Comprehensive Metabolomic Analyses of Diabetic Atherosclerosis

COMPREHENSIVE METABOLOMIC ANALYSES OF

DIABETIC ATHEROSCLEROSIS

By:

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ABSTRACT

The prevalence of diabetes mellitus is increasing dramatically worldwide. Approximately three out of four diabetic patients will die of cerebro- and cardiovascular disease (CVD). Atherosclerosis, a chronic inflammatory disease of the medium-large arteries, is the major underlying cause of most CVDs. Despite the established progressive relationship between diabetes and CVD, the molecular mechanisms by which diabetes promotes atherosclerosis are not well understood. This has impeded the development of strategies to block or slow atherogenesis in diabetic patients. The objective of my thesis is to investigate the molecular alterations by which diabetes accelerates the development of atherosclerosis using comprehensive metabolomics techniques.

We first investigated the development and progression of atherosclerosis at the molecular level in apolipoprotein E-deficient mice. We identified specific changes in plasma-borne metabolites that are associated with the pathogenesis and progression of atherosclerosis. In addition, glycerophospholipid and sphingolipid metabolism were found to be the most significantly altered pathways. Using comprehensive metabolomics techniques, we were able to differentiate atherosclerotic plasma metabolome from healthy control and delineate different stages of atherosclerotic progression.

Next, we characterized multiple mouse models of hyperglycemia-induced accelerated atherosclerosis. We showed that the vascular effects of glucosamine supplementation are comparable to streptozotocin-induced and genetically-induced (Ins2Akita) hyperglycemia in terms of lesional glucosamine, endoplasmic reticulum (ER) stress levels and

atherosclerotic burden. In addition, we showed that a chemical chaperone (4phenylbutyric acid) reduces ER stress levels and attenuates accelerated atherogenesis in each of these models. Together these findings support the mechanism involving glucosamine-induced ER stress in hyperglycemia-induced accelerated atherosclerosis.

Lastly, metabolomics techniques were used to investigate the molecular alterations by which hyperglycemia promotes the accelerated development of atherosclerosis in several disease models. The three mouse models induced both unique and common changes in the plasma metabolome. Identification of the commonly altered metabolite features revealed alterations in glycerophospholipid and sphingolipid metabolisms, and key atherosclerosis-associated processes including inflammation and oxidative stress.

Together, we showed that comprehensive metabolomics techniques can be used to identify specific alterations in the metabolome that are associated with a particular disease genotype and phenotype. These data highlight the important roles of the glycerophospholipid and sphingolipid metabolisms in the pathogenesis of atherosclerosis and diabetic atherosclerosis. The clear difference in the level of several metabolites supports the use of plasma lipid profiling as a diagnostic tool of atherogenesis.

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"Success is 1% inspiration and 99% perspiration." - Old saying

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LIST OF ABBREVIATIONS

4-PBA	4-phenylbutyric acid
ANOVA	analysis of variance
ApoE	apolipoprotein E
Cer	ceramide
СНОР	C/EBP homologous protein
Ctrl	control
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ESI	electrospray ionization
FDR	false discovery rate
GlcN	glucosamine
GluCer	glucosyl-ceramide
GRP78	78 kDa glucose-regulated protein
HAEC	human aortic endothelial cells
HASMC	human aortic smooth muscle cells
HILIC	hydrophilic interaction chromatography
HMDB	human metabolome database
IL	interleukine
Ins2 ^{+/Akita}	Akita point mutation in one copy of the insulin 2 gene
KDEL	lysine-aspartic acid-glutamic acid-leucine
LacCer	lactosyl-ceramide

LC	liquid chromatography
LDL	low-density lipoprotein
m/z	mass-to-charge
MS	mass spectrometry
O-GlcNAc	O-linked N-acetylglucosamine
PCR	polymerase chain reaction
PLS-DA	partial least squares discriminant analysis
QC	quality control
RNA	ribonucleic Acid
RP	reversed-phase
STZ	streptozotocin
TOF	time-of-flight
UDP-GlcNAc	uridine-diphosphate acetylglucosamine
UPR	unfolded protein response
VIP	variable importance in the projection

CHAPTER 1

General Introduction

1.1 Chapter Preface

Portions of the General Introduction are a direct representation of a review article published in *Biomarker Journal*, volume 2, issue 2, March 2016. Vi Dang wrote this review article in collaboration with Dr. Geoff Werstuck.

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1.2 Metabolomics

'Omics' is the comprehensive analysis of systems biology.^{1,2} The three central fields of 'omics' science include genomics, proteomics and metabolomics (Figure 1). Genomics, the study of genes, DNA and RNA, was the first 'omics' to be established. The entire set of proteins encoded by the genome is investigated in proteomics. Metabolomics examines the chemical entities of metabolites, which are small molecules involved in cellular metabolism and are a readout of genetic and proteomic makeup.^{3–5} In contrast to the well-defined fields of genomics and proteomics, the study of metabolomics has only recently emerged in the late twentieth century (Figure 2). The number of PubMed publications containing "metabolomics" as the key concept has increased significantly within the past decade.

Despite its recent emergence, the chemical complexity in metabolomics analyses surpasses the other 'omics' fields (Figure 3). Genomes are made up of only four nucleotide bases – adenine, cytosine, guanine and thymine, whereas proteomes are made up of twenty protein building blocks – the natural amino acids. In contrast, the metabolome, which is the collection of metabolites in a biological specimen, is an extremely complex mixture of chemically diverse compound classes (e.g., amino acids, lipids, nucleosides, etc.), with different chemical properties (e.g., hydrophilic versus hydrophobic) and a dynamic range of molecular concentrations (e.g., low nM to high mM).^{6,7} Metabolites can also undergo different types of chemical modifications including oxidation, glycosylation and methylation, which further enhance the chemical complexity

in metabolomics. As such, metabolomics-based research requires cutting-edge analytical platforms and advanced biochemoinformatic methods.

The profile of specific metabolites provides a direct functional readout of a physiological or pathological state of an organism.^{8–10} Moreover, the levels of metabolites reflect the functional level more accurately than the other 'omics' studies since the metabolic fluxes are regulated not only by gene expression, but also by environmental stresses. Therefore, metabolomics tools hold great potential in clinical applications including identification of disease biomarkers, investigation of disease pathophysiology and drug discovery.^{11–14} Currently, metabolites are used in more than 95% of all diagnostic clinical tests, and approximately 90% of all known drugs are small molecules.¹⁵

Figure 1.1: The 'omics' cascade. The three main fields of omics-science include genomics, proteomics, and metabolomics. Metabolomics is downstream of the central dogma of molecular biology (DNA \rightarrow RNA \rightarrow protein) and is the most sensitive to environmental and dietary influences.



Figure 1.2: Metabolomics is a relatively new field. Total number of PubMed publications containing the term 'metabolomics' from 2001 to 2016 (downloaded from PubMed on September 25, 2017).









1.3 Metabolomics Approach

Many analytical platforms have been developed for metabolomics analyses.^{16–20} Nuclear magnetic resonance spectroscopy, and liquid or gas chromatography coupled to mass spectrometer are most commonly used in metabolomics-based research. However, no single analytical platform is currently capable of detecting the entire metabolome. The choice of employing a specific platform is dependent not only on the scope of the analysis, but also on the nature of the sample and the availability of the analytical techniques. Ideally, multiple platforms should be used to complement each other, since they are capable of separating and detecting different classes of metabolites.

Current advances in technology allow for the characterization of thousands of metabolites, leading to the development of so-called targeted and untargeted (or comprehensive) metabolomics (Figure 4).^{20,21} Targeted metabolomics involves the analysis of a specific set of known metabolites, typically focusing on one or several biological pathways of interest. This type of approach is commonly employed for hypothesis-driven research to answer specific biochemical questions, such as evaluating the effect of a drug on a specific pathway.

Alternatively, untargeted metabolomics performs the global analysis of the metabolome and is capable of revealing novel and unanticipated molecular perturbations.²² As such, this type of approach offers an unbiased examination of the association between the levels of metabolites and their interconnectivity in multiple metabolic pathways, in relation to a phenotype or genotype. Untargeted metabolomics is therefore commonly

used as a discovery tool or as a hypothesis-generating strategy. Depending on the scope of the analysis – targeted versus untargeted, sample preparation, analytical techniques (e.g. separation column(s), ionization method and mass analyzer) and data processing are susceptible to different degrees of challenge, difficulty, and scale of effort.

Figure 1.4: Targeted and untargeted workflow for metabolomics-based research. Targeted metabolomics is a hypothesis-driven approach while untargeted metabolomics is hypothesis-generating and hence requires functional analysis of the identified metabolites, elucidation of novel mechanisms and validation of the hypothesis. Green box: common step between the two approaches, blue box: unique step in untargeted workflow.



1.4 Untargeted Metabolomics Workflow

The typical workflow of untargeted metabolomics consists of the following steps: (i) metabolite extraction, (ii) data acquisition by an analytical platform, (iii) data processing by a biochemoinformatic tool, (iv) data analysis using univariate and multivariate statistics, (v) metabolite identification, and (vi) data interpretation (Figure 5).

- (i) Metabolite extraction: Untargeted metabolomics involves the global analysis of the metabolome. As such, sample preparation in untargeted metabolomics is optimized to extract as many metabolites as possible. An ideal extraction method should be capable of solubilizing all metabolites while excluding proteins and other higher molecular weight components. Recovery standards are commonly added prior to extraction to monitor recovery efficiency, whereas international standards are added prior to data acquisition to correct for instrumental variation.
- (ii) Data acquisition: No single analytical platform is currently capable of detecting the entire metabolome. Mass spectrometry-based techniques are the most commonly used platform in untargeted metabolomics studies due to high sensitivity, high selectivity, and broad dynamic range. Chromatographic separation prior to mass detection enhances data quality (by reducing matrix effects and ionization suppression), increases metabolome coverage and provides additional information for metabolite identification. Pooled samples are usually run in between samples as a quality control check.

- (iii) Data processing: Due to the global scope of its analysis, untargeted metabolomics produces an extremely complex data set that makes the manual examination of the metabolite features impractical. The data processing in this type of metabolomics approach, therefore, requires the assistance of bioinformatic tools. These metabolomics software typically provide algorithms for retention time alignment between multiple analyses, data filtering, metabolite feature detection (mass to charge value and chromatographic retention time), and relative quantification (chromatographic peak area).
- (iv) *Data analysis*: Quality assurance is the first important step in data analysis.
 Redundant metabolite features (e.g. isotopes and adducts) and unreliable features (poor analytical reproducibility in the quality control samples or elute in ion suppression regions) should be removed. Analysis of quality control samples should also be performed to ensure good instrumental reproducibility throughout the period of analysis. Finally, multivariate (supervised or non-supervised) and univariate (e.g. t-test, fold change) analyses are used to determine the metabolite features that are altered significantly in the treatment groups versus the control.
- (v) *Metabolite Identification*: The metabolites of interest are identified based on authentic standards, or their identity can be tentatively assigned based on matching their accurate mass/empirical formula within metabolite databases (putative identification), such as METLIN [42], Human Metabolome Database [43] and LIPID MAPS [44]. In all cases, metabolites should be annotated based on their characteristic mass-to-charge value and retention time. Despite recent

advances in metabolomics technologies, metabolite identification is still the bottleneck step in untargeted metabolomics. Untargeted metabolomics usually produces a large number of unknown metabolites, many of which are putatively identified due to expensive or unavailable authentic standards. Mass accuracy is therefore very important in de novo metabolite identification. As such, in addition to instrumental mass calibration, acquired mass spectra should also be calibrated internally. Internal standards can be added to samples or endogenous compounds such as sodium formate adducts (endogenous sodium is from cells or bio-fluid extracts and formic acid is from the mobile phase) can be used as internal calibrants. The internal calibration can significantly enhance mass accuracy for de novo metabolite identification in metabolomics research.

(vi) *Data interpretation*: Metabolomics data interpretation is the task of understanding the biological functions and system-level effects of the metabolites of interest.
Functional analysis and pathway-mapping tools are usually used to determine the interconnectivity of metabolites within metabolic pathways in relation to an aberrant process or a pathological phenotype. These analyses provide insights into the molecular mechanisms underlying a pathological or aberrant condition.
Further mechanistic studies could also be done in vitro or in vivo to further explore the biological effects of the metabolites of interest.

Figure 1.5: Untargeted metabolomics workflow. The typical workflow of untargeted metabolomics consists of the following steps: metabolite extraction, data acquisition, data processing, data analysis, metabolite identification, and data interpretation. (a) Picture was taken from XCMS (metabolomics suite for data processing).



1.5 Diabetes Mellitus

Burden

Diabetes mellitus is one of the major global health emergencies of our century. Together with cardiovascular diseases, cancer and chronic respiratory diseases, diabetes is one of four priority non-communicable diseases targeted for action by world leaders. It is a debilitating disease that affects millions of individuals worldwide and is increasing in prevalence at a dramatic rate.^{25,26} Globally, the number of people living with diabetes has risen from 108 million in 1980 to 422 million in 2014.²⁷ Global prevalence of diabetes was 8.8% in 2015 and is projected to rise to 10.4% in 2040.²⁸ This leads to an increase in health expenditure from 673 billion in 2015 to 802 billion in 2040.

According to the World Health Organization, high blood glucose is the third highest risk factor for premature mortality, after high blood pressure and tobacco use.²⁹ With its high prevalence and numerous devastating complications, diabetes brings substantial economic loss and poses a heavy burden on the world's healthcare systems.

Types of Diabetes

Diabetes is a complex and heterogeneous disorder. It is a chronic metabolic disease that is clinically defined and characterized by the increase in blood glucose levels, known as hyperglycemia. There are two principle forms of diabetes: type 1 and type 2.

Type 1 diabetes occurs due to selective autoimmune destruction of pancreatic beta cells leading to insulin deficiency and hence hyperglycemia.³⁰ This disease accounts for

approximately 7% to 12% of all diabetes and can affect people of any age, but onset usually occurs in children or young adults.²⁸ Although type 1 is less common than type 2, it is increasing in prevalence by approximately 3% each year globally. Risk factors for this type of diabetes include genetics, infections and other environmental influences. Type 1 diabetes appears very suddenly and is currently incurable.

Type 2 diabetes results from both insulin resistance and impaired beta-cell function.³¹ This type of diabetes has a complex phenotype with defects in insulin secretion, increased hepatic glucose production and resistance to the action of insulin; all of which contribute to the development of overt hyperglycemia. This disease accounts for approximately 87% to 91% of all diabetes and usually occurs in adults, but is increasingly being seen in children.²⁸ Risk factors for this type of diabetes include genetics, obesity, poor nutrition and past history of gestational diabetes. Type 2 diabetes can go unrecognized for years; however, it can often be managed with life styles modifications (i.e. healthy diet and increased physical activity).

Diabetic Complications

The vascular complications of diabetes can be divided into two subtypes: microvascular and macrovascular. Microvascular complications include retinopathy, neuropathy and nephropathy, whereas macrovascular complications include cardio- and cerebro-vascular disease.

The most serious diabetic complication, and the leading cause of death in both type 1 and type 2 diabetes, is cardiovascular disease (CVD).^{32–34} In fact, approximately three out of

four diabetic patients will die of CVD. CV risk in humans is increased by 20% for every 1.5 mM increase in blood glucose.³⁵ Despite established progressive relationship between diabetes and CVD, the lack of understanding of the underlying molecular mechanisms linking diabetes and CVD complicates the development of preventative and treatment strategies.

The relationship between diabetes and CVD is further complicated by a number of metabolic and homeostatic deviations such as obesity, hypertension and dyslipidemia.^{36,37} This complex interplay is one of the reasons why numerous basic research and clinical trials carried out to investigate the connection between hyperglycemia and CVD have failed to formulate a clear and definitive correlation. As such, CVD continues to take away the lives of millions of diabetic individuals. Expanding the knowledge of how diabetes promotes atherosclerosis, which is a major underlying cause of CVD, will greatly assist in the development of prognostic methods, effective therapeutic and preventative strategies for CVD in people with diabetes.

1.6 Atherosclerosis

Atherosclerosis is a multifactorial and progressive disease of the medium-large arteries.³⁸ The hallmarks of this disease are the accumulation of lipid and inflammatory factors within the arterial wall that results in the hardening and narrowing of blood vessels. Decades of research have significantly increased our understanding of the cellular mechanisms that define the pathophysiology of atherosclerosis.^{39–42} Yet, despite these advances, many questions regarding the specific molecular mechanisms underlying the pathogenesis and progression of atherosclerosis remain unanswered. Furthermore, despite great advances in technologies to detect atherosclerosis, the presence of this disease is often not revealed until the latest stage, which presents as a myocardial infarction or stroke.

Pathology

Atherosclerosis is characterized by the accumulation of lipids, inflammatory factors and fibrous elements in the inner lining of the artery wall (Figure 6).³⁸ The pathogenesis of atherosclerosis is initiated by the activation and/or injury at the endothelial cells, which triggers a cascade of inflammatory responses including upregulation of membrane adhesion molecules and enhancement of endothelial permeability.⁴³ This mediates the attachment and infiltration of blood-borne low-density lipoproteins, monocytes and T cells into the sub-endothelial intima. Subsequently, intimal monocytes differentiate into macrophages that endocytose cellular debris and modified lipoprotein particles giving rise to the formation of macrophage foam cells. These cellular events contribute to the

development of fatty streaks, which are the hallmark features of early atherosclerotic lesions.

Within the arterial intima, lipid-engorged macrophages secrete pro-inflammatory cytokines and growth factors, intensifying the local inflammatory environment.⁴⁴ This causes the fatty streak to progress into a more advanced plaque, which is characterized by the migration of medial smooth muscle cells into the intima. Intimal smooth muscle cells proliferate and secret extracellular matrix proteins that form a fibrous cap covering the lesions. Lipid-engorged macrophage foam cells undergo apoptosis and release internal lipid contents, leading to the formation of acellular regions called the necrotic core. Large necrotic cores are associated with destabilized lesions that are prone to rupture.⁴⁵ When lesion rupture occurs, the contents of the lesion come into contact with blood, triggering atherothrombosis that can occlude the artery. Lesion rupture/atherothrombosis is the major cause of acute coronary syndrome and CV death.

Figure 1.6: Pathogenesis and progression of atherosclerosis. A) Inflammatory activation and/or injury at the endothelial cells initiate atherogenesis. This injury triggers the infiltration of monocytes and T cells into the sub-endothelial intima. B) Intimal monocytes differentiate into macrophages that endocytose modified lipoprotein particles forming macrophage foam cells, which is the major component of fatty streaks. C) Advanced plaques are characterized by the migration of medial smooth muscle cells into the intima. Smooth muscle cells proliferate and secret extracellular matrix proteins that form a fibrous cap covering the lesions. The necrotic core, which forms as a result of macrophage foam cell apoptosis, is associated with destabilized lesions. D) Lesion rupture triggers atherothrombosis that can occlude the artery.



1.7 Research Goal

The prevalence of diabetes mellitus is dramatically increasing worldwide. Approximately three out of four diabetic patients will die of cerebro- and cardiovascular disease. However, the molecular mechanisms by which diabetes promotes atherosclerosis are not well understood. This has impeded the development of strategies to block or slow atherogenesis in diabetic patients. Comprehensive metabolomics offers a novel approach to investigate this problem as it is capable of providing a global snapshot of the dynamic intracellular changes associated with a particular pathophysiological state. This allows the identification of novel or unanticipated pathways and biomarkers indicative of metabolic alterations related to pathology of diabetic atherosclerosis.

We hope the findings discovered in this thesis will expand the understanding of specific agents and pathways involved in hyperglycemia-induced accelerated atherosclerosis, which will assist in the development of diagnostic methods and effective therapeutic and preventative strategies for CVD in people with diabetes.
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CHAPTER 2

General Hypothesis and Objective

2.1 Hypothesis

We hypothesize that the accelerated development of atherosclerosis, due to genetic and/or environmental factors, will be associated with specific changes in the metabolome. As such, metabolomics techniques can be used to discover novel and unanticipated metabolic alterations in the pathogenesis and progression of hyperglycemia-induced accelerated atherosclerosis.

2.2 Objective

The global objective of these studies is to use murine models of hyperglycemia-induced accelerated atherosclerosis in combination with metabolomics techniques to explore the association between hyperglycemia and atherosclerosis.

The specific aims of these studies are to:

- Investigate the pathogenesis and progression of atherosclerosis in murine model at different stages of disease progression
- 2. Investigate different mouse models of hyperglycemia-induced accelerated atherosclerosis
- 3. Investigate the molecular alterations by which hyperglycemia promotes accelerated atherosclerosis in three distinct disease mouse models

CHAPTER 3

Comprehensive Plasma Metabolomic Analyses of Atherosclerotic Progression Reveal Alterations in Glycerophospholipid and Sphingolipid Metabolism in Apolipoprotein E-deficient Mice

3.1 Chapter Preface

Atherosclerosis is the major underlying cause of most cardio- and cerebrovascular disease. The objective of this chapter is to investigate the pathogenesis and progression of atherosclerosis in murine model at different stages of disease progression.

In this paper, we identify changes in specific plasma-borne metabolites that are associated with the pathogenesis of atherosclerosis. We show that comprehensive metabolomic techniques can be used to detect changes in plasma composition that are associated with early atherosclerotic progression in murine models of atherosclerosis. We present findings that suggest specific metabolomic profiles can be used to delineate the stage of disease progression. We also discovered specific metabolic pathways that have not been previously linked to atherosclerosis. Together, these results outline a novel approach that can be used to diagnose disease, delineate disease mechanisms, and identify new targets to potentially impede disease progression.

This study was conceived and designed by VTD and GHW. VTD, AH and YS performed mouse experiments. VTD and LHZ conducted and analyzed histochemistry and

immunofluorescence experiments. VTD performed metabolomic experiments and analyses. VTD wrote this manuscript in collaboration with GHW.

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3.2 Abstract

Atherosclerosis is the major underlying cause of most cardiovascular diseases. Despite recent advances, the molecular mechanisms underlying the pathophysiology of atherogenesis are not clear. In this study, comprehensive plasma metabolomics were used to investigate early-stage atherosclerotic development and progression in chow-fed apolipoprotein E-deficient mice at 5, 10 and 15 weeks of age. Comprehensive plasma metabolomic profiles, based on 4365 detected metabolite features, differentiate atherosclerosis-prone from atherosclerosis-resistant models. Metabolites in the sphingomyelin pathway were significantly altered prior to detectable lesion formation and at all subsequent time-points. The cytidine diphosphate-diacylglycerol pathway was up-regulated during stage I of atherosclerosis, while metabolites in the phosphatidylethanolamine and glycosphingolipid pathways were augmented in mice with stage II lesions. These pathways, involving glycerophospholipid and sphingolipid metabolism, were also significantly affected during the course of atherosclerotic progression. Our findings suggest that distinct plasma metabolomic profiles can differentiate the different stages of atherosclerotic progression. This study reveals that alteration of specific, previously unreported pathways of glycerophospholipid and sphingolipid metabolism are associated with atherosclerosis. The clear difference in the level of several metabolites supports the use of plasma lipid profiling as a diagnostic tool of atherogenesis.

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3.3 Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide^{1,2}. Risk factors of CVD include obesity, dyslipidemia, stress, smoking, hypertension and diabetes mellitus³. The major underlying cause of most CVD is atherosclerosis, which is a multifactorial and progressive disease of the medium-large arteries⁴. Atherosclerosis is characterized by the accumulation of lipids and inflammatory factors in the arterial walls. Despite recent advances, the molecular mechanism(s) underlying the pathogenesis and progression of atherosclerosis are still not completely understood⁵⁻⁷. This has complicated the diagnosis, treatment and prevention of atherosclerosis-related diseases.

Metabolomics, which is the study of small molecules, offers a novel approach to investigate disease mechanisms and identify disease biomarkers, as it is capable of providing a global snapshot of the dynamic intracellular changes associated with a particular physiological or pathological state^{8,9}. Moreover, the levels of metabolites can more accurately reflect the functional status of an organism compared to the other 'omics' studies, such as genomics or proteomics, because metabolic fluxes are regulated not only by gene expression, but also by environmental stresses¹⁰. Therefore, the status of specific metabolite levels is a critical indicator of human health or disease.

In this study, comprehensive metabolomic techniques were used to investigate atherosclerosis in chow-fed apolipoprotein E-deficient (ApoE^{-/-}) mice at 5, 10 and 15 weeks of age in order to discover novel metabolic pathways and metabolisms associated

with early stages of atherosclerotic development and progression. Comprehensive metabolomics involves the global analysis of the metabolome^{10,11}. This type of approach is capable of revealing novel and unanticipated molecular perturbations as it represents an unbiased examination of the association between the levels of metabolites and their interconnectivity in multiple metabolic pathways, with relation to a phenotype or genotype.

The ApoE^{-/-} mouse model was chosen to investigate atherosclerosis because it is a wellestablished model in which all recognized stages of atherogenesis can be observed^{12,13}. In addition, the complexity and morphological features of atherosclerotic lesions that develop in this mouse model are very similar to those in humans^{14,15}. Atherosclerosis in ApoE^{-/-} mice is driven by impaired clearance of cholesterol-enriched lipoproteins, which results in elevated levels of plasma cholesterol and atherogenic remnants. In this study, mice were fed a standard chow diet to avoid other metabolic complications associated with high fat diet including weight gain, insulin resistance and non-alcoholic fatty liver disease. We chose to investigate early stages of atherogenesis to address current limitations in our understanding of how the pathogenesis of atherosclerosis initiates, as well as the lack of diagnostic tools for early-stage detection of this disease.

3.4 Results

Atherosclerosis progresses with aging in ApoE^{-/-} mice

No significant differences were observed in body weight, fasting blood glucose or plasma triglycerides levels between atherosclerosis-prone homozygous ApoE^{-/-} mice and agematched atherosclerosis-resistant heterozygous ApoE^{+/-} mice (Supplementary Table S1). Plasma total cholesterol, as well as very low-density, intermediate-density and lowdensity lipoprotein cholesterol were significantly elevated in ApoE^{-/-} mice, relative to ApoE^{+/-} mice, at all ages (Supplementary Fig S1). These atherogenic lipoproteins were undetectable in the control ApoE^{+/-} mice. ApoE^{-/-} mice also showed a moderate decrease in high density lipoprotein levels, compared to ApoE^{+/-} mice at all ages.

Atherosclerotic lesions were not detected in any of the 5-week-old ApoE^{-/-} mice (Fig. 1) or at any age in ApoE^{+/-} mice (Supplementary Fig. S2). Fatty streaks were observed in ApoE^{-/-} mice at 10 weeks of age while more advanced lesions, with areas of necrosis and crystallized cholesterol, were detected in 15-week-old ApoE^{-/-} mice (Fig. 2). Atherosclerotic lesions in the aortic sinus of 15-week-old ApoE^{-/-} mice were approximately 4.2 fold larger (*p*-value <0.0001) than those in 10-week-old ApoE^{-/-} mice (Fig. 1).

Lesional macrophage/foam cell content, adjusted to lesion area, was significantly elevated in ApoE^{-/-} mice at 10 weeks, relative to 15 weeks of age (Fig. 2). Intimal smooth muscle cells, collagen and apoptotic cells, which are indicative of more complex and advanced lesions, were detected only in 15-week-old ApoE^{-/-} mice (Fig. 2 and

Supplementary Fig. S3). Based upon these morphological features, atherosclerotic lesions detected in 10- and 15-week-old ApoE^{-/-} mice were classified as stage I (early) and stage II (intermediate) lesions, respectively^{12,14}.

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Figure 3.1: Atherosclerotic progression in ApoE^{-/-} **mice.** (A) Representative images of aortic cross-sections of 5, 10 and 15-week-old mice stained with Masson's trichrome. Atherosclerotic lesions are indicated by arrows and outlined in yellow. Quantification of atherosclerotic lesion area (B) and volume (C) of 5, 10 and 15-week-old ApoE^{-/-} mice. n=7/group, *p<0.05, ****p<0.0001. Data are presented as the mean ±SD.



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Figure 3.2: Characterization of atherosclerotic progression in ApoE^{-/-} mice. (A)

Representative images of aortic cross-sections of ApoE^{-/-} mice at 5, 10 and 15 weeks of age stained with the indicated chemicals or antibodies. Atherosclerotic plaques are outlined by the dashed yellow line. (B) Quantification of necrotic core, macrophage and intimal smooth muscle cell content in atherosclerotic lesions found in ApoE^{-/-} mice at prestage I, stage I and stage II of atherosclerosis. Percentages were calculated by dividing positively stained areas by total lesion area. n=7/group, **p<0.01. Data are presented as the mean ±SD. Blue: DAPI, green: macrophage, red: smooth muscle cell.



Comprehensive plasma metabolomic profiles differentiate atherosclerosis-prone and atherosclerosis-resistant models

Mouse plasmas were extracted and analyzed by liquid chromatography coupled to a mass spectrometer. We detected 2435 and 1930 metabolite features (n=4365 total) in negative and positive ionization modes, respectively. The principle component analysis score plot showed a tight clustering of quality control samples (Supplementary Fig. S4), indicating good instrumental reproducibility throughout the period of analysis.

Multivariate analyses were used to correlate the metabolomic variables with genotypic and phenotypic patterns. The comprehensive plasma metabolomic profiles of ApoE^{-/-} mice at pre-stage I, stage I and stage II of atherosclerosis were clearly separated from those of age-matched ApoE^{+/-} mice in the partial least squares discriminant analysis (PLS-DA) plot constructed based on the 4365 total metabolite features (Fig. 3A-C). At five weeks of age, prior to any detectable phenotypic differences, the metabolomic profile of ApoE^{-/-} mice was already distinct from that of ApoE^{+/-} mice with moderate prediction accuracy (Q² of 0.53). The prediction accuracy increases significantly at 10 and 15-week time points with Q² value of 0.93 and 0.90 respectively, relative to the 5-week time point. A Q² value greater than 0.9 indicates a highly robust multivariate model. Similarly, the separation component between the metabolomic profiles of ApoE^{-/-} and ApoE^{+/-} mice (component 1 in the PLS-DA score plot) also increases substantially from 5 to 10 and 15week time points (20.2% to 31.2% and 30.7%, respectively).

Volcano plots were used to visualize the number of metabolite features that were significantly altered between ApoE^{-/-} and age-matched ApoE^{+/-} mice (Fig. 3D). Over 200 metabolite features were found to be significantly altered (*p*-value <0.01 and fold change >2) between the two mouse models at each time point. At a more extreme significance cut-offs (*p*-value <0.0001 and fold change >5), we detected 12, 29 and 51 altered metabolite features at the 5-, 10- and 15-week time point, respectively. This indicates that the number of newly emerging significant features, i.e., not observed in the earlier time-point, also increases substantially from pre-stage I to stage I and stage II of atherosclerosis. These findings are consistent with the clinical and physiological observation of the progressive phenotypic differences in atherosclerotic progression.

Figure 3.3: Comprehensive plasma metabolomic profiles differentiate

atherosclerosis-prone and atherosclerosis-resistant models. Direct comparison of the PLS-DA score plots of 4365 total metabolite features detected in plasma of (A) 5-week, (B) 10-week and (C) 15-week ApoE^{-/-} mice versus age-matched ApoE^{+/-}mice. Each dot represents the plasma metabolomic profile of a single mouse. R² and Q² indicate prediction accuracy and model robustness, respectively. (D) Volcano plots indicating the number of metabolite features that were significantly altered between ApoE^{-/-} and ApoE^{+/-} mice at 5, 10 and 15-week time points. Each plot encompasses 4365 metabolite features. Red dots indicate metabolite features with *p*<0.01 and fold change >±2. n=7 per ApoE^{-/-} group and n=4-6 per ApoE^{+/-} group.

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Identification of significantly altered plasma metabolites between the atherosclerosis-prone and age-matched atherosclerosis-resistant models

A Venn diagram, summarizing the identification of extremely different metabolites (p-value <0.0001 and fold change >5) between ApoE^{-/-} and age-matched ApoE^{+/-} mice, is shown in Figure 4A and Supplementary Table S2. The concentrations of several species of diacylglycerol, phosphatidylcholine and sphingomyelin were altered at all three time points in ApoE^{-/-} mice, compared to age-matched ApoE^{+/-} mice. These metabolites are involved in the sphingomyelin biosynthetic pathway (Fig. 4B).

The levels of phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolphosphate were uniquely elevated at 10-week point in ApoE^{-/-} mice, relative to ApoE^{+/-} mice. This suggests an up-regulation in the cytidine diphosphate-diacylglycerol pathway (glycerophospholipid metabolism) during the early stage of atherosclerotic progression (Fig. 4B). At the 15-week time point, we observed an alteration in the concentration of several species of hexosyl-ceramide, dihexosyl-ceramide and phosphatidylethanolamine between the two mouse models. These results suggest that glycosphingolipid (sphingolipid metabolism) and phosphatidylethanolamine pathways (glycerophospholipid metabolism) are altered during the intermediate stage of atherosclerotic progression (Fig. 4B).

Overall, the majority of the metabolites showing the greatest variation between the atherosclerosis-prone and atherosclerosis-resistant models are involved in glycerophospholipid and sphingolipid metabolism. This suggests that these pathways

play important roles in the pathogenesis and progression of early stage atherosclerosis. Approximately half of the extremely altered metabolite features remain unidentified.

Figure 3.4: Identification of significantly altered metabolites between the atherosclerosis-prone and age-matched atherosclerosis-resistant models. (A) Venn diagram showing extremely altered metabolites (*p*<0.0001 and fold change >±5) that were uniquely altered and shared between pre-stage I, stage I and stage II of atherosclerosis. (B) Extremely altered metabolites were mapped into metabolic pathways. Metabolites in green were altered at all three time points, whereas those in orange and blue were altered at 10 and 15-week time point, respectively. The dashed box indicates metabolites that were not detected in the analysis. CAR: acylcaritine, CDP: cytidine diphosphate, Cer: ceramide, CL: cardiolipin, DAG: diacylglycerol, HexCer: hexosylceramide, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PIP: PI-phosphate, SM: sphingomyelin, TAG: triacylglycerol.

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Select plasma metabolomic profiles distinguish different stages of atherosclerotic progression

In order to further investigate the metabolic changes associated with atherosclerotic progression, we examined the metabolite variations over the three stages of atherosclerosis observed in $ApoE^{-/-}$ mice at 5, 10 and 15 weeks of age.

A heat map of the top 2000 metabolite features ranked by *p*-value (two-way ANOVA) is presented in Fig. 5A. This analysis depicts the overall data structure in terms of two factors: genotype and age. A specific subset of metabolite features clearly differentiates the ApoE^{-/-} and ApoE^{+/-} genotypes, independent of age (Fig. 5A-i, iii). A separate subset of features displays similar patterns throughout the three time points, between the two genotypes (Fig. 5A-ii). The features that are similarly altered in both mouse models are most likely associated with age, rather than the pathophysiology of the disease. These age-associated metabolite features were removed to ensure that all detectable metabolic changes are most closely associated to atherosclerotic progression.

A total of 137 metabolites were identified to be significantly altered (ANOVA *p*-value <0.01) between the three atherosclerotic stages in ApoE^{-/-} mice, after correcting for ageassociated features (Supplementary Table S3). Select plasma metabolomic profiles, constructed based on these significantly altered metabolites, differentiate pre-stage I, stage I and stage II of atherosclerosis in ApoE^{-/-} mice (Fig. 5B) with very good prediction accuracy (Q² value of 0.88). The observed separation trend was consistent with the

progression of atherosclerosis in ApoE^{-/-} mice from pre-stage I to stage I and stage II of atherosclerosis.

Figure 3.5: Select plasma metabolomic profiles distinguish different stages of atherosclerotic progression in ApoE^{-/-} mouse model. (A) Heat map indicating the relative levels of the top 2000 metabolite features ranked by between-subject two-way ANOVA *p*-value. The overall data is depicted in terms of genotype and age. Metabolites that exhibit similar (ii) or different patterns (i, iii) between the two genotypes are indicated. Each line represents a single metabolite, coloured by its abundance (red: high abundance and blue: low abundance). (B) PLS-DA score plot of 137 significantly altered metabolites (*p*<0.01) detected in plasma of ApoE^{-/-} mice at pre-stage I, stage I and stage II of atherosclerosis. Each dot represents the plasma metabolomic profile of a single mouse. R² and Q² indicate prediction accuracy and model robustness, respectively. n=7/group.

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Component 1 (32.6%)

Pathway analysis of atherosclerotic progression

Pathway analysis was performed on the 137 significantly altered plasma metabolites between the three atherosclerotic stages observed in ApoE^{-/-} mice. The results from integrating enrichment and pathway topology analyses¹⁶ were used to map out the identified metabolites into specific metabolic pathways. On this basis, 29 pathways were found to be potentially affected during the progression of atherosclerosis (Fig. 6, Supplementary Table S4).

Glycerophospholipid and sphingolipid metabolism were again the most significantly affected pathways based on *p*-values and pathway impact scores. Other impacted pathways included arginine and proline metabolism (amino acid metabolism), nitrogen metabolism (energy metabolism) and pentose phosphate pathway (carbohydrate metabolism). Metabolites from sphingolipid (Fig. 7A) and glycerophospholipid (Fig. 7B) metabolism were mapped into respective metabolic pathways. Most of the key intermediate metabolites involved in these metabolisms were significantly altered between the three stages of atherosclerosis observed in ApoE^{-/-} mice.

Figure 3.6: Pathway analysis of atherosclerotic progression. Pathway analysis was performed on the 137 significantly altered metabolites (p<0.01) between the three stages of atherosclerosis observed in ApoE^{-/-} mice. All matched pathways are plotted according to p-value from pathway enrichment analysis and pathway impact score from pathway topology analysis. Colour gradient and circle size indicate the significance of the pathway ranked by p-value (yellow: higher p-values and red: lower p-values) and pathway impact score (the larger the circle the higher the impact score), respectively. Significantly affected pathways with low p-value and high pathway impact score are identified by name.



Figure 3.7: Sphingolipid and glycerophospholipid metabolism. Metabolites from (A) sphingolipid and (B) glycerophospholipid metabolism were mapped into respective metabolic pathways. All metabolites presented were significantly altered (ANOVA p<0.01) between the three atherosclerotic stages detected in ApoE^{-/-} mice. Each lipid species is showed in one box. In the case of within-species metabolites display different alteration trends between the three stages of atherosclerosis; two metabolites (randomly selected) are shown. Metabolites encircled by a dashed line (sphingosine and cytidine diphosphate diacylglycerol) were not detected. Data are presented as fold change relative to the level of the indicated metabolite detected in plasma of 5-week-old ApoE^{-/-} mice. n=7/group; *5-week vs. 10-week, †5-week vs. 15 week, ^{\$}10-week vs. 15-week; 1, 2, 3 and 4 asterisks indicate p < 0.05, < 0.01, < 0.001 and < 0.0001, respectively. Data are presented as the mean ±SD.

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3.5 Discussion

The objective of this study was to identify alterations in specific plasma-borne metabolites that are associated with the pathogenesis of atherosclerosis. Since atherosclerosis is a progressive disease, age-associated confounding features must be considered in the investigation of disease progression. As such, age-matched atherosclerosis-resistant ApoE^{+/-} littermates were used as controls to account for age-related metabolic changes. The detectable molecular alterations can be attributed to the increase in plasma cholesterol and associated with the pathophysiology of atherosclerosis. This study was performed with female mice because they have faster and more pronounced early atherosclerotic progression, compared to age-matched male ApoE^{-/-} mice¹⁷.

The development and progression of atherosclerosis observed in this study was consistent with previous results in chow-fed ApoE^{-/-} mice^{12,14}. Comprehensive plasma metabolomic profiles differentiated atherosclerosis-prone and atherosclerosis-resistant models. The degree of separation in the metabolomic profiles, as well as the number of extremely altered metabolites between the two mouse models increased substantially from 5 to 10 and 15-week time points, which mirrors the progressive differences in the phenotypes between ApoE^{-/-} and ApoE^{+/-} mice over time. Identification of significantly altered metabolites between the atherosclerosis-prone and age-matched atherosclerosis-resistant models revealed changes in pathways involving glycerophospholipid and sphingolipid metabolism. These lipids are not only structural components of biological membranes,

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but also act as signaling molecules and bioactive mediators in important atherosclerosisrelated cellular processes, including apoptosis, angiogenesis, inflammation and proliferation^{18–22}. Disturbances in their metabolism have been previously observed in atherosclerosis-related diseases^{23–25}; however, to the best of our knowledge, we are the first group to identify the specific pathways within these metabolic systems that are associated with the specific stages of atherosclerotic progression.

Metabolites in the sphingomyelin biosynthetic pathway are significantly altered prior to detectable lesion formation and at all subsequent time points. This pathway is an important link between glycerophospholipid and sphingolipid metabolism. Phosphatidylcholine and sphingomyelin are the two most abundant phospholipids in mammalian plasma^{26,27}. These phospholipids also appear in all major lipoproteins and are abundant in atherosclerotic lesions. Both plasma phosphatidylcholine and sphingomyelin have been identified as independent risk factors for coronary heart disease^{28–31}.

Metabolites in the glycosphingolipid pathway were augmented in ApoE^{-/-} mice with intermediate lesions. Glycosphingolipids are involved in the hydrolytic pathway of ceramide metabolism. Altered levels of hexosyl-ceramide suggests a dysregulation of ceramide synthase, which is an important mediator of sphingolipid metabolism and a key regulator of endoplasmic reticulum (ER) homeostasis³². Indeed, increased levels of hexosyl-ceramide have been associated with the upregulation of C/EBP homologous protein (CHOP), an indicator of ER stress^{33,34}. Previous findings from our lab and others have implicated ER stress in the development and progression of atherosclerosis^{35–38}.

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We further investigated the progression of atherosclerosis within the atherosclerosisprone model. Atherosclerotic progression has previously been explored at the metabolomics level^{39,40}. However, these studies did not appropriately account for ageassociated confounding metabolites in disease progression. Moreover, they did not use a high-throughput, high-sensitivity and comprehensive analytical system, as in this study. This system is capable of simultaneously detecting a wide range of polar and nonpolar molecules from complex mixtures such as bio-fluids⁴¹.

After removing age-associated confounding metabolite features, 29 metabolic pathways associated with multiple metabolisms were found to be potentially affected during the progression of atherosclerosis within ApoE^{-/-} mice. These findings are consistent with our understanding of the multifactorial nature of atherosclerosis, which may reflect the multiple cellular and molecular pathways involved in the development and progression of this disease^{4,6,7}. Glycerophospholipid and sphingolipid metabolism were again the most significantly altered pathways during the progression of atherosclerosis. We also observed a disturbance in arginine metabolism. The level of plasma arginine decreased with atherosclerotic progression. Depletion of arginine is an indicator of systemic inflammation, which is the hallmark of all stages of atherosclerosis^{42,43}.

Since lipids are readily interconverted by complex enzymatic machineries, their specific roles and functions are structure and context specific. In this study, several metabolites from the same lipid species showed different trends of alteration between the three stages of atherosclerosis. For example, within the ceramide species, the concentration of those

with 1,3-*di*hydroxy long-chain bases ('d' designation) increased with atherosclerotic progression, whereas those with 1,3,4-*tri*hydroxy ('t' designation) showed opposite trend. Thus, despite belonging to the same lipid species, metabolites with different structural configuration, degree of saturation and/or length of carbon chain may exert different biological functions.

Lipids have diverse biological roles and their metabolism is extremely complex. As such, it is possible that no single lipid species will be suitable as a disease biomarker. Profiling several lipid species may be of more value in predicting the development and progression of a disease. In this study, the clear difference in the plasma levels of several metabolites, between the atherosclerosis-prone and atherosclerosis-resistant models, supports the feasibility of this concept. Metabolites that are altered prior to detectable lesion formation have the potential to be predictive biomarkers that may be used to facilitate the detection of the disease prior to the appearance of clinical phenotypes. Alternatively, metabolites that were uniquely altered at stage I or stage II of atherosclerosis could potentially be used as prognostic biomarkers in order to aid in the identification of the specific stages of disease progression.

This study identified systemic changes of specific plasma metabolites that are associated with the progression of atherosclerosis in ApoE^{-/-} mice. These molecular alterations are mainly attributed to the increase in plasma cholesterol and atherogenic remnant lipoproteins that can be associated with the pathophysiology of atherosclerosis. As the present study was designed to identify metabolites that are associated with atherogenesis,

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future studies should explore the cause-effect nature of the relationship of these metabolites with atherogenesis in order to determine the roles of these pathways in the development and progression of this disease. In addition, knowledge of the origin (i.e. tissue localization) and source (i.e. free or lipoprotein-associated lipid) of the altered metabolites will require further investigation. As there are no human equivalents to the atherogenic remnant lipoproteins that accumulate in the plasma of ApoE^{-/-} mice, the findings in this study are potentially dependent on the lipoprotein profiles of this mouse model. The relevance of these changes in other models of atherosclerosis as well as in plasma samples from humans with and without atherosclerosis will be explored.

In conclusion, dysregulation of lipid metabolism has been associated with numerous pathological conditions including cardiovascular diseases^{44,45}. Therefore, lipid metabolism has been the target for the prognosis, diagnosis and treatment of these diseases. Based on the results of this study, alterations in glycerophospholipid and sphingolipid metabolism may be a metabolic footprint of atherosclerotic progression. As such, they represent potential targets for further investigation. Our data also suggests that specific branches of these two pathways may be involved at different stages of atherosclerosis. Compared to cholesterol and glycerolipids, our knowledge of the roles of sphingolipid and glycerophospholipid in atherosclerosis is still very limited. Future research will explore the role(s) of each lipid class and individual lipids in atherogenesis, as well as their relevance in human patients with cardiovascular disease.

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3.6 Methods

Animal models

ApoE^{-/-} mice were crossed with ApoE^{+/-} mice to produce ApoE^{-/-} and control ApoE^{+/-} littermates. Litters were genotyped and randomly assigned into age groups. Four week old female ApoE^{-/-} and ApoE^{+/-} mice (B6.129P2-ApoE^{tm1Unc}) in a C57BL6 genetic background were fed a chow diet (TD92078, Harlan Teklad, WI) ad libitum for 1, 6 or 11 weeks and maintained on a 12-hour light/dark cycle throughout the study. Subsets of mice were sacrificed at 5, 10 and 15 weeks of age (n=7 per ApoE^{-/-} group and n=4-6 per Apo $E^{+/-}$ group). Mice were fasted for 6 hours and anaesthetized with 3% isoflurane. Blood was collected by cardiac puncture and mice were euthanized by cervical dislocation. Vasculature was flushed with saline and tissues were collected and prepared for analyses. Plasma total cholesterol and triglycerides were measured using Infinity reagents (Thermo-Scientific). Plasma lipoproteins were separated by fast protein lipid chromatography and total cholesterol was measured in each fraction using the Infinity cholesterol assay. All animal procedures were approved by and performed in accordance with the McMaster University Animal Research Ethics Board and conform with the guidelines of the Canadian Council on Animal Care.

Histochemistry and immunofluorescence analyses

Harvested hearts were fixed in formalin. Aortas, including aortic sinus, were sectioned (5µm/section), as previously described⁴⁶. Atherosclerotic lesions, necrotic core and lesion collagen content were visualized and quantified by staining serial sections with Masson's
Trichrome (Sigma-Aldrich). Apoptotic cell death was measured according to the manufacturer's instructions (Promega DeadEndTM Fluorometric TUNEL System). Different serial sections were stained with primary antibodies against the macrophage marker CD107b (Mac3, BD Pharmingen) or the vascular smooth muscle cell marker α -actin (Santa Cruz). Negative controls were stained with pre-immune rat IgG (Vector) or mouse IgG (Invitrogen), instead of primary antibodies, to correct for non-specific staining. Sections were stained with the nuclear counterstain 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Stained sections were imaged using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Lesion area and positively immunostained areas were quantified using Image J (1.48v) software. Lesion volume was computed as area under the curve of lesion area *versus* distance from the aortic sinus⁴⁶.

Metabolomic analysis

Sample preparation: Ice-thawed plasma was extracted using an ice-cold mixture of 1:1 methanol:ethanol (v/v) containing 10 μ M *L*-phenylalanine-d₈ as the internal standard. The ratio of extraction solvent to plasma was 4:1 (v/v). The resulting mixture was vortexed for 2 min prior to centrifugation at 4°C and 10,000 xg for 10 min. Pooled plasma extracts were run as a quality control measure to assess for system drift.

Chromatographic separation conditions: In order to expand the metabolomic coverage to both polar and non-polar metabolites in plasma, two 2.1 x 50 mm columns (SeQuant ZIC-cHILIC, 3 μ m followed by Halo C8, 2.7 μ m) were directly coupled in series as an

orthogonal separation system under a single compatible solvent elution program, as previously described⁴¹. A 2 μ L plasma extract was injected per run and analyzed with a linear gradient at a flow rate of 200 μ L/min and a column temperature set at 40°C. The LC system used was an Agilent 1200 RR series LC system (Agilent Technologies Inc., CA). Mobile phase A was 100% acetonitrile (LC-MS grade, Sigma Aldrich) and mobile phase B was 10 mM ammonium acetate (Sigma Aldrich) adjusted to pH 3 with formic acid. Gradient elution started with 95% A for 0.5 min and then linearly decreased to 30% A at 13 min, held at 30% A for 2 min followed by a ramping up to 95% A for 1 min. The columns were then re-equilibrated for 15 min at 95% A prior to subsequent sample analysis. Samples were analyzed in a random order.

Mass spectrometer parameters: Plasma extracts were analyzed by TOF-MS using a Bruker micrOTOF II (Bruker Daltonics) mass analyzer equipped with an electrospray ionization (ESI) source (Agilent Technologies Inc.). Each plasma extract sample was run separately in positive and negative electrospray modes. The TOF-MS system was operated with the following settings: capillary potential of +3800 V (ESI-) or -4500 V (ESI+), nebulizer gas (N₂) pressure of 3.0 bar, dry gas (N₂) flow rate of 6.0 L/min, a chamber temperature of 250°C, mass range of 50–1000 m/z, and a scan rate of 2 Hz. A tuning mix solution was used for external mass calibration of the TOF-MS system daily.

Data analysis: The acquired mass spectra were calibrated internally using endogenous sodium formate clusters (Bruker Daltonics DataAnalysis 4.0). Calibrated LC-MS files were then converted to mzXML format (Bruker CompassXport) and processed with the

XCMS and CAMERA software package (Scripps Institute for Metabolomics)⁴⁷. This software provides retention time alignment, metabolite feature detection, feature matching, peak integration, adduct and isotope annotation. Metabolite feature is a chromatographic peak with a unique chromatographic retention time and a unique mass-to-charge (m/z) ratio. Peak areas integrated by XCMS were normalized with the internal standard *L*-phenylalanine-d₈. Metabolite features which showed poor analytical reproducibility (CV >30% in pooled samples) or eluted close to the solvent front that is prone to ion suppression (k'<0.4) were removed. To correct for age-associated features that altered in similar patterns in both mouse models; we removed metabolite features that were significantly altered (ANOVA *p*-value <0.05) between the three time points in the ApoE^{+/-} control mice.

Metabolites were identified based on available authentic standards, tentatively assigned based on structural analogs or by matching their accurate mass/empirical formula with metabolite databases including METLIN, Human Metabolome Database and Lipid Map. In all cases, metabolites were annotated based on their characteristic m/z and retention time.

Statistical analysis

Principal component analysis, partial least squares discriminant analysis, heat map, pathway analysis, one-way ANOVA and between-subject two-way ANOVA were performed using MetaboAnalyst 3.0¹⁶. Auto scaling followed by log transformation was applied in all multivariate analyses. Student t-test (two-tailed, unpaired heteroscedastic)

and one-way ANOVA followed by Tukey's HSD test were performed using Microsoft Excel 2010 and GraphPad Prism (v6.01), respectively.

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CHAPTER 4

Glucosamine-induced ER stress accelerates atherogenesis: A potential link between diabetes and cardiovascular disease

4.1 Chapter Preface

Cardiovascular disease is the one of the leading causes of death worldwide and is responsible for three out of four deaths in diabetic individuals. The objective of this chapter is to investigate different mouse models of hyperglycemia-induced accelerated atherosclerosis. We also investigate the role of glucosamine-induced endoplasmic reticulum stress (ER)/unfolded protein response in diabetic atherosclerosis.

In this paper, we directly compare the glucosamine-supplemented mouse model to two different models of hyperglycemia. We show that the vascular effects of glucosamine-supplementation are comparable to streptozotocin-induced and genetically-induced (Ins2^{Akita}) hyperglycemia in terms of lesional glucosamine, ER stress levels and atherosclerotic burden. In addition, we show that a chemical chaperone (4-phenylbutyric acid) reduces ER stress levels and attenuates accelerated atherogenesis in each of these models. Together, these findings support the mechanism involving glucosamine-induced ER stress in hyperglycemia-induced accelerated atherosclerosis.

This study was conceived and designed by VTD and GHW. VTD, DRB and YS performed mouse experiments. VTD conducted and analyzed histochemistry and

immunofluorescence experiments, with the assistance of AD. VTD wrote this manuscript in collaboration with GHW.

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4.2 Abstract

Background: Cardiovascular disease is the leading cause of death worldwide and is responsible for three out of four deaths in diabetic individuals. Our lack of understanding of the molecular mechanisms linking diabetes and atherosclerosis impedes the development of effective treatment strategies. Hyperglycemia and glucosaminesupplementation have been shown to induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR) in murine models of atherosclerosis. We hypothesize that diabetes/hyperglycemia promotes atherosclerosis by a mechanism involving glucosamine-induced ER stress/UPR activation and that attenuation of ER stress, using the chemical chaperone 4-phenylbutyric acid (4PBA), will slow the accelerated development of atherosclerosis.

Methods: Hyperglycemia was induced in female Apolipoprotein E-deficient (ApoE^{-/-}) mice by multiple low-dose streptozotocin injections or by the introduction of the Ins2^{+/Akita} mutation. Glucosamine-supplementation was achieved by adding different concentrations of glucosamine (0.625–5% w/v) to the drinking water of ApoE^{-/-} mice. Subsets of mice from each group were also treated with 4PBA. The development of atherosclerosis was evaluated based on atherosclerotic lesion area and volume at the aortic sinus. Levels of protein O-linked N-acetylglucosamine (O-GlcNAc) and ER stress markers were determined in atherosclerotic lesions using immunohistochemistry and immunofluorescence staining.

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Results: Hyperglycemic and glucosamine-supplemented mouse models showed similar increases in O-GlcNAc and ER stress/UPR activation levels in atherosclerotic lesions. Lesion area was not significantly different between the three models of accelerated atherosclerosis. Glucosamine supplementation at $\geq 2.5\%$ (w/v) significantly increased lesional O-GlcNAc, UPR activation and atherosclerotic lesion area/volume, independent of changes in any measured metabolic parameters. 4PBA mitigated ER stress and attenuated accelerated atherosclerosis in both hyperglycemic and glucosamine-supplemented mouse models.

Conclusion: These findings suggest that hyperglycemia promotes accelerated atherosclerosis by a mechanism involving glucosamine-induced ER stress. Accelerated atherosclerosis can be attenuated in hyperglycemic ApoE^{-/-} mice by reducing ER stress levels.

4.3 Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. Risk factors of CVD include obesity [2], dyslipidemia [3], smoking [4], hypertension [5] and diabetes mellitus [6]. Diabetes is a debilitating disease that affects millions of individuals and is increasing in prevalence at a dramatic rate [7,8]. Although CVD is responsible for approximately 75% of deaths in individuals with diabetes [9], the development of therapeutic strategies to prevent and/or treat CVD has been impeded due to the lack of understanding of the underlying biochemical mechanisms linking diabetes to CVD. Atherosclerosis is one of the major underlying causes of CVD, and expanding our knowledge of how diabetes promotes atherosclerosis, can facilitate the development of treatment strategies to slow or prevent the development of atherosclerosis.

All forms of diabetes are characterized, and clinically defined, by increased blood glucose levels, known as hyperglycemia. In an attempt to understand the pro-atherogenic effects of hyperglycemia, most of the research has been focussed on determining the pathways affected by the increase in glucose metabolism and the oxidative stress associated with the hyperglycemia [10,11]. Although a number of pre-clinical studies supported the causative role of oxidative stress [12,13], antioxidant treatments have not demonstrated beneficial effects in reducing CVD risk in diabetic population in any of the large clinical trials in which they were tested [14,15]. This observation suggests that there are other causative mechanisms behind hyperglycemia-promoted atherosclerosis that may act independently, or work in parallel to, oxidative stress.

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Impaired endoplasmic reticulum (ER) function has been associated with the development of atherosclerosis in humans and in mouse models [16,17]. The ER is responsible for the folding, modification and trafficking of approximately one-third of all proteins produced in a typical eukaryotic cell [18]. When the influx of newly synthesized proteins exceeds the ER processing capacity, unfolded or misfolded proteins can accumulate and induce ER stress. The unfolded protein response (UPR) is a multifaceted, cellular self-defence mechanism that alleviates ER stress by attenuating *de novo* protein synthesis, increasing protein folding capacity and facilitating the degradation of irreversibly misfolded proteins [18,19]. Chronic ER stress/UPR activation has been associated with various proatherogenic processes including endothelial cell apoptosis, macrophage-foam cell inflammation and lipid accumulation [20].

The objective of this study is to investigate the role of glucosamine-induced ER stress/UPR activation in diabetic atherosclerosis. Previous research in our lab has shown that glucosamine-supplementation accelerates atherosclerosis in ApoE^{-/-} mice [21,22]. We hypothesize that the concentration of glucosamine required to promote ER stress would be similar to that required to promote atherosclerosis. Furthermore, we hypothesize that reducing ER stress levels, using the chemical chaperone 4-phenylbutyric acid (4PBA), will attenuate the accelerated development of atherosclerosis in both hyperglycemic and glucosamine-supplemented mouse models.

4.4 Results

Hyperglycemia and glucosamine-supplementation induce similar increases in vascular O-GlcNAc, ER stress and accelerated atherosclerosis in ApoE^{-/-} mice

Hyperglycemia was induced in female ApoE^{-/-} mice by multiple low-dose STZ injections, or by the introduction of the Ins2^{Akita} mutation. A separate group of ApoE^{-/-} mice were supplemented with glucosamine (5% w/v) in the drinking water. At 10 weeks of age, STZ-injected mice were severely hyperglycemic (FBG=26.9±2.1mM), Ins2^{+/Akita} mice were moderately hyperglycemic (15.0±1.1mM) and glucosamine-supplemented mice were normoglycemic (7.1±0.3mM), relative to ApoE^{-/-} controls (7.4±0.3mM) (Figure 1A). Plasma insulin levels of both hyperglycemic models were significantly lower than that of the control or glucosamine-supplemented ApoE^{-/-} mice (Figure 1B). Up to 15 weeks of age, none of the mice showed any significant changes in plasma cholesterol or triglycerides (Figure 1C, D). Consistent with previous findings, both hyperglycemia and glucosamine-supplementation significantly accelerated atherogenesis at the aortic sinus, relative to controls (Figure 2) [21,22,25]. Atherosclerotic lesion area was not significantly different between the three models of accelerated atherosclerosis.

Protein O-linked N-acetylglucosamine (O-GlcNAc) levels have been directly correlated to intracellular levels of glucosamine [26]. We found that both hyperglycemic mouse models and the glucosamine-supplemented mouse model had similar elevations in the levels of lesional O-GlcNAc, relative to ApoE^{-/-} controls (Figure 2). Glucosamine is a potent ER stress inducer in cultured vascular cells [22]. Both hyperglycemic and the

glucosamine-supplemented mouse models showed similar increases in the levels of UPR proteins GRP78/94 and CHOP within the atherosclerotic lesions. Together these data are consistent with our hypothesis that hyperglycemia promotes atherosclerosis by a mechanism involving glucosamine-induced ER stress.

Figure 4.1: Analysis of metabolic parameters in three models of accelerated atherosclerosis. Fasting (A) blood glucose, (B) plasma insulin, (C) plasma triglycerides, and (D) plasma cholesterol levels of control, STZ-injected, $Ins2^{+/Akita}$ and glucosamine-supplemented ApoE^{-/-} mice. ND indicates "not detected". n=12/group except for ApoE^{-/-} Ins2^{+/Akita} n=4/group, **p*<0.05, ***p*<0.01 relative to control mice.



Figure 4.2: Characterization of three different models of accelerated atherosclerosis. Atherosclerotic lesion area, levels of lesional O-GlcNAc and ER stress markers were compared in STZ injected, $Ins2^{+/Akita}$ and glucosamine-supplemented ApoE^{-/-} mice, relative to controls. (A) Representative images of aortic root cross-sections stained with the indicated chemicals or antibodies. (B) Quantification of atherosclerotic lesions area and immunostaining. H&E indicates hematoxylin and eosin. n=12/group except for ApoE^{-/-}Ins2^{+/Akita} n=4/group, **p*<0.05, ***p*<0.01 relative to control mice.

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Determining the glucosamine threshold level required to accelerate atherogenesis.

If ER stress is necessary to accelerate the development of atherosclerosis, then the concentration of glucosamine required to promote ER stress will be similar to that required to promote atherosclerosis. To determine this threshold level of glucosamine, five-week-old female ApoE^{-/-} mice were supplemented with 0, 0.625, 1.25, 2.5 or 5% (w/v) glucosamine in drinking water for 10 weeks. At 15 weeks of age, all mice were sacrificed and analysed. Glucosamine-supplementation did not alter any metabolic parameters including body weight, fasting glucose concentration, plasma cholesterol and triglycerides levels in ApoE^{-/-} mice (Figure 3).

Supplementation at 0.625 and 1.25% (w/v) did not significantly alter lesional protein linked O-GlcNAc (Figure 4A, Suppl. Figure 1) or UPR protein levels (Figure 4B–C, Suppl. Figures 2–3), nor did they affect atherosclerotic area or volume at the aortic sinus (Figure 5). Glucosamine-supplementation at 2.5 and 5% significantly increased lesional protein linked O-GlcNAc and also activated the UPR, which is indicative of ER stress in the atherosclerotic lesions (Figure 4). Furthermore, significantly larger atherosclerotic lesion area and volume were observed at the aortic sinus in mice supplemented with \geq 2.5% glucosamine, relative to mice receiving <2.5% glucosamine (Figure 5). These results are consistent with the hypothesis that glucosamine-induced ER stress promotes atherosclerosis.

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Figure 4.3: Analysis of metabolic parameters of mice supplemented with different levels of glucosamine in drinking water. (A) Body weight, fasting (B) blood glucose, (C) plasma triglycerides and (D) plasma cholesterol levels of control and 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN)-supplemented ApoE^{-/-} mice. n=4-6/group.



Figure 4.4: Comparison of O-GlcNAc and ER stress levels in atherosclerotic lesions of mice supplemented with different levels of glucosamine in drinking water. Quantification of (A) O-GlcNAc, (B) GRP78 and (C) CHOP levels in aortic root cross-sections of the control and ApoE^{-/-} mice supplemented with 0, 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN). Positively stained areas were normalized to total lesion volume and presented as fold difference relative to control. n=4-6/group, **p*<0.05 relative to control mice.



Figure 4.5: Determine the glucosamine threshold level required to accelerate atherogenesis. (A) Representative images of Masson's trichrome stained aortic cross-sections of mice supplemented with different concentrations of glucosamine. Quantification of atherosclerotic lesions (B) area and (C) volume. n=4-6/group, *p<0.05.



4PBA mitigates ER stress and attenuates accelerated atherosclerosis.

We have identified a correlation between glucosamine-induced activation of the UPR and accelerated atherosclerosis. If elevated ER stress level is necessary for the accelerated development of atherosclerosis in glucosamine-supplemented mice then we would predict that the chemical chaperone, 4PBA, which attenuates ER stress, will also diminish the accelerated development of atherosclerosis in both glucosamine-supplemented and hyperglycemic mouse models. To test this, 4PBA was supplemented in drinking water of five week old ApoE^{-/-} controls, glucosamine-supplemented ApoE^{-/-} mice and ApoE^{-/-} Ins^{+/Akita} mice for 10 weeks. Plasma 4PBA concentration was measured using liquid chromatography coupled to a mass spectrometer. The results indicate that plasma 4PBA levels were not significantly different between any groups of mice treated with 4PBA (Figure 6A). Metabolic parameters including body weight, fasting glucose concentration, plasma cholesterol and triglycerides levels were not significantly different between mice with and without 4PBA treatment (Figure 6B–E).

Glucosamine-supplemented and hyperglycemic mice treated with 4PBA showed significant decrease in the levels of GRP78 and CHOP protein (Figure 7A–B, Suppl. Figure 4–5), relative to mice without 4PBA treatment. 4PBA did not affect the level of ER stress indicators in the control mice. We found that 4PBA also reduced the levels of vascular O-GlcNAc in mice with 4PBA treatment, relative to those without 4PBA treatment, with the exception of control mice (Figure 7C, Suppl. Figure 6).

Atherosclerotic lesion area and volume were significantly decreased in glucosaminesupplemented ApoE^{-/-} mice and ApoE^{-/-}Ins^{+/Akita} mice treated with 4PBA, relative to mice without 4PBA treatment (Figure 8). 4PBA did not reduce atherosclerotic lesion area/volume in the control mice. Collectively, these results indicate that reducing ER stress levels attenuates the accelerated development of atherosclerosis.

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Figure 4.6: Analysis of metabolic parameters of hyperglycemic and glucosamine supplementation models in the absence or presence of 4BPA treatment. (A) Plasma 4PBA concentration of mice treated with 4PBA, (B) body weight, fasting (C) blood glucose, (D) plasma triglycerides and (E) plasma cholesterol levels of the control, 5% glucosaminesupplemented ApoE^{-/-} and ApoE^{-/-}Ins2^{+/Akita} mice with or without 4PBA treatment. n=4-6/group, ****p*<0.001 relative to control mice.



Figure 4.7: 4PBA reduces O-GlcNAc and ER stress levels in atherosclerotic lesions of glucosamine-supplemented and hyperglycemic mouse models. Quantification of (A) GRP78 and (B) CHOP and (C) O-GlcNAc levels in aortic cross-sections of the control, glucosamine-supplemented ApoE^{-/-} and ApoE^{-/-}Ins2^{+/Akita} mice with or without 4PBA supplementation. Positively stained areas were normalized to total lesion volume and presented as fold difference relative to control. n=4-6/group, **p*<0.05 relative to the same group of mice without 4PBA treatment.



Figure 4.8: 4PBA attenuates accelerated atherosclerosis in diabetic and glucosaminesupplemented ApoE^{-/-} mice. Representative images of Masson's trichrome stained aortic cross-sections of ApoE^{-/-} mice supplemented with glucosamine and ApoE^{-/-}Ins2^{+/Akita} mice in the absence or presence of 4PBA treatment. Quantification of atherosclerotic lesions (B) area and (C) volume. n=4-6/group, **p*<0.05 relative to the same group of mice without 4PBA treatment.



4.5 Discussion

In this study, we show that chemically (STZ injection) or genetically (Ins2^{+/Akita})-induced hyperglycemia promotes accelerated atherosclerosis in $ApoE^{-/-}$ mice in a manner that is independent of changes in plasma lipids. Similar increases in atherosclerotic lesion size were induced by supplementing $ApoE^{-/-}$ mice with glucosamine, which is a downstream metabolite of glucose. This study is the first direct comparison of these three mouse models of accelerated atherosclerosis. Each of these models showed a similar elevation in the level of O-GlcNAc and ER stress response proteins in atherosclerotic lesions, relative to $ApoE^{-/-}$ controls. In the titration experiments, we identified the threshold level of glucosamine required to induce ER stress is the same as that required to accelerate atherosclerosis ($\geq 2.5\%$ w/v in drinking water). Finally, we found that a chemical chaperone, which reduces ER stress levels in hyperglycemic and glucosamine-supplemented mice, attenuated the accelerated development of atherosclerosis. Together these findings are consistent with the mechanism in which hyperglycemia-associated atherosclerosis is driven by glucosamine-induced ER stress.

The last few decades have witnessed a dramatic, worldwide increase in the prevalence of type 1 and especially type 2 diabetes that is likely driven by multiple environmental and lifestyle changes [7,8]. CVD is the leading cause of death in both type 1 and type 2 diabetes, even after controlling for other cardiovascular risk factors including dyslipidemia, obesity and hypertension [27,28]. All forms of diabetes are clinically defined by increased blood glucose concentration and there is a progressive relationship

between hyperglycemia and CVD, with CV risk rising approximately 20% for every 1.5mM increase in fasting glucose levels [29], and for every 1% elevation in HbA_{1c} levels [30]. Both epidemiological and pathophysiological studies have shown that hyperglycemia is an independent risk factor for CVD [6,31,32].

Hyperglycemia is known to increase flux through the hexosamine biosynthetic pathway (HBP) leading to increased production of UDP-N-acetylglucosamine (UDP-GlcNAc) – an essential substrate for both N-linked and O-linked protein glycosylation [33,34]. Activation of the HBP has been implicated in the development of diabetic complications including glucotoxicity [35], insulin resistance [36], and cardiomyocyte dysfunction [37]. We have identified an additional consequence of enhanced HBP flux that involves the disruption of protein processing in the ER – a condition known as ER stress. Evidence from our lab and others has implicated ER stress in the development and progression of atherosclerosis in both humans and animal models [16,17]. Nonetheless, the mechanisms by which ER stress and/or UPR activation may promote the induction of pro-atherosclerotic pathways are not fully understood.

The chemical chaperone 4PBA has previously been shown to attenuate atherosclerosis in dyslipidemic mouse models [38]. However, this is the first demonstration that it can block accelerated atherogenesis associated with hyperglycemia. This observation is consistent with our hypothesis that ER stress plays a causative role in diabetes-associated atherosclerosis and further highlights the therapeutic potential of targeting the UPR as a viable strategy to treat and/or prevent atherosclerosis.

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The ability of glucosamine to induce ER stress is interesting because glucosamine is a popular dietary supplement used to treat joint pain associated with osteoarthritis [39]. To determine whether these supplements, when taken as directed (1500mg/day), caused ER stress and/or activated the UPR in humans, we have previously measured markers of UPR activation in circulating peripheral blood cells isolated from fasting blood samples taken from volunteers before and after a 14 day regimen of glucosamine-supplementation. Our results suggested that this glucosamine-supplementation regimen does not result in UPR activation [40]. This is likely because this dose is insufficient to induce ER stress. Relative glucosamine concentrations in our diabetic and glucosamine-supplemented mouse models are estimated to be approximately 40 fold higher.

In conclusion, we have identified a molecular mechanism by which glucosamine-induced ER stress promotes the development of atherosclerosis. We propose that this mechanism may contribute to the accelerated atherosclerosis that is observed in mouse models of hyperglycemia and perhaps, in individuals with diabetes. Expanding the knowledge of how diabetes promotes atherosclerosis will facilitate in the development of novel, more effective therapeutic strategies to slow or stop atherogenesis.

4.6 Materials and Methods

Animal models

Five-week-old female ApoE^{-/-} mice were randomly divided into three groups (n=12/group): control, 5% glucosamine-supplemented and STZ-injected groups. Multiple low-doses (40 mg/kg/day for ten days) of STZ (Sigma-Aldrich) were injected intraperitoneally to induce hyperglycemia. ApoE^{-/-} mice were crossed with Ins2^{+/Akita} mice to produce ApoE^{-/-}:Ins2^{+/Akita} offspring (n=4/group) [25]. A different set of fiveweek-old female ApoE^{-/-} mice were given 0 (n=10), 0.625 (n=4), 1.25 (n=4), 2.5 (n=6) or 5% (n=9) glucosamine (w/v, Sigma-Aldrich) in drinking water. Subsets of control, 5% glucosamine-supplemented ApoE^{-/-} and ApoE^{-/-}Ins2^{+/Akita} mice (n=4-5/group) were treated with 20mM 4PBA (Scandinavian Formulas Inc., PA) in drinking water. This level of 4PBA has previously been shown to attenuate ER stress in mice [24]. All mice were given unrestricted access to water and standard chow diet (TD92078; Harlan Teklad, Madison, WI) and were maintained on a 12-hour light/dark cycle throughout the study. Triglycerides and cholesterol were measured using Infinity reagents (Thermo Scientific). All mice were sacrificed at 15 weeks of age, plasma and tissues were collected for analysis. All procedures were approved by McMaster University Animal Research Ethics Board.

Histochemistry

Formalin-fixed hearts were cut transversely and embedded in paraffin. Aortas including aortic root were sectioned, as previously described [23]. Serial sections (5µm)

were stained with hematoxylin and eosin (Sigma-Aldrich) [23], or Masson's Trichrome (Sigma-Aldrich) to measure atherosclerotic lesion area/volume. Masson's Trichrome staining was performed based on Sigma-Aldrich's instruction in which nuclei, collagen and muscle/erythrocytes are stained in black, blue and red, respectively. Stained sections were imaged using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Lesion area was quantified using Image J (1.48v) software. Lesion volume was computed as area under the curve of lesion area.

Immunohistochemistry and immunofluorescence

Paraffin imbedded serial sections were stained with the primary antibodies against KDEL (StressGen, Canada) for GRP78/GRP94, CTD110.6 (Convance Inc., CA) or RL2 (Affinity Bioreagent) for O-GlcNAc, or GADD153 (Santa Cruz, CA) for CHOP, as previously described [23]. Negative controls were stained with IgG (Sigma-Aldrich) instead of primary antibodies to correct for non-specific staining. Stained sections were imaged using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Positively stained area was quantified using Image J (1.48v) software.

Plasma 4PBA quantification

Plasma was extracted using an ice-cold mixture of 1:1 methanol:ethanol (v/v). *L*phenylalanine-d8 (20µM, Cambridge Isotope Laboratories, MA) was used as the internal standard. An Agilent 1200 RR series liquid chromatography system (Agilent Technologies Inc., CA) coupled to a Bruker micrOTOF II (Bruker Daltonics, MA) mass analyzer equipped with an electrospray ionization (ESI) source (Agilent Technologies

Inc.) was used to quantify plasma 4PBA concentration. A volume of 2μ L of plasma extract was injected per run into a 2.1x50mm Halo C8 column.

Statistical analysis

One-way ANOVA followed by Tukey's HSD test was used to compare results between multiple groups. Data were presented as the mean \pm standard deviation. For all experiments, *p*-value of <0.05 was considered statistically significant, **p*<0.05, ***p*<0.01, ****p*<0.001.

4.7 Acknowledgements

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CHAPTER 5

Glycosphingolipids contribute to pro-atherogenic pathways in the pathogenesis of hyperglycemia-induced atherosclerosis

5.1 Chapter Preface

Three out of every four people with diabetes will die of cardiovascular disease. The objective of this chapter is to investigate the molecular alterations by which hyperglycemia promotes accelerated atherogenesis in three distinct disease mouse models.

In this manuscript, we utilize comprehensive metabolomic analyses to detect and identify specific plasma-borne metabolites that are altered in three distinct mouse models of hyperglycemia-associated accelerated atherosclerosis, relative to normoglycemic controls. Our results suggest that glycerophospholipid and sphingolipid metabolisms (especially the glycosphingolipid pathway) are strongly associated with pro-atherogenic processes including oxidative stress and inflammation, as well as the accelerated progression of atherosclerosis. In addition, we show that glycosphingolipid metabolites induce oxidative stress and increase levels of inflammatory cytokines in cultured human vascular cells. Together, these data highlight the importance of this metabolic pathway in the pathogenesis of atherosclerosis. These studies also identify potential plasma biomarkers for disease detection and potential targets for therapeutic intervention.

This study was conceived and designed by VTD and GHW. VTD and AH performed mouse experiments. VTD and LHZ conducted and analyzed histochemistry experiments. VTD performed metabolomic experiments and analyses. VTD and AD carried out the *in vitro* experiments. VTD wrote this manuscript in collaboration with GHW.

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5.2 Abstract

Three out of four people with diabetes will die of cardiovascular disease. The molecular mechanisms by which diabetes promotes atherosclerosis are not clear. Comprehensive metabolomics were used to investigate the molecular alterations by which hyperglycemia promotes accelerated atherogenesis in three distinct mouse models. Normoglycemic apolipoprotein-E-deficient mice served as atherosclerotic control. Hyperglycemia was induced by multiple low-dose streptozotocin-injections or by introducing a pointmutation in one copy of insulin-2 gene. Glucosamine-supplemented mice, which experience accelerated atherosclerosis to a similar extent as hyperglycemia-induced models without alterations in glucose or insulin levels, were also included in the analysis. Mice with accelerated atherosclerosis showed distinct metabolomic profiles compared to controls. We detected 6369 metabolite features in plasma of each mouse. Second-order analysis of pair comparisons between each disease model and control resulted in 62 commonly altered features (FDR-adjusted p<0.05). Identification of shared metabolites revealed alterations in glycerophospholipid and sphingolipid metabolisms, and proatherogenic processes including inflammation and oxidative stress. Post-multivariate and pathway analyses indicated glycosphingolipid as the most significantly altered pathway. Glycosphingolipid metabolites induced oxidative stress and inflammation in cultured human vascular cells. Treatment with α -tocopherol reduced oxidative stress and inflammation induced by glycosphingolipids. Our findings suggest that glycosphingolipid pathway contributes to pro-atherogenic processes in the pathogenesis of hyperglycemia-

induced atherosclerosis, suggesting it may be a potential therapeutic target to block or slow atherogenesis in diabetic patients.

5.3 Introduction

Omics-based technologies have been used to map pathways and/or mechanisms involved in the etiology and pathogenesis of many human diseases. Metabolomics, the latest omics strategy, offers novel insights into disease mechanisms, as the level of specific metabolites provides a direct functional readout of the physiological or pathological state of an organism.^{1–3} Recent advances in analytical and bioinformatic technologies allow for the characterization of thousands of metabolites, leading to the development of so-called comprehensive (or untargeted) metabolomics. This type of approach involves the global analysis of the metabolome, which is the entire collection of metabolites in a biological specimen. Comprehensive metabolomics enables novel and/or unanticipated discoveries linking molecular pathways to biological phenotypes and/or genotypes. In this study, we used comprehensive metabolomics to investigate molecular alterations in the pathogenesis of hyperglycemia-induced atherosclerosis.

The prevalence of diabetes is increasing dramatically worldwide.^{4,5} Cardiovascular disease (CVD) is the leading cause of death in both type 1 and type 2 diabetes.^{6,7} In fact, approximately three out of four diabetic patients will die of CVD. Atherosclerosis, a chronic inflammatory disease of the medium-large arteries, is the major underlying cause of most CVDs.^{8,9} This disease is characterized by the accumulation of lipids and inflammatory factors in the arterial walls. Despite the progressive relationship between diabetes and CVD, the molecular mechanisms by which diabetes promotes

atherosclerosis are not clear.^{10,11} This has impaired the development of strategies to block or slow atherogenesis in diabetic patients.

Hyperglycemia is the defining characteristic of all types of diabetes mellitus. Although hyperglycemia does not appear to promote atherosclerosis independently of dyslipidemia, it is clear, from animal model studies, that hyperglycemia can accelerate atherosclerosis in dyslipidemic atherosclerosis-prone mice, in a manner that is independent of any changes in the degree of dyslipidemia.¹²

The apolipoprotein E-deficient (ApoE^{-/-}) mouse model was chosen as the atherosclerotic control, as it is a well-established model in which all recognized stages of atherogenesis can be observed.^{13,14} This mouse model also develops complex atherosclerotic lesions that are morphologically very similar to those observed in humans.^{15,16} Hyperglycemia was induced by multiple low-dose streptozotocin (STZ) injections¹⁷ or by introducing a point mutation in one copy of the insulin 2 gene (Ins2^{+/Akita}).¹⁸ The glucosamine-supplemented ApoE^{-/-} mouse model was also included. Glucosamine is a downstream metabolite of glucose and glucosamine-supplemented ApoE^{-/-} mice experience accelerated atherosclerosis to a similar extent as the hyperglycemia-induced models, without alterations in the levels of glucose or insulin.^{19,20} This mouse model serves as a valuable control for insulin in this study.

In this study, comprehensive metabolomic techniques were used to investigate the molecular alterations by which hyperglycemia promotes the accelerated development of atherosclerosis in three disease mouse models. The use of three distinct models facilitates

the identification of commonly altered metabolites and pathways, while avoiding modelspecific changes.

5.4 Results

Mice were fed a standard chow diet to avoid other metabolic complications associated with high fat diet, such as weight gain and non-alcoholic fatty liver disease. We chose to investigate hyperglycemia-induced atherosclerosis in female mice in order to avoid the enhanced dyslipidemia observed in male Ins2^{+/Akita}ApoE^{-/-} mice, relative to Ins2^{+/+}ApoE^{-/-} mice.¹⁸

Hyperglycemia and glucosamine supplementation accelerate atherosclerotic development in ApoE^{-/-} mice

ApoE^{-/-}:Ins2^{+/Akita} mice developed hyperglycemia at five to six weeks of age. STZ and glucosamine were introduced at six weeks of age. Mice were harvested at 15 weeks of age. Plasma insulin was significantly lower in multiple low-dose STZ-injected and Ins2^{+/Akita} mice, relative to the control mice, at 15 weeks of age. Compared to the normoglycemic ApoE^{-/-} control, STZ injections resulted in a 2.4-fold increase in fasting blood glucose, whereas the introduction of the Ins2^{+/Akita} mutation resulted in a 1.8-fold increase in fasting blood glucose (Table 1). Glucosamine supplementation did not alter the level of circulating glucose or insulin in ApoE^{-/-} mice, compared to the non-treated control. No significant differences were observed in plasma triglyceride or cholesterol levels in any of the experimental groups.

Both hyperglycemia and glucosamine supplementation significantly accelerated atherogenesis in ApoE^{-/-} mice (Figure 1). Atherosclerotic lesions in the aortic sinus and ascending aorta of STZ-injected mice were approximately 3.5-fold larger (p<0.001) than

those in the control group. Ins2^{+/Akita} mutation (2.7-fold, p<0.01) and glucosaminesupplementation (2.7-fold, p<0.05) resulted in a similar increase in atherosclerotic lesions in ApoE^{-/-} mice, compared to the control.

Table 5.1: Quantification of blood glucose, plasma insulin, plasma triglycerides andplasma cholesterol level of the control (Ctrl), glucosamine (GlcN)-supplemented, $Ins2^{+/Akita}$ (Akita) and streptozotocin (STZ)-injected mice. Data are presented as mean±SD. n=8/group. **p<0.01 and ****p<0.0001 versus the control.</td>

Group	Glucose (mM)	Insulin (ng/mL)	Triglyceride (mM)	Cholesterol (mM)
Ctrl	8.3 ± 1.4	0.49 ± 0.17	1.2 ± 0.4	7.6 ± 1.5
GlcN	8.7 ± 0.9	$0.52\pm\!\!0.10$	1.1 ± 0.3	8.0 ± 1.6
Akita	$14.7 \pm 1.2^{****}$	$0.31 \pm 0.03^{**}$	1.3 ± 0.3	7.3 ± 2.0
STZ	19.7 ±4.3****	$0.27 \pm \! 0.07^{**}$	1.1 ± 0.5	7.0 ± 1.5

Figure 5.1: Hyperglycemia and glucosamine supplementation accelerate the development of atherosclerosis in ApoE^{-/-} mice. (A) Representative Masson's trichrome stained images of aortic cross-sections of the control (Ctrl), glucosamine (GlcN)-supplemented, Ins2^{+/Akita} (Akita) and streptozotocin (STZ)-injected mice. Atherosclerotic lesions are highlighted by dotted lines. (B) Quantification of atherosclerotic lesion area from the aortic sinus and ascending aorta. (C) Lesion volumes were computed by calculating the area under the lesion area curve. n=8/group, *p<0.05, **p<0.01 and ***p<0.001 versus the control. Data are presented as mean ±SD.



Metabolomics data analysis and quality control

Mouse plasma samples were extracted and analyzed by liquid chromatography (LC) coupled to a mass spectrometer (MS) using two orthogonal analytical columns in tandem. This analytical system allows for the separation and detection coverage of both polar and non-polar metabolites.²⁶ Due to its untargeted nature, comprehensive metabolomics produces complex data sets that require the use of bioinformatic software for its analysis. In this study, metabolomic suite (XCMS²³) was used to extract LC-MS data using an untargeted metabolite profiling approach. XCMS detected 4417 and 6224 metabolite features in negative and positive ionization modes in each plasma sample, respectively. Each metabolite feature represents a LC-MS peak with a unique LC retention time and a mass-to-charge (*m/z*) value.

We then performed metabolomic data reduction (i.e. data cleanup) by removing redundant (isotopes and adducts) and unreliable features (LC-MS peaks eluting in the ion-suppression region). This left a total of 6369 metabolite features, with 2715 features in negative and 3654 features in positive ionization modes. Analysis of the quality control samples by principle component analysis showed a tight clustering of the pooled samples, indicating good instrumental reproducibility throughout the period of analysis (Supplementary Figure S1).

Hyperglycemia and glucosamine supplementation induce similar changes in the plasma metabolomic profiles of ApoE^{-/-} mice

Multivariate analysis was used to correlate plasma metabolite variables with the genotypic and phenotypic patterns of the experimental mice. Partial least squares discriminant analysis (PLS-DA) score plots were constructed using the 6369 total metabolite features detected. The comprehensive plasma metabolomic profiles of glucosamine-supplemented (Figure 2A), Ins2^{+/Akita} (Figure 2B) and STZ-injected (Figure 2C) mice were clearly differentiated from those of the controls.

The magnitude of separation in the metabolomic profiles is indicated as component 1 in the PLS-DA score plot. The multivariate model validation parameters, R^2 (i.e. prediction accuracy) and Q^2 (i.e. model robustness), were used to assess the fitness of the model. A similar magnitude of separation (approximately 22%) and multivariate model robustness $(Q^2 = ~0.8)$ were observed in each treatment group, compared to the control. This suggests that glucosamine, chemically (STZ) and genetically (Ins2^{+/Akita})-induced hyperglycemia, each resulted in similar alterations in the plasma metabolomic profile of ApoE^{-/-} mice.

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Figure 5.2: Glucosamine and hyperglycemia induce changes in the plasma metabolomic profiles of $ApoE^{-/-}$ mice. Direct comparison of the plasma metabolomic profiles of (A) glucosamine (GlcN)-supplemented, (B) $Ins2^{+/Akita}$ (Akita) and (C) streptozotocin (STZ)-injected $ApoE^{-/-}$ mice versus the controls (Ctrl). Each dot in the PLS-DA score plot represents the profiles of 6369 total metabolite features detected in a single mouse. R² and Q² indicate prediction accuracy and model robustness (after cross-validation) of the multivariate analysis, respectively. n=8/group.



Identification of commonly altered metabolite features in three models of hyperglycemia-induced atherosclerosis

In order to identify the metabolite features that were commonly altered in each of the three models of hyperglycemia-induced atherosclerosis, we first performed pair comparison of each model group to the control (Figure 3A–C). Next, a *p*-value filter step (FDR-adjusted p<0.05) was applied to identify the significantly altered metabolite features in each pair comparison (Figure 3D–F). The number of differential features arising from the comparison of the glucosamine-supplemented group and the control was similar to the number found in the comparison of the Ins2^{+/Akita} group versus the control. A larger number of differential features were observed in the STZ-injected group.

The second-order comparison was applied to identify commonly altered metabolite features between the three pair comparisons. The number of uniquely altered and shared features between the glucosamine-supplemented and two hyperglycemic models is summarized in a Venn diagram (Figure 3G). The uniquely altered metabolite features in each model are model-specific and could potentially arise from the confounding effects of the treatment or mutation. To specifically focus on the common molecular alterations that are driving accelerated atherosclerosis in three disease mouse models, the 62 shared metabolite features were identified for further exploration.

Figure 5.3: Identification of commonly altered metabolite features among shared phenotypes using second-order analysis of untargeted metabolomic data from the three model groups. (A-C) Heat map indicating the relative levels of the top 250 metabolite features (ranked by T-test) of the indicated pair groups of analysis. n=8/group. (D-F) T-test analysis was used to identify differential features (filtered by FDR-adjusted p-value smaller than 0.05) between the indicated groups. (G) Second-order analysis was applied to identify commonly altered metabolites between the three pair comparisons. Ctrl: control, GlcN: glucosamine, Akita: Ins2^{+/Akita}, and STZ: streptozotocin.



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Metabolite identification of the commonly altered metabolites in three disease mouse models

A heat map of the 62 commonly altered metabolite features is shown in Figure 4A. A total of 36 metabolites were identified, based on available authentic standards, or tentatively identified, based on structural analogs and their accurate mass/empirical formula (Supplementary Table S1). In all cases, metabolites were annotated based on their characteristic m/z and LC retention time. A total of 26 of the commonly altered metabolite features remain unidentified.

The 36 identified metabolites were associated with several metabolic pathways including inflammation, oxidative stress, glycerophospholipid, and sphingolipid metabolism (Supplementary Table S2). Mice with accelerated atherosclerosis show an upregulation in histamine biosynthesis which is associated with a systemic inflammatory immune activation. The level of several metabolites including oxidized fatty acids, oxidized cholesterol and antioxidant glutathione conjugate were augmented in all three experimental models versus the control, suggesting elevated levels of oxidative stress. We identified 14 and 12 altered metabolites from the glycerophospholipid and sphingolipid metabolism, respectively. These data suggest that these metabolic pathways are strongly associated with the pathogenesis of hyperglycemia-induced accelerated atherosclerosis.

Figure 5.4: Identification and pathway analysis of the 62 commonly altered metabolites among the three mouse models of hyperglycemia-induced atherosclerosis. (A) Heat map indicating the relative levels of the 62 commonly altered metabolites. (B) PLS-DA score plots of control (Ctrl), glucosamine (GlcN)-supplemented, Ins2^{+/Akita} (Akita) and streptozotocin (STZ)-injected ApoE^{-/-} mice, constructed based on the 36 identified metabolites. R^2 and Q^2 indicate prediction accuracy and model robustness (after crossvalidation) of the multivariate analysis, respectively. n=8/group. (C) Variable importance in the projection (VIP) score plot of the top 15 metabolites ranked by VIP scores. (D) Pathway analysis of the 36 identified metabolites. All matched pathways are plotted according to p-value from pathway enrichment analysis and pathway impact score from pathway topology analysis. Colour gradient and circle size indicate the significance of the pathway ranked by p-value (yellow: higher p-value and red: lower p-value) and pathway impact score (the larger the circle the higher the impact score), respectively. Significantly affected pathways with low p-value and high pathway impact score are identified by name. (E) A simplified schematic of the glycosphingolipid pathway.



The glycosphingolipid pathway (sphingolipid metabolism) is the most significantly affected pathway in each model of hyperglycemia-induced atherosclerosis

Select plasma metabolomic profiles, constructed based on the 36 identified metabolites, differentiate the three mouse models of accelerated atherosclerosis from the control (Figure 4B). The variable importance in the projection (VIP) score plot highlights the top 15 metabolites ranked by VIP scores, which is a measure of a variable's importance in the separation of the metabolomic profiles observed in a multivariate model (Figure 4C). Many sphingolipid metabolites, especially from the glycosphingolipid pathway, were among the top 15 metabolites. Additionally, we performed Random Forest classification on the 36 identified metabolites. Mean decrease in accuracy was used to rank metabolites based on their contributions to classification accuracy (Supplementary Figure S2). There is a high degree of consistency (12 out of 15 metabolites) between the top-scoring metabolites identified by the VIP score and those identified by the mean decrease in accuracy score, highlighting the importance of the glycosphingolipid pathway in the pathology of hyperglycemia-induced atherosclerosis.

Pathway analysis was also performed on the 36 identified metabolites. The results from integrating enrichment and pathway topology analyses were used to map out the identified metabolites into specific metabolic pathways (Figure 4D). On this basis, sphingolipid metabolism was found to be the most significantly affected pathway based on *p*-values ($p < 2.4 \times 10^{-8}$) and pathway impact scores (score = 0.38). Pathway view of the sphingolipid metabolism showed alterations around the glycosphingolipid pathway

(Supplementary Figure S3). These results suggest that glycosphingolipid pathway is strongly associated with the accelerated progression of atherosclerosis induced by hyperglycemia and glucosamine supplementation. A simplified schematic of the glycosphingolipid pathway is shown in Figure 4E.

Glycosphingolipid metabolites were elevated at the initial stage of hyperglycemia, prior to accelerated atherogenesis

We have shown that the glycosphingolipid pathway is strongly associated with hyperglycemia-induced accelerated atherosclerosis. However, it is unclear whether these lipids drive the rapid progression of atherosclerosis or they were elevated as a result of accelerated atherosclerosis. Therefore, we measured these glycosphingolipid at the initial stage of hyperglycemia to provide further insights into the cause-effect nature of the relationship between these metabolites and atherogenesis. Aorta and plasma samples from 10-week-old mice were collected and analyzed.

At 10-week time-point, no significant difference was observed in the lesion volume of each treatment groups versus the control (Supplementary Figure S4), although there appears to be a trend toward larger lesions in the treatment groups. The levels of 36 most significantly elevated metabolites that were identified at 15-week time-point were quantified in 10-week-old mice (Supplementary Table S3). All of the glycosphingolipids detected were significantly altered in hyperglycemic and glucosamine-supplemented mice relative to the control at 10 weeks of age. The elevation of glycosphingolipid levels prior to the development of advanced atherosclerosis is consistent with the possibility that glycosphingolipids are actively promoting the observed accelerated atherosclerosis.

Glycosphingolipid metabolites induce oxidative stress and increase levels of inflammatory cytokines in cultured human vascular cells

Atherosclerosis is an inflammatory disease and both endoplasmic reticulum (ER) stress and oxidative stress have a well-established causal role in atherosclerotic development and progression. Correlation analysis of the 36 identified metabolites showed strong association between glycosphingolipids and metabolites involved in oxidative stress and inflammation pathways (Supplementary Figure S5). To the best of our knowledge, there are no known circulating markers of ER stress. Therefore, the effect of glycosphingolipid metabolites on the activation of ER stress, oxidative stress, and inflammation was investigated in human vascular cell lines that are relevant to atherosclerosis, including Thp-1 monocyte-derived macrophages, human aortic endothelial cells (HAEC), and human aortic smooth muscle cells (HASMC).

Macrophages, HAEC, and HASMC were incubated with ceramide, GluCer or LacCer at various concentrations (1, 10 or 50 μ M) for 24 hours. Markers of ER stress (78 and 94 kDa glucose-regulated proteins and C/EBP homologous protein), oxidative stress (catalase and glutathione peroxidase), and inflammation (IL-6 and IL-1 β) were assessed by real-time PCR. Ceramide, GluCer and LacCer were each found to cause a significant increase in the expression levels of transcripts encoding antioxidant genes, including catalase (Figure 5A–C) and glutathione peroxidase (Supplementary Figure S6A-C), in all three cell types. The expression of inflammatory cytokines IL-6 (Figure 5D–F) and IL-1 β (Supplementary Figure S6D-F) were also increased in all three cell types, upon treatment

with ceramide, GluCer and LacCer. All the effects of glycosphingolipid metabolites were in a dose-response manner. No significant effects were observed in the expression of ER stress response genes (data not shown). Together, these results suggest that glycosphingolipid metabolites are potential inducers of oxidative stress and inflammation in atherogenesis.

Figure 5.5: Glycosphingolipid metabolites induce oxidative stress and increase the levels of inflammatory cytokines in human vascular cells. Macrophages, human aortic endothelial cells (HAEC), and human aortic smooth muscle cells (HASMC) were incubated with ceramide, GluCer or LacCer at various concentrations (1, 10 and 50 μ M) for 24 hours. Expression levels of (A-C) catalase and (D-F) IL-6 were assessed by real-time PCR. Data are presented as mean ±SD. n=5/group, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 versus the controls.



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Treatment with α -tocopherol reduces oxidative stress and inflammation induced by glycosphingolipid metabolites

We showed that glycosphingolipid metabolites induce oxidative stress and inflammation *in vitro*. However, it was not clear as to whether glycosphingolipids induced inflammation directly or indirectly via the activation of oxidative stress pathways. To test that, we treated macrophages, HAEC, and HASMC with 50 μ M of the indicated glycosphingolipid simultaneously with the known antioxidant, α -tocopherol²⁷ (50 μ M). Cells were harvested and the expression levels of transcripts encoding catalase, glutathione peroxidase, IL-6, and IL-1 β were assessed.

 α -tocopherol inhibited the increase in the expression of catalase (Figure 6A–C) and glutathione peroxidase (Supplementary Figure S7A-C) induced by ceramide, GluCer and LacCer in macrophages, HAEC, and HASMC. Similarly, the increase in the expression of IL-6 was reduced by α -tocopherol in all three cell types (Figure 6D–F). However, α tocopherol was only able to reduce the increase in IL1- β expression in macrophage, but not in HAEC and HASMC (Supplementary Figure S7D-F). These data showed that treatment with α -tocopherol reduced oxidative stress and inflammation induced by glycosphingolipid metabolites. This suggests that the glycosphingolipids induce inflammation via a pathway that involves oxidative stress.

Figure 5.6: Treatment with α -tocopherol reduces oxidative stress and inflammation induced by glycosphingolipid metabolites. Macrophages, human aortic endothelial cells (HAEC), and human aortic smooth muscle cells (HASMC) were incubated with 50 μ M ceramide, GluCer or LacCer in the presence of absence of 50 μ M α -tocopherol (TOH) for 24 hours. Expression levels of (A-C) catalase and (D-F) IL-6 were assessed by real-time PCR. Data are presented as mean ±SD. n=5/group, *p<0.05, **p<0.01, and ***p<0.001 versus the controls.



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5.5 Discussion

The pathophysiology of diabetes and atherosclerosis has previously been explored individually at the metabolomic level.^{28–30} However, the molecular mechanisms behind the complex interplay between diabetes and its macrovascular complication are not well understood. Currently, there are no ideal experimental models to study diabetic atherosclerosis, as each has its own advantages and disadvantages.^{31,32} To the best of our knowledge, this is the first study that involves the simultaneous comprehensive metabolomic analyses of three distinct murine models of hyperglycemia-induced atherosclerosis so that common metabolic alteration(s) could be evaluated, independent of model-specific changes or artifacts.

"Type 1-like" hyperglycemic mouse models were utilized in this study in order to minimize the potential confounding effects of obesity and insulin resistance that are associated with type 2 diabetes. The glucosamine-supplemented mouse model was included to account for potential confounding complications associated with insulinopenia. In each of the models used in this study, we have shown that accelerated atherosclerosis occurs independent of any changes in plasma triglyceride or cholesterol levels. Therefore, the accelerated progression of atherosclerosis in our mouse models can be mainly attributed to increased levels of glucose and/or downstream metabolites of glucose (i.e. glucosamine).

The two hyperglycemic mouse models exhibited similar degrees of alteration in the plasma metabolome of ApoE^{-/-} mice, compared to glucosamine-supplemented mice. This

suggests that elevated levels of glucosamine accelerate the progression of atherosclerosis, independent from the effect of insulin, which is consistent with hyperglycemia being an independent risk factor for CVD.^{33–35}

Identification of the commonly altered metabolite features between the three experimental models revealed alterations in glycerophospholipid and sphingolipid metabolisms. Previously, our group determined that alteration in these two lipid metabolisms is associated with the development and progression of atherosclerosis in normoglycemic ApoE^{-/-} mice.²² Recently, it has been shown that sphingolipids are elevated in human atherosclerotic plaques associated with symptoms.²⁴ Ceramides, which are the central players in sphingolipid metabolism, were also used to predict CV outcomes at the population level³⁶ and were found to be strongly associated with type 2 diabetes and prediabetes.³⁷ Another lipidomics study revealed associations of sphingolipids with obesity and insulin resistance in young adults.³⁸ Despite the progressive relationship between sphingolipid metabolism and the pathophysiology of atherosclerosis and diabetes, the specific molecular mechanisms and metabolic pathways have not been investigated, especially in the context of hyperglycemia-associated atherosclerosis.

Glycosphingolipid was found to be the most significant pathway in the pathogenesis of hyperglycemia-induced atherosclerosis. Glycosphingolipids are not only ubiquitous building blocks of eukaryotic cell membranes, but are also signalling molecules that regulate a diverse range of cellular processes.³⁹ Inhibiting the synthesis of this pathway
has shown to improve glucose homeostasis in diabetic fatty rats⁴⁰ and reduce atherosclerotic lesions in ApoE*3 Leiden mice.⁴¹ However, the specific roles of the glycosphingolipid pathway in the pathogenesis of hyperglycemia-induced accelerated atherosclerosis are not clear.

Glycosphingolipid metabolites, including ceramide, GluCer and LacCer, were found to induce oxidative stress and inflammation in human vascular cells. This suggests that these glycosphingolipid metabolites are not only strongly associated with the pathology of hyperglycemia-induced atherosclerosis, but may also contribute to the activation of pro-atherogenic processes. Hyperglycemia is known to be an inducer of oxidative stress, which activates pro-inflammatory pathways.⁴² This is consistent with our data that attenuation of oxidative stress, using an antioxidant, reduced the levels of inflammatory cytokines. However, we only observed this effect in macrophages, and not HAEC or HASMC, which may reflect the direct role of macrophages in inflammatory processes. Future studies are required to determine if these lipids are sufficient to induce inflammation and oxidative stress (and promote atherosclerosis) *in vivo*.

In this study, the clear difference in the plasma levels of glycosphingolipid metabolites supports the use of metabolite profiling as a diagnostic tool of accelerated atherogenesis. Since atherosclerosis is a complex metabolic disorder, a single biomarker may not have sufficient sensitivity or specificity for disease diagnosis. Instead, a panel of markers may be a more effective approach. To begin to test this theory, multiple panels of markers were constructed using 2 to 9 glycosphingolipid metabolites that were detected in this

study (Figure 7). As the number of markers in the panel increases, the performance of the diagnostic test also increases. The nine-marker panels increase the area under the curve of the receiver operating characteristic curve by 8.2% (Figure 7A) and increase the predictive accuracy by 11.8% (Figure 7B), compared to the two-marker panel. The nine-marker panel would accurately detect accelerated atherosclerosis in approximately 9 out of 10 cases (89.3% predictive accuracy) in this study. Such a biomarker panel could be used to predict the rapid progression of atherosclerosis for timely intervention to avoid plaque rupture.

Figure 5.7: Evaluation of biomarker panels as a diagnostic tool of accelerated atherogenesis. Biomarker panels were constructed using 2 to 9 glycosphingolipid metabolites detected in this study. Glucosamine supplementation, streptozotocin injections and Ins2^{+/Akita} groups were combined together as the "accelerated atherosclerosis" group. Performance evaluation of a biomarker panel was calculated based on the comparison between the control group versus the accelerated atherosclerosis group. (A) Receiver operating characteristic curves are generated by Monte-Carlo cross validation, using the indicated number of markers. The area under the curve (AUC) and 95% confidence interval (CI) were computed. (B) Overall predictive accuracy was computed for the number of indicated markers in the panel.

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This study utilizes a high-throughput, high-sensitivity, and comprehensive analytical system, which is able to simultaneously detect a wide range of polar and nonpolar metabolites in complex plasma samples.²⁶ The identification of a strong association between glycosphingolipids and the pathogenesis of hyperglycemia-induced atherosclerosis suggests that future studies should focus on lipidomics analysis of plasma samples using lipid-focused sample extraction and analytical techniques in order to increase the detection coverage of the lipidome.

In conclusion, we show that mice with accelerated atherosclerosis present with distinct metabolomic profiles containing elevated levels of circulating sphingolipid metabolites, especially glycosphingolipids. Additionally, treatment of human vascular cells with ceramide, GluCer and LacCer induced oxidative stress and inflammation, suggesting a causal role in disease development. Our findings also provide the first evidence that glycosphingolipid metabolism is a potential link between hyperglycemia and accelerated atherosclerosis. This suggests that the glycosphingolipid pathway may be a potential therapeutic target to block or slow atherogenesis in diabetic patients.

5.6 Methods

Animal models

All animal procedures were pre-approved by, and performed in accordance with, the McMaster University Animal Research Ethics Board and conform with the guidelines of the Canadian Council on Animal Care. Six-week-old female ApoE^{-/-} mice (B6.129P2-ApoE^{tm1Unc}) were randomly divided into three groups: control, streptozotocin-injected or glucosamine-supplemented (n= 14/group). Multiple low-doses (40 mg/kg body weight/day for ten days) of streptozotocin (Sigma-Aldrich) were injected intraperitoneally to induce hyperglycemia. Glucosamine (5% w/v, Sigma-Aldrich) was supplemented in drinking water, which was given *ad libitum*. Female ApoE^{-/-} mice were crossed with male ApoE^{-/-}:Ins2^{+/Akita} mice to produce ApoE^{-/-}:Ins2^{+/Akita} offspring (n= 14/group). All mice were given unrestricted access to water and standard chow diet (Harlan Teklad, Madison, WI) and were maintained on a 12-hour light/dark cycle throughout the study.

Mice were fasted for 6 hours prior to sacrifice at 10 weeks (n=6/group) and 15 weeks (n=8/group) of age. Fasting blood glucose was measured using an UltraMini blood glucose meter (OneTouch). Mice were anaesthetized with 3% isoflurane prior to blood collection by cardiac puncture. Subsequently, mice were euthanized by cervical dislocation. Vasculature was flushed with saline and tissues were collected and prepared for analyses. Fasting plasma insulin was measured by enzyme-linked immunosorbent

assay (Crystal Chem). Plasma total cholesterol and triglycerides were measured using Infinity reagents (Thermo-Scientific).

Histochemistry

Harvested hearts were fixed in 10% formalin. The hearts were cut and paraffin-embedded aortic sinus was sectioned at 5 μ m/section, as previously described.²¹ Atherosclerotic lesions were visualized by staining serial sections with Masson's Trichrome (Sigma-Aldrich). Stained sections were imaged using a Leitz LABORLUX S microscope connected to a DP72 Olympus camera. Lesion areas at the aortic sinus and the ascending aorta were quantified using Image J (1.48v) software. Lesion volume was computed as area under the curve of lesion area *versus* distance from the aortic sinus.

Metabolomic analysis

Sample preparation, liquid chromatographic system, mass spectrometer parameters, metabolomic data analysis, and metabolite identification were performed as previous described.²² Plasma samples were extracted using a mixture of 1:1 methanol:ethanol (v/v) containing 10 μ M L-phenylalanine-d8 (Cambridge Isotope Laboratories Inc.) as the internal standard. The LC system used was an Agilent 1200 RR series LC system (Agilent Technologies Inc., CA). Two analytical columns (SeQuant ZIC-cHILIC, 3 μ m followed by Halo C8, 2.7 μ m) were directly coupled in series as an orthogonal separation system under a single compatible solvent elution program. Plasma extracts were analyzed by Bruker micrOTOF II (Bruker Daltonics) mass analyzer equipped with an electrospray ionization (ESI) source (Agilent Technologies Inc.) in both positive and negative

electrospray modes. Pooled plasma extracts were run as a quality control measure to assess for system drift. Metabolomics software²³ (XCMS and CAMERA) was used to extract and align LC-MS data using untargeted metabolite profiling approach.

Cell culture

Human aortic smooth muscle cells (HASMC) were cultured in Media 231 and smooth muscle growth supplement (Cascade Biologics). Human aortic endothelial cells (HAEC) were cultured in M-200 medium and low serum growth supplement (Cascade Biologics). Human monocytic (Thp-1) cells were cultured in RPMI 1640 medium (Gibco-Life Technologies) containing 10% fetal bovine serum. Monocytes were differentiated into macrophages by exposing the cells to 100 nM PMA for 72 h. All cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Cultured cells were treated with ceramide (C18, Avanti Lipids), glucosyl-ceramide (C18, Avanti Lipids), or lactosyl-ceramide (C18, Avanti Lipids) at the indicated concentrations for 24 hours, with or without the antioxidant α -tocopherol (Sigma). These lipids were dissolved in isopropanol prior to treatment. The final volume of lipids added was 0.5% of the media volume. The glycosphingolipid concentrations used were based on previous publication.²⁴

RNA preparation

Total RNA was extracted from cultured cells using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. Purified RNA was resuspended in RNAse-free

water. RNA concentration and purity were measured by NanoDrop Spectrophotometer (ThermoFisher). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

Real-time quantitative PCR analysis

Quantitative RT-PCR was performed on the StepOne Plus (Applied Biosystems) using 2 μ L of resulting cDNA (corresponding to 0.2 μ g of total RNA used to reverse transcription), 12.5 μ l SensiFAST SYBR No-Rox Kit (Bioline), 1.25 μ l of forward and reverse primers (500 nM), and 8 μ l of RNase-water for a total volume of 25 μ l/well. cDNA was amplified under the following conditions: 10-minute hold at 95 °C, followed by 40 cycles consisting of a 15-second melt at 95 °C, followed by a 1-minute annealing at 60 °C. Relative quantitative analysis (2^{-ddCt}) was performed by normalizing data to the β -actin reference gene. Fold changes in expression levels were calculated relative to the corresponding vehicle-treated cell type.

Gene	Orientation	Sequence
β-actin	Forward	5' ACC GAG CGC GGC TAC AG 3'
	Reverse	5' CTT AAT GTG ACG CAC GAT TTC C 3'
Catalase	Forward	5' GCG GAG ATT CAA CAC TGC CAA TG 3'
	Reverse	5' CTG TTC CTC ATT CAG CAC GTT CAC 3'
Glutathione peroxidase	Forward	5' CGA GGG AGG AAC ACC TGA TCT TAC 3'
	Reverse	5' GGG AAA CTC GCC TTG GTC TGG 3'
IL-6	Forward	5' CCA CAC AGA GAG CCA CTC AC 3'
	Reverse	5' AGG TTG TTT TCT GCC AGT GC 3'
IL-1β	Forward	5' GTG AGC TCT CTC CTT TCA 3'
	Reverse	5' AAT GTG GCC GTG GTT TCT 3'
78 kDa glucose-	Forward	5' CGA GGA GGA GGA CAA GAA GG 3'
regulated protein	Reverse	5' CAC CTT GAA CGG CAA GAA CT 3'

Primer sequences

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94 kDa glucose-	Forward	5' TGG GAA GAG GTT CCA GAA TG 3'
regulated protein	Reverse	5' GTT GCC AGA CCA TCC GTA CT 3'
C/EBP homologous	Forward	5' TGC CTT TCT CTT CGG ACA CT 3'
protein	Reverse	5' TGT GAC CTC TGC TGG TTC TG 3'

Statistical analysis

One/two-way ANOVA followed by Tukey's HSD test were performed using GraphPad Prism (v6.01). The *t*-test (two-tailed, unpaired heteroscedastic) analysis with FDR-adjusted p-value cut-off of 0.05 was used to identify the differential features in the metabolomic analyses. Biomarker analysis, correlation analysis, hierarchical heat map, partial least squares discriminant analysis and the derived VIP score plot, pathway analysis, principal component and Random Forest analysis were performed using MetaboAnalyst 3.0.²⁵

Auto scaling followed by log transformation was applied in all multivariate analyses. Pearson correlation and Ward clustering algorithm were used in correlation analysis and heat map. ROC curves are generated based on Monte-Carlo cross-validation performance of multivariate algorithms using PLS-DA classification and ranking method. Nonparametric re-sampling based approach was used to calculate the confidence intervals. Probability values of <0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

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CHAPTER 6

General Discussion

6.1 Summary and Significance

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide.^{1–3} Risk factors of CVD include obesity⁴, dyslipidemia⁵, smoking⁶, hypertension⁷ and diabetes mellitus⁸. CVD is the most serious complication of diabetes, and three out of every four diabetic patients will die of this disease.⁹ Therefore, the goal of my thesis was to identify plasma biomarkers for disease detection and potential targets for therapeutic intervention in diabetic atherosclerosis.

The specific objectives of my Ph.D. studies were to; **i**) investigate the development and progression of atherosclerosis at the molecular level, **ii**) characterize mouse models to explore the association between diabetes and atherosclerosis, and **iii**) apply the techniques and knowledge acquired in objective (i) in the investigation of the molecular alterations by which diabetes promotes the accelerated progression of atherosclerosis in murine models established in objective (ii).

Decades of research have significantly increased our understanding of the cellular mechanisms that define the pathophysiology of atherosclerosis, the major underlying cause of most CVDs.^{10–13} Despite these advances, many questions regarding the specific molecular mechanisms underlying the pathogenesis and progression of atherosclerosis remain unanswered. Furthermore, despite great advances in technologies to detect

atherosclerosis, the presence of this disease is often not revealed until the latest stage, which presents as a myocardial infarction or stroke.

Current conventional methods to identify patients with atherosclerosis tend to be timeconsuming and expensive.^{14,15} Non-invasive diagnostic techniques such as carotid ultrasound and coronary artery calcium scoring only have moderate predictive values.¹⁶ Alternately, angioplasty, which is the current gold standard of atherosclerosis detection, is invasive and carries significant risks.^{10,17} Therefore, there is a need to identify sensitive and non-invasive biomarkers for the detection of atherosclerosis and ideally, to differentiate the different stages of atherosclerotic progression.

The status of metabolite levels is critical in human health.¹⁸ Metabolomics-based technologies offer a novel approach to investigate disease pathophysiology and discover disease biomarkers. In addition, metabolomics can lead to the identification of novel therapeutic targets, as the relative levels of specific metabolites provide a direct functional readout of a physiological or pathological state of an organism.¹⁹ Moreover, the levels of metabolites reflect the functional level more accurately than the other 'omics' studies since the metabolic fluxes are regulated not only by gene expression, but also by environmental stresses.^{20,21} The genome and proteome tell us what *might* happen while the metabolome tells us both what *might* happen and what *is* happening. As such, metabolomics-based biomarkers do not only indicate the presence or absence of a disease, but are also capable of assessing risks of developing the disease and detecting the disease prior to the appearance of clinical symptoms and/or phenotypes.²² Currently,

metabolites are used in more than 95% of all diagnostic clinical tests and approximately 90% of all known drugs are small molecules.²³

The incorporation of metabolomics-based studies in atherosclerosis research is a relatively new addition to this field of study that has already expanded rapidly within the past decade.^{24–26} A variety of small and large experimental animal models have been used to investigate atherosclerosis, including mouse, pig, rabbit and non-human primates.^{27,28} Murine models are currently the most extensively used due to the relatively low costs, ease of genetic and environmental manipulation, and relatively short time frame for atherosclerotic development.

Apolipoprotein E-deficient (ApoE^{-/-}) model is among the most commonly used murine models of atherosclerosis. Multiple metabolomics-based studies have been carried out in ApoE^{-/-} mice. For instances, choline, taurine, glycine and glucose were identified as potential biomarkers for the early diagnosis of atherosclerosis in ApoE^{-/-} mice.²⁹ Another metabolomics study suggested that urine levels of xanthine and ascorbate were potential markers of atherosclerotic plaque formation in this mouse model.³⁰ Recently, Qingbo *et al* combined metabolomic and proteomic analyses to identify changes in protein and metabolite levels in atherosclerotic vessels of ApoE^{-/-} mice.³¹ Immune activation, oxidative stress and energetic impairment were suggested to be among the earliest alterations in hyperlipidemic animals. The progression of atherosclerosis has been explored in these studies at the molecular levels. However, these investigations did not

appropriately account for age-associated confounding metabolites in disease progression. We have tackled this issue and it is described in Chapter 3 of this thesis.

In Chapter 3,³² we identified specific changes in plasma-borne metabolites that are associated with the pathogenesis and progression of atherosclerosis in ApoE^{-/-} mice at different stages of disease progression including pre-stage I, stage I and stage II of atherosclerosis. We showed that comprehensive metabolomic profiles can be used to differentiate the plasma metabolome of atherosclerosis-prone from atherosclerosisresistant models. Specific metabolic pathways in the glycerophospholipid and sphingolipid metabolisms were found to be associated with specific stages of atherosclerotic progression. Metabolites in the sphingomyelin pathway were significantly altered prior to detectable lesion formation and at all subsequent time-points. The cytosine diphosphate-diacylglycerol pathway was found to be up-regulated during stage I of atherosclerosis, while metabolites in the phosphatidylethanolamine and glycosphingolipid pathways were augmented in mice with stage II lesions.

Glycerophospholipids and sphingolipids are not only structural components of biological membranes, but also act as signalling molecules and bioactive mediators in important atherosclerosis-associated cellular processes including apoptosis, angiogenesis, inflammation and proliferation.^{33,34} In addition, we found that glycerophospholipid and sphingolipid metabolisms were significantly affected during the course of atherosclerotic progression within the atherosclerosis-prone model. Distinct plasma metabolomic profiles can also be used to differentiate the different stages of atherosclerotic progression.

The experiments performed in Chapter 3 revealed alteration of specific and previously unreported pathways of glycerophospholipid and sphingolipid metabolism that are associated with atherosclerosis. The level of several lipids was changing throughout the progression of atherosclerosis, whereas others were uniquely changing at stage I or stage II of atherosclerosis. This suggests that plasma lipid profiling could be used as a diagnostic tool for not only the detection of atherosclerosis, but also for the identification of the specific stages of atherosclerotic progression.

Having established the metabolomics techniques to investigate atherosclerosis, as described in Chapter 3, the next objective was to characterize several murine models to explore the association between diabetes and atherosclerosis. Diabetes is a debilitating disease that affects millions of individuals worldwide and is increasing in prevalence at a dramatic rate.^{35,36} Although CVD is responsible for over 70% of deaths in people with diabetes,⁹ the lack of understanding of the underlying molecular mechanisms linking diabetes and CVD complicates the development of preventative and treatment strategies. Expanding the knowledge of how diabetes promotes atherosclerosis will greatly assist in the development of these strategies.

All forms of diabetes are characterized and clinically defined by hyperglycemia.³⁷ Hyperglycemia is also an independent risk factor for CVD.^{38,39} CV risk in humans is increased by 20% for every 1.5 mM increase in blood glucose.⁴⁰ The progressive association between hyperglycemia and CVD is complicated by a number of metabolic and homeostatic deviations including obesity, hypertension and dyslipidemia.^{41,42} This

complex interplay is one of the reasons why numerous basic research and clinical trials carried out to investigate the connection between hyperglycemia and CVD have failed to formulate a clear and defined association.

A great deal of research to uncover the pro-atherogenic effects of hyperglycemia has been focused on understanding the pathways affected by the increase in glucose metabolism and the oxidative stress associated with the hyperglycemic events.^{43,44} Although a number of pre-clinical studies support the causative role of oxidative stress,^{45,46} antioxidant treatments have not demonstrated beneficial effects in reducing CVD risk in diabetic population in any of the large clinical trials in which they were tested.^{47,48} These observations suggest that there may be other causative mechanism(s) that underlie hyperglycemia-induced atherosclerosis and these mechanisms may act independently, or work in parallel to oxidative stress.

Endoplasmic reticulum (ER) stress has been implicated in the pro-atherogenic pathways by multiple independent studies.^{49–51} Chronic ER stress induces the activation of the unfolded protein response (UPR), which is a cellular mechanism of self-defence that functions to return the ER to homeostasis.^{52–54} Chronic ER stress/UPR activation has been associated with hepatic steatosis, insulin resistance and various pro-atherogenic processes including inflammation, endothelial cell apoptosis and lipid accumulation.⁵⁵

Glucosamine, which is a downstream metabolite of glucose, is a potent ER stressinducing agent in cultured vascular cells,⁵⁶ and promotes atherosclerosis in an ApoE^{-/-} mouse model.^{57,58} Therefore, in Chapter 4, we hypothesized that diabetes and conditions

of hyperglycemia may promote atherosclerosis by a mechanism that involves glucosamine-induced ER stress.

In Chapter 4,⁵⁹ we showed that the vascular effects of glucosamine supplementation are comparable to streptozotocin-induced and genetically-induced (Ins2^{Akita}) hyperglycemia in terms of lesional glucosamine, ER stress levels and atherosclerotic burden. These vascular effects are independent of changes in plasma cholesterol and triglycerides. In addition, we show that a chemical chaperone (4-phenylbutyric acid) reduces ER stress levels and attenuates accelerated atherogenesis in each of these models. Together, these findings support a mechanism involving glucosamine-induced ER stress/UPR activation in diabetic atherosclerosis. Recent study in our lab has indicated that glucosamine disrupts ER homeostasis by interfering with lipid-linked oligosaccharide biosynthesis and consequently impairing N-linked glycosylation.⁵⁹ A working model of glucosamine-induced ER stress in hyperglycemia-induced accelerated atherosclerosis is presented in Figure 6.1.

Figure 6.1: A working model of glucosamine-induced endoplasmic reticulum stress in accelerated atherogenesis. Hyperglycemia is known to increase flux through the hexosamine biosynthesis pathway, leading to increased production of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). This results in the disruption of glycosylation and consequently the induction of endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR). Subsequently, these detrimental processes activate pro-atherosclerotic pathways, which lead to accelerated atherosclerosis.



In Chapter 5, we applied the comprehensive metabolomics techniques, established in Chapter 3, to explore the association between hyperglycemia and atherosclerosis in three disease mouse models, established in Chapter 4.

Currently, there are no ideal experimental models to study cardiovascular complications of diabetes, as each has its own advantages and disadvantages.^{60,61} Chemical toxins, such as STZ, are commonly utilized and allow for hyperglycemia to be induced at different stages of lesion development. However, the toxicity of these chemicals may affect other tissues and organs.^{62–64} Genetic models avoid the potential non-specific effects of toxins; however, the development of diabetes in these mono-genetic models is unlike diabetes in humans, which is multigenic in nature. The simultaneous use of three distinct models facilitates the evaluation/identification of common metabolic alteration(s) and molecular mechanism(s) of atherogenesis, independent of model-specific changes or artifacts.

In Chapter 5, comprehensive metabolomic profiles differentiated mice with accelerated atherogenesis from the control. The three mouse models exhibited both unique and common changes in the plasma metabolome. Identification of the commonly altered metabolite features revealed alterations in glycerophospholipid and sphingolipid metabolisms, as well as key atherosclerosis-associated processes including inflammation and oxidative stress. Glycosphingolipid pathway was found to be the most significantly altered pathway in each mouse model of hyperglycemia-induced accelerated atherosclerosis. Moreover, this pathway was augmented at the initial stage of hyperglycemia, prior to the development of accelerated atherosclerosis. This finding

suggests that glycosphingolipids contribute to the induction of accelerated progression of atherosclerosis in hyperglycemic mice.

We also showed that glycosphingolipid metabolites induce oxidative stress and increase levels of inflammatory cytokines in cultured human vascular cells. Inflammation and oxidative stress pathways are closely interlinked in the pathogenesis of diabetic complications. In fact, hyperglycemia is known to be an inducer of oxidative stress, which activates pro-inflammatory pathways.⁴³ This is consistent with our data that attenuation of oxidative stress, using an antioxidant, reduced the levels of inflammatory cytokines. Together, these data highlight the important involvement of glycosphingolipid pathway in the pathology of hyperglycemia-associated atherosclerosis, suggesting that this metabolic pathway may be a therapeutic target for the development of strategies to block or slow atherogenesis in diabetic patients.

An overall summary of this thesis is presented in Figure 6.2.

Figure 6.2: An overall summary of this thesis.



6.2 Limitations and Future Directions

The metabolome is an extremely complex mixture composed of many classes of metabolites with differences in bioavailability and chemical diversity. Over the past decade, the field of metabolomics has expanded rapidly, thanks to the advances in analytical techniques and biochemoinformatic tools. Nevertheless, there is always a demand for a better and more sophisticated analytical platform that is capable of detecting more diverse array of metabolites with greater dynamic range. The rapid development of metabolite databases such as Human Metabolome Database⁶⁵ with over 40,000 metabolites has greatly assisted in the identification of unknown metabolites and understanding of their biochemical and clinical implications of the metabolites. However, there still remains a large number of 'unknown unknown' metabolites, which could potentially provide great insights into novel metabolic alterations and mechanisms of a disease. Therefore, there remains a need for future advancement in metabolomic techniques including analytical platforms, biochemoinformatic tools and metabolite databases.

Murine models are currently the preferred species for atherosclerosis investigation because of many advantages such as ease of genetic manipulation, which allows for specific gene knockout or knockin through the whole-body or in specific tissue/cell type of interest. All the studies performed in this thesis were done on experimental mouse model with ApoE-deficient background. Atherosclerosis in ApoE^{-/-} mice is driven by impaired clearance of cholesterol-enriched lipoproteins, which results in elevated levels

of plasma cholesterol and atherogenic remnants.^{66–68} Therefore, the detectable molecular alterations could be associated with the ApoE-deficient background. Also, the lipid profile of ApoE^{-/-} mice is significantly different from that of humans. In mice, plasma cholesterol is mainly carried on lipoprotein remnants, whereas in humans, cholesterol is predominately in the form of low-density lipoprotein particles.

Although atherosclerotic lesions observed in ApoE^{-/-} mice are very similar to those that develop in humans, the main site of atherosclerotic development is different in mice (e.g. aortic root), compared to in humans (e.g. coronary arteries). Mice also do not manifest the unstable plaque with overlying thrombosis. In addition, ApoE^{-/-} mice spontaneously develop atherosclerosis (in the absence of hyperglycemia) and therefore the detected changes in the metabolome are indicative of acceleration of the atherogenic process. Future studies should be performed in mice with a different genetic backgrounds or different species of experimental animals (especially the larger models) in order to ensure the findings are translational to different models. Also, due to the fundamental differences between humans and experimental animals, disease mechanisms and biomarkers discovered in animal models will need to be validated in human subjects.

Biomarker discovery in humans is extremely complicated due to the genetic diversity in human population and multiple confounding factors including age, diet, medications and comorbidities. As such, the discovery of biomarkers in human often requires the ability to differentiate subtle clinical symptoms or phenotypes, not only between patients versus healthy individuals, but also within the patient group. Current diagnosis of atherosclerosis

is not suitable for population-based studies because it is invasive, insensitive to early stage disease, time-consuming and expensive. Biomarker discovery in this area either identified specific atherosclerosis biomarkers in small but well-defined phenotype cohorts or proposed indirect biomarkers of atherosclerosis or CV risk in large epidemiological cohorts.

To summarize, animal-based studies offer a great opportunity to gain insight into the pathophysiological mechanisms of the disease and facilitate the discovery of early disease biomarkers prior to the appearance of disease phenotypes. On the other hand, human studies are complicated by confounding factors such as age, diet, medication and other CV risks. Replication and validation of the identified biomarkers are therefore required in multiple human cohorts.

Perhaps ideally, human and animal-based studies should be integrated to translate biomarkers associated with a disease to the molecular mechanisms underlying that association. An excellent example of this type of collaboration was done in the Hazen lab.⁶⁹ In this study, plasma from human patients with a myocardial infarction, stroke or death was compared to age- and gender-matched controls. Comprehensive metabolomic analyses identified choline, trimethylamine N-oxide and betaine to be significantly associated with CVD risk. These metabolites were further validated in an independent cohort and showed significant correlations between one another, suggesting their potential interconnectivity in a common metabolic pathway. Complementary functional studies showed that they are metabolites of dietary phosphatidylcholine and implied the

role of intestinal microbiome in atherogenesis. In the follow-up mechanistic studies, mice supplemented with dietary choline showed accelerated development of atherosclerosis and suppression of intestinal microflora inhibits this accelerated development. This is a very well-designed translational study that demonstrates a wide range of applications of both targeted and untargeted metabolomics in biomarker discovery and novel pathway/mechanism elucidation.

The identification of specific blood-born metabolites associated with the development and progression of atherosclerosis could lead to the establishment of a relatively noninvasive, effective and cost-efficient platform to diagnosis the presence and perhaps the stage of atherosclerosis in human patients. These metabolomics-based biomarkers can also be used to identify those at risk for major adverse cardiac events, independent of traditional risk factors.

Metabolomics measures more than traditional CV risk factors such as cholesterol and triglycerides. It covers many key metabolites and important biological pathways. Metabolomics allows the classification of individuals based on their molecular phenotype or "metabotypes", not their visible phenotype. Therefore, metabolite profiling allows great customization of a given treatment according to their specific biomarker profiles, giving rise to precision medicine.

One of the most successful examples of metabolomics in precision medicine is newborn screening.⁷⁰ This screening system is done shortly after birth to look for treatable diseases, including many metabolic and endocrine diseases, cystic fibrosis and critical

congenital heart disease. These diseases usually show no symptoms in the newborn period. Early diagnosis allows timely intervention that prevents serious health problems later in life and can save lives. For adults, many screening systems have been employed including breast cancer and cervical cancer. Ultimately, metabolite profiling can be part of adult screening for complex metabolic disorders such as diabetes, cardiovascular diseases and cancer. In additional, metabolomics can be combined with other 'omics' studies including genomics, proteomics and the recently emerging microbiomics and epigenomics for precision health. This will truly open a new era of personalized medicine.

6.3 Conclusion

In summary, this thesis determined novel molecular pathophysiological alterations associated with the diseases and identified potential biomarkers of atherosclerosis and/or diabetic atherosclerosis. These findings provide insight for future targeted metabolomicsbased research to further investigate the molecular mechanisms underlying diabetic atherosclerosis and discover novel biomarkers associated with the identified pathways and metabolisms. Ultimately, these findings should be translated and validated in human subjects.

Metabolomics-based discovery has shown great potential in atherosclerosis and diabetes research. It can be used to assess disease risk, diagnose the disease, define pathological mechanisms and identify therapeutic targets. Metabolomics is still a relatively new field which poses some limitations and difficulties in identification of metabolites and interpretation of its data. Emerging analytical and biochemoinformatic techniques will allow a more accurate and comprehensive profiling of the metabolome, which will greatly expand our understanding of the specific agents and pathways involved in diabetes-induced accelerated atherosclerosis. This will allow for the development of fast and non-invasive diagnostic methods of atherosclerosis and diabetic atherosclerosis, and the discovery of novel therapeutic strategies to slow or stop atherogenesis in diabetic population.

6.4 References

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APPENDIX 1

Supplemental Material for Chapter 3

Foreword

Appendix 1 is a direct representation of the supplemental material published in *Scientific Report*, volume 6, article number 35037, October 2016. The experiments were conducted by Vi Dang with the assistance from the co-authors.

The citation for this publication is as follows:

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Supplementary Figures

Supplementary Figure S1.1: FPLC analysis of plasma lipoprotein profiles for ApoE^{-/-} and ApoE^{+/-} mice at 5, 10 and 15 weeks of age. Representative profiles derived from pooled plasma from 3 mice per group are shown.



Supplementary Figure S1.2: Apo $E^{+/-}$ mice do not develop atherosclerosis at any age. Representative images of Masson's trichrome stained aortic cross-sections of Apo $E^{+/-}$ mice at 5, 10 and 15 weeks of age.



Supplementary Figure S1.3: Further characterization of atherosclerotic progression. (A) Representative images of aortic cross-sections of $ApoE^{-/-}$ mice at stage I and II of atherosclerosis, stained with Masson's trichrome or TUNEL. (B) Quantification of lesional collagen content and apoptotic cell death. Percentage of collagen was calculated by dividing positively stained areas by total lesion area. Number of apoptotic cells was measured in the cross-section of the maximal lesion area. Data are presented as the mean \pm SD. n=5/group.



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Supplementary Figure S1.4: Analytical quality assurance of instrumental performance. Tight clustering of pooled samples in the principle component analysis demonstrated good instrumental reproducibility throughout the period of analysis. D and H indicate $ApoE^{-/-}$ and $ApoE^{+/-}$, respectively. n=7 per $ApoE^{-/-}$ group, n=4-6 per $ApoE^{+/-}$ group and n=12 for pooled samples.



Supplementary Tables

Supplementary Table S1.1: Analysis of metabolic parameters of $ApoE^{-/-}$ and $ApoE^{+/-}$ mice at 5, 10 and 15 weeks of age. *p<0.05, n=7 per $ApoE^{-/-}$ group and n=4-6 per $ApoE^{+/-}$ group.

Model	Age (week)	Body weight (g)	Fasting glucose (mM)	Plasma triglycerides (mM)	Plasma cholesterol (mM)
	5	13.5±0.7	8.5±1.4	$0.9{\pm}0.2$	6.5±1.1
ApoE ^{-/-}	10	18.7±1.2	7.7±1.0	1.0±0.2	6.3±1.3
	15	19.9±1.2	8.7±1.4	1.0±0.1	6.6±1.1
	5	13.4±0.6	7.9±0.8	0.8±0.2	2.6±0.4*
ApoE ^{+/-}	10	17.1±1.5	8.9±1.5	0.8±0.1	2.4±0.9*
	15	20.2±1.9	8.8±1.2	0.7±0.2	2.2±0.5*

Supplementary Table S1.2: Identification of metabolites that were altered at a more extreme significance cut-offs (p-value <0.0001 and fold change > \pm 5) in ApoE^{-/-} mice, compared to the age-match ApoE^{+/-} control mice. Fold change is relative to the age-matched control. RT: retention time, FC: fold change, nd: not determined due to undetected levels in [#]ApoE^{-/-} mice or *ApoE^{+/-} mice.

CAR: acylcarnitine, Cer: ceramide, DAG: diacylglycerol, HexCer: hexosyl-ceramide, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PIP: PI-phosphate, SM: sphingomyelin, TG: triacylglycerol.

/	RT	RT metabolita 5		ek 10-w		veek 15-		-week	
111/Z	(min)	metabolite	<i>p</i> -value	FC	<i>p</i> -value	FC	<i>p</i> -value	FC	
(+) 728.525	10.0	PC (32:3)	7.4E-06	5.0	1.7E-06	5.7	1.3E-06	7.7	
(+) 729.586	11.3	SM(d36:2)	7.1E-05	5.7	1.1E-09	9.2	3.3E-07	9.9	
(–) 733.552	10.2	DAG(42:5)	5.5E-06	5.2	1.9E-08	5.8	1.6E-07	7.4	
(+) 747.723	26.0	DAG(O-46:2)	7.4E-05	$\mathrm{nd}^{\#}$	4.3E-07	$\mathrm{nd}^{\#}$	9.4E-10	nd [#]	
(+) 408.267	10.9	CAR(14:1-OH)	-	-	2.6E-11	7.6	4.2E-07	5.7	
(+) 442.260	10.9	PA(16:0)	-	-	3.8E-11	6.4	1.4E-07	5.7	
(+) 725.554	10.8	SM(d34:1)	-	-	1.9E-09	5.6	5.4E-10	6.7	
(+) 731.604	20.0	SM(d36:1)	-	-	3.9E-06	5.0	5.2E-06	6.3	
(+) 826.662	5.6	PC(O-40:3)	-	-	5.2E-06	nd*	2.1E-07	nd*	
(–) 893.699	11.0	PA(50:3)	-	-	1.0E-06	6.9	4.8E-06	9.3	
(+) 797.662	6.5	TG(46:2)	1.0E-06	6.2	-	-	-	-	
(+) 575.511	33.0	DAG(O-34:4)	-	-	1.5E-05	7.9	-	-	
(–) 825.370	11.1	PIP(28:4)	-	-	2.0E-05	5.8	-	-	
(+) 839.478	3.2	PG(40:9)	-	-	2.4E-05	5.8	-	-	
(–) 883.541	10.9	PI(38:5)	-	-	1.1E-10	32.2	-	-	
(+) 624.591	30.3	CAR(32:0)	-	-	-	-	3.7E-05	7.0	
(+) 689.556	10.0	HexCer (d32:1)	-	-	-	-	4.6E-07	6.1	
(+) 691.558	10.0	PA(O-36:0)	-	-	-	-	8.4E-07	6.6	
(+) 700.570	3.6	HexCer (d34:1)	-	-	-	-	2.8E-06	6.7	
(+) 711.539	10.0	PA(O-36:1)	-	-	-	-	4.6E-06	5.9	

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(+) 727.523	10.3	PA(36:0)	-	-	-	-	1.2E-05	5.2
(+) 729.523	10.0	PE(34:4)	-	-	-	-	5.0E-06	5.0
(–) 735.528	9.7	PA(O-40:5)	-	-	-	-	3.2E-06	6.5
(+) 739.529	4.0	DAG(44:10)	-	-	-	-	1.9E-06	5.5
(+) 741.529	10.6	DAG(44:9)	-	-	-	-	2.7E-05	6.8
(+) 742.535	10.6	PE(36:3)	-	-	-	-	1.0E-05	78.5
(+) 772.533	5.3	PE(O-38:6)	-	-	-	-	1.0E-08	6.0
(+) 793.546	11.0	Hex ₂ Cer (d28:2)	-	-	-	-	2.1E-08	5.0

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Supplementary Table S1.3: A total of 137 metabolites were found to be significantly altered (ANOVA p < 0.01 followed by Fisher's LSD *post hoc* test) between the three stages of atherosclerosis in ApoE^{-/-} mice. These metabolites were identified based on available authentic standards (level 1 identification), tentatively assigned based on structural analogs (level 2 identification), or by matching their accurate mass/empirical formula within metabolite databases including METLIN, Human Metabolome Database and Lipid Map (level 3 identification). RT: retention time, LSD: Least Significant Difference, I-L: Identification Level.

CAR: acyl carnitine, Cer: ceramide, CerP: ceramide phosphate, DAG: diacylglycerol, FA: fatty acid, HexCer: hexosyl ceramide, MAG: monoacylglycerol, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, SHexCer: sulfatide, SM: sphingomyelin, TAG: triacylglycerol.

m/z	RT (min)	<i>p</i> -value	Fisher's LSD	Metabolite	I-L
(+)90.092	8.9	9.40E-03	5w-10w; 5w-15w	Dimethylethanolamine	2
(+)110.071	14.4	5.30E-04	5w-10w; 5w-15w	Glycerol	2
(+)112.053	8.4	4.30E-05	5w-10w; 5w-15w	Cytosine	1
(+)116.071	14.9	7.40E-03	5w-10w; 5w-15w	Acetamidopropanal	2
(-)119.041	10.6	6.80E-03	10w-5w; 15w-5w	Deoxythreonic acid	2
(+)130.050	11.9	4.20E-04	5w-10w; 5w-15w	N-Acryloylglycine	2
(+)130.084	8.9	6.50E-03	5w-10w; 5w-15w	Pipecolic acid	2
(+)130.087	14.6	2.10E-04	5w-10w; 5w-15w	Acetylaminobutanal	2
(+)131.082	14	8.90E-03	5w-10w	Pyrroline-carboxylic acid	2
(+)132.066	14	5.70E-03	5w-10w; 5w-15w	Propionylglycine	
(+)132.102	4.6	1.10E-03	5w-10w; 5w-15w	FA(6:1)	
(+)140.068	8.5	7.10E-06	5w-10w; 5w-15w	Dihydroxybenzylamine	2
(-)145.064	11.9	1.70E-04	5w-10w; 5w-15w	Glutamine	
(+)147.113	14.6	2.50E-04	5w-10w; 5w-15w	Lysine	1
(+)148.096	8.8	1.40E-03	5w-10w; 5w-15w	Isoleucine	1
(+)156.042	8.5	8.50E-05	5w-10w; 5w-15w	N-Methylethanolaminium phosphate	2
(+)156.077	14.4	1.30E-03	5w-10w; 5w-15w	Histidine	1
(-)159.067	2.9	9.80E-03	5w-10w; 5w-15w	Methyladipic acid	2
(-)159.115	14	5.30E-03	5w-10w; 5w-15w	Methyl-Lysine	1
(+)161.085	14	4.30E-04	5w-10w; 5w-15w	Alanyl-Alanine	2
(-)164.073	9	2.30E-03	5w-10w; 5w-15w	Phenylalanine	1
(+)169.059	11.9	7.70E-05	5w-10w; 5w-15w	Pyrroloyl-Glycine	2
(-)172.992	2.2	1.50E-04	10w-5w; 15w-5w	Phenol sulphate	2
(+)175.119	14.6	2.00E-04	5w-10w; 5w-15w	Arginine	

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(-)181.072	9.8	2.60E-03	5w-10w; 5w-15w	Hexitol (C ₆ H ₁₄ O ₆)	2
(+)185.033	11.9	4.60E-05	5w-10w; 5w-15w	Hydroxymethylglutaric acid	2
(+)187.073	14	9.20E-04	5w-10w; 5w-15w	Pyroglutamylglycine	2
(-)203.103	14	8.70E-03	5w-10w; 5w-15w	Serinyl-Valine	2
(+)206.049	2.5	7.90E-03	10w-15w; 10w-w	p-Cresol sulfate	3
(+)208.051	12.9	3.20E-04	5w-10w; 5w-15w	Oxalosuccinate	2
(+)217.083	12.5	1.00E-02	5w-10w; 5w-15w	Hydroxyphenyl-valeric acid	2
(+)229.154	9.4	3.60E-03	10w-5w; 15w-5w	Isoleucyl-Proline	2
(-)235.146	4	3.90E-03	10w-5w; 10w- 15w	FA(12:0)	1
(+)241.130	14.4	2.40E-03	10w-5w; 15w-5w	Alanyl-methyl-Histidine	2
(+)247.092	14	7.20E-03	5w-10w; 5w-15w	Aspartyl-Hydroxyproline	2
(-)257.004	3.9	9.20E-03	10w-15w; 5w- 15w	Phosphonoglucono-lactone	2
(+)267.136	11.5	3.20E-03	5w-10w; 5w-15w	Threoninyl-Phenylalanine	2
(+)273.121	8.4	1.30E-03	5w-10w; 5w-15w	5C-Aglycone	2
(+)275.118	8.4	3.00E-03	5w-10w; 5w-15w	Glutamyl-Glutamine	2
(-)282.155	5	3.70E-03	5w-10w; 5w-15w	Lysyl-Histidine	2
(-)309.169	3.3	1.80E-03	5w-10w; 5w-15w	FA(18:5)	2
(+)315.079	14	8.50E-03	5w-10w	Cysteinylglycine disulfide	2
(-)336.099	4.3	2.90E-03	5w-10w; 5w-15w	S-Hydroxy- methylglutathione	2
(+)361.170	8.5	7.80E-03	5w-10w; 5w-15w	Leukotriene derivative	3
(+)370.054	14.4	5.50E-03	10w-5w	Adenosine monophosphate	1
(+)377.144	5.1	6.50E-04	5w-10w; 5w-15w	Riboflavin	2
(+)380.254	9.9	2.90E-03	5w-10w; 5w-15w	Sphingosine 1-phosphate	3
(+)390.958	10.9	3.50E-03	5w-10w; 5w-15w	Phosphoribosyl pyrophosphate	2

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(+)391.283	2.4	1.80E-03	15w-10w; 5w- 10w	Ketolithocholic acid	2
(-)398.124	4.3	3.50E-03	5w-10w; 5w-15w	S-Adenosylmethionine	2
(+)404.313	2.2	2.50E-03	5w-10w; 5w-15w	Glycerol trihexanoate	1
(+)423.299	3.5	3.90E-03	15w-10w; 5w- 10w	DHAP(18:0e)	3
(+)428.372	6.1	9.90E-03	5w-10w; 5w-15w	CAR(18:0)	2
(+)429.294	4.8	6.40E-03	5w-10w; 5w-15w	MAG(22:4)	2
(-)435.240	10.2	1.60E-04	15w-10w; 5w- 10w; 15w-5w	Lyso-PA(18:1)	2
(-)457.234	8.9	3.80E-03	5w-10w; 5w-15w	Sulfodeoxycholic acid	2
(-)463.304	2.2	1.60E-04	5w-10w; 5w-15w	FA(28:6-OH)	2
(-)464.314	8.8	1.70E-03	15w-5w; 15w- 10w	Lyso-PE(P-18:0)	2
(+)470.416	6.4	8.40E-03	5w-15w; 10w- 15w	Cer(t28:1)	3
(-)477.308	2.2	3.60E-04	5w-10w; 5w-15w	Steroid derivative (C29H46O3)	3
(+)532.294	8.1	2.30E-03	5w-10w; 5w-15w	Lyso-PS(P-18:0)	2
(+)539.337	3.6	8.90E-03	5w-10w; 15w- 10w	Lyso-PG(20:1)	2
(+)540.366	8.7	3.70E-03	15w-5w; 15w- 10w	Lyso-PS(O-20:0)	2
(-)552.498	9.3	4.90E-03	5w-10w; 5w-15w	Cer(t34:1)	3
(-)557.458	2.9	8.90E-03	5w-10w; 5w-15w	FA (34:1-OH)	2
(-)558.333	8.3	6.90E-03	10w-5w; 15w-5w	CerP(t26:0)	3
(-)568.362	8.4	4.00E-03	10w-5w; 15w-5w	CerP(d28:1)	3
(+)574.480	2.5	9.40E-03	5w-10w	Cer(t34:2)	3
(+)578.512	3.5	7.90E-03	5w-10w; 5w-15w	Cer(t34:0)	3
(+)580.903	10.9	5.00E-03	5w-10w	Inositol pentaphosphate	2
(-)585.353	8.4	2.20E-03	10w-5w; 15w-5w	PA(28:3)	2

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(+)586.520	4.3	7.10E-05	15w-5w; 15w- 10w	Cer(d36:2)	3
(+)596.369	9.1	3.50E-05	5w-10w; 5w-15w	PS(22:0)	2
(+)606.447	9	4.20E-03	5w-10w	Lyso-PC(24:1)	2
(+)608.463	9.8	4.40E-03	5w-10w; 15w- 10w	Lyso-PC(24:0)	2
(+)613.417	8.3	3.70E-04	5w-10w; 5w-15w	Ubiquinone-6	3
(+)624.591	30.3	4.10E-03	10w-5w; 15w-5w	CAR(32:0)	2
(-)630.425	9.4	8.40E-04	5w-10w; 5w-15w	PE(28:2)	2
(-)632.417	9.4	5.10E-03	5w-10w; 5w-15w	PE(28:1)	2
(+)637.419	8.3	1.10E-03	5w-10w; 5w-15w	PG(26:1)	2
(+)638.406	8.3	7.60E-04	5w-10w; 5w-15w	Lyso-PE(30:6)	2
(+)639.426	8.3	1.60E-04	5w-10w; 15w- 10w	PG(26:0)	2
(-)640.278	2.2	7.00E-04	10w-5w; 15w-5w	Leukotriene	3
(-)641.525	30.5	3.80E-04	10w-5w; 15w-5w	DAG(38:5)	2
(-)643.398	9	1.70E-03	5w-10w; 5w-15w	Lyso-PG(28:4)	2
(-)657.378	9	1.80E-03	5w-10w; 5w-15w	PG(28:4)	2
(-)658.586	27.1	2.70E-03	10w-5w; 10w- 15w	Cer(d40:0)	3
(-)659.376	9.1	1.10E-03	5w-10w; 5w-15w	Lyso-PA(32:5)	2
(+)673.566	2.8	9.90E-03	5w-10w; 5w-15w	DAG(38:1)	2
(-)677.268	2.3	1.00E-03	10w-5w; 15w-5w	Lyso-PIP(18:1)	2
(+)679.631	3.4	4.40E-03	5w-10w; 5w-15w	DAG(40:1)	2
(+)689.556	10	1.90E-04	15w-5w; 15w- 10w	HexCer(d32:1)	3
(+)691.558	10	2.80E-03	15w-5w; 15w- 10w	PA(O-36:0)	2
(+)697.428	3.3	5.50E-03	10w-5w; 15w-5w	Lyso-PG(30:3)	2
(-)698.558	4	2.40E-03	5w-10w; 15w-	CerP(d40:2)	3

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			10w		
(-)706.355	9.4	5.70E-04	5w-10w; 5w-15w	PS(28:4)	2
(+)706.667	2.5	4.90E-03	5w-10w; 5w-15w	DAG(0-42:3)	2
(+)707.547	10.3	8.30E-04	5w-10w; 5w-15w	PE(32:1)	2
(-)708.354	9.4	5.70E-04	5w-10w; 5w-15w	PS(28:3)	2
(+)710.024	2.3	5.80E-03	10w-5w; 15w-5w	Uridine Diphosphate Acetylhexosamine Sulfate	2
(+)711.539	10	7.20E-04	15w-5w; 15w- 10w	PE-Cer(d36:1)	3
(+)717.546	9.7	4.60E-05	5w-10w; 5w-15w	SM(d34:1-OH)	2
(+)717.573	4.5	4.20E-03	5w-10w; 15w- 10w	PA(O-38:1)	
(+)719.567	11.2	2.60E-04	5w-10w; 5w-15w	Cholesteryl Ester (22:6)	
(-)720.523	3.8	3.20E-03	15w-5w; 15w- 10w	Lyso-PS(32:0)	
(+)727.523	10.3	5.10E-03	15w-5w; 15w- 10w	PA(36:0)	
(+)728.557	21.9	8.30E-04	10w-5w; 15w- 5w; 15w-10w	PE(O-36:3)	
(-)728.560	22.9	2.20E-03	10w-5w; 15w-5w	PE(O-36:2)	2
(+)730.535	9	5.00E-04	5w-10w; 5w- 15w; 15w-10w	PC(32:2)	2
(+)731.540	9	6.80E-04	5w-10w; 5w- 15w; 15w-10w	PE(34:3)	2
(+)732.546	9	6.80E-03	5w-10w	PC(32:1)	2
(-)733.552	10.2	8.90E-06	15w-10w; 15w- 5w	DAG(42:5)	2
(+)734.567	21.5	1.50E-03	5w-10w; 5w-15w	PC(32:0)	1
(-)736.527	3.4	1.90E-04	5w-10w; 5w-15w	CerP(d40:1)	3
(+)739.512	9.6	1.90E-03	5w-10w; 5w-15w	PC(32:6)	2
(+)740.533	3.2	9.20E-03	5w-10w; 15w- 10w	PG(32:0)	1

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(+)744.546	9.9	2.10E-06	5w-10w; 5w-15w	PE(36:2)	1
(+)755.554	3.4	8.00E-05	5w-10w; 5w-15w	PA(40:3)	2
(-)768.541	5.3	4.10E-03	10w-5w; 15w-5w	PE(38:3)	2
(+)772.533	5.3	8.60E-03	15w-5w; 15w- 10w	PE(O-38:6)	2
(+)776.492	3.3	3.80E-04	5w-10w; 5w-15w	SHexCer(d34:3)	3
(+)794.511	9.5	3.90E-05	5w-10w; 5w-15w	SHexCer(t34:2)	3
(+)794.601	20.1	8.80E-03	5w-10w	PC(O-36:2)	2
(+)796.523	11.9	2.20E-03	5w-10w; 5w-15w	PE(40:7)	2
(+)797.662	6.5	4.60E-03	15w-10w; 5w- 10w	TAG(46:2)	2
(-)804.576	21.7	4.30E-03	5w-10w; 5w-15w	HexCer(t38:2)	3
(-)806.582	21.8	7.50E-03	5w-10w; 5w-15w	CerP(t44:2)	3
(+)808.663	5.9	6.50E-04	5w-10w; 15w- 10w	HexCer(d42:3)	
(+)809.644	21.4	6.50E-04	5w-10w; 5w-15w	SM(d40:1)	2
(+)818.603	20.6	9.00E-04	15w-5w; 15w- 10w	PC(O-38:4)	2
(-)820.492	3.3	2.30E-03	5w-10w; 5w-15w	PS(36:3)	2
(+)820.617	21.7	2.00E-03	15w-5w; 15w- 10w	PS(38:0)	2
(+)821.561	8.9	9.40E-04	15w-5w; 15w- 10w	PI(O-34:2)	2
(+)821.619	22	3.10E-03	15w-5w; 15w- 10w	PE(O-42:7)	2
(+)848.634	6.7	9.60E-03	15w-5w; 15w- 10w	PS(40:0)	2
(+)871.609	11.9	3.10E-04	5w-10w; 5w-15w	PC(42:10)	2
(-)902.627	11.9	7.90E-03	5w-10w; 5w- 15wpa	PS(P-46:6)	

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Supplementary Table S1.4: Pathway analysis identified 29 affected pathways that were potentially affected during the progression of atherosclerosis.

Pathway Name	<i>p</i> -value	Impact value
Glycerophospholipid metabolism	0.01	0.30
Sphingolipid metabolism	0.02	0.31
Arginine and proline metabolism	0.04	0.09
Pentose phosphate pathway	0.10	0.02
Phenylalanine, tyrosine and tryptophan biosynthesis	0.11	0.50
Steroid biosynthesis	0.27	0.07
Phenylalanine metabolism	0.28	0.41
Purine metabolism	0.31	0.07
Histidine metabolism	0.36	0.24
Glycerolipid metabolism	0.41	0.28
Alanine, aspartate and glutamate metabolism	0.51	0.15
Cysteine and methionine metabolism	0.55	0.07
Inositol phosphate metabolism	0.56	0.06
Steroid hormone biosynthesis	0.89	0.06
Nitrogen metabolism	0.03	0
Lysine biosynthesis	0.11	0
D-Glutamine and D-glutamate metabolism	0.14	0
Biotin metabolism	0.14	0
Linoleic acid metabolism	0.16	0
alpha-Linolenic acid metabolism	0.23	0
Methane metabolism	0.23	0
Riboflavin metabolism	0.28	0

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Pyrimidine metabolism	0.33	0
beta-Alanine metabolism	0.39	0
Citrate cycle (TCA cycle)	0.45	0
Lysine degradation	0.49	0
Galactose metabolism	0.54	0
Arachidonic acid metabolism	0.66	0
Fatty acid biosynthesis	0.72	0

APPENDIX 2

Supplemental Material for Chapter 4

Foreword

Appendix 2 is a direct representation of the supplemental material published in *Journal of Molecular and Genetic Medicine*, volume 9, issue 4, December 2015. The experiments were conducted by Vi Dang with the assistance from the co-authors.

The citation for this publication is as follows:

Dang VT, Beriault DR, Deng A, Shi Y, Werstuck GH. (2015) Glucosamine-induced ER Stress Accelerates Atherogenesis: A Potential Link between Diabetes and Cardiovascular Disease. *J Mol Genet Med* **9**:197. doi:10.4172/1747-0862.1000197.

Supplementary Figures

Supplementary Figure S2.1: Comparison of O-GlcNAc levels in atherosclerotic lesions of mice supplemented with different levels of glucosamine in drinking water.
Representative images of aortic cross-sections of mice supplemented with 0, 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN) stained with antibodies against O-GlcNAc.



Supplementary Figure S2.2: Comparison of GRP78/94 levels in atherosclerotic lesions of mice supplemented with different levels of glucosamine in drinking water.
Representative images of aortic cross-sections of mice supplemented with 0, 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN) stained with antibodies against GRP78/94.



Supplementary Figure S2.3: Comparison of CHOP levels in atherosclerotic lesions of mice supplemented with different levels of glucosamine in drinking water. Representative images of aortic cross-sections of mice supplemented with 0, 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN) stained with antibodies against CHOP.



Supplementary Figure S2.4: 4PBA reduces GRP78/94levels in atherosclerotic lesions of glucosamine-supplemented and hyperglycemic mouse models. Representative images of aortic cross-sections stained with antibodies against GRP78/94.



Supplementary Figure S2.5: 4PBA reduces CHOP levels in atherosclerotic lesions of glucosamine-supplemented and hyperglycemic mouse models. Representative images of aortic cross-sections stained with antibodies against CHOP.



Supplementary Figure S2.6: 4PBA reduces O-GlcNAc levels in atherosclerotic lesions of glucosamine-supplemented and hyperglycemic mouse models. Representative images of aortic cross-sections stained with antibodies against O-GlcNAc.



APPENDIX 3

Supplemental Material for Chapter 5

Foreword

Appendix 3 is a direct representation of the supplemental material submitted in *American Journal of Pathology*, October 2017. The experiments were conducted by Vi Dang with the assistance from the co-authors.

The citation for this manuscript is as follows:

Dang VT, Zhong LH, Huang A, Deng A, Werstuck GH. (2017) Glycosphingolipids contribute to pro-atherogenic pathways in the pathogenesis of hyperglycemia-induced atherosclerosis. *Am J Pathol* (Submitted).

Supplementary Figures

Supplementary Figure S3.1: Analysis of the quality control samples by principal component analysis for analytical quality assurance of instrumental performance. Tight clustering of pooled samples demonstrates good instrumental reproducibility throughout the period of analysis. n=8/group.



Supplementary Figure S3.2: Top 15 metabolites ranked by mean decrease accuracy

from the Random Forest analysis.



Mean Decrease Accuracy

Supplementary Figure S3.3: Pathway view of the sphingolipid metabolism from the pathway analysis of the 36 identified metabolites. Metabolites are shown as KEGG IDs. Red boxes indicate metabolites detected in this study. C00550: sphingomyelin, C00195: ceramide, C02686: lactosylceramide, C01190: glucosylceramide, C06125: sulfatide. Blue boxes indicate metabolites not detected in this study, but used as background for enrichment analysis.



Supplementary Figure S3.4: Quantification of atherosclerotic lesion volume in 10week-old ApoE^{-/-} (Ctrl), glucosamine-supplemented ApoE^{-/-} (GlcN), ApoE^{-/-}:Ins2^{+/Akita} (Akita) and STZ-injected ApoE^{-/-} (STZ). n=6/group, ns: non-significant versus the control. Data are presented as mean \pm SD.



Supplementary Figure S3.5: Metabolite-metabolite correlation heat map of the 36 identified metabolites. Color keys indicate Pearson correlation coefficients.



Supplementary Figure S3.6: Glycosphingolipid metabolites induce oxidative stress and increase the levels of inflammatory cytokines in human vascular cells. Macrophages, human aortic endothelial cells (HAEC), and human aortic smooth muscle cells (HASMC) were incubated with ceramide, GluCer and LacCer at various concentrations (1, 10 and 50 μ M) for 24 hours. Expression levels of (A-C) glutathione peroxidase and (D-F) IL-1 β were assessed by real-time PCR. Data are presented as mean ±SD. n=5/group, **p*<0.05, ***p*<0.01, and ****p*<0.001 versus the controls.



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Supplementary Figure S3.7: Treatment with α -tocopherol reduces oxidative stress and inflammation induced by glycosphingolipid metabolites. Macrophages, human aortic endothelial cells (HAEC), and human aortic smooth muscle cells (HASMC) were incubated with 50 μ M ceramide, GluCer or LacCer in the presence or absence of 50 μ M α -tocopherol (TOH) for 24 hours. Expression levels of (A-C) glutathione peroxidase and (D-F) IL-1 β were assessed by real-time PCR. Data are presented as mean ±SD. n=5/group, **p*<0.05, ***p*<0.01, and ****p*<0.001 versus the controls.


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Supplementary Tables

Supplementary Table S3.1: Abundance (normalized intensity with internal standard) of 36 significantly altered metabolites (FDR-adjusted p<0.05) that were commonly altered between the three experimental groups with accelerated atherogenesis at 15-week time-point.

RT and FC indicate retention time (in minutes) and fold change, respectively. Cer: ceramide, DAG: diacylglycerol, HexCer: hexosyl-ceramide, L(lipid): lyso, LacCer: lactosyl-ceramide, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin.

Matabalita	Abundance (normalized intensity, x10 ²)							
Metadonte	Ctrl	GlcN	Akita	STZ				
Histamine	0.19 ±0.09	0.33 ±0.10	0.37±0.12	0.36 ±0.10				
Imidazole acetol- phosphate	0.22 ± 0.04	0.17 ± 0.04	0.15 ±0.04	0.16 ±0.06				
Histidine	2.05 ±0.31	1.64 ±0.28	1.46 ±0.20	1.23 ±0.11				
Lipoyllysine	1.53 ±0.21	2.63 ± 0.33	2.14 ± 0.16	2.59 ±0.21				
Glutathione conjugate	24.25 ± 1.41	29.14 ±2.76	31.53 ±2.12	28.82 ±2.27				
Fatty acid (20:1-OH2)	2.15 ±0.50	2.80 ± 0.34	2.89 ±0.33	3.62 ±0.93				
Hydroxy-cholesterol	1.84 ±0.32	2.41 ±0.56	2.14 ±0.25	3.34 ±0.96				
DAG(48:2)	0.40 ±0.16	0.68 ±0.26	0.57 ±0.11	1.27 ± 0.90				
LPA(18:1)	5.14 ±0.75	$6.08\pm\!\!0.90$	5.99 ±0.61	6.46 ± 1.61				
LPA(22:2)	4.77 ±0.97	6.49 ± 1.02	5.97 ± 0.98	6.19 ±1.23				
LPA(24:4)	2.10 ± 0.36	2.51 ±0.26	2.58 ± 0.18	3.21 ±0.52				
LPC(20:3)	63.33 ± 7.98	78.79 ± 6.99	75.94 ± 8.65	86.19±9.56				
LPE(18:3)	0.78 ± 0.09	0.99 ± 0.09	0.90 ± 0.11	1.01 ±0.24				
LPE(20:0)	10.15 ± 2.10	13.19 ± 1.22	15.38 ± 1.68	20.23 ± 3.75				
LPE(24:5)	39.65 ±4.90	51.35 ±3.81	46.71 ±3.20	69.59 ±8.92				
PA(O-40:6)	6.74 ± 0.64	7.97 ±1.12	8.57 ± 0.78	12.31 ±2.72				
PC(32:1)	0.28 ± 0.05	0.36 ± 0.08	0.35 ± 0.06	0.55 ±0.15				
PE(38:1)	4.69 ± 1.01	6.20 ± 1.31	6.22 ± 0.70	9.55 ±2.76				
PE(40:7)	0.65 ±0.11	0.77 ±0.13	0.93 ±0.15	1.26 ± 0.43				
PE(42:9)	0.16 ± 0.07	0.23 ± 0.08	0.24 ± 0.06	0.29 ±0.12				
PE(44:11)	0.09 ± 0.02	0.23 ± 0.04	0.17 ± 0.03	0.22 ±0.03				
Cer(t34:1)	0.20 ± 0.04	0.26 ± 0.02	0.34 ±0.03	0.30 ±0.09				
Cer(d36:2)	0.03 ± 0.02	0.07 ± 0.04	0.05 ± 0.04	0.08 ± 0.05				
Cer(d36:1)	0.03 ±0.01	0.04 ± 0.01	0.08 ± 0.01	0.06 ± 0.02				

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GluCer(t34:1)	2.25 ± 0.28	3.89 ± 0.34	2.78 ± 0.43	4.37 ±0.94
GluCer(t36:1)	21.94 ±3.28	28.29 ±3.16	36.20 ± 3.27	39.90 ±4.20
GluCer(d36:1)	0.68 ± 0.09	1.22 ± 0.10	1.82 ± 0.11	1.57 ±0.24
LacCer(d28:2)	0.50 ± 0.11	$0.70\pm\!0.07$	0.91 ± 0.07	1.06 ± 0.10
LacCer(t28:1)	0.86 ± 0.31	1.14 ±0.17	1.43 ±0.22	1.36 ± 0.56
LacCer(d36:1)	0.07 ± 0.03	0.13 ±0.06	0.13 ±0.05	0.14 ± 0.07
SM(d36:2)	0.91 ±0.22	1.28 ±0.22	1.75 ±0.18	2.45 ±0.38
SM(d34:1)	1.43 ±0.11	1.78 ±0.16	2.70 ± 0.17	4.18 ±0.37
SM(d42:1-OH)	0.18 ± 0.05	0.25 ± 0.05	0.23 ± 0.04	0.34 ±0.16
Deoxypyridinoline	0.48 ± 0.12	0.20 ± 0.13	0.24 ± 0.12	0.22 ±0.12
Heparan sulfate glucosaminide	0.39 ± 0.10	0.55 ±0.12	0.53 ± 0.07	0.73 ±0.18
Octaprenyl diphosphate	0.06 ± 0.02	0.09 ± 0.02	0.12 ± 0.03	0.10 ± 0.03

Supplementary Table S3.2: Summary of 36 significantly altered metabolites (FDRadjusted p<0.05) that were commonly altered between the three experimental groups with accelerated atherogenesis at 15-week time-point.

RT and FC indicate retention time (in minutes) and fold change (versus the control), respectively. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 versus the control.

Cer: ceramide, DAG: diacylglycerol, HexCer: hexosyl-ceramide, L(lipid): lyso, LacCer: lactosyl-ceramide, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin.

	RT Metabolite	GlcN vs Ctrl		Akita vs Ctrl		STZ vs Ctrl		Matabalia	
m/z		Metabolite	p-value	FC	p-value	FC	p-value	FC	Metadolism
(+)112.09	14.4	Histamine	*	1.7	*	1.9	*	1.9	
(+)221.03	4.5	Imidazole acetol- phosphate	**	0.8	**	0.7	*	0.7	Inflammation
(+)156.08	13.1	Histidine	*	0.8	*	0.7	*	0.6	
(-)369.11	2.6	Lipoyllysine	**	1.7	*	1.4	*	1.7	
(-)626.31	7.7	Glutathione conjugate	***	1.2	*	1.3	*	1.2	Oxidative
(+)343.28	11.7	Fatty acid (20:1-OH2)	*	1.3	***	1.3	***	1.7	stress
(+)425.34	2.8	Hydroxy-cholesterol	**	1.3	*	1.2	***	1.8	
(+)827.68	2.8	DAG(48:2)	**	1.7	**	1.4	**	3.1	
(+)454.29	8.1	LPA(18:1)	*	1.2	**	1.2	*	1.3	
(-)525.29	7.9	LPA(22:2)	***	1.4	**	1.3	**	1.3	
(+)532.34	7.7	LPA(24:4)	**	1.2	***	1.2	***	1.5	
(+)546.35	7.7	LPC(20:3)	***	1.2	**	1.2	**	1.4	Glycero-
(+)476.27	8.1	LPE(18:3)	*	1.4	*	1.2	**	1.3	phospholipid
(+)510.36	7.7	LPE(20:0)	***	1.3	*	1.5	***	2	
(-)554.32	7.7	LPE(24:5)	***	1.3	*	1.2	***	1.8	
(+)752.55	23.8	PA(O-40:6)	*	1.2	*	1.3	***	1.8	
(+)754.53	4.2	PC(32:1)	**	1.3	**	1.3	****	2	
(+)774.60	24.5	PE(38:1)	**	1.3	***	1.3	**	2	

(+)790.54	9.5	PE(40:7)	*	1.2	***	1.4	***	1.9	
(+)836.51	7.7	PE(42:9)	*	1.5	**	1.5	**	1.8	
(+)855.57	10.2	PE(44:11)	*	2.4	*	1.8	***	2.4	
(+)540.37	9.3	Cer(t34:1)	**	1.3	***	1.5	**	1.3	
(+)586.52	5	Cer(d36:2)	*	2	*	1.4	**	2.3	
(+)588.51	4.4	Cer(d36:1)	*	1.5	**	2.7	*	2	
(+)716.57	4.2	GluCer(t34:1)	***	1.3	*	1.2	***	1.7	
(-)778.57	4.8	GluCer(t36:1)	*	1.4	**	1.6	*	1.8	
(+)745.63	4.4	GluCer(d36:1)	**	1.8	**	2.4	**	2.3	Sphingolinid
(+)793.53	10.5	LacCer(d28:2)	*	1.5	*	1.9	**	2.1	Springonpid
(+)794.52	7.7	LacCer(t28:1)	*	1.4	*	1.7	*	1.5	
(+)907.68	5.4	LacCer(d36:1)	*	1.7	**	1.6	**	1.9	
(+)729.59	11.3	SM(d36:2)	*	1.2	**	1.9	**	2.6	
(+)731.60	19.9	SM(d34:1)	*	1.3	**	2	***	2.9	
(+)829.69	2.8	SM(d42:1-OH)	***	1.4	*	1.3	**	2	
(+)451.16	5.4	Deoxypyridinoline	**	0.4	*	0.5	*	0.5	
(+)795.02	7.7	Heparan sulfate glucosaminide	*	1.4	*	1.4	***	1.9	Unknown
(+)761.40	7.8	Octaprenyl diphosphate	***	1.5	***	2	***	1.7	

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Supplementary Table S3: Summary of 36 metabolites at 10-week time-point. These metabolites were significantly altered in 15-week-old mice as indicated previously in the Supplementary Table II.

RT and FC indicate retention time (in minutes) and fold change (versus the control), respectively. ns: non-significant, *p<0.05, **p<0.01 and ***p<0.001 versus the control.

Cer: ceramide, DAG: diacylglycerol, HexCer: hexosyl-ceramide, L(lipid): lyso, LacCer: lactosyl-ceramide, PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin.

	рт	Matabalita	GlcN v	GlcN vs Ctrl Akita vs (vs Ctrl	s Ctrl STZ vs Ctrl		Matabalian
III/Z	NI	Metabolite	p-value	FC	p-value	FC	p-value	FC	Metadolishi
(+)112.09	14.4	Histamine	*	1.6	*	1.5	**	1.7	
(+)221.03	4.5	Imidazole acetol- phosphate	*	0.5	ns	0.6	**	0.5	Inflammation
(+)156.08	13.1	Histidine	ns	0.6	*	0.6	*	0.5	
(-)369.11	2.6	Lipoyllysine	*	1.5	*	1.6	*	1.6	
(-)626.31	7.7	Glutathione conjugate	**	1.5	*	1.4	*	1.5	Oxidative
(+)343.28	11.7	Fatty acid (20:1-OH2)	ns	1.2	***	2.5	**	2.1	stress
(+)425.34	2.8	Hydroxy-cholesterol	*	1.2	*	1.4	**	2.5	
(+)827.68	2.8	DAG(48:2)	*	1.5	**	1.7	*	1.6	
(+)454.29	8.1	LPA(18:1)	ns	1.0	*	1.3	**	2.3	
(-)525.29	7.9	LPA(22:2)	ns	0.9	ns	0.8	ns	1.1	
(+)532.34	7.7	LPA(24:4)	ns	1.1	**	1.3	**	1.5	
(+)546.35	7.7	LPC(20:3)	ns	0.9	ns	1.1	**	1.8	Glycero-
(+)476.27	8.1	LPE(18:3)	ns	1.2	ns	0.9	*	1.4	phospholipid
(+)510.36	7.7	LPE(20:0)	ns	1.1	ns	1.2	***	2.3	
(-)554.32	7.7	LPE(24:5)	ns	1.0	ns	1.0	*	1.7	
(+)752.55	23.8	PA(O-40:6)	*	1.4	*	1.6	***	2.8	
(+)754.53	4.2	PC(32:1)	*	1.1	*	1.4	**	2.6	
(+)774.60	24.5	PE(38:1)	ns	1.0	*	1.5	*	1.9	

(+)790.54	9.5	PE(40:7)	ns	1.0	ns	1.3	ns	1.5	
(+)836.51	7.7	PE(42:9)	ns	0.9	ns	1.6	*	2.1	
(+)855.57	10.2	PE(44:11)	ns	0.8	ns	1.1	**	2.3	
(+)540.37	9.3	Cer(t34:1)	*	1.5	**	2.0	*	1.5	
(+)586.52	5	Cer(d36:2)	*	1.8	**	2.0	*	1.3	
(+)588.51	4.4	Cer(d36:1)	**	2.2	*	1.6	*	1.2	
(+)716.57	4.2	GluCer(t34:1)	*	1.3	**	2.2	**	2.0	
(-)778.57	4.8	GluCer(t36:1)	*	1.5	**	1.7	**	1.4	
(+)745.63	4.4	GluCer(d36:1)	**	2.0	*	1.4	**	2.5	Sphingolinid
(+)793.53	10.5	LacCer(d28:2)	*	1.3	**	2.1	*	1.5	Springonpid
(+)794.52	7.7	LacCer(t28:1)	*	1.3	**	1.7	*	1.5	
(+)907.68	5.4	LacCer(d36:1)	**	21.9	*	1.7	**	1.7	
(+)729.59	11.3	SM(d36:2)	*	1.2	**	1.3	**	0.9	
(+)731.60	19.9	SM(d34:1)	**	2.0	*	1.8	**	3.2	
(+)829.69	2.8	SM(d42:1-OH)	**	1.4	*	1.5	*	1.7	
(+)451.16	5.4	Deoxypyridinoline	*	0.5	ns	0.6	ns	0.7	
(+)795.02	7.7	Heparan sulfate glucosaminide	ns	1.3	ns	1.2	*	1.4	Unknown
(+)761.40	7.8	Octaprenyl diphosphate	**	1.4	***	2.9	**	1.9	