour. Fifteen minutes later, a second blood flow measurement was made (from -15 to 0 min) followed by the collection of another blood sample (0 min) and the immediate consumption of a 75-gram OGTT drink (Thermo Scientific, Fisher Scientific Company, Middletown, USA). Another five blood draws were at 15, 30, 60, 90 and 120 minutes after ingestion of the glucose drink. At each time point, BHB was measured in whole blood using β-ketone strips (Precision Neo; Abbott Laboratories,
Witney, UK). Two additional blood flow measurements were immediately prior to the 60- and 120-minute blood sample collections. After each trial, participants were asked to complete a gastrointestinal distress questionnaire; a copy of the 24-hour food log was also provided to them and they were asked to repeat the exact diet 24 hours before the following visit.

In a randomized crossover design (randomization was generated by the research coordinator using the online research randomizer: https://www.randomizer.org/), participants returned to the laboratory (≥10 hour fast) at least 48 hours later to complete the alternate condition. Adherence to the 24-hour diet, exercise and alcohol guidelines was confirmed. If no significant deviations were observed, the protocol for the third visit was the same as the previous one, except that participants received the alternate drink. Five female participants were pre-menopausal and completed both experimental conditions in the follicular phase (between 3-9 days after the beginning of their menstrual cycle). Drinks were prepared by the research assistant following randomization sequence, the order of which was not revealed until after data analyses. Participants and study personnel performing laboratory blood sample analyses were blinded to experimental conditions using coding (A for first and B for second visit). Only the researcher preparing the drinks and collecting blood samples was not blinded.

**Blood samples**

Upon collection, serum tubes sat at room temperature for 30 minutes before being centrifuged at 1,500 × g for 15 min at 4°C whereas the P800 K₃EDTA tubes were centrifuged immediately following collection. Serum and plasma samples were then stored at −80°C before blinded batch analyses. Blood metabolites were analyzed using commercially available kits; serum NEFA (Wako Diagnostics HR Series, CA, USA), glucose (Glucose hexokinase, Pointe Scientific INC, MI, USA), lactate (Lactate, Pointe Scientific INC, MI, USA) and triglycerides (Triglyceride, Pointe Scientific INC, MI, USA) on a Chemwell 2910 automated analyzer (Awareness Technologies, Palm City, USA). Plasma C-peptide, insulin, GLP-1, glucagon and leptin were
assessed using a Milliplex MAP Human Diabetes Magnetic Bead Panel (MilliporeSigma, MA, USA) on a Bio-Plex MAGPIX multiplex reader (Bio-Rad Laboratories INC, CA, USA). All assays were run in duplicate, except lactate and triglycerides, which were run singly.

**Common carotid artery measurements**

Right CCA blood velocity and vessel diameter were measured using a 10-MHz multifrequency linear array duplex ultrasound (Terason t3200; Teratech, Burlington, MA, USA). Arterial diameter was measured using B-mode imaging; whereas, pulse-wave mode was used to concurrently measure peak blood velocity. The insonation angle (always 60°) was unchanged throughout each test and all images were optimized in accordance with recent standardized guidelines [371]. Mean blood flow was determined as half of the time-averaged maximal velocity multiplied by the cross-sectional luminal area for a minimum of 12 cardiac cycles [371]. Mean shear rates were calculated using our edge-detection software as four times the peak blood velocity divided by vessel diameter [372]. Our between-day and within-day coefficient of variation for the assessment CCA diameter is 1.4% and 1.3%, respectively.

**Dietary food log**

The 24-hour dietary recall was analyzed using a commercial diet analyses software program (Foodmate, Version 1.1, N.J., USA).

**Visual Analogue Scale**

At the 120 minute time point of each experimental condition, participants were asked to complete VAS [348] assessing the following five symptoms over the last 2.5 hours; nausea, urge to vomit, bloating, belching, and cramps.
Calculations

The OGIS index was computed using the model-based method of Mari et al. [347]. Two-hour AUCs were calculated using GraphPad Prism v.8.0.0 (GraphPad Software Inc., San Diego, CA, USA) and included time points 0 to 120 min, except for triglycerides that included time points 0, 60 and 120 min.

Statistical Analysis

Data were analyzed using SPSS v.23 (SPSS Inc., Chicago, IL, USA). Normality was assessed using Q-Q plots and Shapiro-Wilk tests within each experimental condition. Square root transformations were used on GLP-1 AUCs to achieve a normal distribution. AUCs were compared between experimental conditions using paired Student’s t-tests. VAS differences between conditions were assessed using a Wilcoxon signed-rank test. A linear mixed-effects model including time points -30 to 120 min (Condition and Time as fixed factors and Subject as random factor) was used to determine the treatment effects, except for triglycerides for which only time points -30, 0, 60 and 120 min were used. Significant interactions were followed up with pre-planned contrasts comparing Control drink to KM within each time point using Bonferroni corrections for multiple comparisons. Cohen’s $d$ effect size was calculated for all of the significant pre-planned comparisons. Significance was set at $P < 0.05$. Data in Table 14 and figures (except the recruitment flowchart) are presented as mean (SD); whereas, non-parametric data in Table 15 are presented as median with range.

In order to calculate sample size, we used the effect size of $d = 1.1$ for the difference in glucose AUC (primary outcome) based on our previous KM supplement OGTT study [226]. With a two-tailed alpha level of 0.05 and 90% power a sample size of $n=11$ was calculated using G*Power (v3.1.9.3). In order to preserve power and account for dropouts or missing blood samples we aimed to recruit 15 participants.
RESULTS

The BHB, glucose, insulin, and C-peptide responses over time are presented in Figure 25. A significant condition by time interaction was found for BHB ($P < 0.001$), with all time points except $-30$ ($P = 1.00, d = 0.0$) being higher after KM supplementation compared to control drink (all $P < 0.001$, $d = 4.5$, Figure 25A). Also, significant main effects of time were found for glucose, insulin and C-peptide (all $P < 0.001$). There was a main effect of condition observed for glucose ($P < 0.001$, Figure 25B) with glucose being lower in the KM condition. No significant effects of condition or condition by time interactions were found for insulin ($P = 0.401$ and $P = 0.989$, respectively; Figure 25C) or C-peptide ($P = 0.990$ and $P = 0.702$, respectively, Figure 25D).

Areas under the curve for BHB, glucose, insulin, and C-peptide are presented in Figures 26 and 4. The KM supplement significantly increased BHB AUC compared to control drink (+1565%, $P < 0.001$, $d = 5.4$, Figure 26A). Compared to control drink, the KM supplement significantly decreased glucose AUC (-11%, $P = 0.002$, $d = 1.2$, Figure 26B) and glucose iAUC (-23%, $P = 0.008$, $d = 0.9$, Figure 27A). While no differences were observed between KM and control drink for the insulin AUC: (+5%, $P = 0.207$, $d = 0.4$, Figure 26C), insulin iAUC: (-4%, $P = 0.556$, $d = 0.2$, Figure 27B) or C-peptide AUC (-3%, $P = 0.499$, $d = 0.2$, Figure 26D), C-peptide iAUC showed a significant decrease in the KE condition (-17%, $P = 0.039$, $d = 0.7$, Figure 27C). OGIS index improved by 11% in the KM condition ($P = 0.01$, $d = 0.9$, Figure 28).

A condition by time interaction was observed for serum NEFA ($P < 0.001$; Figure 29A). Pre-planned contrasts comparing the two conditions within each time point revealed significant differences at time 15 ($P < 0.001$, $d = 1.1$) and 30 ($P = 0.003$, $d = 0.8$) but no significant differences at any other time point. NEFA AUC was also decreased by 21% after KM compared to Control drink ($P = 0.009$, $d = 0.8$, Figure 29B).

Significant main effects of time were found for active GLP-1, lactate, and glucagon (all $P < 0.001$). There was a main effect of condition observed for GLP-1 ($P < 0.001$, Figure 29C), lactate ($P = 0.038$, Figure 30A) and glucagon ($P = 0.049$, Figure 30C) with GLP-1 and lactate
being lower while glucagon was higher in the KM condition. No significant condition by time interactions were found for GLP-1 ($P = 0.144$), glucagon ($P = 0.826$), or lactate ($P = 0.462$). The KM supplement significantly decreased GLP-1 AUC and iAUC compared to control drink (-31%, $P = 0.001$, $d = 1.2$, Figure 29D) and -39%, $P = 0.043$, $d = 0.6$, respectively). Compared to control drink, the KM supplement significantly increased glucagon AUC (11%, $P = 0.030$, $d = 0.7$, Figure 30D) while no differences were observed for lactate AUC and iAUC (-5%, $P = 0.213$, $d = 0.3$ and 4%, $P = 0.725$, $d = 0.1$, respectively). There were no significant main effects of condition, time or interaction for triglycerides (All $P \geq 0.210$, Figure 30B). TG AUC was also not significantly different between conditions (2%, $P = 0.741$, $d = 0.1$).

Hemodynamic variables are presented in Table 14. Right CCA diameter and velocity were not different between conditions ($P = 0.330$ and $P = 0.62$, respectively). CCA blood flow and shear rate were also unchanged ($P = 0.19$ and $P = 0.86$, respectively). Although mean arterial pressure (MAP) was lower during the KM trial compared to control drink (main effect of condition: 98±11 vs. 100±9 mmHg, $P = 0.019$); CCA cerebrovascular conductance (i.e., blood flow/MAP) was not different between conditions ($P = 0.160$). Lastly, heart rate was higher in the KM condition at 60 min and 120 min compared to baseline with no changes in control drink ($P = 0.001$).

Wilcoxon signed rank tests revealed no significant differences between conditions for symptoms of nausea, urge to vomit, bloating, belching and cramps (Table 15) (all $P \geq 0.130$).
Table 14. Common carotid artery vascular parameters and systemic cardiovascular responses throughout an oral glucose tolerance test for both ketone and control drink conditions.

<table>
<thead>
<tr>
<th>Variable</th>
<th>-30 mins</th>
<th>0 min</th>
<th>+60 mins</th>
<th>+120 mins</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KM</td>
<td>Ctrl</td>
<td>KM</td>
<td>Ctrl</td>
<td>Time</td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td>0.68±0.05</td>
<td>0.68±0.04</td>
<td>0.69±0.05</td>
<td>0.70±0.02</td>
<td>0.70±0.06</td>
</tr>
<tr>
<td>Velocity (cm·s⁻¹)</td>
<td>33.95±4.75</td>
<td>34.05±6.78</td>
<td>33.76±4.61</td>
<td>33.22±6.06</td>
<td>33.20±5.56</td>
</tr>
<tr>
<td>Flow (mL·min⁻¹)</td>
<td>378±85</td>
<td>380±90</td>
<td>390±88</td>
<td>394±88</td>
<td>396±104</td>
</tr>
<tr>
<td>Shear Rate (1·s⁻¹)</td>
<td>199±25</td>
<td>199±42</td>
<td>194±23</td>
<td>188±31</td>
<td>188±31</td>
</tr>
<tr>
<td>SRAUC (A.U.)</td>
<td>11928±1510</td>
<td>11946±2497</td>
<td>11653±1401</td>
<td>11253±1868</td>
<td>11309±1840</td>
</tr>
<tr>
<td>CVC (mL·min⁻¹·mmHg⁻¹)</td>
<td>4.00±1.16</td>
<td>3.92±1.20</td>
<td>4.09±1.14</td>
<td>4.01±1.17</td>
<td>4.22±1.26</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>98±9</td>
<td>99±8</td>
<td>98±12</td>
<td>101±9</td>
<td>97±13</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>69±7</td>
<td>68±10</td>
<td>67±9</td>
<td>64±10</td>
<td>73±8*</td>
</tr>
</tbody>
</table>

MAP and HR; n=15, all other outcomes n=10. Either ketone or control drinks were consumed at -30 min, respectively. SRAUC; shear rate area under the curve, A.U; arbitrary units, KM; ketone monoester, Ctrl; control drink, MAP; mean arterial pressure, CVC; cerebrovascular conductance (blood flow/MAP), HR; heart rate. *P<0.001 interaction effect; significantly different than KM 0 min. P-values in bold are statistically significant.
Table 15. Gastrointestinal symptoms assessed at the end of the ketone monoester supplement and control drink experimental trials.

<table>
<thead>
<tr>
<th></th>
<th>Nausea</th>
<th></th>
<th>Urge to Vomit</th>
<th></th>
<th>Bloating</th>
<th></th>
<th>Belching</th>
<th></th>
<th>Cramps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Control</td>
<td>0-9</td>
<td>0</td>
<td>0-11</td>
<td>0</td>
<td>0-22</td>
<td>0</td>
<td>0-25</td>
<td>0</td>
<td>0-9</td>
</tr>
<tr>
<td>KM</td>
<td>0-5</td>
<td>0</td>
<td>0-16</td>
<td>0</td>
<td>0-18</td>
<td>0</td>
<td>0-5</td>
<td>0</td>
<td>0-3</td>
</tr>
<tr>
<td>Wilcoxon signed-rank tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P values</td>
<td>0.60</td>
<td>0.71</td>
<td>0.34</td>
<td>0.32</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are presented in millimetres (0-100). KM; ketone monoester, Sample size; n=15.
61 people phone or email screened

41 exclusions
1 over 65 years old
17 Not interested
1 CVD
4 dislike needles
2 on an intermittent fasting protocol
6 did not reach the obesity criteria
1 smoking
6 too busy with work
2 moved out of town
1 did not want to consume the OGTT drink

20 attended screening visit

3 did not meet eligibility criteria for obesity upon measurement
1 inaccessible veins for blood sampling
1 not interested

15 eligible participants randomized

15 completed both conditions in randomized crossover design

Figure 24. Consolidated standards of reporting trials flow diagram. CVD, cardiovascular disease.
Figure 25. Changes over time following a single drink of ketone monoester supplement or control drink. Drinks were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). The black line represents the KM condition while the dotted line represents the control drink. (A) Beta-hydroxybutyrate (BHB) (n=15); (B) Glucose (n=15); (C) Insulin (n=14); and (D) C-peptide (n=14). * P < 0.001 vs. control drink within time point, Bonferroni adjusted post-hoc. † P < 0.001 significant main effect of condition.
Figure 26. Two-hour area under the curve (AUC) following a single drink of ketone monoester supplement or control drink. Drinks were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). The black line represents the KM condition while the dotted line represents the control drink. (A) Beta-hydroxybutyrate (BHB) AUC (n=15); (B) Glucose AUC (n=15); (C) Insulin AUC (n=14); and (D) C-peptide AUC (n=14). *P < 0.001 vs. control drink, **P = 0.002 vs. control drink.
Figure 27. Two-hour incremental area under the curve (iAUC) following a single drink of ketone monoester or control drink. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). The black line represents the KM condition while the dotted line represents the control drink. (A) Glucose iAUC (n=15); (B) Insulin AUC (n=14); and (C) C-peptide iAUC (n=14). *$P < 0.01$ vs. control drink, **$P < 0.05$ vs. control drink.
Figure 28. Oral glucose insulin sensitivity (OGIS) index following a single drink of ketone monoester or control drink. The black line represents the KM condition while the dotted line represents the control drink. Drinks were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT) (n=14). *P = 0.01 vs. control drink.
Figure 29. Non-esterified fatty acids (NEFA) and Glucagon-like peptide 1 (GLP-1) following a single drink of ketone monoester or control drink. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). The black line represents the KM condition while the dotted line represents the control drink. (A) NEFA response over time (n=15); (B) NEFA Two-hour area under the curve (n=15); (C) GLP-1 response over time (n=14); (D) GLP-1 two-hour area under the curve (n=14). *P < 0.005 vs. control drink within time point, Bonferroni adjusted post-hoc. † P < 0.001 significant main effect of condition. **P < 0.01 vs. control drink. ‡ P < 0.001 vs. control drink.
Figure 30. (A) Lactate (n=15), (B) triglycerides (n=15) and (C) glucagon (n=13) responses following a single drink of ketone monoester supplement or control drink. (D) Glucagon two-hour area under the curve. The black line represents the KM condition while the dotted line represents the control drink. Supplements were consumed in the fasted state followed 30 minutes later by a 75-gram oral glucose tolerance test (OGTT). † P < 0.05 significant main effect of condition. *P < 0.05 vs. control drink within time point, Bonferroni adjusted post-hoc.
DISCUSSION
The objectives of this study were to evaluate the acute impact of an oral KM supplement on metabolic control in response to an OGTT in individuals with obesity. We found that a single drink of KM 30 min before an OGTT significantly reduced both glucose AUC and iAUC as well as NEFA AUC. OGIS, an index of insulin sensitivity, was higher in the ketone condition, which is in line with the decrease in C-peptide iAUC. Similar to our work in young, healthy lean adults [226], GLP-1 AUC and iAUC were also lower in the ketone condition compared to control drink. Our results support the notion that the improvement in glucose control following the ingestion of KE is not driven by increased insulin secretion and is related to improved insulin sensitivity. No changes in CCA blood flow or shear rate were observed throughout the OGTT following the KM drink compared to control drink.

Ketone monoester improves glucose tolerance
To the best of our knowledge, this is the first study to directly assess the metabolic effects of an exogenous ketone drink in adults with obesity. We observed that glucose AUC decreased by 11% during a 2-hour OGTT following the KM. We have demonstrated that this same KM supplement and protocol improved the glycemic response by 16% in young, healthy lean adults [226]. Stubbs and colleagues [222] also reported that the KM drink raised BHB levels between 1.3-4.7 mmol/L and reduced circulating glucose by ~15% in both the fasted and fed state in young, healthy participants. Past studies have described the metabolic benefits of BHB infusion in both children and adults with obesity [243, 250]. Binkiewicz and coworkers [243] reported that BHB infusion reduced glucose, with greater effects in lean, compared to obese, children. A decrease of ~10% in steady state glucose was also shown in non-obese and obese adults during a BHB infusion [250]. Both studies were conducted in the fasted state and, importantly, the study conducted by Binkiewicz and colleagues [243] showed an accompanying increase in insulin with ketone infusion, perhaps due to the extremely high levels of BHB achieved (i.e., ~15
mmol/L). In our study, we did not find any significant differences in insulin levels between conditions; however, insulin (155±74 to 207±95 pmol/L, \( P < 0.001 \)) and C-peptide (585±225 to 713±291 pmol/L, \( P = 0.004 \)) increased significantly following KM consumption from time -30 to 0 min (Figure 25C, 25D). The potential for BHB to stimulate beta-cell insulin secretion in the fasting state has been observed in several studies [222, 226, 252] and it has been suggested by Mikkelsen and coworkers [232] to occur when BHB are increased rapidly to levels above 2 mmol/L. Nevertheless, insulin secretion was not be the main driver for the observed fall in glucose and NEFA because: i) no differences in insulin concentrations between conditions were recorded throughout the 2-hour OGTT in either of our KM studies [226]; and ii) the glucose and NEFA-lowering effect of BHB are present in individuals with T1D and in healthy humans who showed no concomitant changes in [243, 244, 250, 373], or even decreased [232], insulin levels with ketone infusion.

Along these lines, our findings suggest that raising BHB through KM supplementation may improve insulin sensitivity, as shown by an 11% increase in the OGIS. This was further supported by lower C-peptide iAUC and reduced active GLP-1. Given that GLP-1 has potent effects on postprandial insulin secretion and glycemic control, the lack of an increase in markers of insulin secretion supports the idea of improved tissue insulin sensitivity. There is no indication that the KM impacts digestion and absorption [221, 222], but we cannot rule out this possibility, which should be assessed in future studies.

**Ketone monoester impacts fat metabolism**

When elevated, BHB binds to GPR109A (encoded by the \( HCAR2 \) gene and also known as the nicotinic acid receptor), which inhibits adipose tissue lipolysis [362]. Balasse and colleagues [374] also reported that GPR109A stimulation increases NEFA uptake, contributing to the NEFA-lowering effect of BHB. In the present study, a 21% decrease in NEFA AUC was seen during the OGTT following KM ingestion. Acutely reducing NEFA levels with acipimox has been
shown to inhibit hepatic glucose output [375], while lipid infusion stimulates glucose release from the liver [376]. Thus, it is likely that the lower levels of NEFA in the KM condition may have contributed to the associated lower glycemic response, although other mechanisms could be involved [377]. Contrary to Stubbs and coworkers (2018), who observed a decrease in triglycerides following KM ingestion, no effects were observed in our study [222]. This could be related to the differences in the fat and protein content of the drinks provided in the two studies.

Mechanistic studies have shown that hepatic glucose output was reduced by ~25-30% in man [247, 378] and ~40-50% in dogs [353, 374] with a concomitant decrease in BHB muscle uptake during ketone infusion [247, 379-381]. Whether the mechanism behind this reduction in hepatic glucose output is hormonally mediated or the result of a direct effect of ketone bodies remains unclear, but might be related to a fall in gluconeogenic precursors [250]. In our study, we found that lactate was slightly, but significantly lower (main effect of condition $P = 0.038$) when compared to the control drink. Other studies have shown inconclusive results on the effect of BHB on lactate concentrations with some reporting no effect [250] or an increase [247] at rest, while others observed a decrease during exercise [227]. Lastly, based on the fall in NEFA concentrations in the ketone condition, it is likely that lipolysis was decreased, reducing the availability of glycerol, as found with BHB infusion [232, 382].

**Role of obesity**

Based on the HOMA-IR score (2.9 ± 1.4), the participants in this study displayed evidence of insulin resistance. Interestingly, compared to the same relative amount of KM in healthy lean adults [19], BHB levels in circulation reached a similar peak (3.4 mM vs. 3.2 mM) but stayed elevated longer throughout the OGTT in participants with obesity. Although this could be related to the higher absolute drink (46 ± 10 vs. 32 ± 5 grams), some evidence suggests that BHB utilization could be affected by age, obesity and/or insulin resistance [365-368]. Although the obese participants experienced a more sustained elevation of BHB in response to the KM
supplement, the relative percent changes in glucose and NEFA appear to be attenuated compared to the young lean individuals. Potential differences in the maximum change in glucose (23.1 vs. 12.3 mg/100 ml) and NEFA (875 vs. 625 uEq/L) following ketone infusion between lean and obese children have been observed [243]. In participants with T2D, sodium acetoacetate infusion altered neither plasma glucose nor hepatic glucose production, but did lower NEFA levels compared to saline infusion [256]. It will be interesting to determine in future studies the impact of KM drinks in individuals with different levels of insulin resistance.

**Impact of ketone ester on hemodynamics**

Acute and chronic hyperglycemia reduces CBF by 7% and 14%, respectively [383], but only chronic hyperglycemia decreases cerebral glucose utilization [384]. Notably, the reduction in cerebral glucose utilization occurs alongside increased cerebral ketone utilization [385], thereby maintaining overall cerebral oxidative metabolism. The decreased resting CBF, coupled with adverse vascular remodelling [386], likely contribute to the incidence of cerebrovascular disease in patients with obesity and T2D; therefore, improving blood flow patterns via KM could prove useful in individuals with obesity. Indeed, systemic ketone levels dictate cerebral ketone uptake and oxidation [232], and acute ketone infusion increased CBF linearly by 30-40% [369, 387]. As a proxy index of CBF, assuming no changes in extra-cranial blood flow [371], we did not observe changes in flow or shear patterns in the CCA. There are two important consideration with these observations. First, although CCA flow was unchanged, the KM rapidly increased plasma ketone bodies within 30 min by 17-fold (up to 3 mmol/L), indicating that delivery of BHB to the brain would be greatly increased. Second, acute hyperglycemia following the OGTT likely counteracted any increases in CBF and related shear patterns induced by the KM [383].

Another noteworthy hemodynamic observation was the 3-4 mmHg reduction in MAP throughout the OGTT in the ketone group that was reflected in a likely baroreflex-mediated compensatory increase in heart rate (HR). Although we cannot find evidence in humans, rodent
work indicates that BHB reduces sympathetic tone via a free-fatty acid receptor 3 pathway at the level of the sympathetic neurons. Such changes in vasomotor sympathetic nervous activity could explain the observed changes in MAP following the KM drink and further support a functional link between nutritional status and autonomic function.

Strengths and limitations
This study was conducted in adults with obesity who were taking neither glucose nor lipid-lowering medications, so it is unknown if our results will apply to individuals with T2D who are on glucose-lowering agents with more advanced metabolic impairment. We were not powered to assess sex differences, but based on exploratory analysis in healthy lean adults and the current study, the effect of KM does not differ between males and females. Our study findings are also limited to the 2-hour period following a single drink of KM and future studies looking at the glucose-lowering effect of repeated KM drinks over the longer-term are needed. Finally, since no tracers were used in our study, we were unable to assess the direct mechanisms that could shed some lights on the observed metabolic effects of KM (i.e., reduced hepatic glucose output, adipocytes lipolysis, etc.).

TAKE-HOME MESSAGE
Consuming an exogenous KM drink lowered both the glycemic and NEFA responses to an OGTT in individuals with obesity. Our results support the notion that the improvement in glucose control following the ingestion of KM was not driven by increased insulin secretion, and is likely related to improved insulin sensitivity and, potentially, a direct effect of BHB on adipose tissue lipolysis. The implications of acute and chronic KM on CBF and related cerebral ketone metabolism, as well as mediating favorable changes in hemodynamic function (i.e., blood pressure), warrants future research. It is possible that the observed metabolic effects of BHB are feedback mechanisms, where BHB partly replaces glucose as fuel while limiting its own
synthesis by inhibiting lipolysis. Future studies in individuals with T2D should be performed to investigate if individuals with even greater insulin resistance could potentially benefit from ketone drinks.
Chapter 6: General discussion and future directions

6.1 Saliva insulin study

6.1.1 Main findings

To date, it is known that salivary insulin increases following the consumption of CHO [281, 282]. Though these studies support the notion that saliva insulin may reflect blood insulin levels, it does not provide insight into whether changes in saliva insulin are sensitive enough to delineate between different meals or between individuals with different metabolic profiles. The objective of Chapter 2 of this thesis was to verify if salivary insulin could be used as a tool to delineate between high and low insulin levels following the ingestion of a high- or low-carbohydrate mixed meal. Our results showed that in both normal weight (NW) and overweight/obese (OO) individuals, plasma and saliva insulin total area under the curve (AUC) and incremental AUC (iAUC) were significantly higher after the HC as compared to the LC meal (all \( p \leq 0.005 \)). Insulin AUC and iAUC in both plasma and saliva were higher in OO than in NW after the HC meal (all \( p \leq 0.02 \)) but only plasma and saliva total AUC were higher in OO after the LC meal (both \( p \leq 0.01 \)). Plasma insulin AUC was significantly correlated with salivary insulin AUC in LC (\( r = 0.821; \ p < 0.001 \)) and HC (\( r = 0.882; \ p < 0.001 \)) suggesting that saliva insulin is representative of plasma insulin. These findings were encouraging first steps for our industry partner, who have continued research on saliva insulin (two subsequent research projects have been funded in our laboratory) in their quest to develop a personal saliva insulin monitor.

6.1.2 Saliva insulin responses to meals

Obesity is generally accompanied by metabolic impairments including insulin resistance and hyperinsulinemia [390]. Accordingly, Chapter 2 found that fasting insulin was higher in both plasma and saliva in the OO as compared to NW. The differences between overweight and normal weight groups in fasting and postprandial saliva insulin were seen despite both groups having glucose levels in the normoglycemic range, highlighting the potential utility of saliva
insulin for detecting underlying insulin resistance and hyperinsulinemia. Fasting saliva insulin concentration was ~30% of the plasma concentration and was positively correlated with plasma insulin levels ($\rho = 0.60$, $p = 0.017$) which is in the same general range to previous reports in adults without diabetes [279, 281, 282]. Small differences in absolute fasting saliva insulin levels could be due to the fact that these earlier studies used a radioimmunoassay technique whereas we used ultrasensitive ELISA. Currently, our ability to monitor insulin is limited to invasive measures that are time consuming and require medical expertise. Finding alternatives to track insulin levels repetitively throughout the day would be greatly beneficial as hyperinsulinemia can be detected years before circulating glucose rises in someone destined for developing T2D. Identifying hyperinsulinemia thus holds the potential to improve metabolic disease detection, prevention and management. To date, most studies that have looked at saliva insulin concentrations have done so in the fasting state or in response to an OGTT [278-280], which does not provide information on postprandial insulin fluctuations following whole-food meals. Correcting postprandial hyperglycemia is considered essential for good overall glycemic control in T2D [64]. Moreover, if saliva is to be used as a fluid to monitor systemic insulin, small but important changes in concentrations such as the ones observed between metabolic status and meals with different carbohydrate contents need to be distinguishable. Our study, by investigating these differences, provided important information to the scarce literature on saliva insulin by showing that differences in salivary insulin can be tracked between meals with different insulinogenic effects and between normal weight and overweight participants.

### 6.1.3 Future directions

In our study, commercial ELISA kits were used to assess insulin concentrations. In order for our results to be translatable, a portable insulin monitor provided with a saliva collection device and/or filter with the capacity to accurately and repeatedly read insulin concentrations could be developed. If combined with glucose monitoring, it would allow individuals to monitor insulin
resistance and their specific responses to different foods in real-time. Contrary to metabolites such as glucose or lactate, which can be measured using biochemical enzymatic assays, peptide hormones like insulin typically require antibody-based techniques to accurately measure, which creates technical challenges when making an assay portable, time-efficient and easily accessible. Substantial engineering will be required to develop a rapid insulin assay for use with saliva. Furthermore, additional studies using larger sample sizes and conducted over a longer period of time are warranted to better explore the kinetics and consistency of salivary insulin responses following mixed-meals to further validate this non-invasive assessment of metabolic health.

### 6.2 Low-carbohydrate high-fat diet in type 2 diabetes study

#### 6.2.1 Main findings

The main objective of Chapter 3 was to determine whether reducing hyperglycemia by following a LCHF diet alone, or in combination with post-meal walking, could improve CGM-derived measures of glycemic control and lower markers of innate immune cell activation and systemic inflammation in people with T2D. Our study showed that while LC and LC+Ex led to superior improvements in glucose control and fasting proinsulin levels as compared to GL, all three diets appeared to lower PBMC p-JNK (a marker of cellular inflammation) over the short-term with no or minimal effect on circulating cytokines levels.

#### 6.2.2 Glycemic control

LCHF diets [296] and exercise [391] have both been shown to help with glucose control in T2D but if their additive effects are currently unknown. Our study suggests that the benefits of these interventions are additive as we found that four-day mean glucose was significantly lower in the LC+Ex as compared to LC (-5%) while both LC+Ex (-16%) and LC (-12%) conditions were lower than GL. Moreover, exercise possesses several cardiometabolic benefits for T2D patients
beyond glycemic control, such as reduced blood pressure and improved blood lipid regulation [392]. Thus, to optimize health, exercise should be an important part of diabetes prevention and management regardless of the type of diet consumed.

Though chronic hyperglycemia is an important part of glucose intolerance, glucose fluctuations and peaks have also been shown to an important aspect of glucose dysregulation [320]. Here again, our results showed the superiority of LCHF with both LC+Ex and LC showing a lower glucose standard deviation (both -53%), MAGE (-61% and -54%) and time over 10 mmol/L (-90% and -83%) when compared to GL. Overall, the observed normalization of glycemia within days, combined with the potential decrease and even withdrawal from medications on the longer-term in individuals with T2D [312, 393], makes the LCHF diets a powerful tool for diabetes management and possibly reversal [394].

6.2.3 Markers of beta-cell function
T2D is characterized by insulin resistance and hyperinsulinemia leading to exhaustion of the beta-cells that cannot keep up with the excessive insulin demand required when CHO are ingested. Insulin processing becomes impaired in individuals with IR/T2D and proinsulin secretion increased. By restricting carbohydrate intake, we posited that beta-cells could get some transient “rest”, allowing for decreased endoplasmic reticulum stress, and improved proinsulin processing [340]. Though no direct (or dynamic) assessments of beta-cell function were performed in our study, proinsulin concentrations were recorded and showed a significant decrease follow both LC and LC+EX. Since beta-cells exhaustion is mandatory for the onset of diabetes and proinsulin is an independent cardiovascular risk factor, interventions such as LCHF diets could hold the potential to improve beta-cell function and cardiometabolic profile in this population. Future studies are warranted to determine if LCHF diets can provide enough beta-cell rest leading to improvement in beta-cell function; such information appears required to solidify true diabetes “reversal” with LCHF diets.
6.2.4 Inflammatory profile

Inflammation is at the crossroad of almost every metabolic disease, including diabetes [395-397]. The benefits of LCHF diets on glycemic control and hyperinsulinemia are well established but effects on inflammation have received less attention. In fact, a substantial body of evidence indicates that different single high-fat meals lead to a pro-inflammatory response, particularly when measured at the cellular level in people with obesity or T2D [299, 337]. Few long-term studies using LCHF diets have studied inflammation at the level of immune cells. However, weight-loss diets, including LCHF, have been shown to reduce circulating inflammatory cytokines and C-reactive protein in populations with IR and obesity [140, 398]. The independent effect of weight loss versus the important reduction in glucose spikes and insulin secretion with LCHF diets is difficult to distinguish. Thus, we decided to conduct a short-term LCHF diet study with the hope that we could help to isolate for the direct immunomodulatory impacts of glucose lowering with LCHF eating in the absence of substantial weight loss.

The pro-inflammatory JNK signaling pathway has been identified as major player in chronic inflammation associated with obesity and T2D [399]. In Chapter 3 of this thesis, phosphorylated-JNK was significantly reduced following all three diets (LC, LC+EX and GL). This could be explained by the fact that food provided to the participants was made of whole food with controlled serving sizes, which might have been healthier than the food participants usually consumed. Moreover, all diets decreased body weight slightly (~1-2 kg), which suggests that despite our efforts, the diets were slightly hypocaloric. This is likely a result of controlling portion sizes and eating opportunities when all food is provided to participants in a study design such as ours.

In addition to inflammatory signaling in circulating PBMCs, we also assessed MPs as circulating molecular biomarkers related to inflammation. While LMPs were unchanged in all three conditions, MMPs were lowered by GL and a tendency for lower MMPs observed for LC ($P = 0.067$). MPs are released in circulation following apoptosis or inflammatory activation of
cells and can act as both mediators and markers of inflammation. MPs are elevated in T2D and based on recent studies, appear to act as messengers between cells via the shuttle of proteins, mRNA and miRNA. The variability in MPs count between participants was quite high in our study and might have contributed to the absence of detectable changes. To the best of our knowledge, MPs have never been quantified following a LCHF intervention and the exact time required to induce significant changes remains to be elucidated. Work on the role of MPs and other extracellular vesicles (e.g., exosomes, apoptotic bodies) in metabolic disease is in its infancy, and further studies are needed to better characterize these novel biomarkers, as well as determine their function as markers or mediators of inflammation, metabolic, or cardiovascular dysfunction [400].

Our results showed that none of the three diets decreased circulating cytokines or total leukocytes. Since multiple cells throughout the body produce cytokines into systemic circulation, small changes can be difficult to detect and longer-term studies with larger effects (e.g., weight loss) might be necessary to observe significant changes. Nevertheless, the findings that PBMC p-JNK was reduced by all diets and that GL (and to a lesser extent LC) reduced LMPs in the absence of changes in plasma cytokines, may suggest that measuring inflammation at the level of immune cells is more sensitive to dietary changes. This is in line with a recent systematic review on the impacts of acute high-fat meals on inflammation in humans [401].

As previously suggested, it is likely that LCHF diets require at least a few weeks of adaptation before their full benefits can be observed. The 4-day intervention thus might not be sufficient to allow the cellular and molecular changes to be reflected in circulating markers (e.g., cytokine) or cell receptors (e.g., toll-like receptors). Overall, the results of Chapter 3 should be interpreted as positive for LCHF diets, with and without exercise, as there were seemingly no detrimental pro-inflammatory effects on participants.
6.2.5 Future directions

Since metabolic adaptations to LCHF can take several weeks to months, and that total energy intake can be affected when on the diet, longer-term studies with ad libitum food intake could be beneficial to deepen our knowledge on their chronic effect on inflammatory and metabolic status. Also, it is possible that the 15-min low-intensity post-meal walks might not have been long or intense enough to trigger additional cardiometabolic modifications on top of the observed improvement in glycemic control. Studies incorporating moderate- or vigorous-intensity exercise sessions should be performed to assess if exercise can provide other additional benefits to LCHF diets [402]. It would also be of interest to perform post-meal walking or other exercise with the GL diet in order to tease out the isolated impacts of exercise and diet within the same study.

6.3 Ketone monoester and metabolic control studies

6.3.1 Main findings

Ketogenic interventions such as LCHF diets and intermittent fasting are known to improve glucose control but their glucose-lowering effect is likely driven by the associated decrease in carbohydrate consumption more than the presence of elevated ketone bodies. Chapter 4 and Chapter 5 of this thesis had as main objective to evaluate the impact of a ketone monoester drink on the glycemic response to a 2-hour OGTT. Additionally, NEFA, insulin and other hormones and metabolites were also measured.

Overall, both studies showed similar results in young lean adults (YLA) and older adults with obesity (Ob). First, we observed that a KM drink can increase BHB levels to approximately 3 mM within 15 minutes and improve glucose AUC by 16% in YLA and 11% in Ob. Along with the attenuation in glycemic response to the OGTT, an important decrease in NEFA of 44% in YLA and 21% in Ob were observed. These metabolic improvements did not appear to be
explained by an increase in insulin during the OGTT, but an improvement of 11% in OGIS index was observed.

6.3.2 Glycemic control and non-esterified fatty acid levels

Our results are in line with previous studies in humans that used BHB infusion [244, 247, 250, 373]. However, our studies are the first to directly confirm the glucose and NEFA-lowering effects using a KM drink before a standardized OGTT in humans. In both studies, these improvements did not appear to be explained by an increase in insulin or C-peptide during the OGTT, but we did observe a higher OGIS index. Since this index is only a surrogate marker of insulin sensitivity computed using certain glucose and insulin times points during the OGTT, it is not possible to be sure if liver or muscle insulin sensitivity were actually improved. The concomitant decrease in GLP-1 and C-peptide iAUC over the 2-hour OGTT in the KM condition seems to point in that direction. Noteworthy, the decrease in C-peptide iAUC is likely explained by the slight increase that occurred at time 0 as opposed to an overall decrease in C-peptide secretion. Based on previous mechanistic studies, liver glucose production is decreased with ketone infusion and might be responsible for the improved glycemic response observed in our study [247]. In support for this, insulin did significantly increase between the KM drink ingestion and the beginning of the OGTT, which we could speculate might have been sufficient to subsequently inhibit hepatic glucose output. Though many studies where KB decreased circulating glucose levels found no increase in insulin in systemic circulation, it is possible that the elevation in insulin was confined to the portal vein. Alternatively, BHB might have a direct and non-hormonal-related effect on the liver that remains to be elucidated. Finally, we cannot discard the possibility that the presence of ketone in the digestive tracks slows down gastric emptying, resulting in a slower release of glucose in circulation.

By acting as a ligand for the GPCR1019A receptor, BHB has been shown to reduce the release of NEFA from adipocytes into the circulation [403]. This decrease in NEFA might have
contributed to a possible improvement in liver insulin sensitivity, which could have led to decreased liver glucose output which has been shown to be inversely correlated to NEFA levels [404]. In T2D and obesity, which are characterized by elevated glucose and lipids such as NEFA, a KM drink could be an option to improve levels of both metabolites. However, it remains to be determined if adding an additional fuel into the mix in individuals with metabolic/mitochondrial overload (i.e., the sum of glucose, lipids, and ketones that cells/mitochondria are exposed to) will be beneficial in the long term.

6.3.3 Future directions

Some pharmacological treatments have shown that simply reducing glucose by itself does not appear to be sufficient to improve cardiovascular disease [149]. For this reason, an important gap that needs to be filled is to discover the mechanisms that underlies the improvement in glucose control following KM consumption. The use of hyperinsulinaemic-euglycaemic clamp or stable isotope glucose tracers could deepen our understanding in that matter by providing better information on tissues insulin sensitivity and the origins of circulating glucose. Such information could then be used to optimize interventions using a KM drink, for example by targeting specific populations that are more likely to respond positively to this supplement. Future studies could also explore gastric emptying (e.g., using acetaminophen) aiming to better understand the mechanisms behind the reduced glycaemic response following the KM supplement.

Insulin resistance is known to affect ketone metabolism and therefore it is still unknown if a KE drink will yield the same benefits in individuals with T2D. So far the only study that has evaluated the effect of acetoacetate infusion in T2D has been able to observe a decrease in NEFA but not glucose [256]. Future studies would also need to evaluate the impact of KM drink following mixed meals over a period of several days to weeks where participants would have to consume the drink multiple times per day. The bitter-taste of the KM can make it difficult to
consume and might limit its application over the long-term as a chronic treatment for metabolic impairments.
Chapter 7: Strength and limitations

7.1 Saliva insulin study

This study has some limitations that should be acknowledged. First, the relatively small sample size limits our ability to fully appreciate the range of achievable postprandial saliva insulin levels in healthy adults. Second, our results are limited to the insulin and glucose responses in the fasting state and following a morning mixed meal, which might differ from meals consumed at other times of the day. Finally, more frequent samples along with a longer postprandial period (e.g., 3-4 hours) would have been useful in order to better compute the insulin curves and determine the timing of when saliva insulin returns to basal levels. Despite the above limitations, our study is strengthened by the fact that we used a randomized crossover design for the meal intervention and assessed metabolic responses to “real-life” mixed-meals.

7.2 Low-carbohydrate high-fat diet in type 2 diabetes study

In this study, despite our efforts to maintain energy balance, a small (~1-2 kg) but significant weight loss occurred in all conditions. The short duration of each intervention did not permit adjusting the caloric intake in attempts to prevent weight loss but energy intake was matched between conditions. Therefore, the small weight loss may be related to an overall increase in diet quality and/or strictly controlling food over the four days. The clinical relevance of this small reduction is unclear but we acknowledge that weight loss could have played a role in the modulation of inflammation. All food consumed by participants was provided and consisted of healthy whole foods. Thus, it is likely that most participants improved the quality of their diet as compared to what they usually eat by removing processed foods. This might have contributed to the absence of significant differences between conditions for some outcomes since each diet seems to have provided a certain degree of improvement over habitual patterns of participants. Furthermore, despite providing fresh whole foods the strict protocol may have reduced generalizability as three participants were unable to complete all conditions citing inability to
comply with study procedures. Like most people with T2D, the participants in the current
investigation were on medications. We made sure to instruct each participant to continue to take
their regular medications throughout the entire study and used a daily log to confirm this but
interaction between medications, diets, and inflammatory outcomes are unknown. Finally, the
relatively small sample size did not allow for examination of interactions with medications or by
sex.

The use of a randomized crossover design and the fact that all food was prepared and
provided ensured a tighter control of the participants’ food intake. Also, the LCHF diets provided
in this study were well formulated with whole food and were very low in carbohydrate (below
10%) to ensure a strong glucose-lowering effect. Moreover, glycemic control, the main outcome
in this study was determined both in the fasting state before and after, as well as throughout,
each 4-day intervention period using a continuous glucose monitor.

7.3 Ketone monoester and metabolic control studies

Some limitations of the KM studies should be acknowledged. First, Chapter 4 was conducted
with healthy young individuals and Chapter 5 was with adults with overweight/obesity so the
results may not apply to clinical populations with advanced metabolic impairments (e.g., those
with T2D). Given that KM are so new, we aimed to investigate the response in healthy
individuals to limit potential confounding from insulin resistance, β-cell dysfunction, and
medications, all of which could influence interpretation of OGTT results. Second, the use of the
OGIS index limits mechanistic interpretation of our findings compared to a more direct
assessment of insulin sensitivity such as a hyperinsulinemic-euglycemic clamp. However, the
OGIS correlates well with clamp-derived measures in healthy individuals and, if KM is to be
used therapeutically to lower glucose, we consider it important to demonstrate efficacy in
reducing glycaemic response because, ultimately, individuals consume food/CHO, which
contributes to hyperglycaemia in real-life. Finally, the present studies could not rule out potential
effects of the KM on the digestion and absorption of the glucose drink. Accordingly, future studies could explore gastric emptying aiming to better understand the mechanisms behind the reduced glycemic response following the KM supplement. Also, only a single dose of KM has been studied and whether the glucose and NEFA-lowering effects would persist throughout the day and after several weeks with chronic consumption is unknown.

These limitations were counterbalanced by the novelty of these studies which are the first investigations to directly assess the effect of the new oral KM drink in humans following a standardized OGTT. We included both males and females in the studies and exploratory analyses confirmed that both sexes appeared to respond similarly to KM. Finally, the key glucoregulatory hormones and metabolites were analyzed in both studies, which contributed to a better global understanding of the metabolic effects of this drink.
Chapter 8: Conclusion

The aims of this thesis were to determine if 1) saliva can be used to track insulin fluctuations following low and high-carbohydrate meal in both lean and obese individuals, 2) a 4-day LCHF diet with or without exercise can improve glycemic control and reduce inflammation in individuals with T2D, 3) a KM drink can reduce the glycemic response and NEFA levels during an OGTT in young lean adults and adults with overweight/obesity. Our first study results suggest that saliva insulin can potentially be used as a fluid to track low and high insulin levels. Future studies should try to develop a saliva insulin monitor that could be an alternative to more invasive methods of monitoring insulin. Our second study using a short-term LCHF diet confirmed that this approach is almost immediately effective for controlling glucose in T2D and that LCHF meals (when made from whole food) do not cause a pro-inflammatory response. Finally, our third and fourth studies concluded that rapidly raising beta-hydroxybutyrate by consuming a KM drink prior to a glucose load improved glycemic control and reduced NEFA levels, which could be of great interest for the prevention and management of hyperglycemia and insulin resistance. Overall, limiting the intake of carbohydrate and consuming a ketone-based drink could hold the potential to improve metabolic health.
Bibliography


