2023

Cardiometabolic proteomics and vascular endothelial health in type 2 diabetes

BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

CARDIOMETABOLIC PROTEOMICS AND VASCULAR ENDOTHELIAL HEALTH IN TYPE 2 DIABETES

by

ERIKA TERESA MINETTI

B.A., Boston University, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2023

Approved by

First Reader

Naomi Hamburg, M.D., M.S.
Professor of Medicine

Second Reader

Jingyan Han, Ph.D.
Assistant Professor of Medicine

Third Reader

Robert Weisbrod, M.A.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my thesis advisor Dr. Naomi Hamburg for her advice, support, and mentorship during my time in the Hamburg lab. Her guidance was incredibly valuable in the writing of my thesis, as well as in my growth as a scientist. I was in awe of her knowledge and expertise, and she's my inspiration in continuing my research endeavors in the future.

I would also like to thank Dr. Jingyan Han for her advice and guidance in writing this thesis, as well as her mentorship since 2017 in furthering my understanding of research practices and encouraging my scientific inquiry.

I am grateful of the members of the Hamburg Lab at Boston University Chobanian and Avedisian School of Medicine for creating a growth-centered and motivating environment where I could learn not only about research, but about their lives as researchers and physicians. I am forever indebted to my supervisor Mr. Robert Weisbrod, who we all call Bob, for his infinite patience and hard work. You created a safe learning space and always provided thoughtful feedback.

I would also like to thank the director of the Master's in Medical Sciences program, Dr. Gwynneth Offner, for her reliable presence throughout this degree, and my degree advisor Dr. Isabel Dominguez for her feedback and constructive criticism.

Finally, I would like to acknowledge everyone mentioned above, as well as Nancy Clinton and my advisors at the International Students and Scholars Office for making sure that I could overcome visa hurdles in these past two years. I was able to write this thesis

and complete my Master's degree thanks to the support I received by the kind and knowledgeable people that I was surrounded by.

CARDIOMETABOLIC PROTEOMICS AND VASCULAR ENDOTHELIAL HEALTH IN TYPE 2 DIABETES

ERIKA TERESA MINETTI

ABSTRACT

Background

Type 2 diabetes (T2DM) is a metabolic disease that arises from insulin resistance and facilitates progression to cardiovascular consequences including myocardial infarction, coronary artery disease, and stroke. A contributor to the cardiovascular complications seen in T2DM is endothelial dysfunction. From a molecular standpoint, studies have shown that the pathophysiology of T2DM involves an altered metabolic milieu and increased oxidative stress, which both arise from insulin resistance, and lead to endothelial dysfunction. There is still much to discover on the pathways that are altered in this disease.

Proteomics is a rapidly improving technique that can elucidate the differences in serum biomarkers, and their relationship to vascular endothelial health to further understand the pathophysiology of T2DM.

Objective

To evaluate the proteomic background and the implicated pathways in T2DM, and to understand how these biomarkers are associated with endothelial cell phenotype and systemic vascular function.

Methods

Age and sex similar individuals with T2DM and control individuals without T2DM between the ages of 30 and 80 were enrolled in this study. Blood was obtained for blood

glucose and insulin levels and two proteomics panels assessing 192 serum biomarkers. Baseline vascular measures were obtained including blood pressure, heart rate, and flow-mediated dilation. Endothelial cells collected from participants were stimulated with insulin *ex vivo* and stained with phosphorylated endothelial nitric oxide synthase (p-eNOS) to measure changes in the insulin-mediated eNOS pathway. Associations between biomarker levels and insulin-stimulated p-eNOS levels were evaluated.

Results

The present study includes 69 subjects including 37 subjects with T2DM (age 57±8 years, 41% female) and 32 control subjects (age 53±9 years, 38% female). Measures of vascular health showed evidence of impairment in patients with T2DM including higher pulse pressure (56±12 mmHg versus 48±11 mmHg, p=0.02) and lower flow-mediated dilation (6.04±3.41% versus 9.1±4.4%, p=0.01).

The proteomic panels revealed 24 serum biomarkers that were significantly upregulated and 2 that were significantly downregulated (adjusted $p<0.05$) in patients with T2DM compared to nondiabetic controls. These biomarkers are mainly involved in metabolism, vascular and fluid homeostasis, immune response, and apoptosis.

Endothelial cell phenotype was abnormal in patients with T2DM compared to controls: mean fold change in insulin-stimulated p-eNOS was 0.34±0.07 for nondiabetic controls and -0.14±0.03 (p=0.01) for patients with T2DM.

Renin and Adrenomedullin were significantly associated with lower insulin stimulated p-eNOS activation (r=-0.38, r=-0.27, and p=0.004, p=0.049 respectively). Whereas Chymotrypsin C (r=0.37, p=0.006), Paraoxonase 3 (r=0.35, p=0.009),

Lipoprotein Lipase (r=0.34, p=0.01), and Superoxide Dismutase 2 (r=0.31, p=0.02) were significantly associated with higher insulin stimulated p-eNOS activation.

Conclusions

We found associations between serum biomarker levels and insulin-stimulated p-eNOS levels which showed that there is a relationship between altered biomarkers and endothelial cell phenotype.

Patients with T2DM had worse vascular endothelial health as shown by measures of endothelial dysfunction and arterial stiffness. Endothelial cell insulin resistance was present in patients with T2DM. In the same group, serum biomarkers showed elevated adiposity, inflammation and oxidative stress, and upregulation of the renin-angiotensin-aldosterone system.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE ..... Angiotensin-converting enzyme

ADH ..... Antidiuretic hormone

AKT ..... AK strain transforming

AMBP ..... Alpha-1-microglobulin/bikunin precursor

ARB ..... Angiotensin receptor blocker

BMI ..... Body mass index

BP ..... Blood Pressure

CAD ..... Coronary artery disease

CV ..... Cardiovascular

CVD ..... Cardiovascular disease

eNOS ..... Endothelial nitric oxide synthase

FMD ..... Flow-mediated dilation

GDF15 ..... Growth differentiation factor 15

GLP-1 ..... Glucagon like peptide-1

Hb A1C ..... Glycated hemoglobin

HDL ..... High density lipoprotein

HOMAGE ..... Heart OMics in AGEing

HR ..... Heart rate

HTN ..... Hypertension

IL-1RN ..... Interleukin-1 receptor antagonist protein

IL-6 ..... Interleukin-6

IR ..... Insulin receptor

IRS ..... Insulin receptor substrate

IRS-1 ..... Insulin receptor substrate 1

IRS-2 ..... Insulin receptor substrate 2

JNK ..... c-jun N-terminal kinase

LDL ..... Low density lipoprotein

MAP ..... Mean arterial pressure

MAPK ..... Mitogen activated protein kinase

MetS ..... Metabolic syndrome

NF-κB ..... Nuclear factor kappa B

NO ..... Nitric oxide

NOS ..... Nitric oxide synthase

NPX ..... Normalized protein eXpression

p-eNOS ..... Phosphorylated endothelial nitric oxide synthase

PAD ..... Peripheral arterial disease

PEA ..... Proximity extension assay

PI3K ..... Phosphoinositide 3-kinase

PKC ..... Protein kinase C

RAAS ..... Renin-angiotensin-aldosterone system

SBP ..... Systolic blood pressure

SD ..... Standard deviation

T2DM ..... Type 2 diabetes

TG ..... Triglycerides

TNF-α ..... Tumor necrosis factor α

TNFRSF11A ..... Tumor necrosis factor receptor superfamily member 11A

TNFRSF1B ..... Tumor necrosis factor receptor superfamily member 1B

Wnt5a ..... Wnt family member 5A

INTRODUCTION

Type 2 Diabetes: Epidemiology and Prevalence

Type 2 Diabetes (T2DM) is a disease characterized by an inability to regulate blood sugar levels due to impaired tissue response to insulin. The pancreas produces elevated levels of insulin leading to hyperglycemia, which if left untreated can lead to end-organ damage and vascular complications.[1] The American Diabetes Association reports that in 2019, 37.3 million Americans had diabetes, of which about 95% had T2DM.[2] The Institute for Health Metrics and Evaluation reports that in the same year, Diabetes Mellitus (both type 1 and 2) accounted for 2.74% of total deaths globally. Nationally, diabetes accounted for 2.64% of total deaths, and was the 7th leading cause of death.[3] As there is no effective cure, current management of T2DM includes pharmaceutical interventions and lifestyle changes such as physical activity and healthy eating, which place a significant burden on patients' lives.[4] Indeed T2DM accounts for 5.5% of total Years Lived with Disability.[3]

Metabolic Consequences of T2DM

T2DM is known to be associated with the Metabolic Syndrome (MetS) through shared cardiovascular (CV) risk factors such as obesity, hypertension (HTN), and insulin resistance.[5] MetS is a series of risk factors that facilitate the progression towards T2DM and cardiovascular disease (CVD). These risk factors include but are not limited to central obesity, elevated blood pressure (BP), insulin resistance, elevated glucose and triglyceride (TG) levels, low levels of high-density lipoprotein (HDL), and a pro-inflammatory state.[6] MetS also becomes more frequent with age.[7]

An evaluation of 11 prospective cohort studies carried out in Europe showed that in nondiabetic adults MetS had a prevalence of 15%, and in the population of which size was 11,512 individuals, 432 of the 1119 deaths that occurred were due to CVD.[8] Persons with MetS are predisposed to CV events such as stroke, congestive heart failure, and chronic kidney disease.[6,9]

Another study found that the prevalence of MetS among a population with T2DM was 66.7% when using the US National Cholesterol Education Programme Adult Treatment Panel III guidelines, and 53.5% when using the guidelines established by the International Diabetes Foundation.[10] Therefore MetS is a problem concerning a significant proportion of the population, and with T2DM the threat is even greater. As such, studying the metabolic changes that occur in T2DM and relating them to CV health can help improve the prognosis of MetS in the context of T2DM.

Due to the interrelationship between MetS and CVD, this constellation of risk factors is also known as the Cardiometabolic Syndrome, and it is connected to the pathogenesis of T2DM through insulin resistance.

Cardiovascular Complications of T2DM

Individuals with T2DM have increased risk for atherosclerosis, which leads to CVDs such as stroke, coronary artery disease (CAD), myocardial infarction, and peripheral arterial ischemia.[11] In a literature review by Einarson et al. that assessed works published between 2007 and 2017, CVD was present in 32.2% of the more than 4.5 million people

with T2DM on whom data was reported. CVD was also the reason for 9.9% of deaths and accounted for half of overall deaths.[12]

It is hypothesized that hyperglycemia is at the basis of poor CV outcomes due to its role in the progression of atherosclerosis. Insulin resistance is the hallmark of T2DM and leads to hyperglycemia through increased gluconeogenesis and glycogenolysis. Hyperglycemia measured, for example, with fasting blood glucose can be a diagnostic test for T2DM.[13]

A study by Park et al. determined that there is a progressive increase in CVD with fasting glucose levels >100 mg/dL. In particular, ischemic heart disease, myocardial infarction, and thrombotic stroke were the CV outcomes associated with higher fasting glucose levels, and in this study myocardial infarction had the most significant increase in hazard ratio in females, and had the second most significant increase in hazard ratio in males, behind hemorrhagic stroke.[14]

Chronically high blood sugar causes end-organ damage in the nervous system, eyes, and kidneys, and the latter is involved in maintaining systemic BP through its release of renin through the renin-angiotensin-aldosterone system (RAAS).[15] The RAAS involves two cleavage reactions: the conversion of angiotensinogen to angiotensin I by renin, and its subsequent conversion to angiotensin II by angiotensin-converting enzyme (ACE) (**Figure 1**).

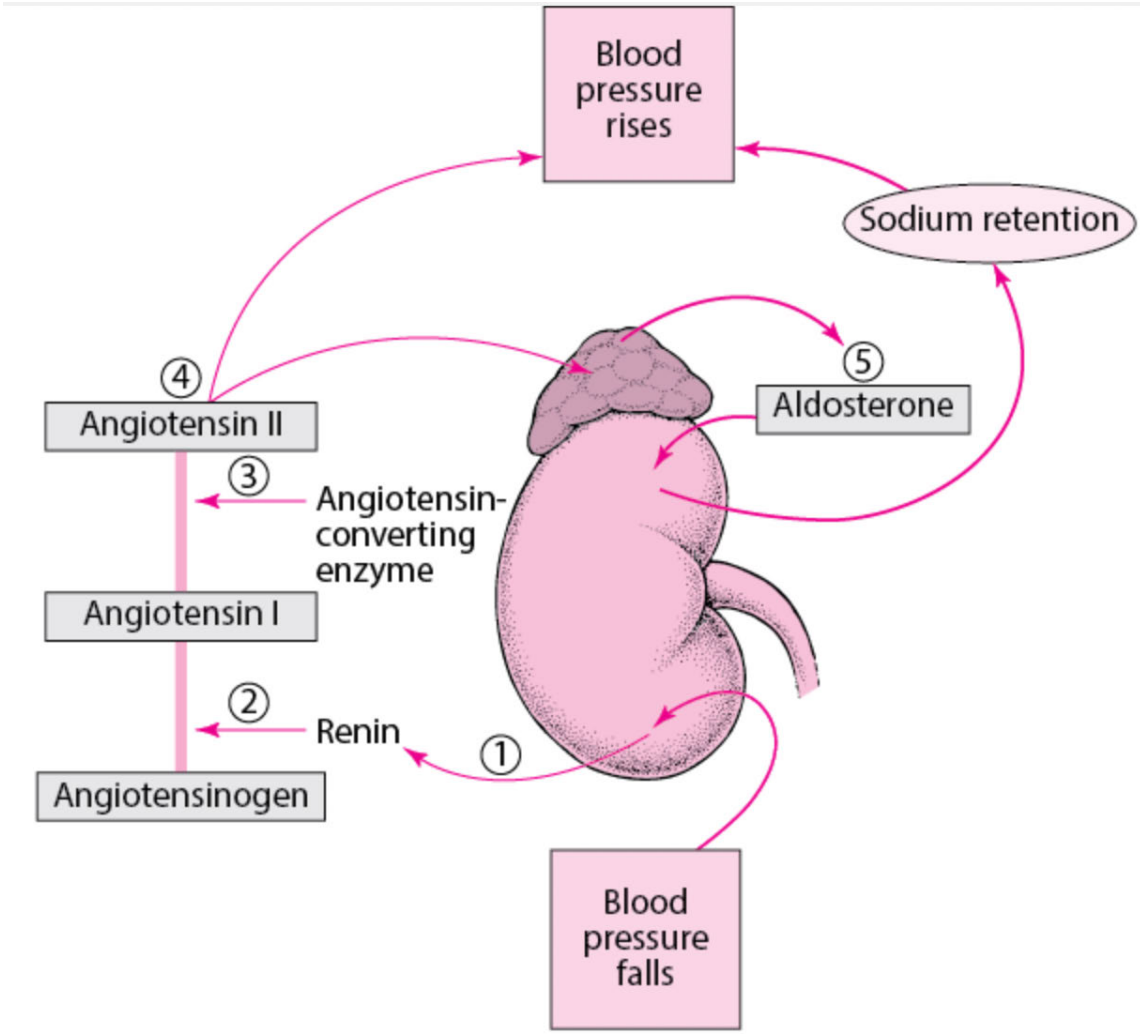

Figure 1: The Renin-Angiotensin-Aldosterone System. This figure was taken from "Regulating Blood Pressure: The Renin-Angiotensin-Aldosterone System", *Merck Manual*, Consumer Version, available at: https://www.merckmanuals.com/home/multimedia/figure/regulating-blood-pressure-the-renin-angiotensin-aldosterone-system. Copyright by 2023 Merck & Co., Inc., Rathway, NJ, USA and its affiliates.[16]

The limiting factor is renin, which is produced by the kidneys when needed.[15] Angiotensin II is a potent vasoconstrictor of systemic arterioles and exerts its effects by increasing resistance in these vessels, which elevates BP. Aside from its vasoconstrictor action, angiotensin II stimulates the zona glomerulosa cells of the adrenal cortex to produce aldosterone.[17] In the distal tubules of the kidneys, Aldosterone increases reabsorption of sodium by increasing its transport through the epithelial sodium channel and sodium-potassium pump. Water follows the movement of sodium and goes down its concentration gradient. The reabsorbed sodium and water then go back to plasma and contribute volume to the extracellular fluid compartment, which in this case leads to an increase in BP as well.[18] Angiotensin II also stimulates the posterior pituitary gland to produce antidiuretic hormone (ADH) also known as vasopressin, which also acts on the distal kidney to minimize excretion, and retain salt and water.[17,18] Because of the importance of maintaining BP within a range that enables important organs like the brain and heart to receive a constant supply of blood, as discussed here the RAAS uses a combination of fluid retention and vasoconstriction to achieve this. However, chronically elevated BP which leads to HTN is also a CV risk factor as outlined by the Framingham heart study.[19] It is also common in patients with T2DM, of whom almost three quarters also have HTN.[20] To lower BP in individuals with HTN, ACE inhibitors are often prescribed, which stops the RAAS at the level of the second cleavage of angiotensinogen, where usually angiotensin I is converted to angiotensin II by ACE. By preventing formation of angiotensin II, both its vasoconstrictor effects and fluid-retention effects mediated by aldosterone and ADH can be hindered.[21]

Returning to the conversation of the effects of hyperglycemia, in addition to facilitating the progression to CVD, it also leads to arterial stiffening and atherosclerotic plaque formation. Hyperglycemia is also directly involved in foam cell development in the vessel wall.[22,23] Atherosclerotic plaques also form from increased oxidative stress in blood vessels which promotes conversion of low-density lipoprotein (LDL) into its oxidized form, and in turn activates platelets and contributes to the recruitment of immune cells such monocytes and neutrophils. These infiltrate the endothelium of blood vessels, which is the cell layer lining the inner wall of the vasculature, and formation of foam cells happens with the gradual accumulation of lipids in macrophages. One molecular mediator of this process is Tumor Necrosis Factor α (TNF-α).[24] Through the Nuclear Factor Kappa B (NF-κB) pathway, the pro-inflammatory cytokine TNF-α causes inflammation in vascular endothelium, and with time, atherosclerosis.[25] Plaque rupture is what ultimately causes thrombosis, which if occurs in coronary arteries results in a myocardial infarction.[26]

T2DM predisposes one to CVD and their interconnection is a meaningful reason to study the vascular health in the context of T2DM.

Insulin in T2DM

Insulin is a hormone secreted by the pancreas, specifically by beta cells in the islets of Langerhans. It is known for its vital metabolic role in internalizing glucose and restoring homeostatic blood glucose levels. Insulin secretion is initiated when blood glucose concentrations exceed 5 mM.[27]

In healthy persons, insulin exerts its effects by binding to cell-surface insulin receptors (IRs) which is a receptor tyrosine kinase.[28] This leads to the phosphorylation of the insulin receptor substrate (IRS). This can then activate the phosphoinositide3-kinase (PI3K)/AK strain transforming (AKT) pathway as well as the mitogen-activated protein kinase (MAPK) pathway. The former pathway mediates cell survival, protein synthesis, and fatty acid and cholesterol synthesis. The latter pathway is involved in cell growth and division, and protein synthesis.[27]

Insulin exerts its effects on adipose by promoting internalization of free fatty acids and their conversion into TGs, their storage form.[29] In skeletal muscle of healthy persons, insulin causes the recruitment of glucose transporter type 4 to the cell membrane, where they interface with blood and are able to internalize glucose.[30] Insulin also exerts its influence on the liver, where it concomitantly stimulates glycogen synthesis and inhibits gluconeogenesis in the postprandial state.[31] (**Figure 2**)

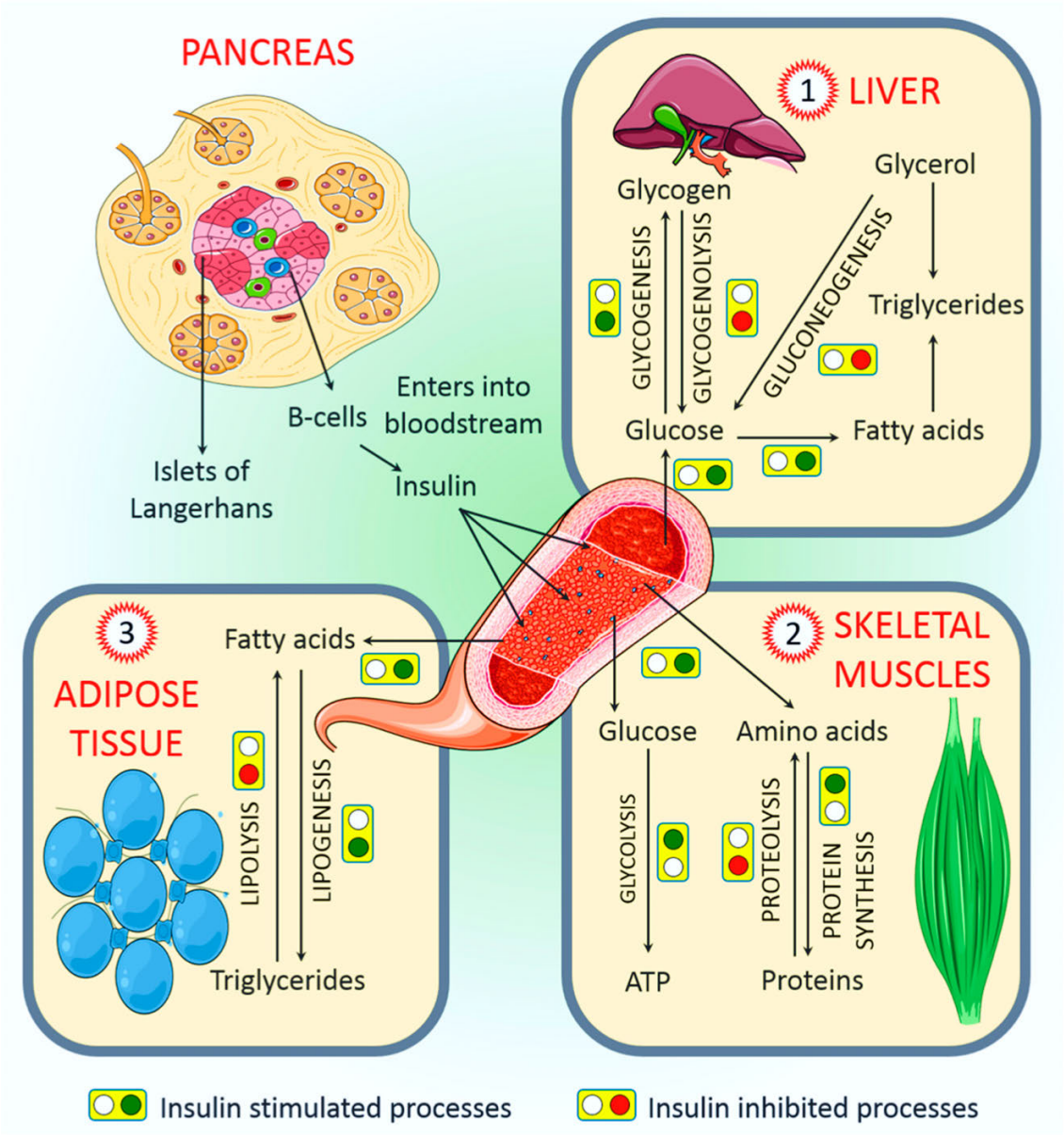

Figure 2: Insulin production and its effects on 1) liver, 2) skeletal muscle, and 3) adipose tissue. This figure was taken from "Role of Insulin in Health and Disease: An Update" by M. S. Rahman et al., 15 June 2021, *International Journal of Molecular Sciences*, 2021, 22, 6403. Copyright by the authors 2021.[27]

In certain instances, such as in type 1 diabetes, there is a lack of production of the hormone insulin due to the autoimmune degradation of the beta cells which are responsible for insulin production. In T2DM, however, although insulin is produced there is resistance to its function at the level of the target tissue.

Insulin resistance is a central aspect of T2DM, and is defined as the dampened response of tissue to insulin which impacts the myriad of functions that are governed by this hormone.[32] Skeletal muscle can become resistant to insulin, and studies have shown that the response to insulin is delayed in addition to its effect being dampened.[33] As a consequence, insulin resistance causes the uptake of glucose into skeletal muscle cells to be impaired, but so are downstream processes such as the immediate phosphorylation of glucose by hexokinase.[34] About 80% of glucose is disposed of from the bloodstream and into skeletal muscle, and with insulin resistant conditions a higher concentration of insulin is required to obtain the same amount of glucose internalization. The decrease in sensitivity to insulin can be explained by the fact that the number of IRs on the surface of skeletal muscle cells is diminished, resulting in a smaller response to the hormone.[33]

In the vasculature, insulin has an important function in nitric oxide (NO)-mediated vasodilation, thus insulin resistance has CV implications. By activating the signaling pathway that involves IRS, PI-3K/AKT, and the phosphorylation of endothelial nitric oxide synthase (eNOS), insulin increases blood flow which can promote the removal of glucose from the blood.[35] (**Figure 3**)

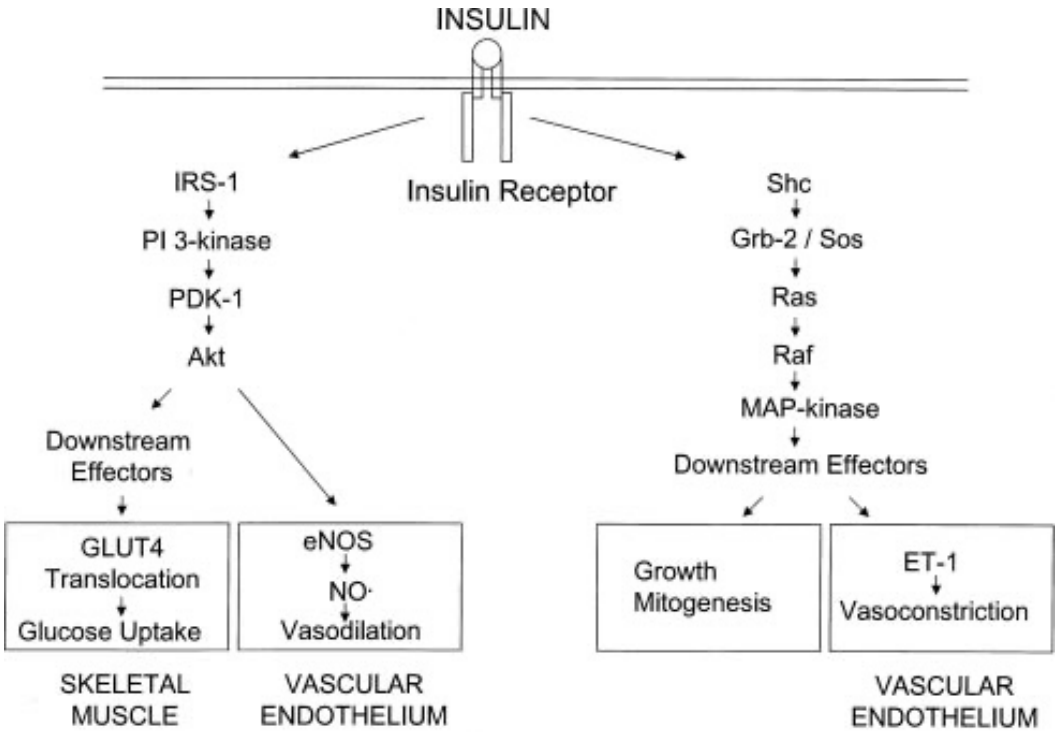

Figure 3: Insulin signaling pathway in skeletal muscle and vascular endothelium. This figure is taken from "Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction" by J. Kim et al, *Circulation*, 2006;113:1888-1904. Copyrights by 2006 American Heart Association.[36]

In skeletal muscle, 25-40% of glucose uptake results from the insulin-stimulated NO-mediated increase in blood flow.[36] With resistance to insulin, this mechanism of vasodilation is impaired and the increase in vascular tone explains why individuals with T2DM are more at risk for HTN.[37] This insulin signaling pathway involving PI-3K/AKT may be activated by insulin receptors substrates 1 and 2 (IRS-1 and IRS-2), but in insulin resistant livers the pathway following IRS-2 is impaired although that following IRS-1 remains active, resulting in concomitant lipogenesis and gluconeogenesis even in interprandial times.[38]

Contrarily to the resistance of some insulin signaling pathways involving PI-3K/AKT, the insulin pathway involving MAPK that leads to glucose transport is not downregulated in instances of insulin resistance.[39] Rather, its persistent activation is part of a vicious cycle that worsens insulin resistance through NF-κB which activates inflammatory cytokines TNF-a, Interleukin 1 beta and Interleukin 6 (IL-6).[40]

This selective resistance to some but not all of the insulin-dependent cascades highlights the intricacy of the pathophysiology of T2DM, especially due to the complexity of the pathways themselves. Studying biomarker levels in serum is thus relevant to elucidate which pathways are differentially modulated in T2DM.

Vascular Endothelial Health in T2DM

As discussed in a previous section, hyperglycemia is involved in the CV pathogenesis of T2DM by facilitating the progression to atherosclerosis. Hyperglycemia also leads to CVD by dysregulating vascular endothelial health.[41]

The endothelium is the innermost vessel layer and has antioxidant, anti-inflammatory, and anti-thrombotic properties, and has an essential role in maintaining vascular tone.[42] Endothelial dysfunction arises from CV risk factors such as aging, hyperglycemia, and HTN, and is a condition where bioavailability of NO is diminished, impairing vasodilation.[43] As mentioned before, this mechanism of NO-mediated vasodilation is insulin-dependent. As such, resistance of vascular endothelial cells specifically to insulin prevents this increase in blood flow.

Reduction in insulin-dependent NO-mediated vasodilation has also been explained by the increased presence of oxygen-derived free radicals in response to fluctuating blood glycemic levels.[41] The mechanism behind this impairment involves Protein Kinase C (PKC) and NFκB and is caused by, but also further promotes oxidative stress.[22] One factor that causes increased activation of PKC is high glucose, and results in a more permeable vascular endothelial layer that lets macromolecules infiltrate more easily in the blood vessel wall, facilitating the progression to atherosclerosis.[44]

To assess whether NO-mediated vasodilation is impaired in patients with T2DM, Tabit et al. obtained vascular endothelial cells from diabetic and nondiabetic controls, and stained them ex vivo with p-eNOS after insulin stimulation.[45] Experimentally, p-eNOS can be used to assess the activation of the signaling pathway that leads to NO production in the endothelium.[46] NO is produced by the NO synthase (NOS) family of proteins, which converts L-arginine into L-citrulline and NO in an oxidation reaction.[47] In cultured endothelial cells, it has been shown that insulin results in phosphorylation of NOS via the PI3K-AKT cascade.[36] Findings by Tabit et al. showed that the basal level of p-eNOS at

serine 1177 was higher in patients with T2DM compared to nondiabetic controls, but stimulation with insulin lead to a significantly smaller increase in phosphorylated eNOS (p-eNOS) levels in patients with T2DM compared to their non diabetic counterparts, which was measured as a ratio of p-eNOS staining detected in insulin-stimulated endothelial cells versus non-stimulated endothelial cells.[45] They also showed that activation of eNOS by insulin correlated with flow mediated dilation suggesting that the endothelial cell abnormality relates to vascular function.

Interestingly, but perhaps unsurprisingly, sheer stress upregulates this pathway of insulin-dependent NO-mediated vasodilation.[48] Shear stress is defined as the force with which blood flowing in a vessel places on the endothelial surface of the wall.[49] (**Figure 4**)

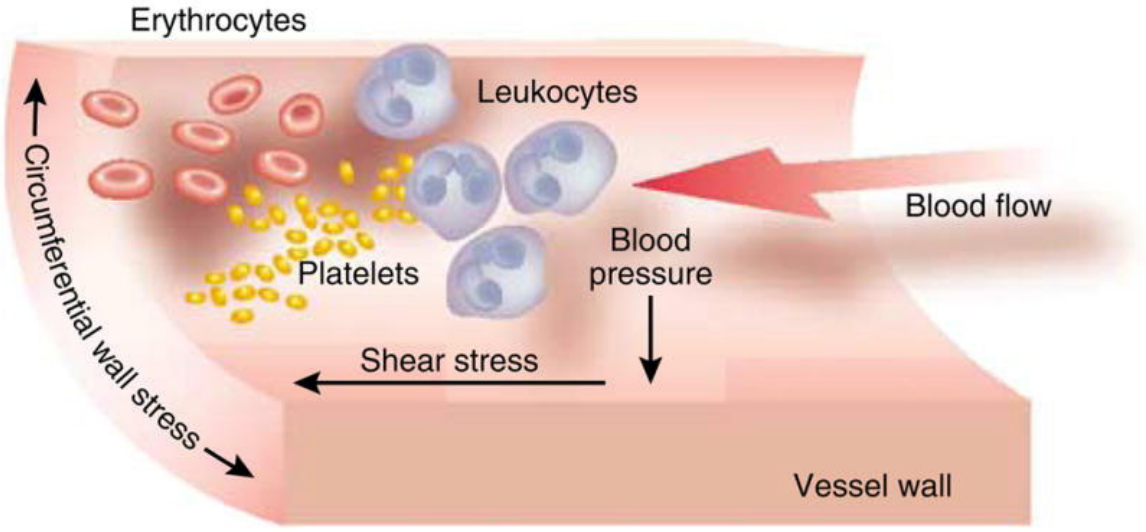

Figure 4: Shear stress caused by blood flow to the vascular endothelial layer of the vessel wall. This figure was taken from "Neointimal Hyperplasia Associated with Synthetic Hemodialysis Graft" by L. Li et al., 2008, Kidney International 2008 Nov;74(10):1247-61. Copyright by 2008 International Society of Nephrology[50]

HTN is an example where shear stress on the endothelial layer is elevated.[51] Because patients with T2DM have a higher incidence of HTN, it can explain why patients with T2DM were found to have higher basal levels of p-eNOS.[12] Shear stress is also significant in atherosclerosis, which once again emphasizes the connection of T2DM and CVD.[49] It has also been found to inhibit the apoptosis of endothelial cells that line the inner vessel wall. Dimmeler et al. demonstrated that this inhibition is mediated by the phosphorylation of AKT, which as mentioned before is part of the cascade that results in vasodilation.[48]

The consequence of impaired vasodilation is increased arterial stiffness and vascular tone, as well as vascular remodeling among other issues of which cumulative effect manifests clinically as HTN. In addition to vasodilation, NO has anti-inflammatory and antioxidant roles, so impairments in its function has widespread consequences.[52] Endothelial dysfunction can also lead to formation of atherosclerotic plaques, which as mentioned before can lead to CVD.[24]

In the studies mentioned above and more generally in clinical research, flow-mediated dilation (FMD) has been used to assess endothelial dysfunction in humans. Although it is not used for diagnostic purposes in hospital settings, FMD results are reflective of endothelial dysfunction, which can progress to atherogenesis and is therefore closely related to CVD.[53] FMD is measured as percent changes in diameter of the brachial artery in response to cuff-induced reactive hyperemia. A low percent change is indicative of endothelial dysfunction. This technique uses ultrasound to image the brachial artery (**Figure 5**).[53,54]

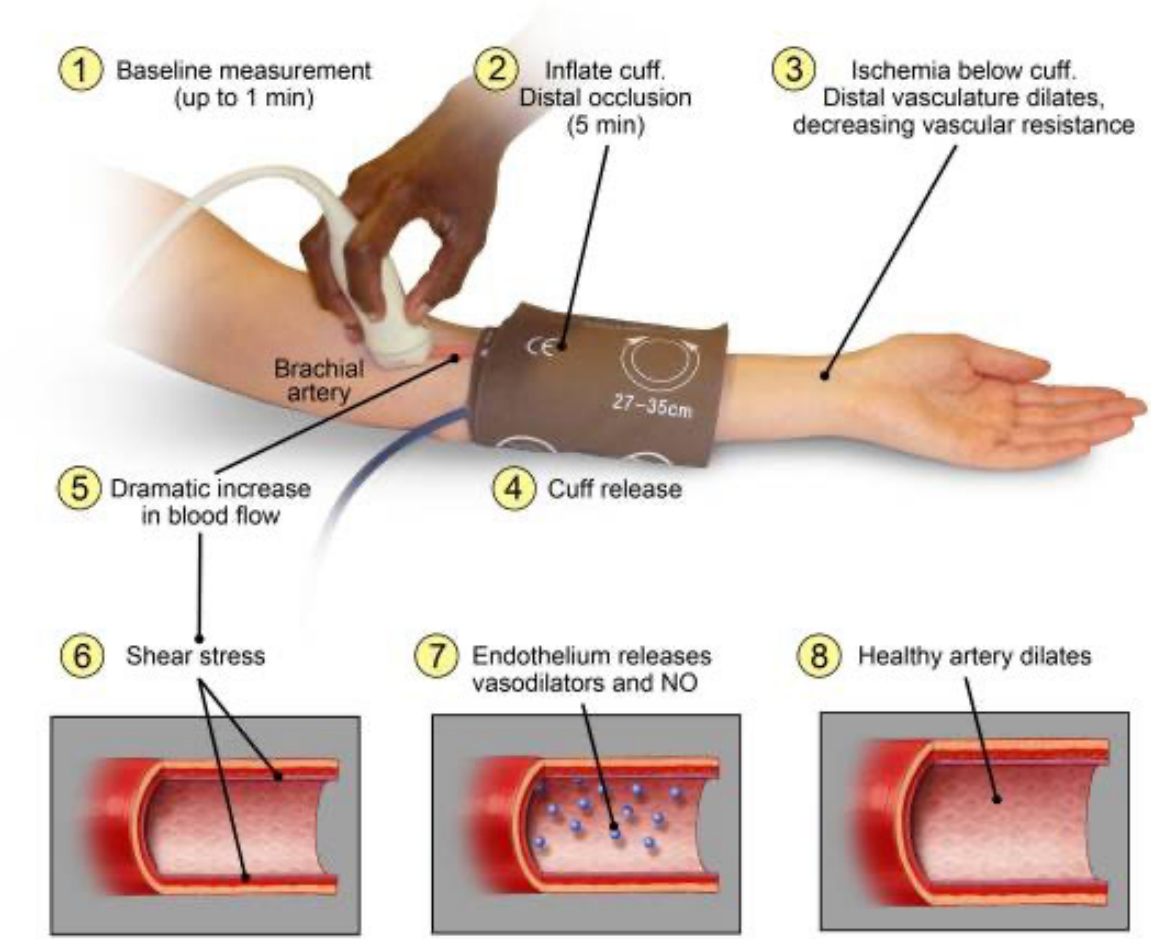

Figure 5: Characteristics of the flow-mediated dilation protocol. Note: the cuff can either be placed on the forearm or on the arm. This figure was taken from "Flow-Mediated Dilation: Can New Approaches Provide Greater Mechanistic Insight into Vascular Dysfunction in Preeclampsia and Other Diseases?" by T. L. Weissgerber, 2014, Current Hypertension Reports 16:487. Copyright by Springer Science+Business Media New York 2014.[54]

Another study, conducted by Bretón-Romero et al., also used FMD to assess endothelial dysfunction in patients with T2DM, this time to determine the levels of Wnt5a and whether there is an association between endothelial dysfunction and Wnt family member 5A (Wnt5a)-c-jun N-terminal kinase (JNK) signaling.[46] Although this signaling pathway is complex due to the multiple receptors that Wnt5a can bind to, more recent studies have shown that it is involved in inflammation such as in atherosclerosis, where Wnt5a is upregulated.[55] Vascular endothelial cells were obtained from patients with T2DM and nondiabetic controls, and both Wnt5a expression as well as JNK activation (but not expression) were significantly higher in patients with T2DM, indicating the higher presence of inflammation in this cohort. This study showed endothelial dysfunction in patients with T2DM by a significantly lower FMD of the brachial artery. Furthermore, the driver of endothelial dysfunction via the Wnt5a-JNK pathway was determined to be insulin resistance by showing that inhibition of this signaling mechanism restored NO production in endothelial cells from patients with T2DM.[46] As such, there are multiple pathways that lead to endothelial dysfunction in insulin-resistant vascular endothelial cells, and as discussed above, result in impairments in insulin-dependent production of NO.

Paralleling the clinical manifestations of T2DM are cellular changes, in particular due to epigenetic mechanisms or changes in protein expression but are not yet fully known. As such, a deeper understanding of how the molecular background of cells correlates with vascular function in T2DM can shed light on the pathways that are involved in connecting T2DM with CVD and provide novel insight on vascular endothelial health. This would provide valuable knowledge to find treatments that alleviate heart disease and poor

vascular health in people with T2DM with the hopes of decreasing mortality due to CV events in this population, which is double compared to that in aged-matched persons.[56]

Proteomics – an Emerging Technique

Proteomics is the study of the entire set of proteins within a cell or organism to better understand the physiological conditions in which these proteins operate.[57,58] It is a broad term that involves the study of protein location, protein-protein interactions, and their quantification with the goal of, for example, determining the pathways that may be upregulated or downregulated within a certain context.[59] According to the central dogma DNA is transcribed into mRNA, which is translated into proteins.[60] As such, proteomics can be a meaningful way to understand more about the gene encoding these proteins, and whether the path from DNA to protein may be differentially regulated in disease states.

It is an increasingly important technique due to its sensitivity and specificity, as well as high throughput, and is particularly relevant in diseases like T2DM where multiple genes, and thus many proteins and signaling pathways, may be involved in its pathogenesis.[61]

Traditionally, proteomics experiments use gel-electrophoresis followed by mass spectrometry to separate and then quantify proteins, enabling scientists to assess post-translational modifications on those proteins as well.[57]

In this study our proteomic analysis uses O-link, and the protocol carried out this company is based on the Proximity Extension Assay (PEA) technology, which has three key steps: an immunoassay, extension, and detection. It is a novel technique where the

immuno reaction involves the binding of the oligonucleotide labeled antibody probe pairs with the protein of interest. The proximal oligonucleotides bind together, creating a DNA reporter sequence that can be amplified and detected using real-time polymerase chain reaction.[62] (**Figure 6**)

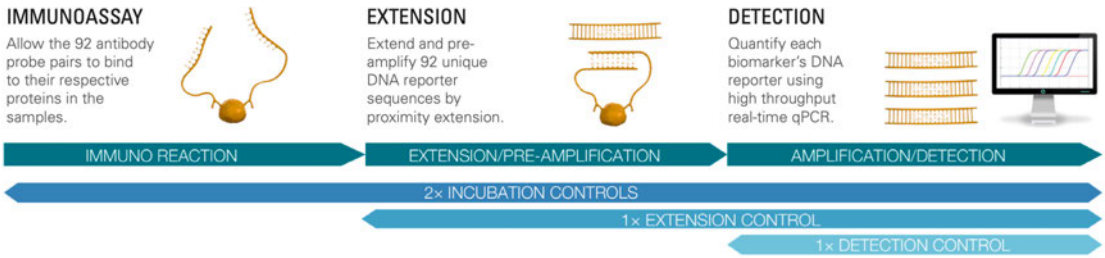

Figure 6: O-Link proteomics protocol. This figure is taken from "Olink Target 96 Cardiovascular Panels", available at https://olink.com/products-services/target/cardiometabolic-panel/. Copyright by Olink 2023.[63]

SPECIFIC AIMS

Hypothesis: It is well known that T2DM has differentially modulated molecular signaling pathways due to the disturbed metabolic milieu as well as elevated oxidative stress and inflammation. We hypothesize that our proteomic panel will reiterate these findings and will expose further differences in biomarkers levels between patients with T2DM and nondiabetic persons. We also hypothesize that our biomarker findings will be related to changes in endothelial cell phenotype.

Aim1: Compare the cardiometabolic proteomic background of persons with T2DM with that of nondiabetic controls to determine which pathways may be differently modulated in the disease state. We will also assess measures of central hemodynamics and arterial stiffness in the same sample population, as well as clinical characteristics that may be significantly altered in T2DM.

Aim 2: Determine the association of circulating protein biomarkers with endothelial cell phenotype and systemic vascular function.

METHODS

Study Population and Eligibility

We recruited nondiabetic self-reported healthy controls and patients with T2DM between the ages of 30 and 80. Persons with the following were considered ineligible to participate: those with uncontrolled HTN, clinically unstable patients that would preclude withholding medication, patients with unstable coronary artery disease or decompensated heart failure within one month of enrollment, pregnant persons, and anyone with a clinically evident major illness including active cancer, end stage renal disease, hepatic failure, and history of pancreatitis. We also deemed ineligible those who were taking the following anticoagulants: Coumadin (Warfarin), Eliquis (Apixaban), or Xarelto (Rivaroxaban). We delayed participation of participants who were hospitalized in the past month, those who got a vaccine in the past two weeks, and those who had COVID-19 in the past 6 weeks. All participants provided written informed consent and the study was approved by the Boston University Chobanian and Avedisian School of Medicine institutional review boards. Participants were required to fast from food and drinks (except water) from 8 hours before the start of the study visit, abstain from tobacco use from 6 hours before, and asked to withhold BP medications the morning of the study.

Vascular Function Measurements

Participants remained in a supine position for all vascular measurements. BP and heart rate (HR) were measured with the Omron automatic BP monitor (model HEM-780N2). FMD was carried out with a Toshiba Xario XG Ultrasound. A BP cuff was inflated for 5 minutes at 50 mmHg above the participant's systolic blood pressure (SBP) to induce

a hyperemic response upon deflation. Baseline diameters and one-minute post cuff release diameters of the brachial artery were measured to calculate a % increase in diameter.

Endothelial Cell Collection and Isolation, Insulin Stimulation, and p-eNOS Activation

Vascular endothelial cells were collected from a forearm vein using 4 j-shaped spring-wires, which were then washed with a dissociation buffer. After isolating vascular endothelial cells and plating them on poly-L lysine coated slides, they were equilibrated in a 5% CO_2 incubator, and stimulated with a 10 nM Insulin PRF EBM solution for 20 minutes and fixed with 4% paraformaldehyde. Slides were stored at -80˚C and stained on a separate day.

Cells that were previously plated on slides were permeabilized with 0.1% Triton X, blocked in a 0.5% BSA solution, and incubated overnight at 4˚C with the recombinant Anti-eNOS (phosphor S1177) antibody by abcam (ab184154) prepared in a 0.5% BSA/50mM Glycine PBS solution at a 1:100 dilution. Cells were cross-stained for von-Willebrand factor, a well-established endothelial marker, ensuring that we were observing endothelial cells. A 1:300 dilution of the Von Willebrand Factor antibody (Dako Omnis) from Agilent was used. Secondary antibodies from Invitrogen targeting rabbit and mouse at wavelengths 594 and 498 were used to detect p-eNOS and von-Willebrand factor respectively. Slides were then incubated at 37˚C for 45 minutes, followed by extensive wash to remove unbound second antibodies. After addition of Vectashield mounting

medium containing Dapi to each well on the slide, samples were subject to fluorescence microscopy.

O-link Proteomics

We collected blood from participants which was centrifuged to obtain serum. 100 μL of serum from each participant were sent to O-link to carry out panels Cardiovascular II and Cardiovascular III which assessed a total of 183 CVD-related human protein biomarkers (**Appendix 1**).

O-link reported Normalized Protein eXpression (NPX) values whereby a higher value indicates higher protein expression. O-link calculated NPX values by normalizing Ct values on a Log2 scale so that a one-point increase in NPX indicates a twofold increase in protein expression.[64] Values that were below the detection limit or that didn't pass the QC threshold determined by O-link were excluded from analysis. The detection limit was defined as three standard deviations (SD) above the background, representing virtually no protein. [62]

Statistical Analysis

To find statistically significant differences in protein expression between patients with T2DM and nondiabetic controls for proteins in the two panels that were assessed, the O-link Insights Stat Analysis—a web-based application offered by the company O-link—was used. The application used a two-sided Welch's t-test in all instances, and an adjusted $p<0.05$ accounting for multiple testing was considered statistically significant.

For all vascular data, averages, and SDs were calculated, followed by two-sided t-tests that assume unequal variances. Consistently, two-sided $p<0.05$ was considered statistically significant in determining differences between patients with T2DM and nondiabetic controls. This analysis used the statistical data analysis package on Excel. Results are described as mean±SD whereas error bars on graphs represent the standard error of the mean.

Chi-squared testing was used for categorical data to compare demographics and medical history across the two groups. For TG levels, the natural logarithm was taken to account for skewed data as revealed by the normality tests Kolmogorov-Smirnov and Shapiro-Wilk. These analyses were done using the SPSS 29.0 software.

RESULTS

Clinical Characteristics

Our study sample size was 69 participants; 37 with T2DM and 32 nondiabetic controls with similar age and sex for whom proteomics data was acquired. There was no statistically significant difference in age between the two groups, but body mass index (BMI) was significantly higher in the diabetic group (p=0.000009). (**Table 1**).

Of the nondiabetic group 69% identified as white, 31% as black/African American, and 0% as Other (p=0.353). In the same group, 6% identified as Hispanic (p=0.204). Of the diabetic group, 49% identified as white, 43% as black/African American, and 5% as Other (p=0.353). In the same group, 12% identified as Hispanic (p=0.204).

Table 1. Clinical characteristics

	Nondiabetic controls (N=32)	**T2DM (N=37)**	**P value**
Age (years)	53±9	57±8	0.05
Female sex, N (%)	12 (38)	15 (41)	0.796
BMI, kg/m2	26.7±4.9	34.0±7.5	0.000009
Race			0.353
White, N (%)	22	18	
Black/African American, N (%)	10	16	
Other, N (%)	0	2	
Ethnicity			
Hispanic, N (%)	2 (6)	4 (12)	0.204
Values are expressed as mean±SD BMI= body mass index			

Metabolic Dysfunction

Patients with T2DM had significantly higher fasting glucose levels (p=0.000009), glycated hemoglobin (Hb A1C) (p=1E-10), and insulin (p=0.01), and significantly lower total cholesterol (p=0.0002) as well as significantly lower HDL and LDL (p=0.0003 and p=0.00008 respectively). The natural logarithm of TG levels was significantly higher in patients with T2DM (p=0.04). (**Table 2**)

Table 2: Fasting plasma glucose levels, lipid panel, Hb A1C, and insulin.

	Nondiabetic controls (N=32)	**T2DM (N=37)**	**P value**
Glucose (mg/dL)	85.3±9.9	150.3±73.4	0.000009
Total Cholesterol (mg/dL)	207.8±39.3	167.6±42.8	0.0002
LDL (mg/dL)	127.3±29.4	93.6±35.5	0.00008
HDL (mg/dL)	58.2±17.0	43.8±13.2	0.0003
***ln(TG) (mg/dL)**	4.57±0.6	5.06±0.8	0.04
Hb A1C (%)	5.4±0.4	7.6±1.4	1E-10
Insulin (uU/mL)	6±5.5	15.4±19.5	0.01
*Natural logarithms of triclyceride levels were taken to account for skewed data revealed by normality tests Kolmogorov-Smirnov and Shapiro-Wilk (p<0.001 in both cases).			

Medical History and Medication

Of nondiabetic controls, 16% had HTN, 41% were ever smokers, 0% had a history of CAD or PAD, and 16% had hypercholesterolemia. Of patients with T2DM, 73% had HTN, 59% were smokers at some point in their life, 11% had a history of CAD, 3% had a history of peripheral arterial disease (PAD), and 78% had hypercholesterolemia. (**Table 3**)

Table 3: Self-reported medical history.

	Nondiabetic controls (N=32)	T2DM (N=37)	P value
Hypertension, N (%)	5 (16)	27 (73)	<0.001
Hypercholesterolemia, N (%)	5 (16)	29 (78)	<0.001
Ever smoker, N (%)	13 (41)	22 (59)	0.119
History of CAD, N (%)	0 (0)	4 (11)	0.055
History of PAD, N (%)	0 (0)	1 (3)	0.328
The above criteria were self-reported by participants			

There were no individuals in the nondiabetic control group who were taking medication for diabetes at the time of the study. In the diabetic group, the most common antidiabetic medication was metformin (62%), followed by sulfonylurea (22%), glucagon like peptide-1 (GLP-1) receptor agonist (16%), and dipeptidyl peptidase-4 inhibitors (11%), with the percentages representing the proportion of patients with T2DM who are taking that medication.

A higher number of patients with T2DM reported taking ACE inhibitors and/or angiotensin receptor blockers (ARB) (51% versus 9%), as well as lipid lowering medications such as statins (59% versus 13%). However, there were still 9% and 13% of nondiabetic participants who reported having a prescription for ACE inhibitors and ARBs, and lipid lowering drugs respectively. (**Table 4**)

Table 4: Self-reported medication

	Non-diabetic controls (N=32)	T2DM (N=37)
Metformin, N (%)	0 (0)	23 (62)
Sulfonylurea, N (%)	0 (0)	8 (22)
Glucagon like peptide-1 receptor agonist, N (%)	0 (0)	6 (16)
Dipeptidyl peptidase-4 inhibitors, N (%)	0 (0)	4 (11)
ACE inhibitor or ARBs, N (%)	3 (9)	19 (51)
Lipid lowering medication, N (%)	4 (13)	22 (59)
The above criteria were self-reported by participants		

Baseline Vascular Measures

There were no significant differences in age, SBP, DBP, and mean arterial pressure (MAP) between patients with T2DM and nondiabetic controls (p=0.3, p=0.4, p=1.0 respectively). Pulse pressure and HR were significantly elevated in the diabetic group (p=0.02 and p=0.0003 respectively) (**Table 5**).

Table 5. Baseline vascular measures

	Nondiabetic controls (N=32)	T2DM (N=37)	P value
Heart rate, bpm	64±10	74±12	0.0003
SBP, mmHg	128±19	133±16	0.3
DBP, mmHg	80±13	78±10	0.4
Pulse Pressure, mmHg	48±11	56±12	0.02
Mean Arterial Pressure, mmHg	96±10	97±12	1.0
Values are expressed as mean±SD SBP=systolic blood pressure, DBP=diastolic blood pressure, bpm=beats per minute			

Endothelial Dysfunction

The average percent dilation of the brachial artery was 9.1±4.4 % for nondiabetics and 6.04±3.41 % for patients with T2DM with a statistically significant difference observed between the two groups (p=0.01). (**Figure 7**)

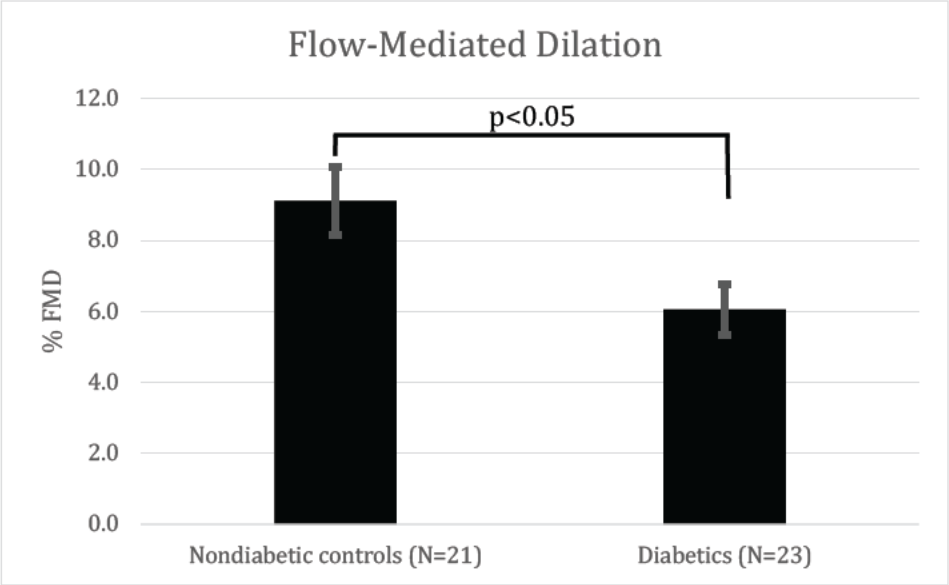

Figure 7: Flow-mediated dilation of the brachial artery. The dilation of the brachial artery in response to hyperemia by cuff inflation and deflation was significantly lower in patients with T2DM then nondiabetic controls (p=0.01).

Our findings showed that 24 biomarkers were upregulated and 2 were downregulated in T2DM participants compared to nondiabetic controls. The upregulated proteins included those involved in metabolism, vascular effects and fluid homeostasis, immune function, apoptosis, and other functions. The two downregulated proteins are involved in metabolism (**Table 6, Figure 8**).

Table 6: List of biomarkers of which levels were statistically significantly altered.

Metabolic Proteins	**Vascular effects & fluid homeostasis**
Growth/differentiation factor 15	Interleukin-6
Leptin	Pro-adrenomedullin
Galectin-3	Renin
Galectin-4	Protein AMBP (redox)
Galectin-9	**Immune function**
Fatty acid-binding protein, adipocyte	Hepatitis A virus cellular receptor 1
Fibroblast growth factor 21	Interleukin-1 receptor antagonist protein
Cathepsin D	C-X-C motif chemokine 16
Retinoic acid receptor responder protein 2	Tumor necrosis factor receptor superfamily member 1B
Serum paraoxonase/lactonase 3*	Tumor necrosis factor receptor superfamily member 11A
Chymotrypsin-C*	**Apoptosis**
Other	Tumor necrosis factor receptor superfamily member 1A
Matrilysin	Tumor necrosis factor receptor superfamily member 10A
Trefoil factor 3	Tumor necrosis factor receptor superfamily member 10B
V-set and immunoglobulin domain-containing protein 2	
*Proteins indicated with * were significantly downregulated, and all other were upregulated in patients with T2DM compared to nondiabetic controls.	

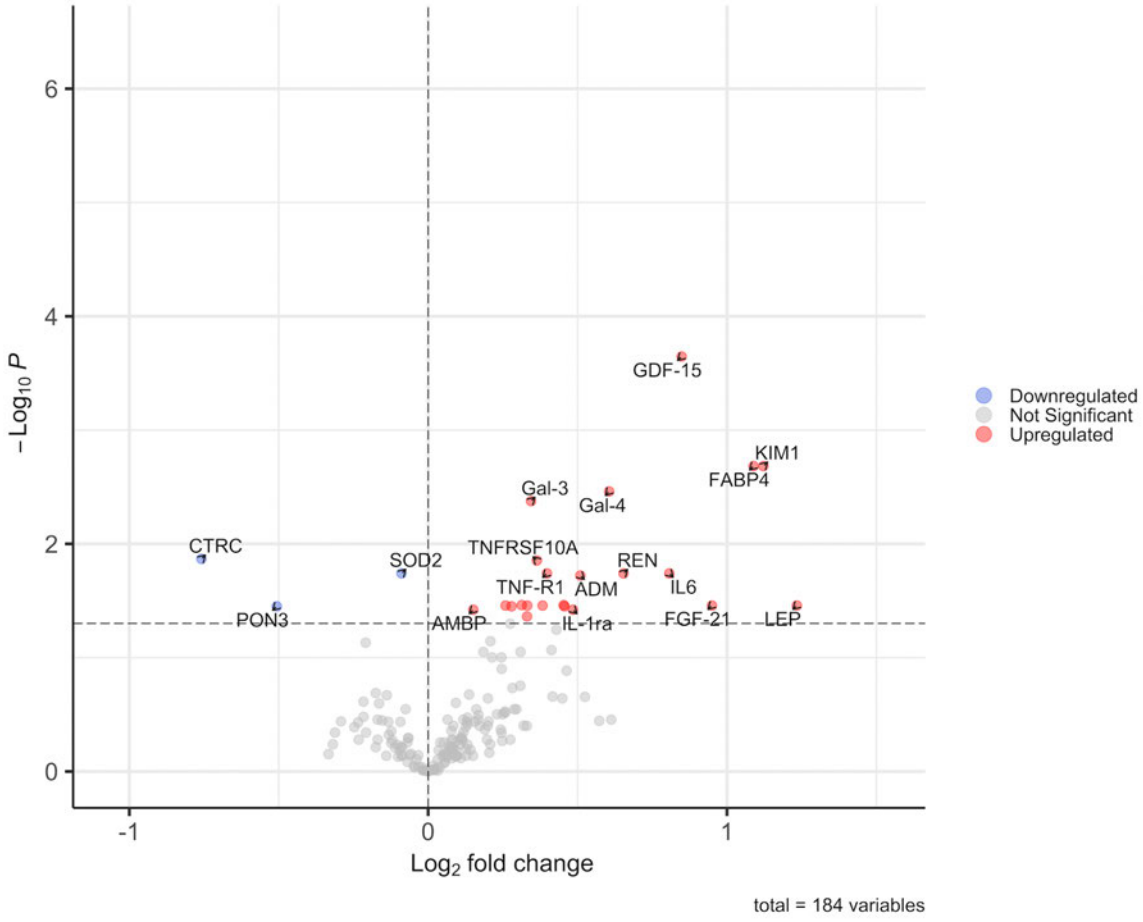

Figure 8: Volcano Plot of significantly altered levels of serum biomarkers in patients with T2DM versus nondiabetic controls. Gene names are labeled for significantly differentially expressed biomarkers. The Y-axis is expressed as a logarithm of the p value (0.05 was considered significant).

Insulin-Stimulated p-eNOS Levels

Out of the 69 participants, vascular endothelial cells from a subpopulation of 50 participants was selected to determine the correlation between insulin stimulated p-eNOS staining, biomarkers. Endothelial NO phosphorylation in DM participants was significantly lower. The mean fold change in insulin-stimulated p-ENOS was 0.34±0.07 for nondiabetic controls, and -0.14±0.03 in patients with T2DM ($p<0.01$) (**Figure 9**).

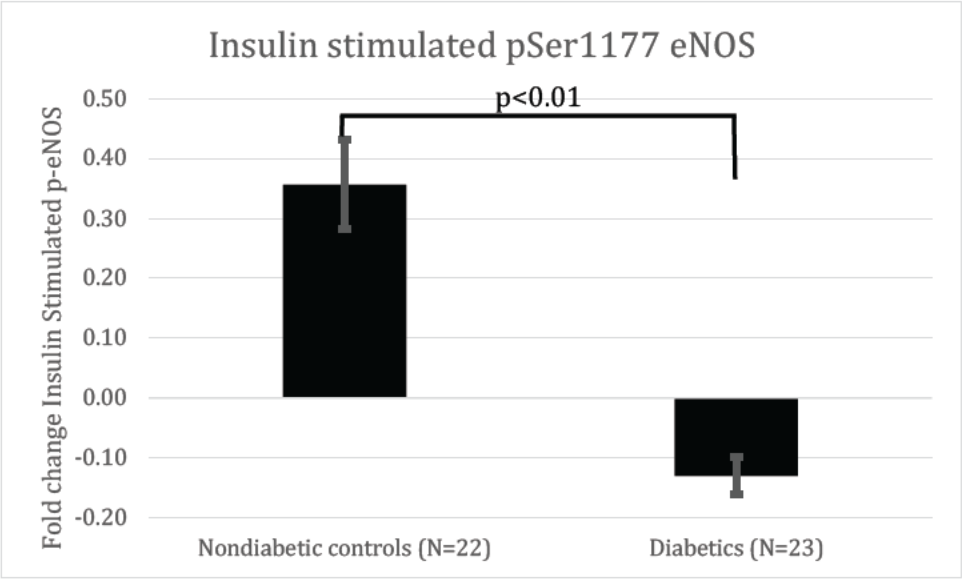

Figure 9: Insulin stimulated activation of p-eNOS. Insulin-stimulated p-eNOS levels measured by p-eNOS staining of human vascular endothelial cells showed significantly lower fold change in patients with T2DM compared to nondiabetic controls.

Correlations Between p-eNOS Levels and Biomarker Levels

NPX values of Renin and Adrenomedullin were significantly associated with lower insulin stimulated p-eNOS levels (r= -0.38, p=0.004 and r=-0.27, p=0.049 respectively). Chymotrypsin C (r=0.37, p=0.006), Paraoxonase 3 (r=0.35, p=0.009), Lipoprotein Lipase (r=0.34, p=0.01), and Superoxide Dismutase 2 (r=0.31, p=0.02) were significantly associated with higher insulin stimulated p-eNOS activation. (**Table 7**).

Table 7: Significant correlations between biomarkers and p-eNOS

BIOMARKER	R WITH p-eNOS	P.VAL
Renin	-0.38	0.004
Chymotrypsin C	0.37	0.006
Paraoxonase 3	0.35	0.009
Lipoprotein Lipase	0.34	0.01
Superoxide Dismutase 2	0.31	0.02
Adrenomedullin	-0.27	0.049

DISCUSSION

Biomarkers and Endothelial Cell Phenotype

Our study was designed to investigate the proteomic biomarkers that are altered in patients with T2DM and how they relate to endothelial cell biology. As expected from our prior work we found differences in vascular function, including HR, pulse pressure, and FMD as discussed in the following paragraphs. In this larger group we also demonstrated differences in the ability of insulin to activate the endothelial cells, as we have shown before. We now describe that there are multiple differences in proteomic biomarkers involving selective pathways that are altered. Some pathways are upregulated such as those involved in oxidative stress, inflammation, and apoptosis, whereas some pathways are downregulated, in directions we might expect.

Importantly, we have early evidence that there are associations between biomarkers and endothelial cell phenotype, showing a link between the altered pathways and abnormal endothelial cell phenotype in T2DM. Taken together, our findings suggest there are novel pathways that are important to investigate for the cell biology of endothelial cell function.

Of the biomarkers that we found to be significantly differentially expressed between patients with T2DM versus nondiabetic controls, eleven are involved in metabolism including Leptin, Growth Differentiation Factor 15 (GDF15), Paraoxonase 3, four are involved in vascular effects and fluid homeostasis including Renin, Adrenomedullin, Protein AMBP (Alpha-1-Microglobulin/Bikunin Precursor), and IL-6, five are involved in immune function including Interleukin-1 Receptor Antagonist Protein (IL-1RN) and Tumor Necrosis Factor Receptor Superfamily Members 1B and 11A

(TNFRSF1B and TNFRSF11A), three are involved in apoptosis, and three are involved in other functions.

Our data also showed significantly lower p-eNOS levels in vascular endothelial cells in patients with T2DM, from which we can deduce that insulin-dependent NO-mediated vasodilation is also impaired in this group. This finding is consistent with what is reported in the literature, and explains the reduced disposal of glucose from the bloodstream, which is a key feature of T2DM.[45,65] Combined with our observation that brachial artery dilation is significantly impaired in patients with T2DM, our findings show impaired endothelial cell activation in this group.

Surprisingly basal levels of p-eNOS upon insulin stimulation trended higher in patients with T2DM and this is once again consistent with previous studies.[45] This can be explained by the fact that sheer stress upregulates this pathway of insulin-dependent NO-mediated vasodilation, resulting in higher basal levels although there is impairment in the response to insulin in patients with T2DM, and pulse pressure was also higher in this group which was also found to correlate with shear stress.[48,66]

Upon analyzing the fold change in p-eNOS levels with the serum biomarkers, we found a negative correlation of Renin and Adrenomedullin with insulin stimulated p-eNOS and may explain the higher risk of HTN in patients with T2DM. Renin, as mentioned before, controls systemic arteriolar tone as well as blood volume and therefore modulates BP by influencing sodium and water retention in the kidney.[15,17,67] Adrenomedullin instead has a role in lowering BP and its dysregulation has been found as a feature of T2DM.[68] Our findings showed higher insulin-stimulated NO activation when Adrenomedullin was low,

and vice versa. Renin, which has a role in fluid homeostasis, is involved in the progression to HTN as it promotes an increase in BP and fluid retention, especially through the RAAS.[17]

Analyzing the biomarker levels with p-eNOS levels, we found that Chymotrypsin C, Paraoxonase 3, Lipoprotein Lipase, and Superoxide Dismutase 2 had a positive correlation with insulin stimulated p-eNOS activation. Two of these biomarkers are involved in metabolism, Chymotrypsin C and Lipoprotein Lipase, highlighting the altered metabolic milieu. Also, Lipoprotein Lipase is an enzyme involved in the breakdown of TGs and uptake of the resulting free fatty acids from blood.[69] TG levels in patients with T2DM trended higher than in nondiabetic controls, which may explain the significantly higher levels of this biomarker. Interestingly, although Lipoprotein Lipase is initially anchored to cell membranes of cells such as vascular endothelial cells, with intravenous heparin infusion, this enzyme can detach from the cell membrane and be consequently found in circulation.[69] Perhaps it is because it can detach from endothelial cells that it was possible for us to detect it through the O-link panel.

Furthermore, as the following paragraphs outline, our data confirms that people with T2DM have abnormal vascular health measures and evidence of multiple metabolic changes that may be drivers of proteomic and endothelial cell phenotype changes. These are unsurprising findings but were nevertheless a necessary step to be able to interpret our serum biomarker findings with metabolic and vascular changes, which are connected at the level of vascular endothelial health.

The diabetic group had an average fasting glucose of 150.3±73.4 mg/dL, which is above the level needed to diagnose T2DM (>126 mg/dL), as well as an average Hb A1C value of 7.6±1.4 %, which is within the diabetic range.[70] The nondiabetic group had an average fasting glucose and Hb A1C that would be considered normal (85.3±9.9 mg/dL and 5.4±0.4 % respectively). This confirms that the two groups can be compared in terms of their diabetic or nondiabetic status. On the other hand, age was not significantly different. Having age-matched groups is relevant as age is an independent risk factor for CVD and T2DM.[71]

The significantly higher levels of fasting glucose and Hb A1C in patients with T2DM is indicative of insulin resistance in these persons, which was further confirmed by their significantly higher insulin levels.

The lipid panel showed that patients with T2DM had significantly higher TG and significantly lower HDL, which aligns with the characteristics of MetS in this group.[72] Patients with T2DM also had significantly lower LDL and total cholesterol, which is the opposite of what we would expect from a group that is more likely to have MetS and its associated CV complications. However, both these findings can be explained by their use of lipid lowering medications, which was reported as a prescribed drug in 59% of patients with T2DM in concordance with the diagnosis for hypercholesterolemia in 78% of patients with T2DM. Statins are known to be able to reduce LDL levels by 25-55% and reduce TG levels as well, thus lowering the risk of CVD.[73]

Furthermore, the diagnosis of HTN was reported 4.5 times more frequently in patients with T2DM who also had 5.7 times higher use of antihypertensive agents such as

ACE inhibitors and ARBs. This data, combined with the significantly higher BMI and insulin resistance in patients with T2DM, demonstrates metabolic dysfunction in this group despite its attenuation with a combination of antidiabetic, antihypertensive, and lipid lowering drugs.[5,74]

Prior Findings of Differentially Regulated Proteins in Obesity and T2DM

A study by Alfadda et al. assessed the proteomics of obese patients in mature adipocytes. They found elevated levels of biomarkers involved in apoptosis and inflammation in older compared to younger patients, which as they explain are both involved in obesity and aging.[75] A study by Benabdelkamel et al. also studied protein levels in mature adipocytes but compared lean, overweight, and morbidly obese individuals. They found significantly different levels in proteins involved in metabolism, energy regulation, and reduction-oxidation pathways.[76]

Other studies have instead looked at proteins in circulation. One study compared protein levels before and after treatment with Liraglutide, which is a GLP-1 receptor agonist. They observed improvement in biomarkers associated with inflammation and oxidative stress.[77] Another found Leptin, Renin, and IL-1RN to be associated with insulin resistance, and the latter was also associated with T2DM.[61]

There have also been researchers who took advantage of the O-link technology to assess proteomics in T2DM, using panels cardiovascular II and III which are the same ones used in our study. The HOMAGE (Heart OMics in AGEing) trial found significantly differentially present proteins in patients with diabetes compared to those without. Many are the same proteins that we found differentially expressed, including Cathepsin D,

GDF15, and Galectin 4, which were positively associated with T2DM, and Lipoprotein Lipase, and C-X-C Motif Chemokine 10, which were negatively associated.[78]

So far, studies of protein levels in T2DM have provided meaningful insight on the proteome changes that occur in obesity and in T2DM. Our study extends the prior work by comparing comprehensive biomarkers to the endothelial cell phenotype. Furthermore, because we study biomarkers in serum, we are able to make inferences on vascular endothelial health, and connect these findings with vascular health and endothelial cell phenotype.

Interpretation of Our Proteomic Analysis

Levels of the metabolic biomarker Leptin were significantly higher in patients with T2DM (adjusted p=0.02) and correlated with increased adiposity, which was confirmed by a significantly greater BMI in the diabetic group (p=0.00002). Leptin, encoded by the obese *(ob)* gene is a hormone that is released by and thus directly correlates with the amount of adipose in the body. Its signaling mechanism relayed to the brain serves to detect satiety, but has a role in the immune system as well.[79]

GDF15 levels were also significantly higher in patients with T2DM. Increased levels of this biomarker are indicative of tissue hypoxia, inflammation, and oxidative stress, all of which are features of T2DM.[80] Concomitantly to the elevated levels of GDF15, we observed significantly higher levels of five biomarkers involved in immune function. For example we found significantly higher serum levels of IL-1RN, which protects from immune dysregulation and systemic inflammation.[81]

In addition to significantly differentially expressed biomarkers that have a role in metabolism, immune function, and inflammation, we also observed significant differences in biomarkers that mediate fluid homeostasis and vascular effects. Notably, renin was significantly elevated in patients with T2DM. This upregulation of renin in patients with T2DM is associated with the significantly higher risk for HTN in patients with T2DM via the renin-angiotensin-aldosterone system (RAAS).[67] Aldosterone and anti-diuretic hormone, which as mentioned previously are activated by this system, both act on the kidney to increase sodium and water retention, thus expanding blood volume and increasing BP. [17] Our data showed that SBP trended slightly higher in patients with T2DM although no significant difference was found in SBP or DBP. However, there was a significant difference in pulse pressure between patients with T2DM and nondiabetic controls. A study showed that pulse pressure is correlated with plasma aldosterone concentration, which is consistent with our finding of significantly higher renin (the hormone involved in RAAS) and pulse pressure in patients with T2DM.[82]

Another biomarker that we found to be significantly upregulated in patients with T2DM is protein AMBP, which protects the vasculature against reactive oxygen species.[83] This upregulation may be a protective mechanism in T2DM to counter the vulnerability to oxidative stress.[84]

It is necessary to further analyze inflammation and oxidative stress as they are significant drivers in the mechanisms of the pathogenesis of T2DM.[84] Our findings show that IL-6, TNFRSF1B and TNFRSF11A are upregulated in patients with T2DM, and are consistent with this notion of elevated inflammation. IL-6 an inflammatory cytokine of

which levels may increase through the insulin-mediated MAPK pathway involving NF-κB.[40] This cytokine can lead to a multitude of effects, and relevant to this topic is the increase in vascular permeability through the stabilization of VEGF, which can facilitate the progression of atherosclerosis and facilitate progression to CVD as mentioned before.[25,85] Our findings of elevated TNFRSF1B and TNFRSF11A are also relevant due to their effect on the NF-κB pathway. Although the effect of binding these receptors leads to inhibition of apoptosis, their overlap with NF-κB may have implications in inflammation, which is consistent with T2DM.[25,86,87]

Some of these findings have already been established before as mentioned in the previous subsection. Leptin, renin, and IL-1RN have emerged as differentially present in insulin resistance, and the latter in T2DM, already highlighting the altered metabolic milieu and changes in systemic fluid homeostasis.[61] The HOMAGE trial also had found GDF15 and Galectin-4 to be higher in diabetes, which our study also confirmed, and they hypothesize that the former is due to the stimulatory effects of metformin, whereas the latter further worsens insulin resistance.[78] Our study is consistent with previous findings of altered metabolic milieu and upregulation of inflammatory markers, but we also found higher levels of proteins involved in vascular function and immune function that may suggest the upregulation of mechanisms to protect against immune dysregulation and systemic inflammation.

Taken together, the differential expression of these biomarkers may therefore be involved in the manifestation of HTN, altered metabolic milieu, and inflammation, which

are all known to be pathogenic factors in T2DM, but we also found some biomarkers that have protective effects.

Association of Biomarkers with Flow-Mediated Dilation

As mentioned in the previous subsection, GDF15 levels were significantly higher in patients with T2DM. Increased levels of this biomarker are indicative of tissue hypoxia.[80] According to Miura et al. T2DM impairs vasodilation in coronary arterioles in response to hypoxia, the mechanism by which normally occurs via the opening of ATP-sensitive K^+ channels and leads to the hyperpolarization of the vascular smooth muscle cells surrounding the endothelial layer.[88] Aligning with this information is our finding of significantly lower vasodilation in the brachial artery in response to hyperemia, as measured by FMD.

We also observed higher biomarker levels of renin, which increases fluid retention through RAAS and contributes to HTN.[89] In turn HTN and arterial stiffness are related, which explains why FMD was significantly lower in persons with T2DM, and endothelial dysfunction has been hypothesized to be a precursor to HTN, which further confirms this notion.[90] Interestingly, levels of the vasodilator Adrenomedullin were elevated in patients with T2DM, which could potentially explain why basal p-eNOS levels were higher. Assuming that vascular tone can be inferred from p-eNOS levels, the brachial artery of persons with T2DM would have a wider diameter at baseline, and a smaller percent increase upon hyperemia. Finding elevated levels of a vasodilator may also indicate the increased drive to dispose glucose in conditions of insulin resistance.

Arterial Stiffness and Endothelial Dysfunction

Much of our vascular function data reiterates findings that are already in the literature, but confirming the presence of arterial stiffness and endothelial dysfunction in patients with T2DM was nonetheless valuable to compare endothelial cell phenotype with biomarker levels.

Our data showed that HR was significantly elevated in patients with T2DM versus their nondiabetic counterparts. A study by Whelton et al. showed that higher HRs have been associated with stiffness in carotid and aortic arteries, which is associated with CVD events. We found that pulse pressure, which is also an indicator of arterial stiffness, was significantly higher in patients with T2DM, reiterating their vulnerability to CVD.[91] Pulse pressure is also correlated with shear stress and makes the vascular system vulnerable to atherogenesis and subsequent atherogenic diseases such as CAD and PAD, which a handful of patients with T2DM, but no controls, reported having.[66]

We did not detect significant differences in SBP, DBP, or MAP despite a 4.6 times higher proportion of patients with T2DM with a diagnosis for HTN. This may potentially be explained by the pharmacokinetics of antihypertensive medication. For example, for Olmesartan medoxomil (CS-866) which is an ARB, the elimination half-life, which is defined as the time it takes for the concentration of a drug to decrease to half its administered dose, ranges from 12 to 15 hours.[92,93] The terminal elimination half-life reported in the same source was between 12 and 18 hours, and is defined as the time it takes for the drug concentration in plasma to decrease by half from its level at pseudo-equilibrium.[93,94] Although we required all participants to withhold medication the morning

of the study, depending on when the last dose of Olmesartan or other antihypertensives was taken, it may have affected our baseline BP readings.

FMD of the brachial artery was significantly lower in patients with T2DM, indicating endothelial dysfunction and impaired arterial compliance in this group.[95] As mentioned previously, endothelial dysfunction is an indicator of CV risk factors and its progression results in atherosclerosis.[96] This is particularly relevant in the context of T2DM as insulin resistance is a key factor in making patients with T2DM more vulnerable to atherosclerotic diseases, in particular through dyslipidemia, HTN, and chronic inflammation.[97]

Overall, our vascular data indicates significantly worse vascular endothelial health in patients with T2DM compared to the nondiabetic group.

Conclusion

We observed significantly differentially regulated biomarkers that demonstrated an altered metabolic milieu, oxidative stress, inflammation, and altered vascular and fluid homeostasis, as well as the upregulation of protective mechanisms against increased vascular tone and inflammation in persons with T2DM. We found associations between biomarker levels and insulin-stimulated p-eNOS levels which showed that there is a relationship between altered biomarkers and endothelial cell phenotype.

Our vascular data showed significantly worse arterial stiffness and endothelial dysfunction in persons with T2DM compared to controls, and these findings reflect the biomarker changes associated with fluid homeostasis and oxidative stress.

Study Limitations

Despite the significance of the findings in this study, there are certain limitations that need to be acknowledged. Firstly, although we have determined correlations between serum biomarkers and insulin-stimulated p-eNOS activation, the direction of this association, i.e. whether changes in endothelial cell phenotype alter the serum biomarkers or vice versa, is still unknown. This is due to the cross-sectional nature of the study. Determining whether changes in vascular endothelial health lead to altered serum biomarker levels or whether the opposite is true could provide significant insight on how to address and treat patients who suffer from T2DM and its associated pathophysiology.

Another limitation of our study is the potential for confounding due to risk factors and medication. Some of the results we observed, such as lower LDL levels in patients with T2DM, may be attributable to their lipid-lowering medications as mentioned before. The drug regimen of patients with T2DM and even nondiabetic controls is diverse, with participants taking multiple medications at different doses and varying frequencies, potentially skewing our results as we saw in the lipid panel. We do not have enough participants on specific medications to look at the associations between medication and biomarker levels. Risk factors such as high lipid levels and high blood pressure may also be confounders.

Furthermore, we used venous endothelial cells to assess insulin stimulation of p-eNOS, whereas our vascular measurements assessed endothelial dysfunction in arteries. Our O-link biomarker assay was used to examine serum biomarkers and it cannot be stated with certainty that the differential presence of these biomarkers in patients with T2DM

versus nondiabetic controls reflects the health of the arterial endothelium. Our analyses and correlations still hold significance, however, because of the systemic effects of these biomarkers and their implication in the context of vascular endothelial health, and more broadly MetS and T2DM.

Future Directions

More vascular measurements will be added, including carotid-radial and carotid-femoral pulse wave velocity, which are measures of arterial stiffness, and nitroglycerin-mediated dilation, which similarly to FMD is a measure of vascular dysfunction and arterial compliance.[91,95] Correlating these measurements with the insulin-stimulation activation of p-eNOS as well as with the proteomics data can further elucidate the issue of insulin resistance within the context of vascular endothelial health, and can provide better insight on the association between endothelial cell biology and alterations in signaling pathways.

Furthermore, we will determine the association of circulating protein biomarkers with endothelial cell phenotype as well as systemic vascular function in a more comprehensive way, carrying out multivariable analysis.

Author's Role

I was a research assistant in this study from July 2022, but I have previously worked on this study when I was part of the Hamburg lab between July 2020 and August 2021. I recruited and consented eligible participants, carried out the study visits where I collected their medical history, performed vascular measurements, and assisted with blood and cell collections. I processed these samples in the lab, which were then used to acquire proteomics data and biomarker levels. With the proteomics NPX values that we received from O-link, I carried out statistical analysis to determine proteins which were statistically significant. I assembled, analyzed, and quality controlled the vascular data, and subsequently carried out statistical analyses under the guidance of Dr. Naomi Hamburg.

APPENDIX 1: Targets of Proteomics Panel CV II and CV III

Panel Cardiovascular II			
BMP-6	IL-27	MERTK	AGRP
ANGPT1	IL-17D	KIM1	HB-EGF
ADM	CXCL1	THBS2	GDF-2
CD40-L	LOX-1	TM	FABP2
SLAMF7	Gal-9	VSIG2	THPO
PGF	GIF	AMBP	MARCO
ADAM-TS13	SCF	PRELP	GT
BOC	IL18	HO-1	BNP
IL-4RA	FGF-21	XCL1	MMP12
SRC	PIgR	IL16	ACE2
IL-1ra	RAGE	SORT1	PD-L2
IL-6	SOD2	CEACAM8	CTSL1
TNFRSF10A	CTRC	PTX3	hOSCAR
STK4	FGF-23	PSGL-1	TNFRSF13B
IDUA	SPON2	CCL17	TGM2
TNFRSF11A	GH	CCL3	LEP
PAR-1	FS	MMP7	CA5A
TRAIL-R2	GLO1	IgG Fc receptor II-b	HSP 27
PRSS27	CD84	ITGB1BP2	CD4
TIE2	PAPPA	DCN	NEMO
TF	SERPINA12	Dkk-1	VEGFD
IL1RL2	REN	LPL	PARP-1
PDGF subunit B	DECR1	PRSS8	HAOX1

Panel Cardiovascular III			
TNFRSF14	CNTN1	PI3	CPB1
LDL receptor	CDH5	Ep-CAM	CHI3L1
ITGB2	TLT-2	AP-N	ST2
IL-17RA	FABP4	AXL	t-PA
TNF-R2	TFPI	IL-1RT1	SCGB3A2
MMP-9	PAI	MMP-2	EGFR
EPHB4	CCL24	FAS	IGFBP-7
IL2-RA	TR	MB	CD93
OPG	TNFRSF10C	TNFSF13B	IL-18BP
ALCAM	GDF-15	PRTN3	COL1A1
TFF3	SELE	PCSK9	PON3
SELP	AZU1	U-PAR	CTSZ
CSTB	DLK-1	OPN	MMP-3
MCP-1	SPON1	CTSD	RARRES2
CD163	MPO	PGLYRP1	ICAM-2
Gal-3	CXCL16	CPA1	KLK6
GRN	IL-6RA	JAM-A	PDGF subunit A
NT-proBNP	RETN	Gal-4	TNF-R1
BLM hydrolase	IGFBP-1	IL-1RT2	IGFBP-2
PLC	CHIT1	SHPS-1	vWF
LTBR	TR-AP	CCL15	PECAM-1
Notch 3	GP6	CASP-3	MEPE
TIMP4	PSP-D	uPA	CCL16

BIBLIOGRAPHY

1. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 2013;36(Supplement_1):S67-S74. doi:10.2337/dc13-S067

2. Statistics About Diabetes | American Diabetes Association. Accessed March 28, 2023. https://diabetes.org/about-us/statistics/about-diabetes

3. Global Burden of Disease Compare. Institute for Health Metrics and Evaluation. Accessed December 14, 2022. http://vizhub.healthdata.org/gbd-compare

4. Centers for Disease Control and Prevention. What is Diabetes? Centers for Disease Control and Prevention. Published July 7, 2022. Accessed December 14, 2022. https://www.cdc.gov/diabetes/basics/diabetes.html

5. Govindarajan G, Whaley-Connell A, Mugo M, Stump C, Sowers JR. The Cardiometabolic Syndrome as a Cardiovascular Risk Factor. *The American Journal of the Medical Sciences*. 2005;330(6):311-318. doi:10.1097/00000441-200512000-00009

6. Bovolini A, Garcia J, Andrade MA, Duarte JA. Metabolic Syndrome Pathophysiology and Predisposing Factors. *International Journal of Sports Medicine*. 2021;42(3):199-214. doi:10.1055/a-1263-0898

7. Desroches S, Lamarche B. The Evolving Definitions and Increasing Prevalence of the Metabolic Syndrome. *Applied Physiology, Nutrition, and Metabolism*. 2007;32(1): 23-32. doi:10.1139/h06-095

8. Hu G, Qiao Q, Tuomilehto J, et al. Prevalence of the Metabolic Syndrome and its Relation to All-Cause and cardiovascular Mortality in Nondiabetic European Men and Women. *Archives of Internal Medicine*. 2004;164(10):1066-1076. doi:10.1001/archinte.164.10.1066

9. Papakonstantinou E, Lambadiari V, Dimitriadis G, Zampelas A. Metabolic Syndrome and Cardiometabolic Risk Factors. *Current Vascular Pharmacology*. 2013;11(6): 858-879. doi:10.2174/15701611113116660176

10. Biadgo B, Melak T, Ambachew S, et al. The Prevalence of Metabolic Syndrome and Its Components among Type 2 Diabetes Mellitus Patients at a Tertiary Hospital, Northwest Ethiopia. *Ethiopian Journal of Health Sciences*. 2018;28(5):645-654. doi:10.4314/ejhs.v28i5.16

11. Martín-Timón I. Type 2 diabetes and cardiovascular disease: Have All Risk Factors the Same Strength? *WJD*. 2014;5(4):444. doi:10.4239/wjd.v5.i4.444

12. Einarson TR, Acs A, Ludwig C, Panton UH. Prevalence of Cardiovascular Disease in Type 2 Diabetes: a Systematic Literature Review of Scientific Evidence from Across the World in 2007-2017. *Cardiovascular Diabetology*. 2018;17(1):83. doi:10.1186/s12933-018-0728-6

13. Chao JH, Hirsch IB. *Initial Management of Severe Hyperglycemia in Type 2 Diabetes*. MDText.com, Inc.; 2018. Accessed February 5, 2023. https://www.ncbi.nlm.nih.gov/books/NBK278997/

14. Park C, Guallar E, Linton JA, et al. Fasting Glucose Level and the Risk of Incident Atherosclerotic Cardiovascular Diseases. *Diabetes Care*. 2013;36(7):1988-1993. doi:10.2337/dc12-1577

15. Persson PB. Renin: Origin, Secretion and Synthesis. *Journal of Physiology*. 2003; 552(Pt 3):667-671. doi:10.1113/jphysiol.2003.049890

16. Figure: Regulating Blood Pressure: The Renin-Angiotensin-Aldosterone System - Merck Manuals Consumer Version. Accessed March 16, 2023. https://www.merckmanuals.com/home/multimedia/figure/regulating-blood-pressure-the-renin-angiotensin-aldosterone-system

17. Fountain JH, Lappin SL. Physiology, Renin Angiotensin System. In: *StatPearls*. StatPearls Publishing; 2022. Accessed January 25, 2023. http://www.ncbi.nlm.nih.gov/books/NBK470410/

18. Scott JH, Menouar MA, Dunn RJ. *Physiology, Aldosterone*. StatPearls Publishing; 2022. Accessed March 15, 2023. https://www.ncbi.nlm.nih.gov/books/NBK470339/

19. Stokes J, Kannel WB, Wolf PA, D'Agostino RB, Cupples LA. Blood Pressure as a Risk Factor for Cardiovascular Disease. The Framingham Study--30 Years of Follow-Up. *Hypertension*. 1989;13(5 Suppl):I13-18. doi:10.1161/01.hyp.13.5_suppl.i13

20. Naha S, Gardner MJ, Khangura D, Kurukulasuriya LR, Sowers JR. *Hypertension in Diabetes*. MDText.com, Inc.; 2021. Accessed March 15, 2023. https://www.ncbi.nlm.nih.gov/books/NBK279027/

21. What Do ACE Inhibitors Do for Heart Health? Mayo Clinic. Accessed March 16, 2023. https://www.mayoclinic.org/diseases-conditions/high-blood-pressure/in-depth/ace-inhibitors/art-20047480

22. Chia CW, Egan JM, Ferrucci L. Age-Related Changes in Glucose Metabolism, Hyperglycemia, and Cardiovascular Risk. *Circulation Research*. 2018;123(7):886-904. doi:10.1161/CIRCRESAHA.118.312806

23. Sherwani SI, Khan HA, Ekhzaimy A, Masood A, Sakharkar MK. Significance of HbA1c Test in Diagnosis and Prognosis of Diabetic Patients. *Biomarker Insights*. 2016;11:95-104. doi:10.4137/BMI.S38440

24. Javadifar A, Rastgoo S, Banach M, Jamialahmadi T, Johnston TP, Sahebkar A. Foam Cells as Therapeutic Targets in Atherosclerosis with a Focus on the Regulatory Roles of Non-Coding RNAs. *International Journal of Molecular Sciences*. 2021;22(5):25–29. doi:10.3390/ijms22052529

25. Gao W, Liu H, Yuan J, et al. Exosomes Derived from Mature Dendritic Cells Increase Endothelial Inflammation and Atherosclerosis via Membrane TNF-α Mediated NF-κB Pathway. *Journal of Cellular and Molecular Medicine*. 2016; 20(12):2318-2327. doi:10.1111/jcmm.12923

26. Shah PK. Plaque Disruption and Thrombosis: Potential Role of Inflammation and Infection. *Cardiology in Review*. 2000;8(1):31-39.

27. Rahman MS, Hossain KS, Das S, et al. Role of Insulin in Health and Disease: An Update. *International Journal of Molecular Sciences*. 2021;22(12):6403. doi:10.3390/ijms22126403

28. Nystrom FH, Quon MJ. Insulin Signalling: Metabolic Pathways and Mechanisms for Specificity. *Cellular Signaling.* 1999;11(8):563–574

29. 100th Anniversary of the Discovery of Insulin Perspective: Insulin and Adipose Tissue Fatty Acid Metabolism. doi:10.1152/ajpendo.00620.2020

30. Glucose Transporters and Insulin Action — Implications for Insulin Resistance and Diabetes Mellitus | *New England Journal of Medicine.* Accessed March 15, 2023. https://www.nejm.org/doi/full/10.1056/nejm199907223410406

31. *Insulin*. National Institute of Diabetes and Digestive and Kidney Diseases; 2018. Accessed March 16, 2023. https://www.ncbi.nlm.nih.gov/books/NBK548016/

32. Saltiel AR, Kahn CR. Insulin Signaling and the Regulation of Glucose and Lipid Metabolism. *Nature*. 2001;414(6865):799-806. doi:10.1038/414799a

33. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of Insulin Resistance in Skeletal Muscle. *J Biomedicine & Biotechnology*. 2010:476279. doi:10.1155/2010/476279

34. Bajaj M. Metabolic and Molecular Basis of Insulin Resistance. *Journal of Nuclear Cardiology*. 2003;10(3):311-323. doi:10.1016/S1071-3581(03)00520-8

35. Olver TD, Grunewald ZI, Ghiarone T, et al. Persistent Insulin Signaling Coupled with Restricted PI3K Activation Causes Insulin-Induced Vasoconstriction. *American Journal of Physiology. Heart and Circulatory Physiology*. 2019;317(5):H1166-H1172. doi:10.1152/ajpheart.00464.2019

36. Kim J a, Montagnani M, Koh KK, Quon MJ. Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction: Molecular and Pathophysiological Mechanisms. *Circulation*. 2006;113(15):1888-1904. doi:10.1161/CIRCULATIONAHA.105.563213

37. Reynolds LJ, Credeur DP, Manrique C, Padilla J, Fadel PJ, Thyfault JP. Obesity, type 2 diabetes, and impaired insulin-stimulated blood flow: role of Skeletal Muscle NO Synthase and Endothelin-1. *Journal of Applied Physiology*. 2017;122(1):38-47. doi:10.1152/japplphysiol.00286.2016

38. Ferré P, Foufelle F. Hepatic Steatosis: a Role for De Novo Lipogenesis and the Transcription Factor SREBP-1c. *Diabetes, Obesity and Metabolism*. 2010;12:83-92. doi:10.1111/j.1463-1326.2010.01275.x

39. Bengal E, Aviram S, Hayek T. p38 MAPK in Glucose Metabolism of Skeletal Muscle: Beneficial or Harmful? *International Journal of Molecular Sciences*. 2020;21(18):6480. doi:10.3390/ijms21186480

40. Cui X, Qian DW, Jiang S, Shang EX, Zhu ZH, Duan JA. Scutellariae Radix and Coptidis Rhizoma Improve Glucose and Lipid Metabolism in T2DM Rats via Regulation of the Metabolic Profiling and MAPK/PI3K/Akt Signaling Pathway. *International Journal of Molecular Sciences*. 2018;19(11):3634. doi:10.3390/ijms19113634

41. Strain WD, Paldánius PM. Diabetes, Cardiovascular Disease and the Microcirculation. *Cardiovascular Diabetology*. 2018;17:57. doi:10.1186/s12933-018-0703-2

42. Xu S, Ilyas I, Little PJ, et al. Endothelial Dysfunction in Atherosclerotic Cardiovascular Diseases and Beyond: From Mechanism to Pharmacotherapies. Ma Q, ed. *Pharmacological Reviews*. 2021;73(3):924-967. doi:10.1124/pharmrev.120.000096

43. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in Diabetes Mellitus: Molecular Mechanisms and Clinical Implications. *Reviews in Endocrine & Metabolic Disorders*. 2010;11(1):61-74. doi:10.1007/s11154-010-9134-4

44. Pricci F, Leto G, Amadio L, et al. Oxidative Stress in Diabetes-Induced Endothelial Dysfunction Involvement of Nitric Oxide and Protein Kinase C. *Free Radical*

Biology and Medicine. 2003;35(6):683-694. doi:10.1016/S0891-5849(03)00401-5

45. Tabit CE, Shenouda SM, Holbrook M, et al. Protein Kinase C-β Contributes to Impaired Endothelial Insulin Signaling in Humans With Diabetes Mellitus. *Circulation*. 2013;127(1):86-95. doi:10.1161/CIRCULATIONAHA.112.127514

46. Bretón-Romero R, Feng B, Holbrook M, et al. Endothelial Dysfunction in Human Diabetes Is Mediated by Wnt5a–JNK Signaling. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2016;36(3):561-569. doi:10.1161/ATVBAHA.115.306578

47. Phospho-eNOS (Ser1177) Monoclonal Antibody (H.83.2) (MA5-14957). Accessed March 15, 2023. https://www.thermofisher.com/antibody/product/Phospho-eNOS-Ser1177-Antibody-clone-H-83-2-Monoclonal/MA5-14957

48. Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM. Fluid Shear Stress Stimulates Phosphorylation of Akt in Human Endothelial Cells: Involvement in Suppression of Apoptosis. *Circulation Research*. 1998;83(3):334-341. doi:10.1161/01.res.83.3.334

49. Paszkowiak JJ, Dardik A. Arterial Wall Shear Stress: Observations from the Bench to the Bedside. *Vascular and Endovascular Surgery*. 2003;37(1):47-57. doi:10.1177/153857440303700107

50. Li L, Terry CM, Shiu YTE, Cheung AK. Neointimal Hyperplasia Associated with Synthetic Hemodialysis Grafts. *Kidney International*. 2008;74(10):1247-1261. doi:10.1038/ki.2008.318

51. Lu D, Kassab GS. Role of Shear Stress and Stretch in Vascular Mechanobiology. *Journal of the Royal Society. Interface*. 2011;8(63):1379-1385. doi:10.1098/rsif.2011.0177

52. Cyr AR, Huckaby LV, Shiva SS, Zuckerbraun BS. Nitric Oxide and Endothelial Dysfunction. *Critical Care Clinics*. 2020;36(2):307-321. doi:10.1016/j.ccc.2019.12.009

53. Raitakari OT, Celermajer DS. Flow-Mediated Dilatation. *British Journal of Clinical Pharmacology*. 2000;50(5):397-404. doi:10.1046/j.1365-2125.2000.00277.x

54. Weissgerber TL. Flow-Mediated Dilation: Can New Approaches Provide Greater Mechanistic Insight into Vascular Dysfunction in Preeclampsia and Other Diseases? *Current Hypertension Reports*. 2014;16(11):487. doi:10.1007/s11906-014-0487-z

55. Bhatt PM, Malgor R. Wnt5a: a Player in the Pathogenesis of Atherosclerosis and Other Inflammatory Disorders. *Atherosclerosis*. 2014;237(1):155-162. doi:10.1016/j.atherosclerosis.2014.08.027

56. Laakso M. Cardiovascular Disease in Type 2 Diabetes from Population to Man to Mechanisms. *Diabetes Care*. 2010;33(2):442-449. doi:10.2337/dc09-0749

57. Al-Amrani S, Al-Jabri Z, Al-Zaabi A, Alshekaili J, Al-Khabori M. Proteomics: Concepts and Applications in Human Medicine. *World Journal of Biological Chem*istry. 2021;12(5):57-69. doi:10.4331/wjbc.v12.i5.57

58. Definition of proteomics - NCI Dictionary of Cancer Terms - NCI. Published February 2, 2011. Accessed March 13, 2023. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/proteomics

59. EMBL-EBI. What is Proteomics? | Proteomics. Accessed March 13, 2023. https://www.ebi.ac.uk/training/online/courses/proteomics-an-introduction/what-is-proteomics/

60. Central Dogma. Genome.gov. Published September 14, 2022. Accessed March 13, 2023. https://www.genome.gov/genetics-glossary/Central-Dogma

61. Chen ZZ, Gerszten RE. Metabolomics and Proteomics in Type 2 Diabetes. *Circulation Research*. 2020;126(11):1613-1627. doi:10.1161/CIRCRESAHA.120.315898

62. olink-cvd-ii-validation-data-v2.1.pdf. Accessed January 18, 2023. https://www.olink.com/content/uploads/2021/09/olink-cvd-ii-validation-data-v2.1.pdf

63. Olink Target 96 Cardiovascular panels. Olink. Accessed March 16, 2023. https://olink.com/products-services/target/cardiometabolic-panel/

64. What is NPX? Olink. Accessed January 7, 2023. https://olink.com/faq/what-is-npx/

65. Williams SB, Cusco JA, Roddy MA, Johnstone MT, Creager MA. Impaired Nitric Oxide-Mediated Vasodilation in Patients with Non-Insulin-Dependent Diabetes Mellitus. *Journal of the American College of Cardiology*. 1996;27(3):567-574. doi:10.1016/0735-1097(95)00522-6

66. Mishani S, Belhoul-Fakir H, Lagat C, Jansen S, Evans B, Lawrence-Brown M. Stress Distribution in the Walls of Major Arteries: Implications for atherogenesis.

Quantitative Imaging in Medicine and Surgery. 2021;11(8):3494-3505. doi:10.21037/qims-20-614

67. Takahashi H, Yoshika M, Komiyama Y, Nishimura M. The Central Mechanism Underlying Hypertension: a Review of the Roles of Sodium Ions, Epithelial Sodium Channels, the Renin–Angiotensin–Aldosterone System, Oxidative Stress and Endogenous Digitalis in the Brain. *Hypertension Research*. 2011;34(11):1147-1160. doi:10.1038/hr.2011.105

68. Wong HK, Tang F, Cheung TT, Cheung BMY. Adrenomedullin and Diabetes. *World Journal of Diabetes*. 2014;5(3):364-371. doi:10.4239/wjd.v5.i3.364

69. Wang H, Eckel RH. Lipoprotein Lipase: From Gene to Obesity. *American Journal of Physiology. Endocrinology and Metabolism*. 2009;297(2):E271-E288. doi:10.1152/ajpendo.90920.2008

70. CDC. Diabetes Testing. Centers for Disease Control and Prevention. Published February 28, 2023. Accessed March 20, 2023. https://www.cdc.gov/diabetes/basics/getting-tested.html

71. Mordarska K, Godziejewska-Zawada M. Diabetes in the elderly. *Przeglad Menopauzalny*. 2017;16(2):38-43. doi:10.5114/pm.2017.68589

72. Hamburg NM, Larson MG, Vita JA, et al. Metabolic Syndrome, Insulin Resistance, and Brachial Artery Vasodilator Function in Framingham Offspring Participants Without Clinical Evidence of Cardiovascular Disease. *The American Journal of Cardiology*. 2008;101(1):82-88. doi:10.1016/j.amjcard.2007.07.053

73. Grundy SM, Cleeman JI, Merz CNB, et al. Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. *Circulation*. 2004;110(2):227-239. doi:10.1161/01.CIR.0000133317.49796.0E

74. Lakka HM, Laaksonen DE, Lakka TA, et al. The Metabolic Syndrome and Total and Cardiovascular Disease Mortality in Middle-aged Men. *JAMA: The Journal of the American Medical Association*. 2002;288(21):2709-2716. doi:10.1001/jama.288.21.2709

75. Alfadda AA, Benabdelkamel H, Masood A, et al. Proteomic analysis of Mature Adipocytes from Obese Patients in Relation to Aging. *Experimental Gerontology*. 2013;48(11):1196-1203. doi:10.1016/j.exger.2013.07.008

76. Mature Adipocyte Proteome Reveals Differentially Altered Protein Abundances Between Lean, Overweight and Morbidly Obese Human Subjects | Elsevier Enhanced Reader. doi:10.1016/j.mce.2014.11.021

77. Ekhzaimy AA, Masood A, Benabdelkamel H, Elhassan T, Musambil M, Alfadda AA. Plasma Proteomics Reveals an Improved Cardio-Metabolic Profile in Patients with Type 2 Diabetes Post-Liraglutide Treatment. *Diabetes & Vascular Disease Research*. 2022;19(3):14791641221094322. doi:10.1177/14791641221094322

78. Verdonschot JAJ, Ferreira JP, Pellicori P, et al. Proteomic Mechanistic Profile of Patients with Diabetes at Risk of Developing Heart Failure: insights from the HOMAGE trial. *Cardiovascular Diabetology*. 2021;20(1):163. doi:10.1186/s12933-021-01357-9

79. Dornbush S, Aeddula NR. *Physiology, Leptin*. StatPearls Publishing; 2022. Accessed March 13, 2023. https://www.ncbi.nlm.nih.gov/books/NBK537038/

80. PubChem. GDF15 - Growth Differentiation Factor 15 (human). Accessed March 21, 2023. https://pubchem.ncbi.nlm.nih.gov/gene/GDF15/human

81. IL1RN - Interleukin-1 Receptor Antagonist Protein - Homo Sapiens (Human) | UniProtKB | UniProt. Accessed March 21, 2023. https://www.uniprot.org/uniprotkb/P18510/entry

82. Yao X, Li N, Zhang Y, et al. Plasma Aldosterone Concentration Is Positively Associated With Pulse Pressure in Patients With Primary Hypertension. *Medicine (Baltimore)*. 2015;94(10):e614. doi:10.1097/MD.0000000000000614

83. AMBP - Protein AMBP - Homo sapiens (Human) | UniProtKB | UniProt. Accessed March 21, 2023. https://www.uniprot.org/uniprotkb/P02760/entry

84. Halim M, Halim A. The Effects of Inflammation, Aging and Oxidative Stress on the Pathogenesis of Diabetes Mellitus (Type 2 Diabetes). *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2019;13(2):1165-1172. doi:10.1016/j.dsx.2019.01.040

85. Tanaka T, Narazaki M, Kishimoto T. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspectives in Biology*. 2014;6(10):a016295. doi:10.1101/cshperspect.a016295

86. Tumor Necrosis Factor Receptor Family Members in the Immune System |Elsevier Enhanced Reader. doi:10.1006/smim.1998.0144

87. PubChem. TNFRSF1B - TNF Receptor Superfamily Member 1B (Human). Accessed March 16, 2023. https://pubchem.ncbi.nlm.nih.gov/gene/TNFRSF1B/human

88. Miura H, Wachtel RE, Loberiza FR, et al. Diabetes Mellitus Impairs Vasodilation to Hypoxia in Human Coronary Arterioles. *Circulation Research*. 2003;92(2):151-158. doi:10.1161/01.RES.0000052671.53256.49

89. Brown MJ. Renin: friend or foe? *Heart*. 2007;93(9):1026-1033. doi:10.1136/hrt.2006.107706

90. Konukoglu D, Uzun H. Endothelial Dysfunction and Hypertension. In: Islam MdS, ed. *Hypertension: From Basic Research to Clinical Practice*. Advances in Experimental Medicine and Biology. Springer International Publishing; 2017:511-540. doi:10.1007/5584_2016_90

91. Niiranen TJ, Kalesan B, Mitchell GF, Vasan RS. Relative Contributions of Pulse Pressure and Arterial Stiffness to Cardiovascular Disease. *Hypertension*. 2019;73(3):712-717. doi:10.1161/HYPERTENSIONAHA.118.12289

92. Hallare J, Gerriets V. *Half Life*. StatPearls Publishing; 2022. Accessed March 21, 2023. https://www.ncbi.nlm.nih.gov/books/NBK554498/

93. Schwocho LR, Masonson HN. Pharmacokinetics of CS-866, a New Angiotensin II Receptor Blocker, in Healthy Subjects. *The Journal of Clinical Pharmacology*. 2001;41(5):515-527. doi:10.1177/00912700122010393

94. Toutain PL, Bousquet-Mélou A. Plasma Terminal Half-Life. *Journal of Veterinary Pharmacology and Therapeutics*. 2004;27(6):427-439. doi:10.1111/j.1365-2885.2004.00600.x

95. Ghiadoni L, Salvetti M, Muiesan ML, Taddei S. Evaluation of Endothelial Function by Flow Mediated Dilation: Methodological Issues and Clinical Importance. *High Blood Pressure & Cardiovascular Prevention*. 2015;22(1):17-22. doi:10.1007/s40292-014-0047-2

96. Hadi HA, Carr CS, Al Suwaidi J. Endothelial Dysfunction: Cardiovascular Risk Factors, Therapy, and Outcome. *Vascular Health and Risk Managment*. 2005;1(3):183-198.

97. Watson KE, Peters Harmel AL, Matson G. Atherosclerosis in Type 2 Diabetes Mellitus: the Role of Insulin Resistance. *Journal of Cardiovascular Pharmacology and Therapeutics*. 2003;8(4):253-260. doi:10.1177/107424840300800402

CURRICULUM VITAE