



University of Saskatchewan

# **D-Lactate and its related metabolic intermediates: Potential biomarkers for diabetic vascular complications**

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By

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## **ABSTRACT**

Micro- and macrovascular diseases are the main causes of mortality and morbidity among patients with Type 2 Diabetes Mellitus (**DM2**). Routine surveillance for vascular complications in these individuals is important to optimize their health outcomes. Endothelial dysfunction is one of the known root causes of micro- and macrovascular problems of all patients including diabetic patients, yet endothelial function is not directly tested in diabetic individuals. Intracellular Adhesion Molecule (**ICAM**), Vascular Adhesion Molecule (**VCAM**), and E-selectin are three important research biomarkers of endothelial function. D- and L-Lactate and methylglyoxal are three natural glucose metabolites and have known toxicity to the endothelium. We aimed to explore the correlation of these three key glucose metabolites in diabetic patients with the biomarkers of endothelial dysfunction to determine if glucose control is reflective of endothelial health. Metformin, a common oral diabetic medication, may increase D-lactate levels in serum and may directly damage endothelium, which was also explored in this study.

The study involved an open label randomized design with four unique groups of participants. Two groups were patients with a DM2 diagnosis who either were prescribed metformin (**DM2 plus MET**) or were not treated with metformin (**DM2 no MET**). The remaining two control groups did not have DM2, but one group was prescribed metformin for unrelated indications (i.e. women with PCOS) (**NonDM plus MET**) and the other group was not prescribed metformin (**NonDM no MET**). Multiple fasting blood samples were collected from each individual over the 1-year study at the Royal University Hospital in Saskatoon. In total 76 persons participated in the study, 6 in DM2 plus MET, 1 in DM2 no MET, 39 in NonDM plus MET and 30 in NonDM no MET groups. In the non-DM2 groups, 35 of 39 participants were women treated for >6 months

with metformin for polycystic ovarian syndrome (**PCOS**) and were originally recruited as part of another study.

We hypothesized that the hyperglycemia associated with DM2 correlates with relatively high levels of glucose metabolites and endothelial damage markers. To test this correlation, fasting glucose, hemoglobin A1C, and fasting insulin levels of all participants were measured. All these measurements revealed that levels of these factors among diabetic patients are higher than non-diabetic patients consistent with their underlying hyperglycemia. The mean for fasting glucose levels was 9.4 mmol/L for diabetic participants and 5.0 mmol/L in non-diabetic participants over three independent testing times. The mean levels for HbA1C were 7.2% in the diabetic groups and 5.2% for non-diabetic participants (normal  $\leq 7\%$ ). These findings revealed suboptimal long- (reflected by HbA1c) and short-term (fasting glucose) glucose control among diabetic participants.

As predicted, the levels of three glucose metabolites, D-lactate, L-lactate, and methylglyoxal, were higher in the diabetic population than in non-diabetic participants. We did not detect D-lactate elevations above normal for any of our individual cases or in grouped analysis including those taking metformin. However, the mean D-lactate level in diabetic participants was significantly higher than non-diabetic participants (P-value  $<0.05$ ), although still in the normal range and less than 0.5 nmol/ $\mu$ L. This important finding included our study group using metformin for diabetic control. This was one of the major concerns for metformin users and led us to formally assess this. The average L-lactate level in diabetic participants was significantly higher than in non-diabetic participants (P-value  $<0.05$ ), yet the methylglyoxal level in diabetic participants was not statistically different from non-diabetic participants (P-value  $>0.05$ ). Our fasting samples did not provide enough examples of hyperglycemia to conduct a comparison between the levels of D-

lactate, L-lactate, and methylglyoxal among participants with high and normal levels of blood glucose.

To address if the levels of the glucose metabolite, D-lactate, directly correlated with markers of endothelial damage, we explored the association of three endothelial biomarkers, ICAM, VCAM, and E-selectin with D-lactate levels. We detected a significant positive association between D-lactate and both ICAM and E-selectin (P-value <0.05), but not VCAM and D-lactate (P-value >0.05). This positive correlation of 2 out of 3 markers of endothelial dysfunction with D-lactate levels provides opportunity to consider D-lactate as a potential biomarker for endothelial dysfunction in diabetic patients. Measuring D-lactate needs simpler instruments and in general can improve access for monitoring cardiovascular consequences in DM2 patients. In addition, except for a few differential diagnoses, D-lactate may provide a reliable opportunity for identification of the cardiovascular consequences.

A number of limitations existed in this study included the small number of recruited patients as the demographic and health-related differences of diabetic and non-diabetic patients, including sex, age, race, weight, and BMI all of which have the potential to affect the results of the study. All the NonDM plus MET group were women so that the sex was not equally represented and may impact the outcomes. Furthermore, we need to consider that the diabetic participants were much older than the control group which can influence the results. These exciting and novel results in our pilot study will require a larger study to confirm the generalizability of D-lactate as a potential biomarker of endothelial damage.

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## List of Abbreviations

ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AGE	Advanced glycation end-products
ANG	Angiotensin
AIF	Apoptosis inducing factor
AKT1	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANG-II	Angiotensin II
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bcl-2	Apoptosis regulator Bcl-2 alpha isoform
Bcl-XL	B-cell lymphoma-extra large
Bp	Base pair
Ca <sup>2+</sup>	Calcium
CAD	Coronary artery disease
Cr	Creatine
CrP	Creatine phosphate
CVD	Cardiovascular disease
P450	Cytochrome P450
DHAP	Dihydroxyacetone phosphate
DISC	Death inducing signaling complex
DLA	D-Lactic acid
DLD	D-Lactate dehydrogenase
DKA	Diabetic ketoacidosis
DM2	Diabetes mellitus type 2
eNOS	Endothelial nitric oxide synthase
GI	Gastrointestinal tract
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GPX	Glutathione peroxidase
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HbA1c	Haemoglobin A1c
HSP	Heat shock protein;
HT	Hypertension
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis
ICAM	Intracellular adhesion molecule-1
IFG	Impaired fasting glucose
IGT	Insulin glucose tolerance
LAM	Leukocyte adhesion molecule
LLA	L-Lactic acid
L-LDH	L-Lactate dehydrogenase
LTA	Lymphotoxin alpha
MCP-1	Monocyte chemotactic protein-1
MCTs	Monocarboxylate transporters
MG	Methylglyoxal
ml	Milliliter
mM	Millimole

MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF $\kappa$ B	Nuclear factor $\kappa$ B
NO	Nitric oxide
NOS	Nitric oxide synthase
O <sub>2</sub> <sup>-</sup>	Superoxide
OD	Optical density
PAD	Peripheral arterial disease
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PDH	Pyruvate dehydrogenase
PECAM	Platelet endothelial cell adhesion molecule
PGI-2	Prostacyclin
pH	power of hydrogen
PI3K	Phosphoinositide kinase-3
ROS	Reactive oxygen species
SBS	Short bowel syndrome
TCA cycle	Tricarboxylic acid cycle
TNF $\alpha$	Tumor necrosis factor $\alpha$
UA	Unstable angina
VCAM	Vascular adhesion molecule-1
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
$\mu$ M	Micromole

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# 1. Introduction

## Problem Statement

Patients with Type 2 diabetes mellitus (DM2) are at significantly greater risk of developing both microvascular and macrovascular disease over time (M. D. Sharma, Farmer, & Garber, 2011). Although the clinical association between sustained hyperglycemia and development of chronic vascular damage is established, the molecular factors that result in vascular damage are only beginning to be identified, with reactive oxygen species (ROS) as the best-known factor (Giacco & Brownlee, 2010). Yet, ROS are not sufficient to fully explain the chronic damage to endothelial cells. Previous studies have demonstrated that byproducts of glucose metabolism also have the potential to damage blood vessels, including the lining endothelial cells, which have a significant role in vascular homeostasis. Recent literature proposes that other elements such as methylglyoxal and its metabolic end-product, D-lactate, may cause additional adverse effects on the endothelium and contribute to chronic vascular damage (Tikellis et al., 2014). This study aims to explore the correlation between glucose control and glucose metabolites levels as well as the association of D-lactate, L-lactate, and methylglyoxal levels in diabetic and non-diabetic patients with other well-known markers of endothelial dysfunction including vascular adhesion molecule-1 (VCAM), intracellular adhesion molecule-1 (ICAM), and E-selectin. The correlation of serum glucose level and HbA1c with glucose metabolites will also be explored. Finding a positive association might contribute to better screening, monitoring, and management of cardiovascular complications in DM2 patients.

## **2. Literature Review**

### **Diabetes Mellitus**

Diabetes is a very important public health concern all around the globe and one of the four priority Non-Communicable Diseases (NCDs), which has been targeted for action by global health authorities. As per the 2016 WHO report on diabetes, the estimated number of diabetic patients is 422 million (Roglic & Organization, 2016). In reference to the same report the global age-standardized prevalence of diabetes has doubled in adults between 1980 and 2014 (4.7% vs 8%).

Diabetes mellitus (DM) is defined as a fasting plasma glucose level equal or higher than 7.0 mmol/L. This disease consists of three main types: Type 1 diabetes, Type 2 diabetes, and gestational diabetes (Clark, 1994). Type 1 diabetes mellitus (DM1) is an autoimmune disease and results when the immune system turns against the insulin-producing beta cells in the pancreas and destroys them. The pancreas then produces little or no insulin. A person who has DM1 must take insulin daily to live (Witek, Witek, & Pankowska, 2012). The exact reason that causes the body's immune system to attack the beta cells are not completely understood, but it is believed that autoimmune, genetic, and environmental factors, and possibly viruses are involved (Zhang et al., 2008). DM1 develops most often in children and young adults but can appear at any age (Zhang et al., 2008). Symptoms of DM1 include increased thirst and urination, constant hunger, weight loss, blurred vision, and extreme fatigue. These symptoms and signs develop over a short period, while beta cell destruction can begin years earlier. Undiagnosed and untreated DM1 may lead to diabetic ketoacidosis, a life-threatening situation (Witek et al., 2012) and other comorbidities such as retinopathy, nephropathy, peripheral neuropathy and cardiovascular disease, and associated

autoimmune conditions such as coeliac disease, hypothyroidism/hyperthyroidism and Addison disease, mental disorders, epilepsy and pulmonary disease (Fazeli Farsani et al., 2015)

Type 2 diabetes mellitus (DM2) is the most common form of diabetes worldwide. About 90 to 95 percent of people with diabetes have type 2. DM2 is typically associated with insulin resistance, and appears in populations with older age, a family history of diabetes, previous history of gestational diabetes, physical inactivity, and ethnicity. About 80 percent of people with DM2 are overweight (Cheng, 2005). Unfortunately, DM2 is increasingly diagnosed in children and youth due to their Insulin resistance as a result of rising obesity rates, but nationally representative data on the current prevalence of DM2 in adolescents are not available (Viner, White, & Christie, 2017). Upon initial diagnosis of DM2, the pancreas usually produces enough insulin, but the body cannot use insulin effectively, a condition called insulin resistance. With prolonged disease course, though, insulin production usually decreases. Symptoms can include fatigue or nausea, frequent urination, unusual thirst, weight loss, blurred vision, frequent infections, and slow healing of wounds or sores. Some people have no symptoms. The symptoms of DM2 develop gradually, unlike the sudden onset of symptoms associated with DM1 (Done, 1977).

Gestational diabetes (GDM) is defined as hyperglycemia being first recognized during pregnancy. GDM occurs more often in African Americans, American Indians, Hispanic Americans, and in those with a family history of diabetes. Women who have had GDM have a 20 to 50 percent chance of developing DM2 within 5 to 10 years (Clark, 1994). GDM can also affect the unborn child in their future life. The negative consequences are high birth weight, impaired glucose tolerance and higher probability of DM2, and higher adiposity indicators (Dabelea, 2010; Jiménez Cruz, Ortega Cisneros, & Bacardí Gascón, 2014).

Distinction between DM1 and DM2 is not easy in every case and sometimes sophisticated tests for pancreatic function are required. In most cases, it is observed that most type 1 occurs at a younger age and type 2 at middle age, but this is not a scientific distinction between these two types as the prevalence of type 2 is increasing among children and youth. (Roglic & Organization, 2016). The importance of insulin resistance in DM2 has been well studied and the role of reduced effectiveness of insulin in control of blood sugar has been reported (S. Sharma & Taliyan, 2016). This factor has been challenged by the new definition of double diabetes where individuals share characteristics of both DM1 and DM2 (Cleland, Fisher, Colhoun, Sattar, & Petrie, 2013). These factors have complicated the differentiation between DM1 and DM2.

At present, prevention of type 1 is not possible, while the situation is different for type 2. Lack of exercise, overweight, and obesity are risk factors of diabetes type 2. During the past ten years the prevalence of diabetes has been growing much faster in low to middle income countries than high income countries (Roglic & Organization, 2016). Exercise leads to an increase in glucose uptake in skeletal muscles. This happens through the glucose transporter (GLUT4). The level of glucose uptake and GLUT4 translocation gets impaired in DM2 and exercise contributes to reduction of this impairment (Thent, Das, & Henry, 2013).

In 2012, approximately 1.5 million people died because of diabetic related complications (Roglic & Organization, 2016). Microvascular and macrovascular origins contribute principally to the excess morbidity and mortality in diabetes (Souto, Souto, Braga, & Medina, 2011). Diabetic related vascular diseases can be categorized into two major groups, macrovascular and microvascular. Macrovascular problems in DM2 are similar to atherosclerotic lesions from a morphologic and functional point of view, including myocardial infarcts, strokes and heart failure. Microvascular problems emerge as nephropathy, retinopathy, and circulation problems in the

extremities and can lead to visual problems, kidney failure, stroke, cardiomyopathy, and dysfunction of lower extremities (Shi & Vanhoutte, 2017). Solid evidence shows the association between tight glucose control and prevention of micro- and macrovascular problems (Kinaan, Ding, & Triggie, 2015). However, despite consistently good glucose control, vascular problems of these patients still remain a considerable challenge. This suggests that there might be other contributing factors in micro- and macrovascular problems rather than plasma glucose levels alone (Bagi, 2017).

## **The Role of Diet in Diabetes Type 2**

The relationship between diet and DM2 has been well studied and reviewed (Khatib, 2004). The high circulating fatty acids leads to impairment of insulin metabolism and absorption in liver and skeletal tissues. In muscles, insulin stimulated glycogen synthesis decreases and this problem in liver leads to a decreased hepatocellular glucose production and output (Radulian, Rusu, Dragomir, & Posea, 2009). This is the basis the relationship between high glycemic index and insulin resistance in diabetic patients (Kopelman, 2007). The excess intake of energy without suitable and required exercise can lead to excess adiposity (McAuley & Mann, 2006). The best predictor of this excess adipose tissues in the body is waist circumference which provides a better understanding about the fat distribution in the body (Steyn et al., 2004). Adipose tissue excrete a number of cytokines such as TNF- $\alpha$ , Resistin, and Adiponectin that can impair the function of insulin (Saxena et al., 2019). The role of diet in prevention and control of DM2 has been accepted for a long time and many studies have been conducted to recommend the most suitable diet for control of diabetes (Ley, Hamdy, Mohan, & Hu, 2014).

## **Therapeutic and Lifestyle Management of Diabetes**

Successful management of newly diagnosed diabetes often requires pharmacological therapy and lifestyle improvements. Effective management of diabetes leads to decreased medication use, lower rate of comorbidities, and higher life expectancies (Levesque, 2017). Nutritional planning is one of the major steps in diabetes management. Nutritionists believe there is not a generic “diabetic diet”, rather there are some rules which require consideration in meal planning for diabetic persons (Garber et al., 2016). Diabetic patients are recommended to eat a more plant-based diet and use polyunsaturated and monounsaturated fatty acids and no trans-fat and reduced free sugar intake (Organization, 2015). Furthermore, when patients are overweight and obese (body mass index >25 and 30, respectively), weight losses of 5-10% of their body weight helps prevent irreversible and severe dysfunction of pancreatic  $\beta$ -cells (Garber et al., 2016) and improves the ability of individuals to reach glucose targets.

Diabetic patients also are encouraged to have regular physical activity. This not only reduces the risk of cardiovascular disease but helps patients to have better control on their glucose level. In general, physical activity reduces the risk of all-cause mortality in DM2 (Thent et al., 2013). Although solid evidence is lacking regarding the effect of physical activity on improved glucose control in DM1 patients, they are also recommended to have regular physical activity in order to reduce the risk of cardiovascular disease (Levesque, 2017). Diet plays a very important role in control and prevention of DM2, and based on the different dietary regimens in the world, a wide range of recommendations have been proposed. Studies has showed that the risk of DM2 reduces when people consume low energy density food. In addition, fermented dairy products are more suitable for prevention of DM2 than non-fermented products. On the other end, refined grains and beverages with high level of sugars can lead to obesity and diabetes (Kolb & Martin, 2017). The

other major components for successful management of diabetes are blood glucose monitoring, medication management, prevention of acute complications, and foot care (Levesque, 2017).

## **Pathophysiology of Vascular Diseases in Diabetes**

Vascular problems of diabetes can be simply categorized into two major groups, macro- and microvascular complications. The most important macrovascular problems of diabetes are myocardial infarction (MI) and stroke. These are the major causes of mortality and morbidity in both DM1 and DM2 (Holt, Cockram, Flyvbjerg, & Flyvbjerg, 2010). Studies have shown that diabetes has a positive association with macrovascular complication rates even after adjustment for other risk factors including hypertension and hyperlipidemia (Kengne, Amoah, & Mbanya, 2005; Zimmet, 2009). Both hemodynamic and metabolic factors contribute to the macrovascular consequences of diabetes. The Renin Angiotensin System (RAS) is considered to be the most important hemodynamic factor contributing to the macrovascular problems in diabetic patients; however, other factors including endothelin, urotensin system, TNF apoptosis inducing ligands, and complement system are also important contributing factors (Holt et al., 2010).

One of the major differences between macro- and microvascular problems is the role of hyperglycemia. Inadequate glycemic control which can be the result of poor control on diet is more likely to result in microvascular complications such that an increase in HbA<sub>1C</sub> from 5.5% to 9.5% enhances the risk of microvascular complications by 10 times while only 2 times for macrovascular problems (Holt et al., 2010).

The risk of microvascular complications of diabetes (i.e. nephropathy, retinopathy, erectile dysfunction and neuropathy) increases with disease duration and glucose elevations. High glucose

levels activate multiple molecular pathways leading to microvascular complications. These pathways include higher flux through the polyol pathway, enhanced advanced glycation end-products (AGEs), greater shift towards the hexosamine pathway and protein C kinase signaling pathway activation (PKC) (Cagliero, 2016). It is also known that reactive oxygen species (ROS) are common elements of these pathways. Hyperglycemia leads to enhanced ROS production by mitochondria and increases in oxidative stress (Cagliero, 2016). Collectively, these pathways enhance signaling by transforming growth factor beta (TGF $\beta$ ) and lower the level of endothelial nitric oxide synthase (eNOS) and prostacyclin synthase, resulting in enhanced activation of pro-inflammatory pathways and microvascular complications (Cagliero, 2016).

### **Role of Endothelium in Cardiovascular Homeostasis**

The endothelium is a semi-permeable polarized epithelium that lines the lumen of all blood vessels. The endothelium plays a very important role in vascular homeostasis including vascular tone, cellular adhesion, activity of platelets, inflammation of vessel wall, angiogenesis, and proliferation of muscle cells of vessels (Bagi, 2017). Endothelial cells regulate the vascular system through a number of vasoactive molecules including nitric oxide (NO), endothelium derived hyperpolarized factor (EDHF), prostacyclin (PGI<sub>2</sub>), and endothelin (ET-1) (Bryan, You, Golding, & Marrelli, 2005).

Nitric Oxide (NO) is the most important mediator of the endothelium and is produced by endothelial nitric oxide synthase (eNOS) from L-arginine in the presence of a tetrahydrobiopterin cofactor. NO regulates vascular relaxation. NO activates guanylyl cyclase in smooth muscle cells in vessels, which produces cyclic guanosine monophosphate (cGMP), and activates the responsible

kinases which control vascular relaxation ultimately resulting in vasodilatation (Betteridge & Nicholls, 2014; Levick, 2003). Other vasodilator factors that affect the vascular system independently from NO include prostacyclins and EDHF. Their role is more prominent in situations where the bioavailability of NO is decreased such as patients with cardiovascular risk factors (Ozkor et al., 2014). In order to maintain a normal and balanced situation, an equilibrium between these vasoconstrictive (such as ET-1 and angiotensin II) and vasodilator factors (such as NO and PGI<sub>2</sub>) is necessary (Davignon & Ganz, 2004).

Stress on the vessel wall by the blood flow as well as adenosine, bradykinin, serotonin and vascular endothelial growth factor can also separately activate eNOS. These contribute to the control of the vasodilation/vasoconstriction equilibrium. The secretion of these molecules is regulated in response to different kinds of stresses. For example, serotonin is released in response to platelet aggregation and vascular endothelial growth factor is mostly secreted in response to hypoxia (Betteridge & Nicholls, 2014).

Endothelial cells also play a key role in homeostasis (clotting) of the vascular system through control of inflammatory pathways. The role of NO is not limited to vasodilation, but NO also opposes the inflammatory pathways by downregulating key components of these pathways. Furthermore, NO reduces the production of ET-1, a very potent vasoconstrictive factor. In addition to its vasoconstrictive role, ET-1 also has pro-inflammatory, pro-oxidant and pro-proliferative effects. Consequently, the action of NO will diminish all these effects (Bourque, Davidge, & Adams, 2011). Prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), thromboxane A<sub>2</sub> (TXA<sub>2</sub>), and reactive oxygen species (ROS) are other vasoconstrictive factors produced by endothelial cells (Roberts & Porter, 2013) and contribute to vasoconstriction, which can be opposed by NO.

Finally, NO also plays a very important antithrombotic role by inhibiting the proliferation of smooth muscle cells (SMC) and decreasing nuclear transcription of leukocyte-adhesion molecules such as VCAM and ICAM (Levick, 2003). Angiotensin II (in addition to its effect on smooth muscles and vasoconstriction), selectins, and adhesion molecules (all of which are produced by endothelial cells) affect binding and trans-endothelial migration of inflammatory cells. When activated, VCAM and ICAM promote the adhesion of leukocytes and endothelial cells produce Tumor Necrosis Factor  $\alpha$  (TNF- $\alpha$ ), key pro-thrombotic factors that oppose the action of NO (Sumpio, Riley, & Dardik, 2002). Endothelial cells produce two major factors to oppose thrombosis, namely tissue-type plasminogen activator (t-PA) and its plasminogen activator inhibitor I (PAI-1) (Betteridge & Nicholls, 2014). Balance between coagulation and fibrinolysis, then, is another key role of endothelial cells in homeostasis of vascular system.

## **Endothelial Dysfunction**

Endothelial dysfunction occurs when there is an impaired vasodilation, deficiency of nitric oxide or an activated endothelium. Generally, all cardiovascular risk factors including diabetes, hyperlipidemia, hypertension, and smoking affect this equilibrium (Hadi, Carr, & Al Suwaidi, 2005). In the process of endothelial dysfunction, an increase in expression and secretion of pro-inflammatory molecules induces further activation of endothelial cells. This leads to an inflammatory response and vessel wall damage as a first step in triggering plaque formation. Plaque buildup eventually can lead to arterial blockage and ischemia or infarction within key organs and tissues (Betteridge & Nicholls, 2014).

The most important cause for endothelial activation is low NO bioavailability as a result of “eNOS uncoupling” and enhanced oxidative stress. Under pathological conditions, eNOS shifts to

an increased production of superoxide (reactive oxygen species or ROS) and less NO production. Superoxide dismutase metabolizes ROS to hydrogen peroxide and, in turn, hydrogen peroxide affects the function of cellular regulatory proteins and promotes inflammatory gene transcription (Betteridge & Nicholls, 2014). Under pathological conditions such as hypoxia, hyperglycemia or high levels of free fatty acids, the careful balance of ROS production by the mitochondrion is also shifted resulting in an increase in generation of oxygen radicals (Matough, Budin, Hamid, Alwahaibi, & Mohamed, 2012). ROS, in addition to other sources of oxidative stress in endothelial cells such as xanthine oxidase and NADPH oxidase activity, can increase considerably in pathological states such as coronary artery disease. ROS activates a proinflammatory state within the endothelium leading to various micro- and macrovascular sequelae (Kayama et al., 2015).

### **Effects of Diabetes on Endothelium**

Several discrete mechanisms associated with chronic hyperglycemia can affect the equilibrium of endothelial cells and lead to dysfunction (Figure 2.1). In diabetic patients, hyperglycemia can have detrimental effects on endothelial homeostasis. High levels of intracellular glucose activates four major pathways in the endothelial cell – hexosamine and polyol pathways, protein kinase C isoforms, and formation of the reactive carbonyl compounds, methylglyoxal and glyoxal. (Betteridge & Nicholls, 2014). In this process, accumulation of triosephosphate intermediates ( $TP_{INT}$ ) forms methylglyoxal (MG), which is a reactive  $\alpha$ -dicarbonyl and very potent glycating agent. In experimental diabetes, the circulating level of MG is 3-5 times higher than normal situations, especially among patients with vascular problems (Tikellis et al., 2014). Reactive  $\alpha$ -dicarbonyls are toxic to cells as they modify proteins, lipids, and nucleic acids in the process of Maillard reaction. This modification by  $\alpha$ -dicarbonyls produce advanced

glycation end products (AGEs). AGEs play a very important role in initiation and progression of atherosclerosis and can be found in extra- and intracellular atherosclerotic lesions (Hanssen et al., 2014). In the presence of the hyperglycemic situation, control of dicarbonyl production is vasculoprotective (Watson et al., 2011). Consequently, enhanced exposure to MG regardless of the source (i.e. internal or external) results in enhanced endothelial adhesion and risk of atherogenicity (Tikellis et al., 2014).

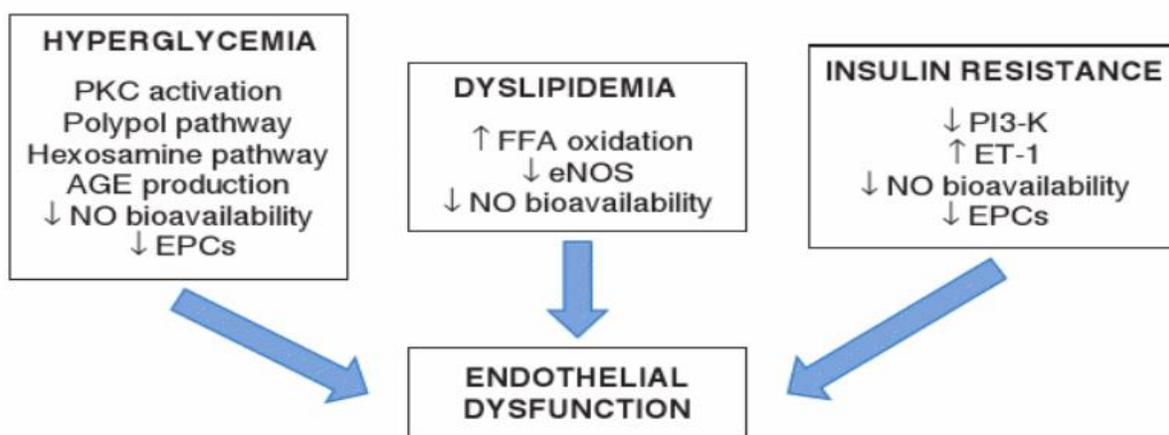


Figure 2.1. Mechanisms that increase oxidative stress and result in endothelial dysfunction in diabetes. Adapted from (Betteridge & Nicholls, 2014).

PKC: Protein Kinase C, AGE: Advanced Glycation End-products, NO: Nitric Oxide, EPC: Endothelial Progenitor Cell, FFA: Free Fatty Acids, eNOS: Endothelial Nitric Oxide Synthase, PI3-K: Phosphoinositide 3-Kinase, ET-1: Endothelin.

The effect of  $\alpha$ -dicarbonyl modification can result in alterations in the structure, function, and stability of susceptible proteins, lipids, and DNA molecules. For example, MG can modify LDL to increase its density and propensity to bind to proteoglycans of arterial walls, which consequently promotes atherogenicity. In a similar process, MG can inhibit the tyrosine kinase activity of platelet derived growth factor receptors (PDGFR) and change the PDGF-BB binding to the receptors, altering its mitogenic function and consequently increasing atherogenic effects

(Tikellis et al., 2014). In addition, AGEs can activate proatherogenic pathways through interaction with receptors for AGEs (RAGE). In hyperglycemic situations, RAGE receptors are up-regulated, a phenomenon partly mediated by MG (Tikellis et al., 2014). Activation of RAGE causes the enhancement (repetitive) of various cellular responses to different stress situations that ultimately lead to cell damage. Thus, a wide number of proatherogenic mediators including ICAM, ICAM, E-selectin, NADPH oxidase are up-regulated following the activation of RAGE (Yao & Brownlee, 2010). RAGE also activates NF $\kappa$ B, which in turn, increases the production of ROS (Betteridge & Nicholls, 2014).

### **Endothelial Health and Atherogenicity in Diabetic Patients**

In addition to up-regulation of the polypol pathway, intracellular hyperglycemia results in activation of the hexosamine pathway and reductions in circulating levels of Endothelial Progenitor Cells (EPC). Activation of hexosamine increases the expression of PAI-1 and activates protein kinase C (PKC) and NF $\kappa$ B to result in more inflammation and increased risk for atherogenicity (Kayama et al., 2015). Activation of PKC also promotes vascular permeability and angiogenesis through the action of vascular endothelial growth factor (VEGF) (Ringvold & Khalil, 2017). Reduction in EPC levels affects vascular repair capacity of endothelial cells, further enhancing the risk for atherogenesis (Kolluru, Bir, & Kevil, 2012). The atherogenic risk is further compounded by the elevated free fatty acid levels frequently associated with DM1 and DM2 (Wu et al., 2017).

The insulin resistance associated with DM2 also contributes to endothelial dysfunction. Some studies conclude that this problem in diabetic patients occurs because of the dysfunction of

mitochondria of muscular cells. Therefore, even DM2 can be considered as a clinical feature of this problem at the cellular level (Taylor, 2012). In physiological situations, insulin activates a signaling cascade through PI3K-AKT that phosphorylates eNOS and leads to increased release of NO (Kayama et al., 2015). Insulin resistance impairs this cascade resulting in a reduction in the release of NO (leading to reduce vascular relaxation), increased secretion of ET-1, and the abnormal overexpression of adhesion molecules, including ICAM and ICAM, which accelerates adhesion of leukocytes and negatively affects endothelial function (Shi & Vanhoutte, 2017). Furthermore, proliferation and differentiation of EPC is impaired in the presence of insulin resistance (Betteridge & Nicholls, 2014).

The vasoconstrictive consequences of hyperglycemia and insulin resistance are predominantly present in larger vessels with smooth muscles. In small vessels and capillaries, the importance of endothelial dysfunction relates to failure of the cellular vascular barrier and increased permeability of small vessels and capillaries. These small vessels do not have smooth muscle cells and vasoconstriction is therefore not an issue (Shi & Vanhoutte, 2017). In these vessels, hyperglycemia damages the barriers between blood and tissues (including brain, retina and kidney/glomerular tissues) by reductions in the production of connexins 30.2 and 43, claudins-5 and -11, and zonular occludens-1, and further expression of integrin (Shi & Vanhoutte, 2017). These changes increase the leakage and permeability from vessels into tissues and leads to underlying tissue dysfunction (Fu, Lee, Chuang, Liu, & He, 2015; Y. J. Lee et al., 2016; Poittevin et al., 2015).

## **Methylglyoxal and Endothelial Health**

Methylglyoxal is produced under normal physiological conditions and is carefully maintained at low levels through the action of the glyoxalase system. The normal level of MG is 50-150 nM in plasma and 1-4  $\mu$ M in the tissues (Rabbani & Thornalley, 2014). The highest amount of MG has been reported from aorta followed by heart, liver, kidney and blood (Randell, Vasdev, & Gill, 2005). The Elevations of plasma and tissue MG can cause a wide range of health problems such as the acceleration of cellular aging process in DM2, cardiovascular disease, cancer, and CNS disorders (Maessen, Stehouwer, & Schalkwijk, 2015). Glyoxalase 1 and 2 (GLO-1 and GLO-2) are responsible for the detoxification of MG and its two-carbon analog, glyoxal, to D-lactate (Queisser et al., 2010). Since MG is a major source of advanced glycation end products (AGEs), lack of the required enzymes for MG detoxification, especially the GLO-1 enzyme, leads to high level of AGEs. These MG-derived AGEs make a considerable contribution to the microvascular and macrovascular diseases associated with diabetes (Queisser et al., 2010).

MG plays an important role in multiple diabetic complications. One of the most important consequences of high plasma levels of MG and failure of the glyoxalase system in diabetic patients is the effects of MG on the vascular system (Maessen et al., 2015). The basic mechanism for micro- and macrovascular problems in diabetic individuals arises from endothelial dysfunction (Schalkwijk & Stehouwer, 2005). Unlike other cells, transport of glucose into the endothelial cells is not dependent on insulin. In other words, high plasma glucose levels, accumulation of glucose occurs in endothelial cells without regulation. This leads to higher concentrations of glucose and its metabolites such as MG inside the endothelial cells (Abebe & Mozaffari, 2010). The higher level of ICAM, VCAM and E-selectin (Su, Lei, Wu, & Liu, 2012). This higher concentration of

MG impairs the balance of vasodilation/vasoconstriction in macrovascular systems and leads to higher vasoconstriction. Higher MG levels are also prothrombotic because of platelet aggregation and accelerated thrombogenesis processes (Nigro et al., 2017). MGO accumulation affects the microvascular system as well. The AGEs produced from MG have a role in initiation of nephropathy and neuropathy (Nigro et al., 2017). MG accumulation also impairs the blood-brain barrier and reduces the integrity of the blood-brain-barrier through damaging effects on brain microvascular endothelia cells (BMEC) (Nigro et al., 2017).

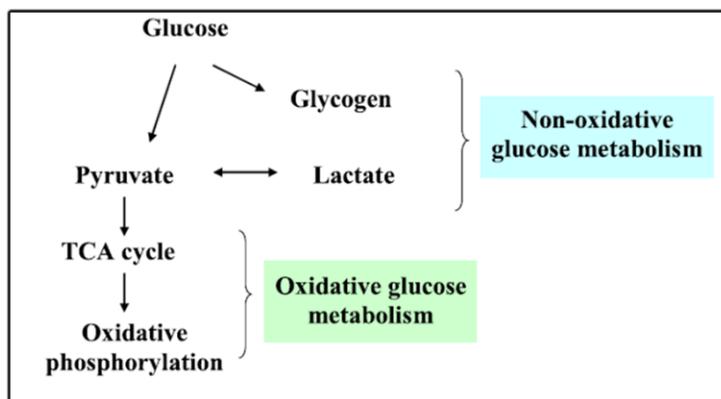


Figure 2.2. Oxidative and non-oxidative metabolism of glucose

### D-Lactate Metabolism

Under physiological conditions glucose undergoes oxidative metabolism within the Citric Acid Cycle (TCA/Krebs) to generate ATP or can proceed through glycolysis to generate lactate. Excess glucose might also be stored as glycogen in cells (Figure 2.2) (Adeva-Andany et al., 2014). In the normal situation after every meal, the proportion of oxidation, storage, and non-oxidative glycolysis is 43.5%, 33%, and 23.5%, respectively (Woerle et al., 2003). Lactate production is primarily the L-lactate isomer and the steady-state concentration of D-lactate is 1% of total concentration of L-lactate. The main source of D-lactate production is from MG metabolism by intestinal bacteria (Chou et al., 2015; Scheijen et al., 2012). When there is a problem in these processes, the level of D-lactate increases pathologically (Scheijen et al., 2012). There are also

exogenous sources for D-lactate and L-lactate including fermented foods such as yogurt, pickles and sauerkraut, which do not create any acid-base problems in normal situations (Ewaschuk, Naylor, & Zello, 2005)

In healthy individuals, a major proportion of exogenous D-lactate is cleared through metabolism and the body has a considerable capacity for this purpose (Andersen et al., 2013). Metabolism mostly occurs through the oxidation of D-lactate to pyruvate, which re-enters the oxidative metabolic pathways. A very minor and negligible proportion is excreted by kidney, highlighted by the failure of D-lactate to accumulate even in patients with low (or almost zero) urine production who require hemodialysis (Adeva-Andany et al., 2014). Since metabolism of D-lactate and glucose are interconnected, any situation such as diabetes or obesity, which affects glucose metabolism, subsequently affects the metabolism of D-lactate (Adeva-Andany et al., 2014).

### **Diabetes and D-Lactate**

In people with diabetes the concentration of D-lactate in plasma and urine is higher than normal, typically around two times that of a non-diabetic person, presumably as a consequence of hyperglycemia (Talasniemi, Pennanen, Savolainen, Niskanen, & Liesivuori, 2008). In hyperglycemia, the level of MG is increased, which is the main precursor of D-lactate (Maessen et al., 2015). Under physiological conditions, about 99% of MG is metabolized by the glyoxalase system, which converts MG into D-lactate (Maessen et al., 2015). As MG is a very reactive compound, the elevated level of D-lactate reflects the increase of MG as a means of detoxification of MG (Scheijen et al., 2012). However, in pathological situations, such as diabetes, the increase in intracellular formation of MG is not exactly matched by detoxification processes due to an

impairment in balance between MG and Glyoxalase 1 (Glo1) activity (Maessen et al., 2015; Nigro et al., 2017). This leads to more leakage of MG into the plasma (Maessen et al., 2015).

### **Biomarkers of Endothelial Vascular Diseases**

The incidence of cardiovascular disease is detectably higher among diabetic populations. The identification of appropriate biomarkers, then, is a very important step in monitoring the risk of vascular complications among diabetic patients. The use of biomarkers would allow one the ability to devise appropriate screening and treatment programs which, in turn, can substantially influence the survival of diabetic patients, especially patients who have other cardiovascular risk factors (Derosa & Maffioli, 2016). A wide range of biomarkers have been studied and their association with diabetes have been shown. These are summarized in Table 2.1 (Derosa & Maffioli, 2016). For any biomarker it is essential to demonstrate the validity and clinical utility across different sets of patients. However, this will not suffice and all the biomarkers should have ease of measurement, preferably at point-of-care, as well as adequate precision and accuracy and low intra-individual variability. Biomarkers should have the potential to explain the pathophysiologic process of endothelial dysfunction and also provide meaningful information about prognosis and assist in clinical decisions. The glucose metabolites are possible biomarkers as their measurement is easy and accessible with adequate precision and accuracy. In addition they can explain the pathophysiology of the disease and can be very helpful in making clinical decisions (Dhingra & Vasan, 2017).

Table 2.1. Biomarkers of vascular diseases. Reproduced from Stoner *et al* and Kampoli *et al* (Stoner et al., 2013) (Kampoli et al., 2011)

Biomarker	Role
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CRP	<ul style="list-style-type: none"> <li>• marker of acute inflammation</li> <li>• prognostic risk factor for the development of DM</li> <li>• independent factor for future development of DM in essential hypertensive patients</li> <li>• major predictor of the outcome in diabetic patients with cardiovascular disease</li> <li>• increased CRP increases the risk of coronary artery disease in the metabolic syndrome subjects through cardiovascular inflammation</li> <li>• elevated CRP levels and reduced glomerular filtration rate on admission is associated with excessive hospital mortality</li> </ul>
TNF- $\alpha$	<ul style="list-style-type: none"> <li>• cytokine regulating immune cells, inducing apoptotic cell death, inflammation, and inhibiting tumorogenesis and viral replication</li> <li>• elevated TNF-alpha is found in acute and chronic inflammatory conditions</li> <li>• increased serum concentrations of TNF-<math>\alpha</math> are associated with increased intensive care unit mortality rate of diabetic patients</li> <li>• increased levels of TNF-<math>\alpha</math> in diabetics with hypertension and cardiovascular disease</li> <li>• in type 1 diabetic patients elevated levels of TNF-<math>\alpha</math> are associated with microangiopathy and worsening outcomes in cardiovascular disease</li> </ul>
IL-6	<ul style="list-style-type: none"> <li>• a pro-inflammatory and anti-inflammatory cytokine secreted by T cells and macrophages</li> <li>• combined elevation of IL-1 and IL-6 independently increases the risk of type 2 diabetes</li> </ul>

	<ul style="list-style-type: none"> <li>elevated IL-6 levels have strong predictive value for poor outcome in end-stage renal disease diabetic patients</li> </ul>
IL-1a	<ul style="list-style-type: none"> <li>cytokine with metabolic, physiological, haematopoietic activities, and plays a central role in regulation of the immune response</li> <li>increase of pro-inflammatory cytokines such as IL-1<math>\alpha</math> and oxidative stress leads to beta-cell damage and promotes beta-cells apoptosis in types I and II diabetes mellitus</li> <li>IL-1 provokes the synthesis and release of chemokines and these chemokines attract neutrophils, macrophages, and lymphocytes that cause tissue inflammation</li> <li>IL-1Ra reduces the inflammatory effects of IL-1 and preserves cell function in both types of diabetes</li> <li>IL-1Ra and MSCs act as modulators of diabetogenesis</li> </ul>
PAI-1	<ul style="list-style-type: none"> <li>the principal inhibitor of tissue plasminogen activator and urokinase</li> <li>increased circulating levels of PAI-1 in diabetic patients</li> <li>prognostic value of PAI-1 indicating reduced likelihood of reperfusion in diabetic patients with elevated PAI-1 levels</li> <li>inhibits fibrinolytic/proteolytic system in diabetic nephropathy</li> </ul>
ICAM and VCAM	<ul style="list-style-type: none"> <li>responsible, in part, for the adherence of hematopoietic cells to endothelium</li> <li>increased plasma levels of ICAM and VCAM are associated with increased risk for future coronary events they both facilitate the proliferation of endothelial cells</li> <li>elevated in diabetics with endothelial dysfunction</li> </ul>
AGEs	<ul style="list-style-type: none"> <li>Macrophage activation</li> </ul>

ANG II	<ul style="list-style-type: none"> <li>• Induce production of ROS, cytokines and adhesion molecules</li> </ul>
E-selectin	<ul style="list-style-type: none"> <li>• Leukocyte recruitment</li> </ul>
HSP	<ul style="list-style-type: none"> <li>• Amplify cytokine production</li> </ul>
MMPs	<ul style="list-style-type: none"> <li>• Plaque rupture</li> </ul>
MPO	<ul style="list-style-type: none"> <li>• LDL-uptake</li> <li>• MMP activation</li> <li>• plaque rupture; endothelial dysfunction</li> </ul>
PECAM-1	<ul style="list-style-type: none"> <li>• Leukocyte migration/motility</li> </ul>
<p>CRP: C-reactive protein, TNF-<math>\alpha</math>: Tumor Necrosis Factor <math>\alpha</math>, IL-6: Interleukin 6, IL-1a: Interleukin 1a, PAI-1: Plasminogen Activating Inhibitor 1, ICAM: Intercellular Adhesion Molecule 1, ICAM: Vascular Cell Adhesion Molecule, AGEs: Advanced Glycation End-products, ANG II: Angiotensin II, HSP: Heat Shock Proteins, MMPs: Matrix Metalloproteinases, MPO: Myeloperoxidase, PECAM-1: Platelet Endothelial Cell Adhesion Molecule 1.</p>	

## D-Lactate as a Biomarker

Hyperglycemic situations lead to higher levels of MG, which result in an imbalance of vasodilation/vasoconstriction and prothrombotic environment in endothelial cells of the macrovascular system and nephropathy and neuropathy within the microvascular systems. (Nigro et al., 2017). Although D-lactate is an end product of MG detoxification, its elevation in diabetic/hyperglycemic patients suggests it may have potential as a predictor of endothelial

dysfunction. In general, the blood level of D-lactate is very low. Several pathological situations, independent of diabetes, are associated with high levels of D-lactate including short bowel syndrome and intestinal ischemia (Nielsen, Kirkegård, Erlandsen, Lindholt, & Mortensen, 2015). High D-lactate levels can also create some pathological outcomes. For example, there are reports of D-lactate encephalopathy with symptoms of delirium, ataxia, and slurred speech, as the consequence of short bowl syndrome (Chou et al., 2015). High levels of glucose can also result in elevated D-lactate. No study has evaluated the association of level D-lactate with biomarkers of endothelial dysfunction (Nigro et al., 2017).

### **Metformin and D-Lactate**

DM2 patients have a wide range of medications available to manage their blood glucose levels. A popular oral medicine for DM2 patients is metformin. Metformin has been available in the pharmaceutical market since 1958 and used worldwide due to its strong safety profile and lack of associate hypoglycemia. Today metformin is a first line treatment for DM2 and Polycystic Ovarian Syndrome (PCOS), and active research suggests it has potential as an adjuvant treatment in some cancers (Kinaan et al., 2015). Metformin is used in non-obese PCOS patients in order to induce ovulation (Johnson, 2014). Metformin, a dimethylbiguanide, is currently the only oral biguanide used alone or with other medications for the treatment of insulin resistance and/or hyperglycemia. Biguanides mediate blood glucose control through reductions in gastrointestinal absorption of carbohydrates, strong inhibition of hepatic gluconeogenesis, and increased cellular uptake of glucose through increased insulin signaling. (Sangeeta, 2012). Oral metformin is absorbed relatively quickly, is not metabolized, and 90% of the drug is eliminated by the kidney (via glomerular filtration and tubular secretion). Plasma protein binding of metformin is minor (Timmins, Donahue, Meeker, & Marathe, 2005).

Metformin is the only biguanide available in the U.S. Another biguanide, phenformin, had been used since the 1950s, but was declared an “imminent hazard” in 1976 because of lactic acidosis. At the time of its removal from the market, 306 documented cases of phenformin-associated lactic acidosis were reported (Bando et al., 2010). The risk of lactic acidosis in patients taking phenformin was much higher than the risk in patients taking metformin (Irsigler, Kritz, Regal, & Foltin, 1978). Several studies found no evidence for the role of metformin in the development of lactic acidosis (Stades, Heikens, Erkelens, Holleman, & Hoekstra, 2004). Latest reports in 2017 indicate that the lactic acidosis rate is less than 1 per 10,000 (Trinkley, Anderson, Nair, Malone, & Saseen, 2018) and previous studies had reported an incidence of metformin-induced lactic acidosis as 3-9 per 100,000 people per year. In clinical practice, 22-94% of patients using metformin have contraindications to metformin (most commonly severely reduced kidney function), so the incidence of acidosis may be higher than reported (van Berlo-van de Laar, Vermeij, & Doorenbos, 2011). As well, others suggest that most cases of metformin-associated lactic acidosis are related to underlying conditions such as infection, cancer, liver failure, and renal failure, rather than to metformin itself (Duong et al., 2013). Interestingly, in patients with DM2, the rate of lactic acidosis is reported to be similar in patients who are taking metformin and in patients who have never taken metformin (Duong et al., 2013). Furthermore, diabetic patients are prone to develop other medical conditions that lead to lactic acidosis (van Berlo-van de Laar et al., 2011). Considering this data and the lack of correlation between lactate levels and metformin levels in lactic acidosis strongly suggests that metformin is often an innocent bystander (van Berlo-van de Laar et al., 2011). Most importantly a meta-analysis in 2017 did not find any relationship between metformin use and high level of lactate or lactic acidosis (E. Y. Lee et al., 2017). Although the molecular mechanism(s) of action of metformin is controversial its putative ability to increase

NO bioavailability by eNOS deacetylation (Kinaan et al., 2015) would result in reductions in MG, and possibly D-lactate, further refuting an associating between elevated D-lactate and metformin use.

## **Rationale**

The purpose of this study is to investigate the correlation of blood concentrations of D-lactate (a normal organic acid that present in the human body at low concentrations) and its related metabolic intermediates (molecules involved in normal D-lactate metabolism including methylglyoxal, L-lactate and pyruvate) with endothelial dysfunction biomarkers. People living with diabetes are at a higher risk of developing diabetic complications including those related to endothelial function, both macrovascular (i.e., cardiovascular disease) and microvascular (i.e., vision and kidney damage). How and why these complications occur is not yet fully understood. Normally, humans have very low levels of a D-lactate in their blood. In clinical conditions such as diabetes, the level of this compound may be elevated. Use of the medication, metformin, has also been shown may lead to increased blood D-lactate levels. This research aimed to evaluate and examine correlations between serum levels of glucose and its metabolites in patients with and without a diagnosis of DM2 and with or without metformin, by measuring blood levels of widely used markers specific to damaged endothelium (inflammatory and adhesion molecules). A significant correlation between D-lactate levels and endothelial biomarker levels would support the idea that excess D-lactate may contribute to endothelial damage.

## **Hypothesis**

The hypothesis of this study was that chronically elevated blood glucose levels correlate with increases in serum glucose metabolites and biomarkers of endothelial damage. We also hypothesized that metformin did not increase endothelial damage through excess D-lactate production. Lastly, we anticipated that elevated blood D-lactate levels were associated with increased blood glucose levels and levels of biomarkers of blood vessel damage.

## **Study Objective**

This study aimed to evaluate and seek correlations between serum levels of glucose and its metabolites in patients with and without a diagnosis of Diabetes Mellitus type 2 (DM2) and with or without using metformin, with blood levels of widely used markers specific to damaged endothelium (inflammatory and adhesion molecules).

These data were generated through participants recruited specifically for this study and secondary use data obtained from a separate study that recruited volunteers consistent with one of the current study's control groups (i.e. Women living with PCOS) (Approved Biomedical Research Ethics Board (BIO-REB # 15-280) Dr. Zello).

## **Specific Objectives**

The objectives of this study were to determine whether:

1. Elevated glucose metabolites levels correlated with chronic glucose level elevations, measured by HbA1c.

2. Elevated glucose metabolites levels correlated with acute glucose level elevations, measured by fasting glucose.
3. Biomarkers of blood vessel damage correlated with elevations of blood glucose or glucose metabolites.
4. Metformin exposure correlated to increased endothelial damage markers and/or D-lactate levels.

## **3. Methodology**

### **3.1. Study Subjects**

This observational study consisted of a prospective analysis of participants assigned to one of 4 study groups over a 1 year time period. The four groups of participants were divided into two groups of patients with diagnosis of Type 2 Diabetes Mellitus (DM2) who were either prescribed metformin (at least 6 months prior to sampling) as a DM2 plus MET group or not treated with metformin as a DM2 no MET group (Table 3.1). For the other two groups of participants, one group was without DM2 diagnosis, but medically prescribed (>6 months) metformin (i.e. women with PCOS) as NonDM plus MET group, while the second group was a no metformin, no DM2 group (but could have other disease such as hyperthyroid on unrelated therapy) as a NonDM no MET group. Data from volunteers with PCOS enrolled in a separate clinical trial (Approved Biomedical Research Ethics Board (BIO-REB # 15-280) Dr. Zello) formed the group of non-DM2 with metformin treatment. This clinical trial received approval from the U of S Biomedical Research Ethics Board. Active recruitment of participants occurred primarily between 2013 January and 2013 March.

Based on previous studies, 15-30 participants would undergo recruitment for every group. The inclusion criteria for the study subjects included: Age 20-70, male or female, on insulin or not, DM2 patients who are either treated with metformin (from 500 to 2600 mg/day for more than 6 months) or not treated with metformin, patients without diabetes with or without metformin treatment, healthy subjects without any medical condition for negative control. Exclusion criteria included: acute or chronic infectious or inflammatory disease, renal impairment, significant liver and/or heart disease, or on anti-inflammatory agents. Metformin use for medically indicated

therapy is a pre-existing criterion and was not initiated in any patient for our study. The baseline data was collected when metformin treatment was instituted for more than 6 months. At the time of the recruitment, this was considered that none of the participants had less than 6 months history of diabetes. Four blood samples were collected every four months over a 1 year time frame beginning January 2013 till February 2014. All of the participants were contacted and invited to participate.

Table 3.1. Baseline age and sex of all participants in the study.								
Group definition	DM2 recruits				NonDM2 recruits			
	under treatment with metformin**		not under treatment with metformin		under treatment with metformin**		not under treatment with metformin	
Abbreviation	DM2 plus MET Group		DM2 no MET Group		NonDM plus MET Group		Non DM no MET Group	
	Male	Female	Male	Female	Male	Female	Male	Female
Number	5	1	0	1	0	39	21	9
Mean age (y)	60.9	72.1	-	52	-	33.3	30.4	33.8
*n/a: Not applicable, as the age of NonDM2 plus MET group from PCOS patients was not available.								
** Average dose of metformin was 1500 mg per day								

### **3.2. Strategies for Recruitment:**

Three strategies were used for recruitment. Participants were recruited under the supervision of approved endocrinologists or by the Diabetic Nurse Educators in the Live Well Diabetes program at the Royal University Hospital. Furthermore, recruitment posters were posted on information boards in hospitals and community pharmacies. The participants were recruited and selected based on the agreed strategy. Relevant consent forms were signed by participants afterwards (refer to appendix 1 and 2)

### **3.3. Sample Collection and Handling**

Blood samples were collected in pre-chilled PST tubes (Tubes with spray-coated lithium heparin and a gel for plasma separation) from fasting participants (food withheld at least 12-14 hours prior to blood sampling). Approximately 5 mL of blood was drawn by venipuncture, the blood was centrifuged for 10 min at 3000 rpm, and plasma aliquots were transferred by plastic transfer pipette into 9 separate eppendorf tubes and immediately stored at -70°C for later biomarker and glucose byproducts determination. Samples were collected at 4-month intervals for up to 12 months at hospital by nurses.

### **3.4 Determination of Clinical Biochemical Variables**

A wide range of clinical biochemical variables were measured in blood collected from participants during the study and immediately analyzed in the hospital lab. Biochemical variables included plasma electrolytes, serum creatinine, serum glucose, HbA1, C-reactive protein (CRP), triglycerides, total cholesterol, HDL-C, LDL-C. Variables were measured using chemistry analyzers at the Royal University Hospital clinical biochemistry laboratory. The CRP was

measured as the only inflammatory factor, since the process was easy and accessible in the hospital lab. All clinical biochemistry parameters were routinely measured every 4 months.

One of the calculated factors was the Homeostasis Model Assessment – estimated Insulin Resistance (HOMA-IR). The applied formula for calculation of HOMA-IR was:

$$\frac{\text{Fasting insulin} \left( \frac{\text{microIU}}{\text{L}} \right) \times \text{Fasting glucose (nmol/L)}}{22.5}$$

As insulin measurement in the test was in pmol/L, this value was divided by 6.945 before calculation of the HOMA-IR. It should be noted that none of the cases were under treatment with insulin. The mean levels in all groups at all-time point collections fell within the reference range.

### **3.5. Quantitation of Glucose Metabolite Abundance in Serum:**

#### **3.5.1. Quantitation of D-lactate Abundance**

D-Lactate was analyzed using enzymatic kits from Biovision (Biovision Inc. California, USA). A 100 mM D-lactate standard included in the kit was diluted to 1 mM by adding 10 µL of the standard to 990 µL of kit assay buffer. The resulting working solution was mixed well and 0, 2, 4, 6, 8, 10 µL was added into wells of a 96-well plate to create a standard curve. The well volume was adjusted to 50 µL/well by addition of assay buffer to generate a D-lactate standard curve of 0, 2, 4, 6, 8, 10 nmol/well. Test samples in volumes varying from 1 - 50 µL were added to wells and the volume was adjusted to 50 µL/well with assay buffer as appropriate. A reaction mix was prepared by adding 46 µL D-lactate assay buffer with 2 µL D-lactate substrate mix and 2 µL D-lactate enzyme mix were mixed well. To each well, 50 µL of the reaction mix was added and the 96-well plate was kept at room temperature for 30 minutes before measurement. D-Lactate concentrations were measured by using OD 450 nm in microplate reader and concentration was

calculated as  $C = La/Sv$  (nmol/ $\mu$ L), where La is the D-lactate amount (nmol) interpolated from the standard curve, and Sv is the sample volume ( $\mu$ L) added into the well.

### **3.5.2. Quantitation of L-Lactate Abundance**

L-Lactate concentrations were determined using a Lactate Colorimetric/Fluorometric assay kit from Biovision (Biovision Inc. California, USA). After addition of 2-50  $\mu$ L test samples to a 96-well plate, the volume was adjusted to 50  $\mu$ L/well with lactate assay buffer. The lactate standard (100 nmol/ $\mu$ L) was diluted to 1 nmol/ $\mu$ L with assay buffer. The resulting working solution was mixed well and 0, 2, 4, 6, 8, 10  $\mu$ L was added into wells of a 96-well plate to create a standard curve. The well volume was adjusted to 50  $\mu$ L/well by addition of assay buffer to generate an L-lactate standard curve of 0, 2, 4, 6, 8, 10 nmol/well. Required reagents were subsequently added to each well. After incubation in the dark for 30 minutes at room temperature, the plate was read at an absorbance of 570 nm using a microplate reader. The sample lactate concentration (C) was determined as follows:

$$\text{Sample Lactate concentration (C)} = (B/V) \times D \text{ (nmol}/\mu\text{L)}$$

Where: B is the amount of lactate in the sample well (nmol); V is the sample volume added into the reaction well ( $\mu$ L); D is the sample dilution factor.

### **3.5.3. Quantitation of Methylglyoxal Abundance from Serum**

Methylglyoxal was measured using a competitive enzyme-linked immunosorbent assay (ELISA) (OxiSelect™ Methylglyoxal (MG) Competitive ELISA Kit, Cell Biolabs, INC). All reagents were prepared and thoroughly mixed before use according to kit instructions. A 50  $\mu$ L sample or standard was added to the wells of a 96-well plate and 50  $\mu$ L of the diluted anti-MG antibody was added to each well and incubated at room temperature for 1 hour. After 3 washings

with 250  $\mu\text{L}$  of 1X Wash Buffer, 100  $\mu\text{L}$  of diluted Secondary Antibody-HRP Conjugate was added to all wells and incubated for 1 hour at room temperature. Substrate Solution (100  $\mu\text{L}$ ) at room temperature was then added to each well. The enzyme reaction was stopped by adding 100  $\mu\text{L}$  of Stop Solution. Absorbance of each well was read on a micro-plate reader at 450 nm.

### **3.6. Quantitation of Protein Biomarkers for Endothelial Damage ICAM, ICAM, and E-Selectin Levels:**

ICAM and VCAM, and E-selectin were measured using enzyme-linked immunosorbent assays (ELISA) specific for each biomarker (Research & Development Systems, Minneapolis, MN, USA). In order to measure these factors, 100  $\mu\text{L}$  of serum or standard was added in Reagent Diluent to multi-well plates. This was covered with an adhesive strip and incubated for 2 hours at room temperature. Then, 100  $\mu\text{L}$  of the Detection Antibody was added to each well containing Reagent Diluent. This was covered with an adhesive strip and incubated for 2 hours at room temperature. After this, 100  $\mu\text{L}$  of the working dilution of Streptavidin-HRP was added to each well. This was covered with an adhesive strip and incubated for 20 minutes at room temperature. Subsequently, 100  $\mu\text{L}$  of Substrate Solution was added to every well and covered with an adhesive strip and incubated for 20 minutes at room temperature. Finally, 50  $\mu\text{L}$  of Stop Solution was added to each well and the optical density of each well was determined immediately using a micro-plate reader at absorbance of 450 nm.

### **3.7 Statistical Analysis**

For comparison of the results of biochemical and hematological tests, the treatment group means and medians were calculated and compared with each other as well as with the reference range for every test. For the main study variables including D-lactate, L-lactate, methylglyoxal, ICAM, VCAM and E-selectin, since the distribution was not normal, a non-parametric one-way

ANOVA (Kruskal Wallis) test was used to explore the significant differences in D-lactate levels between the four study groups at all-time point collections. The Bonferroni-adjusted Mann-Whitney test was also used for post hoc. For special situations including HbA1C, Insulin and HOMA-IR the average for all tests of the diabetic groups were also compared with the non-diabetic groups.

D-Lactate correlation with other biomarkers of endothelial dysfunction, including ICAM, VCAM and E-selectin, was explored by Spearman correlation test, because the distribution of data was not normal. All the test and statistical analysis were conducted by using STATA 14.1. A P value less than 0.05 was considered significant.

## **4. Results**

### **4.1. General Characteristics of Participants in the Study**

The baseline characteristics of all participants were listed in table 3.1 (please refer to page 27). An uneven number of participants were recruited into each of the four study groups due to difficulty with recruitment. Of the 39 participants in the NonDM plus MET group, 35 were women under treatment with metformin for polycystic ovarian syndrome (PCOS) and part of another unrelated study (Kazemi et al., 2019).

The mean age for the NonDM no MET group was 31.4 years (95% CI: 29.0 – 33.9), which was much younger than the mean age of the DM2 plus MET group (60.9 years with 95% CI: 52.0 – 69.8). The age of the only participant in the DM2 no MET group was 52 years at the beginning of the study. In the NonDM no MET group 30% of participants were female, while participants of NonDM plus MET group were just females. In the DM2 plus MET group 17% were female and the only participant of DM2 no MET group was female.

### **4.2. Baseline Biochemical Parameters in the Study Groups.**

Although all of the participants in the study were intended to have standard clinical biochemistry and hematological evaluations, this was not conducted for all participants. Clinical biochemical and hematological values are reported in table 4.1 and all values fell within the normal reference ranges. This should be noted that DM2 no MET had one participant without this data and the following biochemical data was available just for one person of group DM2 plus MET. Therefore, calculation of confidence intervals was not possible.

Table 4.1. Baseline biochemical parameters for participants.

	Non DM no MET mean (95% CI)	Non DM plus MET mean (95% CI)	DM plus MET mean (95% CI)	Normal range
N	28	3	1	
Sodium (mmol/L)	141.3 140.5 - 142.0	138.3 137.7 - 139.0	140.0 n/a	135 - 146
Potassium (mmol/L)	4.1 4.0 - 4.2	4.0 3.1 - 4.9	4.4 n/a	3.5 - 5.1
Chloride (mmol/L)	103.3 102.4 - 104.1	100.7 98.2 - 103.1	102.0 n/a	100.0 - 110
CO2 (mmol/L)	25.6 24.6 - 26.7	25.7 23.2 - 28.1	23.0 n/a	22 - 31
Creatinine ( $\mu$ mol/L)	77.9 72.7 - 83.0	60.7 55.3 - 66.0	75.0 n/a	53 - 115
Anion gap (mmol/L)	12.4 11.5 - 13.2	12.0 10.8 - 13.2	15.0 n/a	8 - 16
Triglyceride (mmol/L)	1.0 0.8 - 1.2	1.2 0.9 - 1.6	1.6 n/a	0.6 - 2.3
Cholesterol (mmol/L)	4.7 4.4 - 5.0	4.3 4.1 - 4.5	5.2 n/a	3.8 - 5.2
HDL (mmol/L)	1.4 0.7 - 1.8	1.6 1.1 - 5.4	1.4 n/a	0.9 - 1.6
LDL (mmol/L)	2.9 2.6 - 3.2	2.2 1.7 - 3.1	3.1 n/a	2 - 3.4
Ratio Total Cholesterol:	3.6	2.7	3.7	Different
HDL-C	3.2 - 4.1	2.3 - 3.1	n/a	

### 4.3. Determination of Glucose Regulation and Related Factors

#### 4.3.1. Long Term Glucose Control: Plasma Hemoglobin A<sub>1</sub>C (HbA<sub>1</sub>c) Levels in the Study Groups.

Plasma hemoglobin A<sub>1</sub>C was measured on three occasions separated by three months between blood collections. Plasma hemoglobin A<sub>1</sub>C values are illustrated in figure 4.1. The mean

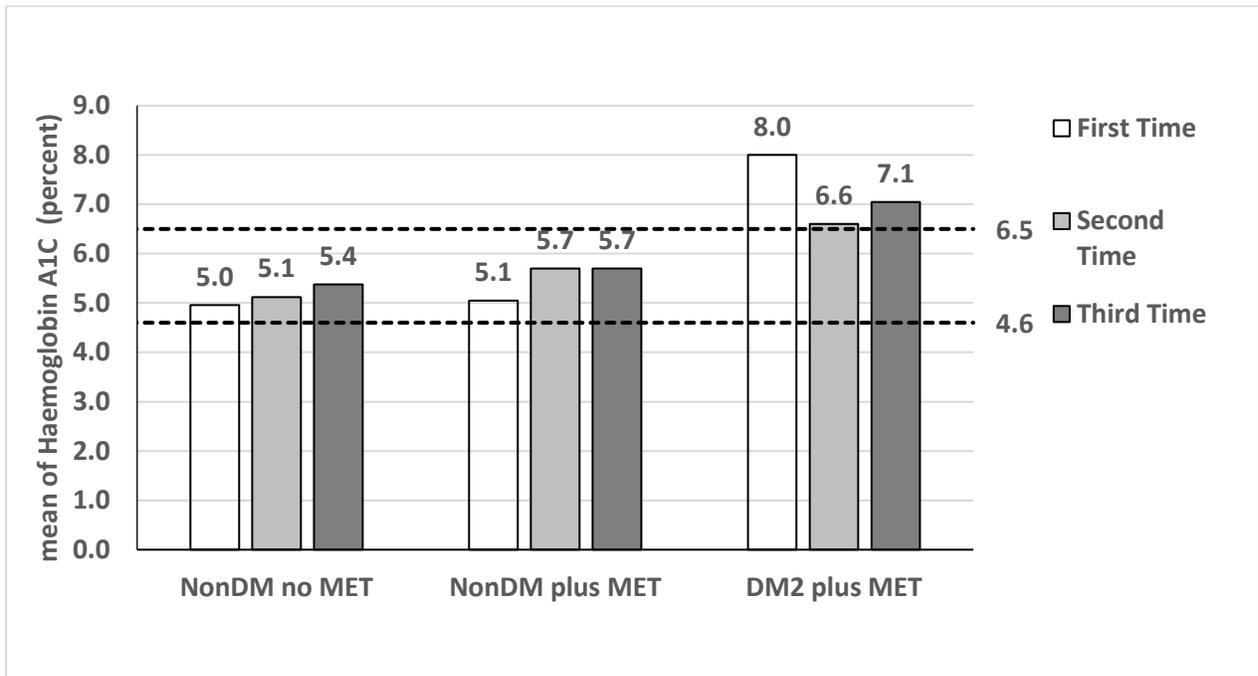


Figure 4.1: Mean of Haemoglobin A<sub>1</sub>C levels in the different treatment groups (NonDM no MET – Non-diabetic patients who were not under treatment with metformin, NonDM plus MET– Non-diabetic patients using metformin, and DM2 plus MET – Diabetic patients under treatment with metformin) at baseline and at 4 and 8 months into the study. Dashed lines represent the upper and lower limit of the normal reference range of Haemoglobin A<sub>1</sub>C. It should be noted that HbA<sub>1</sub>c was not measured at fourth time blood collection

of HbA<sub>1</sub>C in diabetic patients who were under treatment with metformin (DM2 plus MET group) fell above the reference range at all-time point collections, while values fell within the reference range for NonDM no MET and NonDM plus MET groups.

The diabetic and non-diabetic participants were also compared with each other and the result of this comparison is illustrated in figure 4.2. Hemoglobin A1C in diabetic group is higher than non-diabetic group in all three blood sampling periods and also above the reference range.

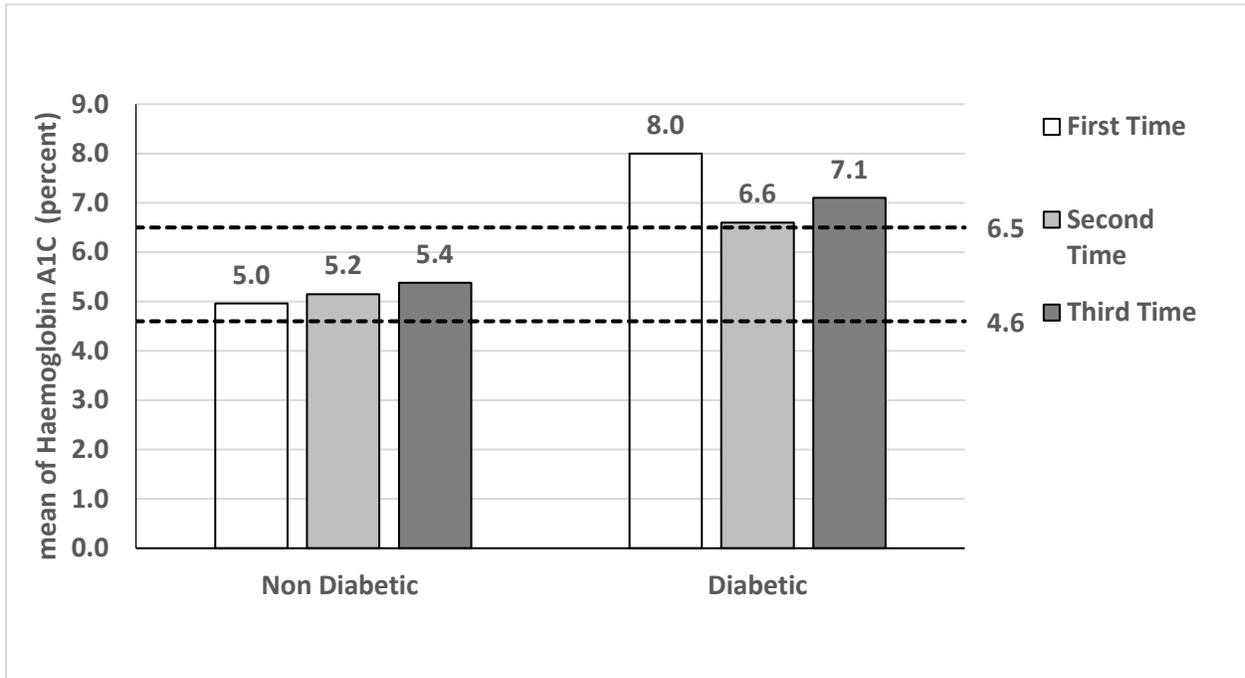


Figure 4.2: Mean of Haemoglobin A1C levels in the diabetic and non-diabetic participants at baseline and at 4 and 8 months into the study. Dashed lines represent the upper and lower limit of the normal reference range of Haemoglobin A1C. It should be noted that HbA1c was not measured at fourth time blood collection.

### 4.3.2. Assessment of Acute Glucose Control: Plasma Fasting Glucose Levels in the Study Groups.

Plasma fasting glucose levels were measured on three occasions separated by three months between blood collections. Plasma glucose values are illustrated in figure 4.3. The mean of fasting glucose levels in diabetic patients who were under treatment with metformin (DM2 plus MET group) fell above the reference range at all-time point collections, while values fell within the reference range for NonDM no MET and NonDM plus MET groups.

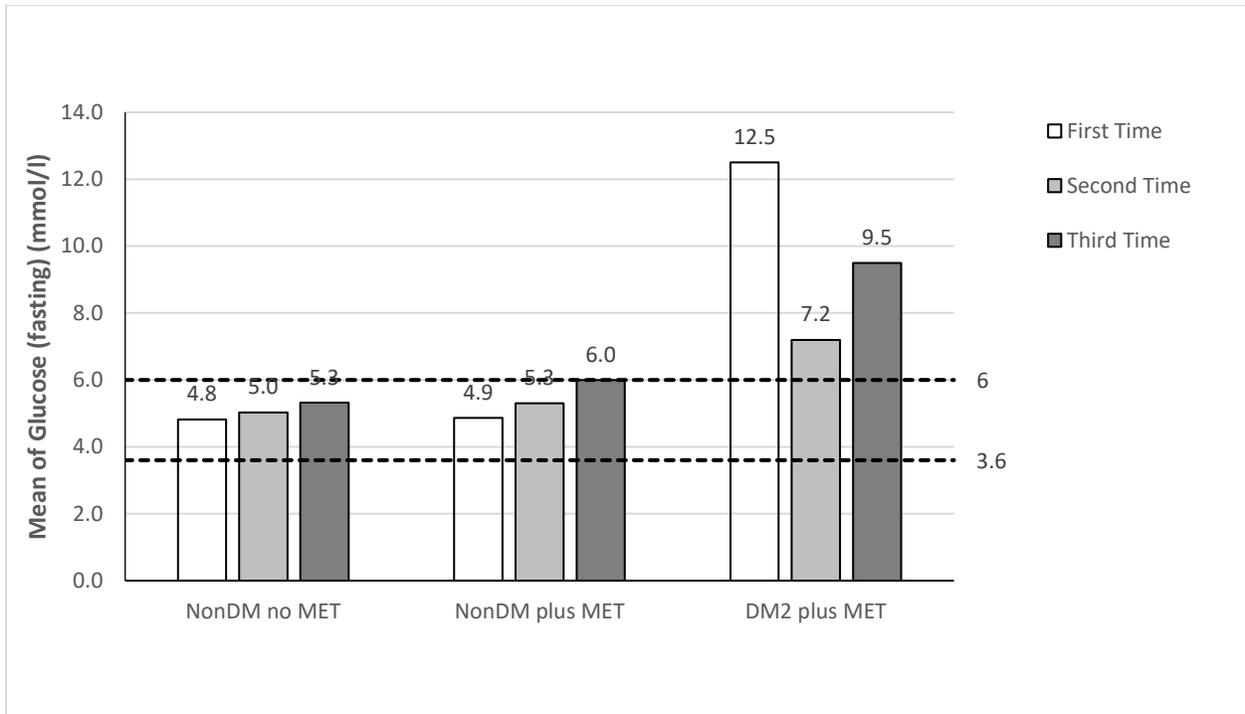


Figure 4.3: Mean fasting glucose levels at baseline and at three and six months into the study (NonDM no MET – Non-diabetic patients who were not under treatment with metformin, NonDM plus MET – Non-diabetic patients using metformin, and DM2 with MET – Diabetic patients under treatment with metformin). Dashed lines represent the upper and lower limit of the normal reference range of fasting glucose. It should be noted that fasting glucose was not measured at fourth blood collection

### 4.3.3. Determination of Insulin Resistance in the Study Groups Using Homeostasis Model Assessment – Estimated Insulin Resistance (HOMA-IR) Calculation

Plasma fasting insulin levels were measured on three occasions separated by three months between blood collections. Plasma fasting insulin values are illustrated in figure 4.4. The mean of fasting insulin levels in all groups at all-time point collections fell within the reference range.

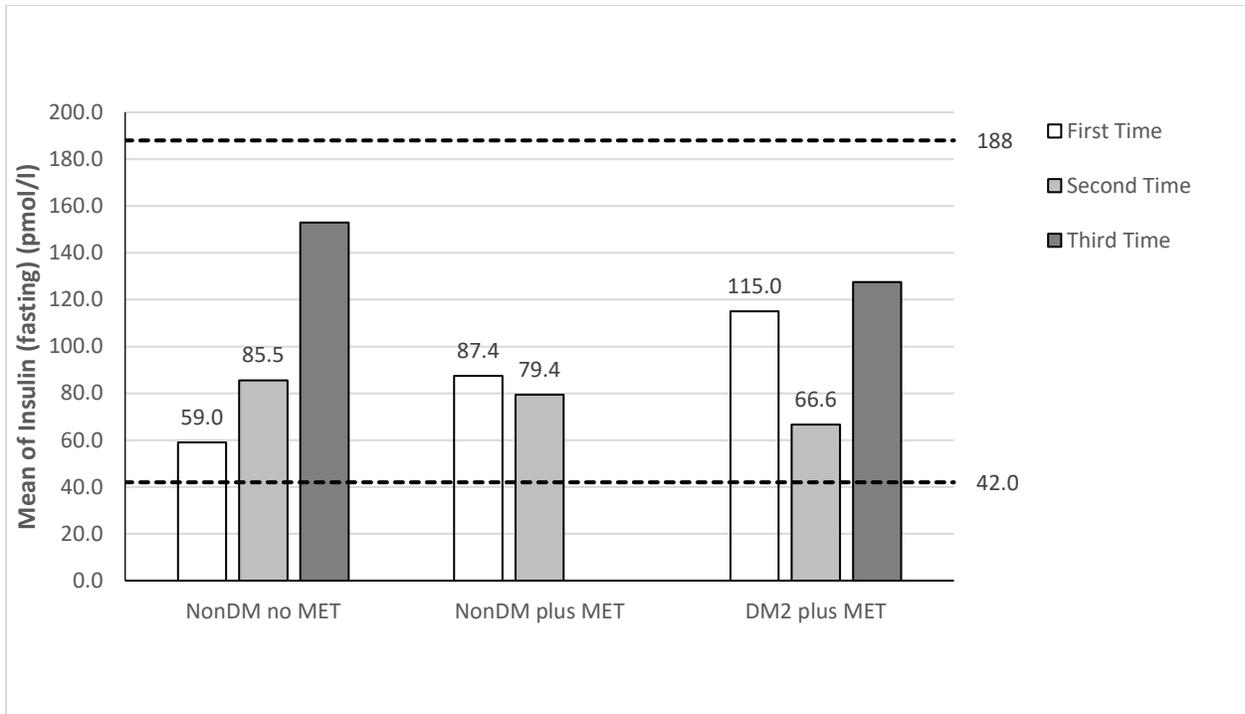


Figure 4.4. Mean fasting insulin levels at baseline and at three and six months into the study (NonDM no MET – Non-diabetic patients who were not under treatment with metformin, NonDM plus MET – Non-diabetic patients using metformin, and DM2 plus MET – Diabetic patients under treatment with metformin). Dashed lines represent the upper and lower limit of the normal reference range of fasting insulin. It should be noted that fasting Insulin was not measured at fourth time blood collection. No case for the last measurement in NonDM plus MET group.

When participants were pooled into diabetic or non-diabetic groups and the mean of three time point measurements was calculated and compared between these two groups, the mean of fasting insulin in the diabetic group was 116.1 (pmol/L), markedly higher than 72.8 (pmol/L) among non-diabetic group (figure 4.5), but not statistically significant.

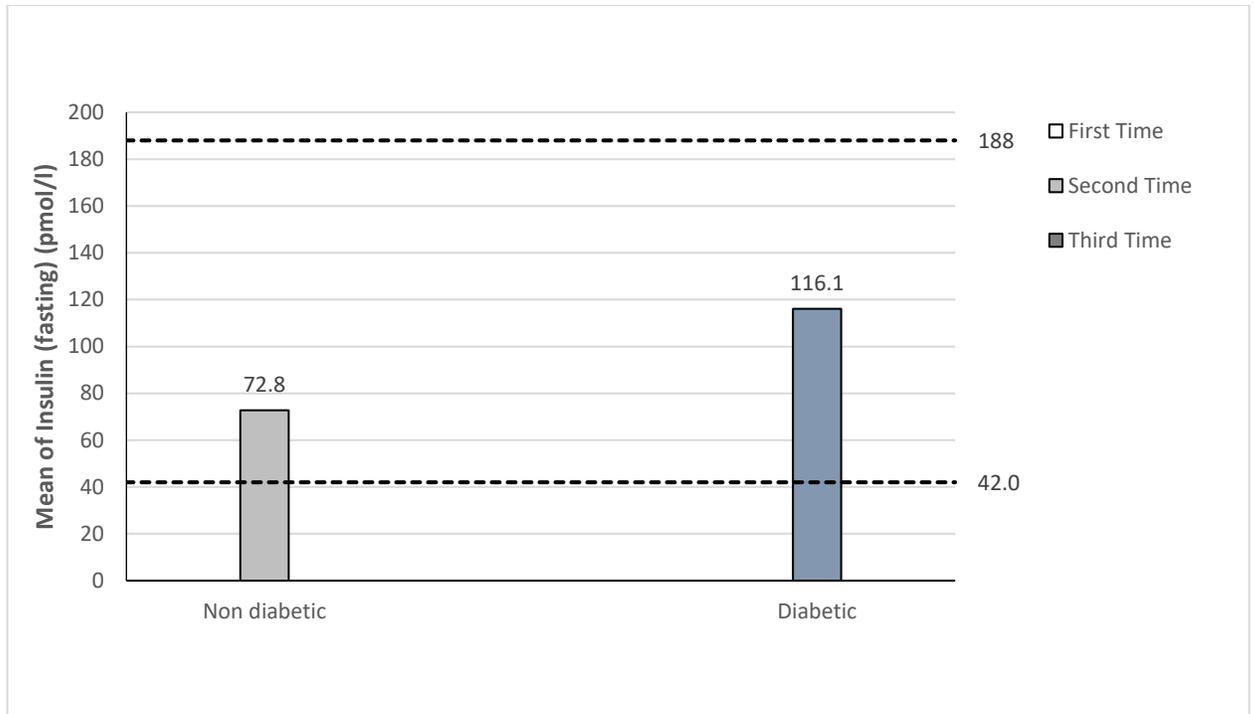


Figure 4.5. Mean fasting insulin levels in three different testing times compared between diabetic and non-diabetic participants. Dashed lines represent the upper and lower limit of the normal reference range of fasting insulin. It should be noted that fasting insulin was not measured at a fourth blood collection

In order to have a better understanding about insulin resistance among the groups, the HOMA-IR was also compared among the groups with hyperglycemia, or without a diagnosis of type 2 diabetes. The mean of the results is illustrated in figure 4.6. It should be noted that none of the participants were under treatment with exogenous insulin. The mean levels of HOMA-IR in all groups at all-time point collections fell within the normal reference range.

The level of HOMA-IR in DM2 plus MET is higher than in the non-diabetic groups. Of the three time points tested, the first and last data collection periods were above the HOMA-IR cut-off limit. In the last round of sampling (figure 4.6), the HOMA-IR level in NonDM no MET is unexpectedly also above the cut-off limit.

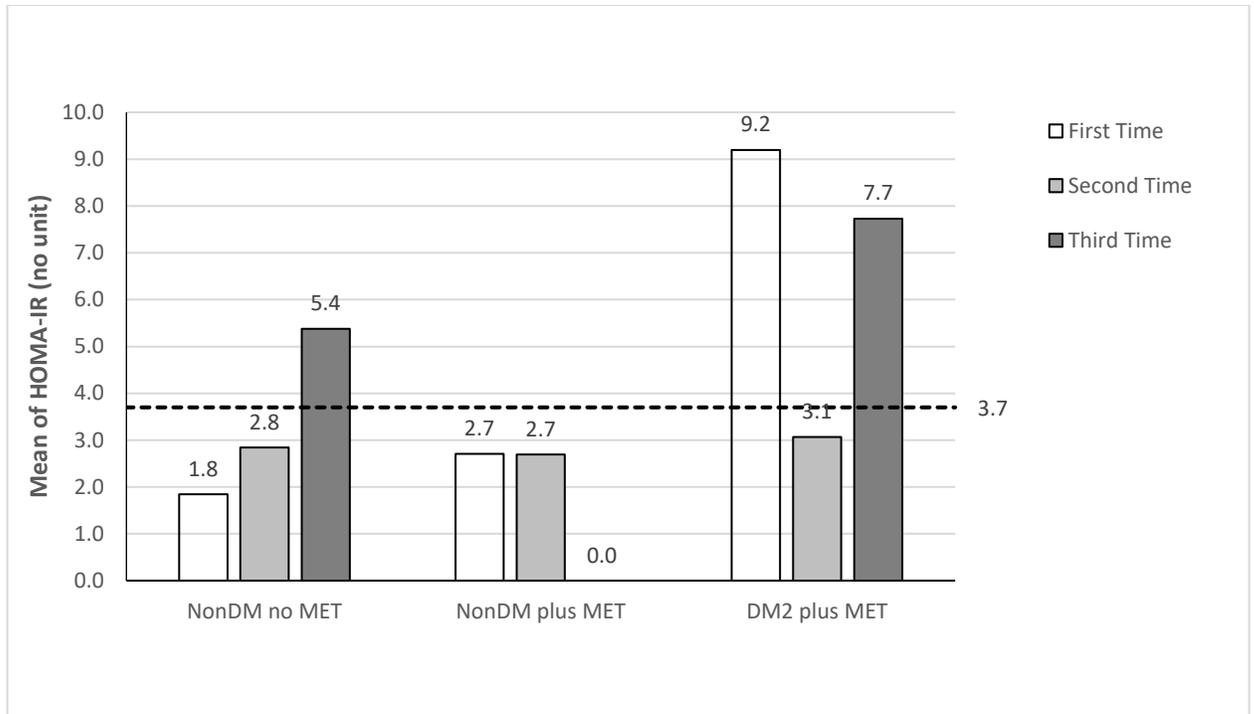


Figure 4.6. Mean Homeostasis Model Assessment (HOMA-IR) in order to explore the insulin resistance level at baseline and at three and six months into the study. NonDM no MET – Non-diabetic patients who were not under treatment with metformin, NonDM plus MET – Non-diabetic patients using metformin, and DM2 plus MET – Diabetic patients under treatment with metformin). Dashed line represents the cut-off limit of the normal reference range of HOMA-IR. It should be noted that fasting insulin was not measured a fourth time.

#### 4.3.4. C-Reactive Protein-High Sensitivity (CRP-HS) Levels among the Study Groups.

The CRP-HS levels were measured on three occasions separated by four months between blood collections. Mean CRP-HS values are illustrated in figure 4.7. The mean levels in all groups at all-time point collections fell within the expected normal range. This is an unexpected finding as CRP is generally higher than normal among diabetic populations. However, from another point of view this can be a proxy for lack of inflammation among all participants, especially diabetic patients.

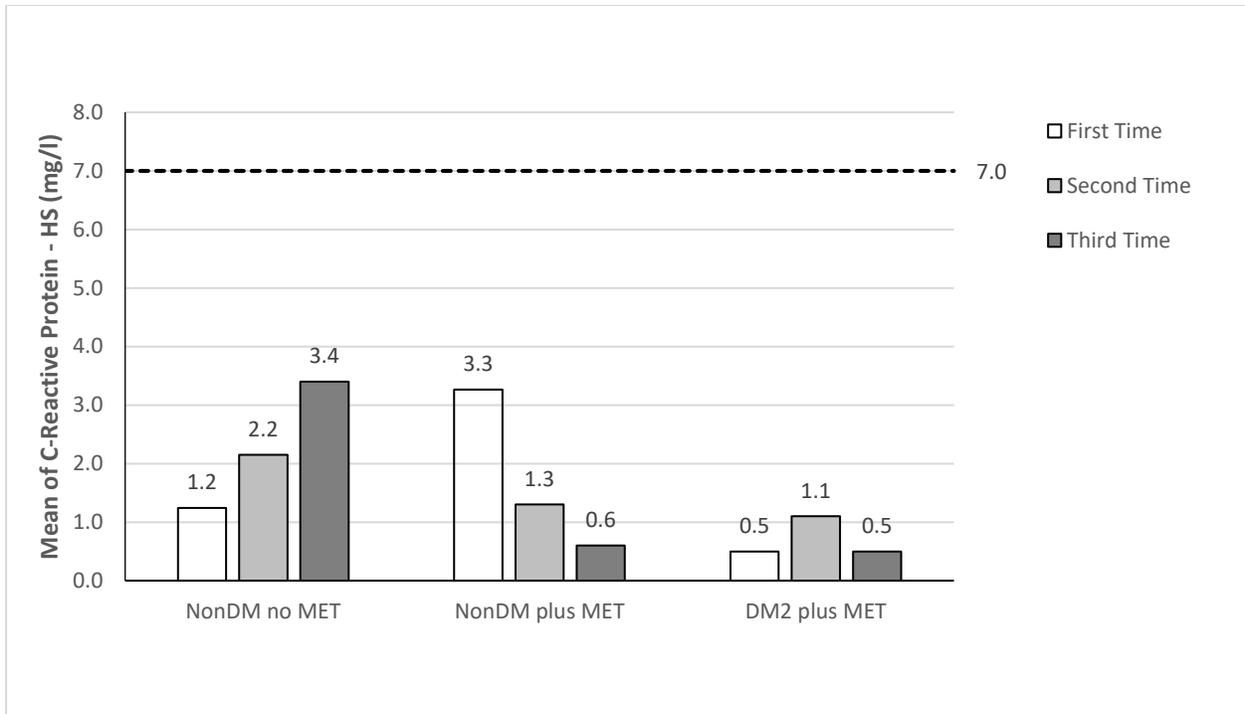


Figure 4.7. Mean fasting C-reactive protein–HS at baseline and at three and six months into the study (NonDM no MET – Non-diabetic patients who were not under treatment with metformin, NonDM plus MET – Non-diabetic patients using metformin, and DM2 plus MET – Diabetic patients under treatment with metformin). Dashed line represents the upper normal reference cut-off point of C-reactive protein–HS.

#### 4.4. Glucose Metabolites and Their Correlations with Specific Indicators of Diabetes

##### 4.4.1. Higher Levels of Plasma D-Lactate are Detected in Diabetic Participants.

Plasma D-lactate levels were measured on four occasions separated by four months between blood collections. Mean and median of D-lactate values were illustrated in table 4.2. A non-parametric one-way ANOVA (Kruskal Wallis) test indicated significant differences in D-lactate levels between the four study groups at all-time point collections. The Bonferroni-adjusted Mann-

Table 4.2. Plasma D-lactate levels (nmol/ $\mu$ L) in the four different study groups collected at baseline and at 4 months intervals.

	Baseline			Second time			Third time			Fourth time			Mean of the four times			
	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	
*NonDM no MET	29	0.27 (0.23-0.31)	0.22	18	0.32 (0.26-0.39)	0.29	17	0.57 (0.47-0.67)	0.54	2	0.29 (0.27-0.31)	0.29	29	0.36 (0.31-0.40)	0.31	
*NonDM plus MET	36	0.17 (0.15-0.19)	0.15	24	0.15 (0.14-0.16)	0.15	23	0.16 (0.14-0.18)	0.16	0			36	0.16 (0.15-0.18)	0.15	
*DM2 no MET	1	0.4	0.4	0			0			0			1	0.4	0.4	
*DM2 plus MET	3	0.34 (0.21-0.47)	0.37	1	0.31	0.31	1	0.43	0.43	1	0.85	0.85	3	0.42 (0.37-0.47)	0.44	
P-value (Non-parametric one way ANOVA)		<0.05			<0.05			<0.05			>0.05			<0.05		

Whitney test was used for post hoc analysis. At baseline, D-lactate in NonDM no MET group was significantly higher than the NonDM plus MET group (P-value<0.05). Also, D-lactate level of the DM2 plus MET group was significantly higher than NonDM plus MET group. The difference between NonDM no MET and DM2 plus MET groups was not statistically significant (table 4.2). The differences between mean and median were very small. This can be the result of the lack of any outliers in the study.

For the other blood collection times, Mann-Whitney test was not conducted as it was clear that the difference between NonDM no MET and NonDM plus MET was statistically significant, and statistical comparisons for DM2 plus MET group were not done since this group only had one participant. None of the participants displayed D-lactic acidosis at any time point during the study period (> 3 mM D-lactate in plasma).

For this analysis, participants were also pooled and divided into two groups: diabetic and non-diabetic groups, and the plasma D-lactate level was compared. The mean of D-lactate in the

pooled diabetic group was significantly higher than the non-diabetic group (0.43 vs 0.25 nmol/ $\mu$ L, P-value<0.05). A similar process was conducted by categorizing participants into users and non-users of metformin. Unexpectedly, the D-lactate level in non-users of metformin was significantly higher than users (0.36 vs 0.18 nmol/ $\mu$ L, P-value<0.05). In other words, use of metformin did not lead to higher levels of D-lactate.

Table 4.3. Comparison of D-lactate levels between pooled diabetic and non-diabetic participants as well as users and non-users of metformin

Type of group	Number of participants	D- lactate (nmol/ $\mu$ L)		P-value
		Mean	Median	
Diabetic	7	0.43	0.40	<0.05
Non-diabetic	149	0.25	0.19	
Users of metformin	89	0.18	0.16	<0.05
Non-users of metformin	67	0.36	0.32	

#### **4.4.2. Higher L-lactate Levels are Present in Diabetic Patients under Treatment with Metformin**

Plasma L-lactate levels were measured on four occasions separated by four months between blood collections. Mean L-lactate values are illustrated in table 4.4. L-lactate levels were not available for PCO patients. A non-parametric one-way ANOVA test did not detect significant differences in L-lactate levels between the four study groups at any time point. In comparing the median of L-lactate from the four collection times, the L-lactate plasma level was higher among diabetic patients who were under treatment with metformin, but this difference was not significant.

Table 4.4. Plasma L-lactate levels (nmol/ $\mu$ L) in different study groups collected at baseline and at 4 months intervals

	Baseline			Second time			Third time			Fourth time			Mean of the four times		
	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median
*NonDM no MET	13	1.01 (0.77-1.26)	0.83	20	1.20 (0.98-1.41)	1.10	23	0.84 (0.65 – 1.03)	0.81	11	0.71 (0.51 – 0.90)	0.80	30	0.91 (0.79-1.02)	0.89
*NonDM plus MET	1	1.03	1.03	0			0			0			1	1.03	1.03
*DM2 no MET	1	0.57	0.57	0			0			0			1	0.57	0.57
*DM2 plus MET	3	1.35 (0.45 – 2.26)	1.43	2	1.21 (0.22 – 2.20)	1.21	1	1.93	1.93	1	1.56	1.56	3	1.53 (0.95-2.10)	1.44
P-value (Non-parametric one way ANOVA)		>0.05			>0.05			>0.05			>0.05			>0.05	

In addition, participants were pooled and first divided into two major groups (diabetic and non-diabetic) and the plasma L-Lactate level was compared (table 4-5). The L-lactate in the pooled diabetic group was not significantly different from the non-diabetic group (0.1.32 vs 0.96 nmol/ $\mu$ L, P-value>0.05). A similar process was conducted by categorizing participants into users and non-users of metformin (table 4.5). The L-lactate level in users of metformin was significantly higher than non-users (1.38 vs 0.95 nmol/ $\mu$ L, P-value<0.05).

Table 4.5. Compare the L-lactate between pooled diabetic and non-diabetic as well as users and non-users of metformin

Type of group	Number of participants	L- lactate (nmol/ $\mu$ L)		P-value
		Mean	Median	
Diabetic	8	1.32	1.49	>0.05
Non-diabetic	68	0.96	0.91	
Users of metformin	8	1.38	1.49	<0.05
Non-users of metformin	68	0.95	0.90	

### 4.4.3. Methylglyoxal Levels Do not Shift Significantly in Diabetic Patients or Metformin Users.

Plasma methylglyoxal levels were measured on four occasions separated by four months between blood collections. Mean and median of methylglyoxal values are illustrated in table 4.6. Methylglyoxal levels were not available for PCO patients who were included in our study group (Non DM plus MET). None of the differences were statistically significant for methylglyoxal. This should be noted that in a number of the situations the median of methylglyoxal was zero which means that a considerable number of cases did not have detectable methylglyoxal. This was predictable because of the unstable structure of this glucose metabolite.

Table 4.6. Plasma methylglyoxal levels (ng/mL) in different study groups collected at baseline and at 4 months intervals.

	Baseline			Second time			Third time			Fourth time			Mean of the four times			
	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	
*NonDM no MET	21	0.35 (0.02 – 0.68)	0	19	0.20 (0.12 – 0.40)	0.00	13	0.24 (- 0.18- 0.66)	0	9	0.11 (- 0.14 – 0.37)	0.00	29	0.28 (0.05- 0.51)	0	
*NonDM plus MET	3	0	0	1	0.44	0.44	0			0			3	0.74 (- 0.08- 0.22)	0	
*DM2 no MET	0			0			0			0			0			
*DM2 plus MET	2	0.29 (- 0.31 – 0.89)	0.29	2	0.40 (- 0.43 – 1.23)	0.4	2	0	0	1	0	0	3	0.28 (- 0.06- 0.63)	0.27	
P-value (Non-parametric one way ANOVA)		>0.05			>0.05			>0.05			>0.05			>0.05		

As done for the other metabolite analyses, participants were pooled and first divided into two major groups (diabetic and non-diabetic) and the plasma methylglyoxal level was compared (table 4.7).

The mean of the methylglyoxal levels in pooled diabetic group was not significantly different from the non-diabetic group (0.20 vs 0.24 ng/mL, P-value>0.05). A similar process was conducted by categorizing participants into users and non-users of metformin (table 4.7). The methylglyoxal level in users of metformin was lower than non-users, but the difference was not statistically significant (0.17 vs 0.25 ng/ml, P-value>0.05).

Table 4.7. Comparison of the methylglyoxal levels between pooled diabetic and non-diabetic as well as users and non-users of metformin

Type of group	Number of participants	Methylglyoxal (ng/mL)		P-value
		Mean	Median	
Diabetic	7	0.2	0.0	>0.05
Non-diabetic	66	0.24	0.0	
Users of Metformin	11	0.17	0.0	>0.05
Non-users of Metformin	62	0.25	0.0	

#### 4.4.4. Assessment of Correlations between Fasting Glucose and D-lactate Levels.

In order to explore the correlation between fasting glucose levels and D-lactate, we used the Spearman correlation test and reported the results in table 4.8.

Table 4.8. Correlation of fasting glucose with D-lactate in blood collection time points in all groups.

	D-lactate (first round)		D-lactate (second round)		D-lactate (third round)	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
Fasting glucose	0.51	>0.05	-0.07	>0.05	0	-

The total number of data point observations was 29, 16 and 3 in first, second and third rounds of data collections, respectively. The correlations were not significant. Since the number of diabetic individuals was very limited and statistical analysis was not possible just in diabetic participants, the analysis was instead conducted among all participants.

In order to better refine the effect of fasting glucose on D-lactate levels, another statistical analysis was conducted based on the cut-off level for normal fasting glucose at 7 mmol/L. However, out of 69 observations with at least one report of D-lactate, only 2 had glucose levels higher than 7 mmol/L, which greatly limited our ability to perform this analysis in a meaningful way. The mean D-lactate level in the group with glucose levels higher than 7 mmol/L was 0.41 nmol/  $\mu$ L whereas this was 0.25 nmol/ $\mu$ L in the group with less than or equal to 7 mmol/L. The difference was not significant (P-value>0.05) although a trend may be reinforced were there more samples available.

#### **4.4.5. Assessment of Correlations between Fasting Glucose and L-lactate Levels.**

In order to explore the correlation of fasting glucose level with L-lactate, the associations of fasting glucose with L-lactate were explored by Spearman correlation test and reported in Table 4.9. Since the number of diabetic individuals were very limited and statistical analysis was not possible just in diabetic participants the analysis was conducted among all participants. Out of 34 observation with at least one report of L-lactate, just 2 had glucose levels higher than 7 mmol/L. The mean L-lactate level in the group with glucose levels higher than 7 mmol/L was 1.26 nmol/  $\mu$ L whereas this was 0.93 nmol/ $\mu$ L in the group with less than or equal to 7 mmol/L. The difference was not significant (P-value>0.05).

Table 4.9. Correlation of fasting glucose with L-lactate in all blood collection time points.

	L-lactate (first round)		L-lactate (second round)		L-lactate (third round)	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
Fasting glucose	-0.44	>0.05	-0.08	>0.05	0.52	>0.05

#### 4.4.6. Assessment of Correlations between Fasting Glucose and Methylglyoxal Levels.

In order to explore the correlation between fasting glucose and methylglyoxal, the associations of fasting glucose with methylglyoxal were explored by Spearman correlation test and reported in table 4.10.

Table 4.10. Correlation of fasting glucose with methylglyoxal levels.

	Methylglyoxal (first round)		Methylglyoxal (second round)		Methylglyoxal (third round)	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
Fasting glucose	-0.02	>0.05	0.19	>0.05	-	-

The total number of observations was 29, 19, and 2 in first, second and third rounds of data collections, respectively. The correlations were not significant at any time point. Since the number

of diabetic individuals were very limited and statistical analysis was not possible just in diabetic participants, the analysis was conducted among all participants.

Out of 32 observation with at least one report of methylglyoxal, just 2 had glucose levels higher than 7 mmol/L. The mean methylglyoxal level in the group with glucose levels higher than 7mmol/L was 0.13 ng/mL whereas this was 0.30 ng/mL in the group with less than or equal to 7 mmol/L. The difference was not significant (P-value>0.05).

#### 4.4.7 Correlation of HOMA-IR with Glucose Metabolites in Diabetic Patients

In order to explore the correlation of fasting of HOMA-IR level in diabetic patients with glucose metabolites, the associations of HOMA-IR with D-lactate, L-lactate and methylglyoxal were explored by Spearman correlation test and reported in table 4.11.

Table 4.11. Correlation of HOMA-IR levels with glucose metabolites in all blood collection times

	D-lactate (all collection times)		L-lactate (all collection times)		Methylglyoxal (all collection times)	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
HOMA-IR	-	-	0.5	>0.05	-	-

Our analysis of the HOMA-IR levels in diabetic patients was independent from all glucose metabolites. The relation between HOMA- IR and endothelial dysfunction biomarkers was also explored. This data is presented in table 4.12.

Table 4.12. Correlation of HOMA-IR levels with endothelial dysfunction biomarkers for all blood collection times

	ICAM-1 (all collection times)		VCAM-1 (all collection times)		E-selectin (all collection times)	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
HOMA-IR	0.5	>0.05	- 0.8	>0.05	0.5	>0.05

As noted from our analysis of glucose metabolite correlations with plasma level of biomarkers for endothelial dysfunction, the HOMA-IR was also independent.

#### **4.5. Endothelial Dysfunction Biomarkers and Their Correlation with Metabolites of Glucose.**

##### **4.5.1. Plasma ICAM Levels Differed Significantly among Study Participant Groups Taking Metformin.**

Plasma ICAM levels were measured on four occasions separated by four months between blood collections. These measurements were taken to explore ICAM as a biomarker of endothelial dysfunction among the different groups and its associations with glucose/diabetes in the study participants. Mean and median of ICAM values are illustrated in table 4.13. Plasma ICAM levels were not available for PCOS patients. A non-parametric one-way ANOVA test indicated significant differences in ICAM levels between the study groups at baseline and at the total pooled collection. In order to identify the difference between these groups, a post hoc test was conducted for every treatment group pairing. At baseline, the plasma ICAM level differed depending on

whether metformin was used or not. The NonDM plus MET and DM2 plus MET groups were significantly higher than the NonDM no MET group (P-value <0.05); however, among those taking metformin, the diagnosis of diabetes did not contribute to changes in ICAM levels (NonDM plus MET and DM2 plus MET groups, P-value>0.05). At the other blood collection times, there were no statistically significant differences between groups. For all time comparisons, the post hoc test was conducted. Group NonDM no MET was significantly lower than all groups. Group NonDM plus MET was significantly higher than diabetic groups. There was not any significant difference between diabetic groups.

Table 4.13. Plasma ICAM levels (ng/mL) in different study groups collected at baseline and at 4 months intervals.

	Baseline			Second time			Third time			Fourth time			All times			
	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	
*NonDM no MET	30	205.5 (191.4 - 219.6)	197.4	29	205.4 (193.9 - 217.0)	201.30	27	213.4 (197.0 - 229.7)	207.9	18	211.2 (192.3 - 230.0)	216.40	104	208.5 (201.3 - 215.7)	203.0	
*NonDM plus MET	4	294.0 (256.9 - 331.1)	287.4	1	294	294	1	276.1	276.1	1	283.8	283.8	7	290.0 (269.9 - 310.2)	283.8	
*DM2 no MET	1	248.9	248.9	1	243	243	0			0			2	246.1 (240.7 - 251.6)	246.1	
*DM2 plus MET	5	266.8 (217.6 - 316.1)	246.2	4	233 (176.3 - 288.8)	248.2	3	239 (210.1 - 268.0)	245.5	3	213.5 (134.8 - 292.2)	226.7	15	241.5 (215.3 - 267.6)	245.5	
P-value (Non-parametric one way ANOVA)		<0.05			>0.05			>0.05			>0.05			<0.05		

Table 4.14. Comparison of ICAM levels between pooled diabetic and non-diabetic participants as well as users and non-users of metformin

Type of group	Number of participants	ICAM (ng/mL)		P-value
		Mean	Median	

Diabetic	17	242	245.5	< 0.05
Non-diabetic	111	213.6	207.9	
Users of Metformin	22	256.9	265.6	< 0.05
Non-users of Metformin	106	209.2	205.3	

Participants were pooled and first divided into two groups (diabetic and non-diabetic) and the plasma ICAM levels were compared (table 4.14). The mean ICAM levels in the pooled diabetic groups was significantly higher than in the non-diabetic group (242.0 vs 213.6 ng/mL, P-value <0.05). A similar process was conducted by categorizing participants into users and non-users of metformin (table 4.14). The ICAM level in users of metformin was higher than non-users and the difference was statistically significant (256.9 vs 209.2 ng/mL, P-value <0.05).

#### 4.5.2. Plasma VCAM Levels Differed Significantly among Participant Groups Taking Metformin.

Mean and median of VCAM values are illustrated in table 4.15. Plasma VCAM levels were not available for PCOS patients. A non-parametric one-way ANOVA test indicated no statistically significant differences in VCAM levels between the study groups at any time point or at all times.

Table 4.15. Plasma VCAM levels (ng/mL) in different study groups collected at baseline and at 4 months intervals

	Baseline			Second time			Third time			Fourth time			All times		
	n	Mean (CI 95%)	Media n	n	Mean (CI 95%)	Media n	n	Mean (CI 95%)	Media n	n	Mean (CI 95%)	Media n	n	Mean (CI 95%)	Media n

*NonDM no MET	30	643.4 (584.5 - 702.3)	606.4	29	641.7 (599.3 - 684.2)	633.00	27	662.1 (609.5 - 714.7)	661.6	18	626 (546.6 - 705.3)	603.5	104	644.8 (617.6 - 672.0)	620.9
*NonDM plus MET	4	770.5 (483.8 - 1057.2)	654.4	1	589.3	589.3	1	610.7	610.7	1	609.6	609.6	7	698.8 (534.6 - 863.1)	610.7
*DM2 no MET	1	593.7	593.7	1	515.5	515.5	0			0			2	554.6 (477.3 - 631.9)	554.6
*DM2 plus MET	5	776.3 (603.1 - 949.5)	737.2	4	791.2 (503.1 - 1079.3)	755.3	4	759.9 (592.0 - 927.8)	731	3	674.3 (600.7 - 747.9)	674.5	16	756.8 (666.1 - 847.5)	731
P-value (Non-parametric one way ANOVA)		>0.05			>0.05			>0.05			>0.05			>0.05	

Table 4.16. Comparison of VCAM levels between pooled diabetic and non-diabetic participants as well as users and non-users of metformin

Type of group	Number of participants	VCAM (ng/mL)		P-value
		Mean	Median	
Diabetic	18	734.3	701.1	> 0.05
Non-diabetic	111	648.2	619.7	
Users of Metformin	23	739.2	647.5	< 0.05
Non-users of Metformin	106	643.1	617.6	

As noted for the ICAM analysis above, participants were pooled and first divided into two groups (diabetic and non-diabetic) to look for correlations with changes in plasma VCAM levels (table 4.16). The mean of the VCAM levels in the pooled diabetic group was higher than in the non-diabetic group but the difference was not statistically significant (734.3 vs 648.2 ng/mL, P-value >0.05). A similar process was conducted by categorizing participants into users and non-users of metformin (table 4.16). The VCAM level in users of metformin was significantly higher

than non-users (739.2 vs 643.1 ng/mL, P-value <0.05).

### 4.5.3. Plasma E-selectin Levels Differed Significantly among Study Participant Groups.

Plasma E-selectin levels were measured on four occasions separated by four months between blood collections. Mean and median of E-selectin values are illustrated in table 4.17. Plasma E-selectin levels were not available for PCO patients. A non-parametric one-way ANOVA test showed statistically significant differences in E-selectin levels between the study groups at

Table 4.17. Plasma E-Selectin levels (ng/mL) in different study groups collected at baseline and at 4 months intervals.

	Baseline			Second time			Third time			Fourth time			All times		
	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median	n	Mean (CI 95%)	Median
*NonDM no MET	29	28.0 (23.6 - 32.5)	25.1	29	32.1 (26.1-38.0)	25.00	27	27.5 (22.3 - 32.8)	25.9	18	27.5 (21.1-33.9)	25.1	103	28.9 (26.3 - 31.6)	25.1
*NonDM plus MET	4	37.2 (18.9 - 55.4)	39.3	1	14.5	14.5	1	31.7	31.7	1	36.0	36.0	7	33.0 (21.5 - 44.4)	31.7
*DM2 no MET	1	43.5	43.5	1	55.7	55.7	0			0			2	49.6 (37.5 - 61.6)	49.6
*DM2 plus MET	5	54.1 (39.3 - 68.9)	51.1	4	38.5(16.7 -60.4)	41.7	2	44.6 (30.0 - 59.1)	44.6	2	26.3(9.1 -43.5)	26.3	13	43.6 (33.6 - 53.5)	40.8
P-value Non-parametric one way ANOVA		<0.05			>0.05			>0.05			>0.05			<0.05	

baseline. In order to identify the difference between these groups, a series of post hoc tests were conducted. At baseline, the plasma E-selectin level was statistically different between the NonDM

no MET and DM2 plus MET groups and the E-selectin level of the DM2 plus MET group was significantly higher than the NonDM no MET group. In the collective comparison, groups were significantly different (P-value<0.05). Post hoc tests revealed that difference is just significant between NonDM no MET group and both diabetic groups (i.e., DM2 no MET and DM2 plus MET).

Table 4.18. Comparison of E-Selectin levels between pooled diabetic and non-diabetic as well as users and non-users of metformin.

Type of group	Number of participants	E-selectine (ng/mL)		P-value
		Mean	Median	
Diabetic	15	44.4	43.5	< 0.05
Non-diabetic	110	29.2	25.9	
Users of Metformin	20	39.9	37.4	< 0.05
Non-users of Metformin	105	29.3	25.8	

As we measured for ICAM and VCAM levels in study participants, participants were pooled and divided into two groups (diabetic and non-diabetic) and the plasma E-selectin levels were compared (Table 4.18). The mean of E-selectin levels in the pooled diabetic group was significantly higher than in the non-diabetic group (44.4 vs 29.2 ng/mL, P-value <0.05). A similar process was conducted by categorizing participants into users and non-users of metformin (table 4.18). The E-selectin level in users of metformin was significantly higher than non-users (39.9 vs 29.3 ng/ml, P-value <0.05).

#### **4.5.4. Review of Associations between Glucose Metabolites and Endothelial Dysfunction Biomarkers.**

In order to explore the correlation of D-lactate, L-lactate and methylglyoxal levels with the

levels of biomarkers of endothelial dysfunction, we explored ICAM, VCAM and E-selectin association with those metabolites using the Spearman correlation test and reported in table 4.19.

Table 4.19. Correlation between D-lactate, L-lactate and E-selectin with ICAM, VCAM and E-selectin compiled from all blood collection time points.

	ICAM		VCAM		E-Selectin	
	Spearman $\rho$	P-value	Spearman $\rho$	P-value	Spearman $\rho$	P-value
D-lactate	0.1977	>0.05	0.1621	>0.05	0.2009	>0.05
L-lactate	- 0.0568	>0.05	0.1366	>0.05	0.1635	>0.05
Methylglyoxal	- 0.0525	>0.05	0.0317	>0.05	- 0.0580	>0.05

No significant positive association was identified between D-lactate, L-lactate and methylglyoxal levels with ICAM, VCAM and E-selectin (table 4.19).

For further exploration of D-lactate as a potential biomarker the correlation of mean of all blood collection times and baseline values of D-lactate with endothelial dysfunction biomarkers were studied (table 4.20 and 4.21)

Table 4.20. Correlation between mean D-lactate, mean ICAM, mean VCAM and mean E-selectin compiled from all blood collection time points.

	ICAM (mean-total)		VCAM (mean-total)		E-Selectin (mean-total)	
	Spearman $\rho$	p-value	Spearman $\rho$	p-value	Spearman $\rho$	p-value
D-Lactate (mean-total)	0.1649	>0.05	0.0833	>0.05	0.3788	<0.05

A significant positive association was identified between D-lactate and E-selectin (Table 4.20).

Table 4.21. Correlation between baseline values of plasma D-lactate levels and the endothelial damage biomarkers ICAM, VCAM, and E-selectin.

	ICAM (first test-total)		VCAM (first test-total)		E-Selectin (first test-total)	
	Spearman $\rho$	p-value	Spearman $\rho$	p-value	Spearman $\rho$	p-value
D-Lactate (first test-total)	0.3904	<0.05	-0.01	>0.05	0.4223	<0.05

At baseline a significantly positive association was identified between D-lactate and ICAM as well as between D-lactate and E-selectin. This association was not significant at other blood collection times.

## **5. Discussion**

### **5.1. Biomarkers for Cardiovascular Consequences in Patients with DM2**

Macrovascular and microvascular complications of diabetes are the main causes of mortality and morbidity of diabetic patients (Souto et al., 2011). Early diagnosis of these complications can help improve length and quality of life among diabetic patients as well as reduce costs to the health system through preventative health measures. Biomarkers could provide a reliable basis for prediction or progression of these complications or indicate “how well the body responds to a treatment for a disease or condition” (Group., 2001). These may be direct, molecules that are an endpoint or a part of the disease pathophysiology, or indirect, molecules that are not directly a part of disease progress or endpoint but rather a surrogate to those factors (Lyons & Basu, 2012). In order to find the appropriate biomarkers for diabetic vascular problems one may turn to the critical role of the endothelium (Libby, Aikawa, & Jain, 2006). Finding relevant factors that can provide evidence of endothelial damage might provide a reliable and practical biomarker to follow endothelial health in diabetic patients or other at-risk populations (Libby et al., 2006). Two important factors were considered in this new exploration. First, the potential biomarker should have a comparable accuracy to the currently used endothelial dysfunction biomarkers. In addition, accessibility to that test needs to be reasonable and measurement can be conducted with minimum instrumentation.

### **5.2. Utility of Measuring Glucose Metabolites**

Using glucose metabolites as biomarkers has a long history and different metabolites have been used for diagnosis and/or follow up for a wide range of diseases such as septic shock, infections, cardiovascular disease, and Duchenne muscular dystrophy (Scheijen et al., 2012; Schneider et al., 2018). Methylglyoxal is one of the metabolites that has a clear correlation to

endothelial dysfunction and has been used as an indirect biomarker for endothelial dysfunction (Alomar et al., 2016; Vulesevic et al., 2016); however, it has limitations for generalized use. Methylglyoxal is an unstable metabolite and its measurement is complicated making it not useful for widespread use. In contrast, D-lactate, a product of methylglyoxal metabolism in pathological situations, is a more stable metabolite and can be more easily tracked in blood and urine. Until recently, two obstacles were considered barriers for using D-lactate as a biomarker of endothelial dysfunction. First, high levels of D-lactate in the blood are not specific for endothelial dysfunction or diabetes. It can be associated with a number of pathological states or induced by diet and is not specific to a diagnosis of diabetes (Cağlayan, Cakmak, Cağlayan, & Cavuşoglu, 2003; Herrera, Morris, Johnston, & Griffiths, 2008; Jeejeebhoy, 2002; Poeze, Solberg, Greve, & Ramsay, 2003). We avoided this complication by carefully selecting our recruits and applying strict exclusion criteria that eliminates the majority of secondary causes of D-lactate elevations. Second, measurement techniques were not accurate enough for widespread applicability (Scheijen et al., 2012). Improvements in measurement methods of D-lactate has recently increased the accuracy over older quantitative methodologies. The previous techniques were liquid chromatography with UV or fluorescence, enzymatic assays and gas chromatography. Those methods suffered from low sensitivity, necessity to have a large sample, and lengthy sample preparation processes (Talasniemi et al., 2008). In the new automated assay, most of these problems are controlled, and improvements in reliability of the measured levels (Scheijen et al., 2012).

In addition, other diseases can be ruled out by different clinical and laboratory testing approaches. These improvements in patient selection and optimized assays may enhance the utility of D-lactate as a more specific biomarker (Scheijen et al., 2012).

In our study to assess the appropriateness of the glucose metabolites, D-lactate, L-lactate and

methylglyoxal, as biomarkers of endothelial dysfunction, our first objective was to explore the correlations in the abundance of glucose metabolite levels between diabetic and non-diabetic patients, expecting to see higher levels with elevated glucose levels. Different studies have shown that levels of these metabolites are different between diabetic and non-diabetic patients (Adeva-Andany et al., 2014; Chou et al., 2015; Kong et al., 2014; Turk, Nemet, Varga-Defteardarović, & Car, 2006). In our study we expanded this perspective and investigated the correlation of these factors with biomarkers of endothelial dysfunction.

### **5.3. D-lactate as a Potential Biomarker for Endothelial Dysfunction**

In our study, the level of D-lactate was significantly higher among diabetic patients (table 4.3). This finding was in the presence of suboptimal glucose control in the participants, based on their levels of HbA1C and fasting glucose. The levels were not only significantly higher than non-diabetic patients (figure 4.2) but also above the target range for well controlled diabetic patients (normal HbA1c <6.5%). This is a reliable sign of poor diabetic control (American Diabetes, 2010). In other words, neither short-term/acute control (fasting glucose) nor long-term diabetic control (HbA1c, reflecting previous 3 months) in participants was optimal.

Our exploration of associations between fasting insulin levels and HOMA-IR had a similar finding to that of D-lactate, as both were higher among diabetic patients (figure 4.4, 4.5 and 4.6). Using a normal cut-off level for fasting glucose (7 mmol/L) also revealed that D-lactate and L-lactate levels were higher among participants with high glucose levels (independent of a diagnosis of diabetes) compared to participants with a lower cut-off level, but the difference was not significant because of the limited number of participants. Therefore, this discussion supports the previous observations that high levels of glucose metabolites are an expected consequence of both diabetes by itself or high levels of glucose. We were unable to determine if acute glucose control

resulted in significant elevations of glucose metabolites as we had too few hyperglycemic events to perform an analysis. Our finding, though, was in line with other studies, which showed that D-lactate levels are higher in DM2 patients (Chou et al., 2015; Claus et al., 2017; Scheijen et al., 2012; Shah & Brownlee, 2016). This also can be explained by biochemistry of D-lactate production pathways (Adeva-Andany et al., 2014).

The correlation between levels of fasting glucose and D-lactate was not significant (table 4.8). This difference between our finding and some other studies on glucose metabolite production can be explained by the small number of recruited patients as well as the demographic and health-related differences among our groups of diabetic and non-diabetic patients including sex, age, race, weight and BMI. This was one of the main limitations that was faced in this study. Studies about D-lactate levels in reference to sex and age differences are very limited. However, none of them reported any difference based on age and sex. Some studies found differences in lactate level in diabetic patients based on their race, BMI, waist circumference. (Crawford et al., 2010; Ohkuwa, Miyamura, Andou, & Utsuno, 1988; Sandoval & Matt, 2002).

Our study was not limited to a comparison between diabetic and non-diabetic patients. Because of the importance of metformin in the treatment of diabetic patients and some concerns about increased lactic acidosis risk with use of metformin (Chang, Chen, Fang, & Huang, 2002), we also compared the level of lactate-relevant glucose metabolites between metformin users and non-users in diabetic and non-diabetic participants. The important finding in our study is about the non-diabetic patients who were under treatment of metformin for their diagnosis of PCOS. The level of D-lactate in the PCOS group was significantly lower than diabetic patients who were also under treatment with comparable doses of metformin (table 4.3). Our finding is in agreement with a number of studies which show that the probability of high levels of D-lactate (and therefore the

risk of precipitating lactic acidosis) is low with metformin use, regardless of glucose control (Aharaz et al., 2018; van Berlo-van de Laar et al., 2011).

#### **5.4. Differences in L-lactate Level between Diabetic and Non-diabetic Participants**

L-lactate is the other optical isomer of lactate. L-lactate concentrations in the blood under physiological situations are about 100 times greater than that of D-lactate. L-lactate is normally produced through anaerobic glycolysis by lactate dehydrogenase (Ewaschuk et al., 2005). Metabolism of glucose in vascular cells is very important and can affect vascular function. In diabetic patients, production of L-lactate increases in vascular cells, which can be a source of subsequent vascular dysfunction. L-lactate interferes with normal regulators of vascular function including VEGF and leads to abnormal angiogenesis (Sawada & Arany, 2017). In the presence of metformin, the effect of insulin in the tissues increases and this can have a positive effect on vascular function. In one study, researchers found that metformin use was associated with normalization of ICAM levels, as a proxy for better vascular function (Jager et al., 2013). In our study the level of L-lactate in diabetic patients was significantly higher than in non-diabetic patients (table 4.5). This is consistent with other studies which have found high levels of L-lactate in diabetic patients (Scheijen et al., 2012). This also can be explained by biochemistry of L-lactate production (Brownlee, 2001; Hasegawa et al., 2003). Furthermore, similar to D-lactate, this finding, high level of L-lactate, was in the presence of poor diabetic control.

#### **5.5. Differences in Methylglyoxal Level between Diabetic and Non-diabetic Participants**

Methylglyoxal is a reactive product of glycolysis and lipid peroxidation and the most important precursor of AGEs. Production of AGEs leads to a cascade of reactions at the vascular level with a negative outcome on endothelial function. There are many studies that have investigated the association between high levels of MG and endothelial dysfunction (Alomar et

al., 2016; Vulesevic et al., 2016). Our assessment of correlations between MG and biomarkers of endothelial dysfunction did not reveal a similar association (table 4-19). Similar to D-lactate and L-lactate associations, the levels of MG showed higher trends among diabetic patients, but this difference was not significant (table 4.7). MG is a well-studied metabolite and there are a considerable number of studies that confirm the high level of MG in diabetic patients (Alomar et al., 2016; Giacco & Brownlee, 2010; Vulesevic et al., 2016).

The difference between our finding and some other studies on MG serum levels can be explained by the small number of recruited individuals as well as differences in the demographics between diabetic and non-diabetic patients. This is one of the main limitations that was faced in this study. Notwithstanding, the differences in MG level with age and sex of the participants in studies were not reported (Srikanth et al., 2013). Some studies that investigated the role of MG on inflammatory and autoimmune diseases such as multiple sclerosis, have reported age and sex difference in MG levels in those diseases (Wetzels, Wouters, Schalkwijk, Vanmierlo, & Hendriks, 2017). This can be a proxy for either different levels of MG in different age and sex groups or at least different consequences of MG on people from various age and sex groups (Wetzels et al., 2017).

## **5.6. Biomarkers of Endothelial Damage**

Endothelial dysfunction is considered as one of the main causes of vascular problems in the diabetic patient. (Dimitris, Anna-Maria, & Costas Tentolouris Nikolaos Papageorgiou and Christodoulos, 2012). Based on different potential theories for endothelial dysfunction in diabetic patients the following factors might be involved in vascular complication of diabetes: insulin resistance, abnormal cluster of hyperglycemia, increased oxidative stress, increased inflammatory status, elevated free fatty acids, glycosylated end products, vascular smooth muscle dysfunction,

and endothelial dysfunction (Tousoulis et al., 2013). Considering all possible causes for the vascular consequences, different biomarkers within *in vivo* and *in vitro* studies are used to find the most appropriate marker for diagnosis and follow up of vascular complications. Three major markers of endothelial dysfunction (ICAM, E-selectin and VCAM), inflammation (CRP, IL-6, IL-1 $\beta$  and TNF- $\alpha$ ), and pro-coagulation factors (PAI, fibrinogen and selectin) have all been used as disease biomarkers (Tousoulis et al., 2013).

To assess endothelial dysfunction, we chose ICAM, VCAM, and E-selectin. Our aim was to investigate the association between glucose metabolites and these biomarkers of endothelial dysfunction, with the assumption that chronic hyperglycemia could result in endothelial damage and subsequent elevations in ICAM/VCAM and E-selectin. In our study the ICAM and E-selectin levels for diabetic patients were higher than non-diabetic patients. This difference was not significant for VCAM. These high levels of biomarkers were reliable proxies for endothelial dysfunction of diabetic patients in our study, although we did not perform gold standard assays to prove endothelial health (vessel biopsy and pathology). It is critical to note that data for these biomarkers were not available for the PCOS cases, and few DM2 individuals were recruited. This might explain the insignificant VCAM difference among the groups.

CRP in our study was used as a simple and available test to explore the existence of inflammation among the participants. The results revealed that participants do not have any specific inflammation that can affect the analysis. However, studies have shown that metformin users may have lower levels of CRP (Chen et al., 2017), and this can overshadow the existence of inflammation. In future studies, the use of more than one inflammatory biomarker can resolve this problem.

## **5.7. Correlation between Glucose Metabolites and Biomarkers of Endothelial Damage**

The goal of the study was to investigate whether glucose metabolites can be appropriate biomarkers of endothelial dysfunction and to provide insight into the risk of subsequent vascular complications. A comparison of the levels of glucose metabolites and endothelial dysfunction biomarkers between groups was required to explore the correlation of these differences between participant groups. This correlation was explored at baseline and at all blood collection times. In both scenarios, the correlation between D-lactate as a glucose metabolite was significantly positive with ICAM and E-selectin in baseline data collection, whereas a significant correlation was found only for E-selectin in pooled data samples (table 4.20 and 4.21). A correlation was not significant between D-lactate and VCAM.

Since ICAM and E-selectin are well-studied biomarkers of endothelial dysfunction and their high levels in serum are important alarms for atherosclerosis (Favero, Paganelli, Buffoli, Rodella, & Rezzani, 2014; Lawson & Wolf, 2009; Liao, 2013; Sun, Li, Graziani, Filion, & Allan, 2013), this correlation between D-lactate and ICAM and E-selectin might suggest D-lactate is a good candidate for screening of endothelial dysfunction. However, high levels of D-lactate are associated with a number of clinical diagnoses, which affects the specificity of D-lactate as a biomarker for vascular disease. D-lactate serum concentrations may be elevated in patients with short bowel syndrome (Herrera et al., 2008), patients on parenteral nutrition (Jeejeebhoy, 2002), with appendicitis (Cağlayan et al., 2003; Unverir & Karcioğlu, 2011), septic shock (Poeze et al., 2003), and other rare diseases such as triosephosphate isomerase deficiency and necrotizing enterocolitis (Herrera et al., 2008). Furthermore, this is further complicated by observed correlations between D-lactate and ICAM in some situations such as inflammatory bowel disease (Song et al., 2009), where a recent study that showed the presence of endothelial dysfunction in

inflammatory bowel disease (Gravina et al., 2018). In conclusion, D-lactate has some potential to be a biomarker for endothelial dysfunction, yet further knowledge and larger controlled studies are required for final determination.

The other factor which needs to be considered in final analysis is the cardiovascular risk of PCOS. There are some studies that refer to PCOS as a risk factor for cardiovascular diseases (Scicchitano et al., 2012). Metabolism of lipid/glucose changes in this patients and there is higher prevalence of inflammations and vascular injuries among them (Scicchitano et al., 2012). This could affect the final analysis of our study since their cardiovascular situation has not been included in our analysis. Therefore, the conclusions included this group needs to be treated cautiously.

## **5.8. Limitations of the Study**

Although a number of studies about health and welfare are increasing world-wide, the issue of recruitment and retention of participants is an important challenge that is well recognized. The challenges of patient recruitment have been assessed by a number of different organizations, which has resulted in a wide range of recommendations particularly by leading research organizations. Nonetheless, patient recruitment remains an important challenge (Bower et al., 2014).

While conducting our research, several different approaches were applied to enhance recruitment and retention. In order to recruit the participants, posters were distributed in different places including the hospital and possible locations that participants may visit. Non-monetary incentives were used for NonDM no MET participants. A researcher was present in the office of the research medical doctor in order to provide extra information for any potential participants who were introduced by the medical doctor. Finally, data from another study on PCOS patients with a history of metformin use was used as the major part of NonDM plus MET group of our

study.

However, the final number of participants did not meet the required sample size, mainly because of subject burden. Various factors may have contributed to this situation. First, participation in the study lasted one year and participants were supposed to visit the hospital for blood sample collection four times. Some participants might have felt overburdened with this requirement. In addition, there was just one center for blood collection and participants had to come to that center. This could be a strong deterrent for those participants who lived far from that center. Furthermore, the research was conducted in a small city and considering the small target population, access to the predicted sample size was difficult.

To resolve poor recruitment issues some approaches/solutions can be proposed. The study can be implemented in more than one place and the research team can create a network to pool participants and increase the total number of the cases. All the findings and progress with participants can be synchronized by using on-line network approaches. The other solution is the expansion of partner medical doctors. For example, by cooperating with family physicians, access to diabetic patients could be increased since many diabetic patients refer to their family physicians for day to day problems. Finally, to increase the number of participants mechanisms for easing blood collection may facilitate recruitment and retention. In our study, all participants had to refer to the hospital. Alternative blood collection locations would encourage participation particularly with, participants who are old or suffer from some comorbidities.

Additional limitations to our analysis also exist. The mean age for the NonDM no MET group was 31.4 years (95% CI: 29.0 – 33.9), which was much younger than the mean age of the DM2 plus MET group (60.9 years with 95% CI: 52.0 – 69.8). The age of the only participant in the DM2 no MET group was 52.4 at the beginning of the study. In the NonDM no MET group,

70% of participants were male, in comparison to the DM2 plus MET group which 20% were female and to the DM2 no MET group which was female only. Hence, sex and age of participants may have contributed to bias in some of collected data.

Different statistical methods are applied in case of high missing values including list wise or case deletion, pairwise deletion, mean substitution, regression imputation, maximum likelihood and multiple imputation. Unfortunately none of these methods was appropriate for our research (Kang, 2013), because the number of participants in two of the groups (namely DM2 plus MET and DM2 no MET) was very small and information about the other group (i.e., NonDM plus MET) was limited to the level of D-lactate.

In order to conduct a statistic analysis and glean useful information from the small sample sizes, nonparametric tests were applied. In our research, we mainly relied on non-parametric one-way ANOVA for comparing the means and Spearman correlation to determine the association between variables. This is the only possible solution for doing the statistics, where the conventional (parametric) tests are not possible (Nahm, 2016). The use of nonparametric tests had some advantages when we have a small sample size. These tests are conservative, in other words the probability of a wrong conclusion in these tests is low (Treister et al., 2015).

## **6. Summary of Findings**

With the caveat of small sample size and age and gender bias among study groups, the major finding of our research is the significant correlation between the levels of D-lactate and that of the endothelial dysfunction biomarkers ICAM and E-selection. This positive correlation provides an opportunity to consider D-lactate as a potential biomarker for endothelial dysfunction in diabetic patients. However, the specificity of D-lactate to diabetes is questionable and would require the need to rule out other possible diagnoses that can result in elevated blood levels of D-lactate. Furthermore, this finding needs to be supported with larger sample size research.

An additional important finding involves the lack of elevation in D-lactate levels with metformin use. This is one of the major historic concerns for metformin users as it may precipitate metabolic acidosis and diabetic ketoacidosis, both of which are medically important illnesses. Our failure to detect serum elevations of D-lactate with normal metformin use in diabetic and on diabetic populations is reassuring.

## **7. Future Directions**

Assessing D-lactate as a potential biomarker for vascular complications was the major aim of this study and we were able to detect a significant correlation between these factors. In our study we did not find a direct association between high levels of blood glucose or poor control of hyperglycemia (i.e., high levels of HbA1C) with the main glucose metabolites (D- and L-lactate and MG). However, our study confirmed a positive association between D-lactate from one end and ICAM and E-selectin on the other end. Based on this finding, some possible future considerations include the need for a larger sample size to confirm the findings of the study. This is particularly important to allow assessment of the influence of other covariates such as age, sex,

waist circumference versus BMI, diet, exercise, time of DM2, history of cardiovascular disease, comorbidities, and socio-economic status (SES).

Given the likelihood of other confounding factors for D-lactate elevations and/or endothelial damage such as L-lactate or Methylglyoxal, the study would need to be expanded to explore the association of additional metabolites of glucose with more biomarkers of vascular complications. This expanded study can provide further knowledge about the relationship between the potential biomarkers and vascular complications in diabetic patients and perhaps yield insight into the underlying mechanisms causing this complication.

Another aim of this study was to measure the effect of glucose control on vascular complications through endothelial biomarkers. Trend analysis of biomarkers and glucose metabolites can be very helpful in better understanding the pathophysiology of vascular complications in diabetic patients and consequently prevent vascular complications in such patients. A cohort study might be the ideal study design for this purpose; however, historical cohort study designs can also be useful including health registries or reliable past medical history.

The other finding of this study was about the effect of metformin use on D-lactate levels in both diabetic and non-diabetic cohorts. In our study, non-diabetic metformin users had the lowest level of D-lactate while there were some studies that contradict this finding. However, this may be the result of their existing health situation because of their PCOS and this can also be explored with other cohort studies with adequate sample size.

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## APPENDIX A STUDY GROUPS SUBJECT INFORMATION AND CONSENT FORM

**STUDY TITLE:** Plasma concentrations of D-lactate and its related metabolic intermediates in diabetic patients with or without metformin: potential biomarkers for diabetic vascular complications

**PRINCIPAL INVESTIGATOR:** Dr. T. Arnason

**SUB-INVESTIGATORS and/or STUDENT RESEARCHERS:**

Dr. G. Zello, Dr. J. Alcorn, Dr.G.Dala, Dr.D.Chizen, Dr. R. Ostad Ali Dehaghi (graduate student), Dr. P. Rafiei (graduate student)

**FUNDING AGENCY:** Royal University Hospital Foundation, Natural Sciences & Engineering Research Council of Canada (NSERC)

**CONTACT NUMBER:** Please call or leave message on 966-7911 regarding “Diabetes Study”

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### INTRODUCTION

You are being asked to take part in this research as a study group because you have been diagnosed with Type 2 Diabetes. You may, or may not, be taking metformin as part of your treatment. Participation is voluntary and it is up to you if you wish to be enrolled in this study. If you do decide that you will take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision. If you do not wish to participate, you will not lose the benefit of any medical care to which you are entitled or presently receiving.

### WHO IS CONDUCTING THE STUDY?

The Division of Endocrinology/Department of Medicine and the College of Pharmacy and Nutrition at the University of Saskatchewan are conducting this study. The investigators or staff will not receive any direct financial benefit from conducting this study, other than their regular salary.

### WHY IS THIS STUDY BEING DONE?

The purpose of this study is to investigate the blood concentrations of D-lactate (a normal organic acid that present in human body at low concentration) and its related metabolic intermediates (molecules that involve in normal D-lactate metabolism including Methylglyoxal, L-lactate and pyruvate). People living with diabetes are at a higher risk of developing diabetic complications including cardiovascular disease. How and why these complications occur is not yet fully understood. Normally, humans have very low levels of a D-lactate in our blood. In clinical conditions such as diabetes, the level of this compound may be elevated. Use of a medication called Metformin has also shown to at times increase blood D-lactate levels. This research is to determine if there are measureable changes to D-lactate levels in the blood when comparing people with or without Diabetes and whether they take Metformin or not.

### WHAT DOES THE STUDY INVOLVE?

We are requesting to take two extra tubes of blood (this will not involve an extra needle poke) during your visit, which will happen every **four** months for a period of one year. **Your medical care will not be delayed or affected in any way.** If you agree, two extra tubes of blood sample will be drawn in addition to other blood samples that are ordered by your physician as part of your routine care (routine blood work up to every four month for up to 12 months). Blood samples will be transported to third Floor of D-wing of Health Sciences Building and analyzed for D-, L-Lactate, Methylglyoxal (a normal byproduct of human sugar usage) and pyruvate (a normal measurable end-product of sugar metabolism) content. The vascular endothelial markers, including human soluble intercellular adhesion molecule 1 (sICAM-1) and human soluble vascular cell adhesion molecule-1 (sVCAM-1), TNF-alpha, PAI-1 and human sE-selectin that will

be tested using commercial available ELISA kits. Your medical chart will also be reviewed for the results of the routine blood tests (electrolytes, creatinine, glucose, HgA1C, cholesterol, triglycerides, C-reactive protein (CRP), HDL and LDL) to compare with the lactic acid levels measured as part of this study. Blood samples will be destroyed at the end of the study.

**WHAT ARE THE RISKS AND BENEFITS OF PARTICIPATING IN THIS STUDY?**

There are no risks from participation in this study. There will not be any direct benefits to you. It is hoped the information gained from this study can be used in the future to benefit others with Type 2 diabetes.

**WHAT HAPPENS IF I DECIDE TO WITHDRAW?**

Your participation in this research is voluntary. If you choose to enter in the study and then decide to withdraw later, you may ask that the samples be destroyed by contacting the study doctor. All data collected about you during your enrolment will be retained for analysis. Please inform us immediately if you plane to withdraw.

**WHAT WILL THE STUDY COST ME?**

You will not be charged for any research-related procedures. You will not be paid for participating in this study. You will not receive any compensation, or financial benefits for being in this study, or as a result of data obtained from research conducted under this study.

**WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?**

In Saskatchewan, *the Health Information Protection Act (HIPA)* protects the privacy of your personal health information. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator and the University of Saskatchewan Research Ethics Board for the purpose of monitoring the research. However, no records, which identify you by name or initials, will be attached to any information. The results of this study may be presented in a scientific meeting or published, but your identity will not be disclosed.

**WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY?**

If you have any questions or desire further information about this study before or during participation, you can contact anyone of the researchers: Razieh Ostad Ali Dehaghi (306)881-7817, Dr. G Zello (306) 966-5825, Dr. T. Arnason (306) 844-1119, Dr. J. Alcorn (306) 966-6365 ,Pedram Rafiei (306) 715-7966

If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the Chair of the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan, at 306-966-4053. The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan.

**CONSENT TO PARTICIPATE**

I agree to participate in this study. I give permission to the use and disclosure of my de-identified personal health information collected for the research purposes described in this form. I understand that by signing this document I do not waive any of my legal rights. I will be given a signed copy of this consent form.

Printed name of study participant

Signature

Date

.....

.....

.....

Printed name of person obtaining consent:

Signature

Date

**CONSENT TO PARTICIPATE**

- I have read (or someone has read to me) the information in this consent form.
- I understand the purpose and procedures and the possible risks and benefits of the study.
- I was given sufficient time to think about it.
- I had the opportunity to ask questions and have received satisfactory answers.
- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care.
- I have been informed there is no guarantee that this study will provide any benefits to me.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form
  
- My family physician can [or will] be informed about my participation in this study, and, if required, consulted regarding my health and treatment.
  - Yes, you may contact my primary care physician
  - No, please do not contact my primary care physician
  - I do not have a primary care physician.
  
- I grant the Saskatchewan Ministry of Health permission to disclose my health care information to the study researchers  Yes  No

I agree to participate in this study:

Printed name of participant:

Signature

Date

Printed name of person obtaining consent:

Signature

Date



## **CONTROL GROUPS SUBJECT INFORMATION AND CONSENT FORM**

**STUDY TITLE:** Plasma concentrations of D-lactate and its related metabolic intermediates in diabetic patients with or without metformin: potential biomarkers for diabetic vascular complications

**PRINCIPAL INVESTIGATOR:** Dr. T. Arnason

**SUB-INVESTIGATORS and/or STUDENT RESEARCHERS:**

Dr. G. Zello, Dr. J. Alcorn, Dr.G.Dala, Dr.D.Chizen, Dr. R. Ostad Ali Dehaghi (graduate student), Dr. P. Rafiei (graduate student)

**FUNDING AGENCY:** Royal University Hospital Foundation, Natural Sciences & Engineering Research Council of Canada (NSERC)

**CONTACT NUMBER:** Please call or leave message on 966-7911 regarding “Diabetes Study”

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### **INTRODUCTION**

You are being asked to participate in this study to serve as a control group. You may, or may not, be taking metformin as part of your medical treatment. Participation is voluntary and it is up to you if you wish to be enrolled in this study. If you do decide that you will take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision. If you do not wish to participate, you will not lose the benefit of any medical care to which you are entitled or presently receiving.

### **WHO IS CONDUCTING THE STUDY?**

The Division of Endocrinology/Department of Medicine and the College of Pharmacy and Nutrition at the University of Saskatchewan are joining to conduct this study. The investigators or staff will not receive any direct financial benefit from conducting this study, other than their regular salary.

### **WHY IS THIS STUDY BEING DONE?**

The purpose of this study is to investigate the blood concentrations of D-lactate (a normal organic acid that present in human body at low concentration) and its related metabolic intermediates (molecules that involve in normal D-lactate metabolism including Methylglyoxal, L-lactate and pyruvate). People living with diabetes are at a higher risk of developing diabetic complications including cardiovascular disease. How and why these complications occur is not yet fully understood. Normally, humans have very low levels of a D-lactate in our blood. In clinical conditions such as diabetes, the level of this compound may be elevated. Use of a medication called Metformin has also shown to at times increase blood D-lactate levels. This research is to determine if there are measureable changes to D-lactate levels in the blood when comparing people with or without Diabetes and whether they take Metformin or not,

### **WHAT DOES THE STUDY INVOLVE?**

We are requesting your permission to take two extra tubes of blood (this will not involve an extra needle poke) during your visit, which will happen every four months for a period of one year. Blood samples will be transported to third Floor of D-wing of Health Sciences Building and analyzed for D-, L-Lactate, Methylglyoxal (a normal byproduct of human sugar usage) and pyruvate (a normal measurable end-product of sugar metabolism) content. The vascular endothelial markers, including human soluble intercellular adhesion molecule 1 (sICAM-1) and human soluble vascular cell adhesion molecule-1 (sVCAM-1), TNF-alpha, PAI-1 and human sE-selectin that will be tested using commercial available ELISA kits. Your medical chart will also be reviewed for the results of the routine blood tests (electrolytes, creatinine, glucose, HgA1C, cholesterol, triglycerides, C-reactive protein (CRP), HDL and LDL) to compare with the lactic acid levels measured as part of this study. Blood samples will be destroyed at the end of the study.

### **WHAT ARE THE RISKS AND BENEFITS OF PARTICIPATING IN THIS STUDY?**

There are no risks from participation in this study. There will not be any direct benefits to you. It is hoped the information gained from this study can be used in the future to benefit others with Type 2 diabetes.

**WHAT HAPPENS IF I DECIDE TO WITHDRAW?**

Your participation in this research is voluntary. If you choose to enter in the study and then decide to withdraw later, you may ask that the samples be destroyed by contacting the study doctor. All data collected about you during your enrolment will be retained for analysis. Please inform us immediately if you plane to withdraw.

**WHAT WILL THE STUDY COST ME?**

You will not be charged for any research-related procedures. You will not be paid for participating in this study. An honorarium gift card of \$10 will be provided at three blood sampling visits to reimburse your time.

**WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?**

In Saskatchewan, *the Health Information Protection Act (HIPA)* protects the privacy of your personal health information. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator and the University of Saskatchewan Research Ethics Board for the purpose of monitoring the research. However, no records, which identify you by name or initials, will be attached to any information. The results of this study may be presented in a scientific meeting or published, but your identity will not be disclosed.

**WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY?**

If you have any questions or desire further information about this study before or during participation, you can contact anyone of the researchers: Razieh Ostad Ali Dehaghi (306)881-7817, Dr. G Zello (306) 966-5825, Dr. T. Arnason (306) 844-1119, Dr. J. Alcorn (306) 966-6365, Pedram Rafiei (306) 715-7966

If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the Chair of the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan, at 306-966-4053. The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the Biomedical Research Ethics Board (Bio-REB) of the University of Saskatchewan.

**CONSENT TO PARTICIPATE**

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Printed name of study participant	Signature	Date
.....	.....	.....

Printed name of person obtaining consent:	Signature	Date
.....	.....	.....

### CONSENT TO PARTICIPATE

- I have read (or someone has read to me) the information in this consent form.
  - I understand the purpose and procedures and the possible risks and benefits of the study.
  - I was given sufficient time to think about it.
  - I had the opportunity to ask questions and have received satisfactory answers.
  - I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care.
  - I have been informed there is no guarantee that this study will provide any benefits to me.
  - I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
  - I understand that by signing this document I do not waive any of my legal rights.
  - I will be given a signed and dated copy of this consent form
- 
- My family physician can [or will] be informed about my participation in this study, and, if required, consulted regarding my health and treatment.
    - Yes, you may contact my primary care physician
    - No, please do not contact my primary care physician
    - I do not have a primary care physician.
  
  - I grant the Saskatchewan Ministry of Health permission to disclose my health care information to the study researchers  Yes  No

I agree to participate in this study:

Printed name of participant:

Signature

Date

Printed name of person obtaining consent:

Signature

Date