VASA VASORUM IN HYPERGLYCEMIA INDUCED ATHEROSCLEROSIS

INVESTIGATING THE ATHEROGENIC POTENTIAL OF CHRONIC HYPERGLYCEMIA: IS DIABETIC ATHEROSCLEROSIS A MICROVASCULAR COMPLICATION?

By

KALEY JENNIFER VEERMAN, B.H.Sc. (Hons.)

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

© Copyright by Kaley J. Veerman, 2011

MASTER OF SCIENCE (2011) McMaster University

(Medical Sciences) Hamilton, Ontario

TITLE: Investigating the pro-atherogenic potential of chronic hyperlgycemia: is diabetic atherosclerosis a m complication?

AUTHOR: Kaley Jennifer Veerman, B.H.Sc. (Hons.) (McMaster University)

SUPERVISOR: Geoff H. Werstuck, Ph.D.

NUMBER OF PAGES: xvi, 122

ABSTRACT

Diabetes mellitus (DM) is associated with a significant
microvascular complications, such as retinopathy, nephrop
as well as macrovascular disorders, including cardiovascular disease.
Traditionally, micro- and macrovascular complications of DM have bee
considered distinct and independent disorders; however, da
epidemiological and pathophysiological studies suggest the
been suggested that the vasa vasorum, a microvascular network which nouri
the walls of large muscular arteries, may play a role in ma
atherosclerosis. The effect of hyperglycemia on the microv vasa
vasorum and the potential impact of these effects on macrova
atherosclerosis are not known.

Here, we use a low-dose multiple streptozotocin (STZ) injected
apolipoprotein E-deficient mouse model to investigate the effects
hyperglycemia on the vasa vasorum, and to correlate such effects to
atherosclerotic plaque progression. Hyperglycemia signific
size and necrotic area (3- and 4-fold, respectively) relative to controls by
weeks of age. However, the density of vasa vasorum microv
wall of hyperglycemic mice was reduced at each time point
vasa vasorum deficiency was also observed in STZ-induced hyperglycemic C57Bl/6J
mice and hyperglycemic $Ins2^{Akita}$ mice, and microvessel density could be
corrected by insulin-mediated glucose normalization, suggesting a h

specific effect. A localized deficiency in VEGF appears to
reduced neovascularisation. Lastly, hyperglycemic mice fed
supplemented with benfotiamine, a drug used to treat microvascular disor
DM, appear to have reduced atherosclerosis.

These findings provide the first indication that, in add
glomerular capillary beds, hyperglycemia alters the microv
vasa vasorum. Such microvascular changes directly correlate
and progression of atherosclerosis in hyperglycemic ApoE-deficient mice.

ACKNOWLEDGEMENTS

This project would not have been possible without the contributions of many people. First and foremost, I would like to thank my superv Werstuck, for his endless and unwavering support over the guidance has been insurmountable; words cannot express my appreciation encouragement, mentorship, and friendship.

This work would not have been possible without Dr. Moham and Dr. Peter Shi, who provided technical expertise and advice throughout the duration of this project. I would also like to thank the mem lab: past graduate students Andrew Sage, Brian Pichna, an welcomed me into the lab during my undergraduate studies Cameron McAlpine, Daniel Beriault, and Nicole Banko, who successes and frustrations of basic science research and helped to cr work environment. I would also like to thank Maryam Ramar Wu, and Andrea Wan for their assistance in analysis and q

I am very grateful for the guidance of my committee members, Dr. Be Gross and Dr. Bernardo Trigatti. Thank you for challenging me with your and advice; they have been truly invaluable.

Lastly, I would like to acknowledge my parents, Debbie and Perry Veerman, my siblings, Brett Veerman, Emily Veerman, and Jonathan Corle They have provided endless and overwhelming love, encour appreciation, and gave me the strength to complete this wo

TABLE OF CONTENTS

1 INTRODUCTION 1

1.1 DIABETES MELLITUS 1

1.1.1 Epidemiology 1

1.1.2 Clinical diagnosis 1

1.1.3 Classification 2

1.2 VASCULAR COMPLICATIONS OF DIABETES 2

1.2.1 Micro versus macro vascular complications 2

1.2.2 Microvascular disorders predict macrovascular outcome 3

1.2.3 Hyperglycemia and vascular disease 3

1.3 MECHANISMS OF HYPERGLYCEMIC-INDUCED DAMAGE 4

1.3.1 The polyol pathway 5

1.3.2 The hexosamine biosynthesis pathway 5

1.3.3 Activation of protein kinase C 6

1.3.4 Advanced glycation end-products 7

1.3.5 Mitochondrial superoxide production 7

1.4 MECHANISMS OF MICRO AND MACRO VASCULAR DISEAS 10

1.4.1 Structure of micro versus macro vessels 10

1.4.2 Angiogenesis 11

1.4.2.1 Mechanisms of angiogenesis 12

1.4.2.2 Diabetes, microvascular disease, and angiogenesis 21

1.4.3 Atherosclerosis 23

1.4.3.1 Mechanisms of atherosclerosis 23
1.4.3.2 Diabetes, macrovascular disease, and atherosclerosis 27
1.5 THE VASA VASORUM 27
1.5.1 Vasa vasorum structure and function 27
1.5.1.1 Functional geometry 27
1.5.1.2 Physiologic activity 31
1.5.2 Neovascularisation in atherosclerosis 31
1.5.3 Neovascularisation and plaque progression 32
1.5.4 Angiogenic modulation and atherosclerosis 34
1.6 BENFOTIAMINE AND DIABETIC MICROVASCULAR DISEASE 35
2 RATIONALE, HYPOTHESIS AND OBJECTIVES 36
2.1 RATIONALE 36
2.2 HYPOTHESIS 36
2.3 OBJECTIVES 37
3 EXPERIMENTAL PROCEDURES 38
3.1 MATERIALS 38
3.1.1 Animals 38
3.1.2 Diets 38
3.1.3 Reagents 38
3.2 ANIMAL MODELS 39
3.2.1 Streptozotocin-induced hyperglycemia 39
3.2.2 Genetically induced hyperglycemia 40

3.2.3 Diet induced dyslipidemia 40
3.2.4 Benfotiamine treatment in hyperglycemic ApoE-deficient mice 41
3.3 PLASMA ANALYSIS 44
3.4 HISTOLOGY AND IMMUNOHISTOCHEMISTRY 44
3.4.1 Tissue preparation 44
3.4.2 Immunohistochemistry and immunofluorescence 44
3.5 AORTIC LESION ANALYSIS 45
3.6 RETINAL CAPILLARY DENSITY 45
3.7 VASA VASORUM QUANTIFICATION 49
3.7.1 Vasa vasorum density 49
3.7.2 Microfil resin casting 49
3.7.3 Fluorescent microangiography (FMA) 50
3.8 HYPOXIA AND ANGIOGENIC PROTEIN EXPRESSION 50
3.8.1 Hypoxia analysis 50
3.8.2 Endoglin quantification 51
3.8.3 VEGF-A, VEGF receptors, and caspase-3 expression 51
3.9 IMAGING 52
3.10 STATISTICAL ANALYSIS 53
4 RESULTS 54
4.1 MICRO AND MACRO VASCULAR COMPLICATIONS IN HYPERGLYCEMIC APOLIPOPROTEIN E-DEFICIENT MICE 54

4.1.1 MLD-STZ injection induces chronic and progressive hyperglycemia in ApoE$^{-/-}$ mice 56
4.1.2 Hyperglycemic ApoE$^{-/-}$ mice have accelerated atherosclerosis at root 54
4.1.3 Hyperglycemic ApoE$^{-/-}$ mice show indications of microvascular c the retina 59
4.2 EFFECTS OF HYPERGLYCEMIA ON THE VASA VASORUM 62
4.2.1 Hyperglycemic ApoE$^{-/-}$ mice have reduced vasa vasorum density aortic root 62
4.2.2 Normolipidemic, hyperglycemic C57Bl/6J Ins2^{Akita} mice also have reduced vasa vasorum density at the aortic root 65
4.2.3 Fluorescent microangiography (FMA) imaging confirms vasa vasorum deficiency in the aortic arch of hyperglycemic ApoE$^{-/-}$ mice 67
4.2.4 Acclerated atherosclerosis is associated with vasa vas neovascularisation in dyslipidemic LDLR$^{-/-}$ and normoglycemic Apo mice 70
4.3 MARKERS OF ANGIOGENESIS AND APOPTOSIS IN HYPERGLYCEMIC APOLIPOPROTEIN E-DEFICIENT MICE 74
4.3.1 Hyperglycemic ApoE$^{-/-}$ mice have elevated levels of hypoxia and at the aortic root 74
4.3.2 Hyperglycemic ApoE$^{-/-}$ mice have increased endothelial cell acti the aortic root 77

4.3.3 Hyperglycemic ApoE$^{-/-}$ mice have a relative deficiency of VEGF-A and VEGF receptors at the aortic root 80

4.3.4 Hyperglycemic ApoE$^{-/-}$ mice have reduced expression of cleaved
3 at the aortic root 83

4.4 EFFECT OF BENFOTIAMINE ON THE DEVELOPMENT OF M
AND MACRO VASCULAR COMPLICATIONS IN HYPERGLYCEMIC APOLIPOPROTEIN E-DEFICIENT MICE 86

4.4.1 Benfotiamine supplementation reduces atherosclerosis
and hyperglycemic ApoE$^{-/-}$ mice 86

5 DISCUSSION 89

5.1 CLASSICAL MICRO AND MACRO VASCULAR COMPLICATIO
HYPERGLYCEMIC APOLIPOPROTEIN E-DEFICIENT MICE 89

5.2 HYPERGLYCEMIA AND VASA VASORUM DEFICIENCY 90

5.3 VASA VASORUM DEFICIENCY AND ACCELERATED ATHEROGENESIS 92

5.4 HYPOXIA-INDUCED EXPRESSION OF ENDOGLIN, VEGF, AND RECEPTORS-1 AND -2 93

5.5 BENFOTIAMINE AND ATHEROSCLEROSIS 94

5.6 CRITICAL APPRAISAL AND SIGNIFICANCE OF WORK 95

5.7 FUTURE DIRECTIONS 96

6 CONCLUSIONS 99

7 REFERENCES 100

LIST OF FIGURES

Figure 1. Pathways of hyperglycemia-induced damage 8

Figure 2. Hypoxia inducible factors 13

Figure 3. Angiogenic sprout formation 17

Figure 4. Mechanisms of atherosclerosis 25

Figure 5. Structure of the vasa vasorum 29

Figure 6. Injection timeline and experimental design 42

Figure 7. Retinal capillary density template 47

Figure 8. Hyperglycemia is associated with accelerated develop

atherosclerosis at the aortic root in ApoE$^{-/-}$ mice 57

Figure 9. Hyperglycemia is associated with vascular changes in the retina

.... 60

Figure 10. Hyperglycemia is associated with reduced vasa vaso

aortic root in ApoE$^{-/-}$ mice 63

Figure 11. Microfil casting and Fluorescent microangiography (F

vasa vasorum in ApoE$^{-/-}$ mice 68

Figure 12. Western Fed ApoE$^{-/-}$ and LDLR$^{-/-}$ mice have accelerated

atherosclerosis accompanied by vasa vasorum proliferation 72

Figure 13. Hyperglycemia is associated with elevated levels of hypoxia and HIF-1α at the aortic root in 15 week old ApoE$^{-/-}$ mice 75

Figure 14. Hyperglycemia is associated with increased activation endothel

the aortic root 78

Figure 15. Hyperglycemia is associated with reduced VEGF-A and VEGF receptor expression at the aortic root 81

Figure 16. Cleaved caspase-3 expression within the lesions of ApoE$^{-/-}$ 84

Figure 17. Benfotiamine supplementation reduces atherosclerosis in normoglycemic and hyperglycemic ApoE$^{-/-}$ 87

LIST OF TABLES

Table 1. Metabolic parameters in $ApoE^{-/-}$ mice 55

Table 2. Metabolic parameters in $Ins2^{Akita}$ and C57Bl/6J mice 66

Table 3. Metabolic parameters in $ApoE^{-/-}$ and $LDLR^{-/-}$ mice fed Control or high fat Western diet 71

LIST OF ABBREVIATIONS

AGE	Advanced glycation end-product
Ang-1(-2)	Angiopoietin-1(-2)
ApoE	Apolipoprotein E
AR	Aldose reductase
BM	Basement membrane
CH-1	Cysteine-histidine rich domain
CHD	Coronary heart disease
CVD	Cardiovascular disease
DAG	Diacylglycerol
DHAP	Dihydroxyacetone phosphate
DLL4	Delta-like-4
DM	Diabetes mellitus
EC	Endothelial cell
ECM	Extracellular matrix
EDG1	Endothelial differentiation sphingolipid G-protein-coupled receptor-1
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
F-6-P	Fructose-6-phosphate
FIH-1	Factor inhibiting HIF-1
FMA	Fluorescent microangiography

FPG	Fasting plasma glucose
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAT	L-glutamine:D-fructose-6-phosphate amidotransferase
GlcN-6-P	Glucosamine-6-phosphate
GlcNAc	N-acetyl glucosamine
GSH	Glutathione
HBP	Hexosamine biosynthesis pathway
HDL	High density lipoprotein
HG	Hyperglycemic
HIER	Heat-induced epitope retrieval
HIF	Hypoxia-inducible factor
HRE	Hypoxia response elements
HSP	Heparan sulphate proteoglycan
ICAM-1	Intercellular adhesion molecule-1
IF	Immunofluorescence
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
MA	Microalbuminuria
MI	Myocardial infarction
MLD	Multiple low dose

Abbreviation	Definition
MMP	Matrix metalloproteinases
NAD^+	Nicotinamide adenine dinucleotide
NADH	Hydrogenated nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor-kappa B
NG	Normoglycemic
NO	Nitric oxide
NRP	Neuropilin
OGTT	Oral glucose tolerance test
oxLDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PDGF-B	Platelet-derived growth factor B
PDGFRβ	Platelet-derived growth factor receptor beta
PECAM-1	Platelet endothelial cell adhesion molecule-1
PHD	Prolyl-4-hydroxylase
PKC	Protein kinase C
RAGE	Receptor for advanced glycation end products
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SEM	Standard error of the mean
STZ	Streptozotocin
T1D	Type 1 diabetes

T2D	Type 2 diabetes
TGF-β	Transforming growth factor beta
TGFβR-I,(II)	Transforming growth factor beta receptor-I,-II
TIMP	Tissue inhibitor of matrix metalloproteinases
TK	Transketolase
TSP-1	Thrombospondin-1
UDP	Uridine diphosphate
uPA	Urokinase plasminogen activator
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
VEGFA	Vascular endothelial growth factor A
VEGFR-1,(2)	Vascular endothelial growth factor receptor-1,-2
VHL	von Hippel-Lindau
VSMC	Vascular smooth muscle cell
Vv	Vasa vasorum
WHO	World Health Organization

1.0 INTRODUCTION

1.1 DIABETES MELLITUS

1.1.1 Epidemiology

The World Health Organization (WHO) estimates that o
people worldwide currently live with diabetes mellitus (DM).[1] An additional 314
million people are expected to have impaired glucose toler
fasting glucose (IFG), and many live with the disease unkn
.[2] These numbers are projected to exceed 450 million by 2030, rising obesity
rates, increasingly sedentary lifestyles, and an aging glob
.[2] Diabetes is a major cause of global illness and disability, and is the
cause of death in most-income countries.[2] As such, DM has become a glob
epidemic and worldwide health concern.

1.1.2 Clinical Diagnosis

Diabetes is a heterogeneous metabolic disease that is
chronic hyperglycemia and glucose intolerance, a state collective
dysglycemia.[3] Clinically, DM is diagnosed by a fasting plasma
value ≥ 7.0 mmol/L, a casual plasma glucose value ≥ 11.0 mmol/L, or a 2
plasma glucose (2hPG) value ≥ 11.0 mmol/L in a 75g oral glucose toler
(OGTT).[3] Elevated blood glucose levels below the diabetic thr

FPG values from 6.1 to 6.9 mmol/L or 2hrPG values betwee
are diagnostic of IGT and IFG.[3]

1.1.2 Classification

Hyperglycemia arises from deficient insulin action. In
(T1D), auto-immune mediated pancreatic beta cell destruction leads
insulin production and chronic hyperglycemia. By contrast, type 2 diabetes (T2
results from impaired insulin effect, and may involve a com
insulin resistance and defective beta cell secretion. Pre-diabetes is a term used
to describe IGT and IFG, and is recognized as an intermediate stage between
normalcy and incipient DM.[3,4] Each form of the disease is characterize
impaired insulin-mediated glucose uptake, chronic hyperglycemia, and a ra
potentially life threatening vascular complications.

1.2 THE VASCULAR COMPLICATIONS OF DIABETES

1.2.1 Micro versus Macro Vascular Complications

The vascular complications of DM have traditionally be
either micro- or macro-vascular in origin. Microvascular complicatio
retinopathy, nephropathy, and neuropathy, predispose peop
blindness, chronic kidney disease, and foot ulcers requirin
amputation.[1,5] By contrast, macrovascular complications involve
development of atherosclerosis and an increased risk of th

DM is associated with a cardiovascular disease (CVD) mortality rate that
70%, and a 2-4-fold increased risk of dying from myocardial infa
stroke compared to non-diabetics.[6]

1.2.2 Microvascular Disorders Predict Macrovascular Outcomes

Although the vascular complications of DM have traditi
viewed as distinct and independent disorders, data from se
studies show that microvascular abnormalities predict macr
Microvascular changes in the retina and kidney, in particular, are
correlated to CVD outcome. Proliferative retinopathy is a s
cause mortality, CVD death, and coronary heart disease (C
with T1D and T2D, independent of cardiovascular risk factor.[7-9]
Microalbuminuria (MA) is also a major independent risk fac
persons with diabetes, and has been reported to increase the risk of CVD dea
100%-150% depending on the level of increase in albuminuria.[10,11] Collectively,
these data suggest that similar pathways may underlie mic
macrovascular disease in diabetes.

1.2.3 Hyperglycemia and Vascular Disease

Despite the possible existence of common underlying f
fundamental difference in how microvascular and macrovas
respond to glucose lowering. It is well established that lowering fasting blo

glucose levels below 7.0mmol/L significantly reduces the in
retinopathy, nephropathy, and neuropathy in patients with[5, 12-15] In fact, the
glycemic parameters that define/diagnose diabetes were ch
effectively differentiate individuals at high risk for developing retinopathy from
individuals at low risk.[16] However, the effect of glucose normalizatio
cardiovascular risk remains controversial. While accumulat
suggest that blood glucose normalization improves CVD outcome, a similar
glucose threshold does not exist for CVD risk.[17, 18] Furthermore, intensive glucose
lowering was associated with increased mortality in the AC[15]
indicating that the relationship between hyperglycemia and
possible explanation for these differences is that microvas
contribute to the pathogenesis of cardiovascular outcomes.

1.3 MECHANISMS OF HYPERGLYCEMIA INDUCED DAMAGE

High blood glucose concentrations may induce tissue d
and macro vessels through four major mechanisms: (1) incr
through the polyol pathway, (2) increased hexosamine path
increased activation of protein kinase C (PKC) isoforms, and (4) increase
intracellular formation of advanced glycation end-products (AGEs). These four
cytoplasmic pathways are thought to be amplified by a fifth
process, namely, overproduction of superoxide. Together, t

oxidative stress and endoplasmic reticulum (ER) stress in
promote the development of micro and macro vascular comp

1.3.1 The Polyol Pathway

In the polyol pathway, glucose is metabolized to sorbit
fructose, by aldose reductase (AR) and sorbitol dehydroge
respectively. These reactions are accompanied by oxidatio
adenine dinucleotide phosphate (NADPH) to nicotinamide a
(NAD^+), and reduction of NAD^+ to hydrogenated NAD (NADH). Excess gl
flux through the polyol pathway leads to NADPH depletion
accumulation. NADPH consumption limits the regeneration
glutathione (GSH), leaving the cell vulnerable to reactive o
species (ROS)[19,20]. Moreover, NADH accumulation inhibits the glycolytic act
glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to a
flux of glucose metabolites further down through branching pathways.

1.3.2 The Hexosamine Biosynthesis Pathway

In the hexosamine biosynthesis pathway (HBP), fructose-6-phosphate (fructos
6-P) is converted to glucosamine-6-P (GlcN-6-P), and finally to uridine
diphosphate-N-acetyl glucosamine (UDP-GlcNAc) through the action of th
rate-limiting enzyme L-glutamine: D-fructose-6-phosphate amidotransferase
(GFAT). Cytoplasmic O-linked GlcNAc transferases catalyze the

of GlcNAc to specific residues on nuclear proteins,[21] transcription factors,[22] and signalling factors,[23] thereby altering their functional activity and capacity. Excessive glycosylation and improper protein folding have been implicated in activation of the unfolded protein response (U ER stress, which dysregulates lipid metabolism, activates inflammatory pathways, and induces apoptosis in vascular smooth muscle and endothelial cells (ECs).[24-26]

1.3.3 Activation of Protein Kinase C

Hyperglycemia increases de novo synthesis of diacylglycerol (DAG), a key activator of PKC isoforms, from glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP). Indirect activation of PKC is mediated by AGE-RAGE and polyol signalling pathways.[19] Glucose-induced activation of PKC inhibits endothelial nitric oxide synthase (eNOS) expression and decreases nitric oxide (NO) generation.[27] PKC isoforms in vascular cells have also been shown to increase endothelial permeability, alter gene expression, promote capillary occlusion, and increase ROS formation, which are implicated in the pathogenesis of macro- and microvascular disorders.[19,28]

1.3.4 Advanced Glycation End Products

Elevated intracellular glucose levels also lead to the ge
by way of the Maillard reaction, a nonenzymatic process in
sugars react with amino groups from proteins. Reactive int
methylglyoxal, 3-deoxyglucosone, and glyoxal are produced during the Mail
reaction and contribute to carbonyl stress independent of A
precursors can damage the cell by modifying the function o
by abnormally cross-linking extracellular matrix (ECM) components, and/or by
interacting with the receptor for AGE (RAGE) on cells such
ECs, and VSMCs. AGE-RAGE binding induces ROS production and nu
factor-kappaB (NF-κB) activation, which increase the transcription
molecules and pro-inflammatory genes and potentiate vascular dam[19,20,29]

1.3.5 Mitochondrial Superoxide Production

These four cytoplasmic processes are thought to be am
mitochondrial process, namely, overproduction of superoxi
glycolysis directly increases the flux of electrons through
electron transport chain (ETC). This flux leads to an accumulation of elec
within the ETC and increases the generation of superoxide
the activity of the GAPDH. By inhibiting GAPDH, hyperglycemia-induced
superoxide production increases the flux of glycolytic intermediates through the
four key pathways involved in vascular endothelial damage[19,28]

Figure 1. Pathways of **Hyperglycemia**-Induced Damage

Potential mechanisms by which hyperglycemia damage. Increased tiss
glucose flux through the polyol pathway consumes NADPH a
regeneration of glutathione. Increased conversion of fructose-6-phosphate (Fruc of fructo
6-P) to UDP-N-acetyl glucosamine (UDP-GlcNAc) leads to increased protein
modification by O-linked glycosylation and endoplasmic reticulum
Excess glyceraldehyde-3-phosphate (G3-P) increases de novo synthesis of
diacylglycerol (DAG), an activator of protein kinase C (PKC
formation of methylglyoxal, the intracellular AGE precursor. Mitocho
overproduction of superoxide inhibits the activity of glycer
dehydrogenase (GAPDH), diverting upstream glucose metab
damaging pathways.

Adapted from Brown Nature (2001)[28]

1.4 MECHANISMS OF MICRO AND MACRO VASCULAR DISEA

Excess glucose flux through the above described pathw
the pathogenesis of both micro and macrovascular complic in DM
However, hyperglycemia-associated disease processes in the small a
blood vessels differ greatly. Small blood vessels of the mi
aberrant phases of vasoregression and angiogenesis, while
the macrocirculation develop atherosclerotic lesions.

1.4.1 Structure of Micro versus Macro Vessels

The vascular microcirculation consists of first and sec
(diameter of 20-200 µm), terminal arterioles (10-30 µm), capillaries (3
postcapillary venules (10-30 µm), and first and second order venules (20-200 µm)
[30]. Arterioles and large venules are comprised of three dis
endothelial lining and basement membrane (BM), known as the tunica intima;
medial VSMC layer, called the tunica media; and an outer r
tissue, known as the tunica adventitia. By contrast, non-muscular capilla
venules consist of only ECs, BM, and sparsely interspersed
function as primary exchange vessels, and are classified a
central nervous system), fenestrated (ex. glomeruli), or sin
depending on the nature of their endothelial junctions and
BM[31].

Large blood vessels of the macrocirculation, including
coronary arteries, have thick muscular walls that also cons
and adventitial layers. The luminal intima of macro vessels is composed of a
single, continuous layer of ECs surrounded by subendothel
that is rich in proteoglycan.[32] The media consists of many layers (lame
circumferentially arranged VSMCs embedded within a matrix of collagen and
elastin. The outer adventitia also contains collagen and el
fibroblast and SMC cell types.[31] In contrast to microvessels, the advent
large blood vessels also contains two structurally unique c
specialized microvascular network known as the vasa vasorum, and a system
of autonomic nerve fibres.[33]

1.4.2 ANGIOGENESIS

Unlike large vessels, microvessels have the capacity t
in response to local tissue ischemia or injury, and can do s
mechanisms. Vasculogenesis refers to the de novo formation of blood v
from vascular progenitor cells,[34] angiogenesis describes the sprouting of new
capillary branches from existing vasculature, and arteriogenesis describes the
remodelling of conduit vessels through increases[34,35] in luminal c
Physiological angiogenesis is controlled by a balance of pr-and anti-angiogenic
factors, many of which are upregulated under conditions of hypoxia. Disrupt
this balance, as occurs in diabetes, leads to abnormal ves

1.4.2.1 Mechanisms of Angiogenesis

Angiogenesis is a complex physiological process consi
overlapping phases[36]: (1) formation, (2) stabilization, (3) branching
and pruning, and (4) specialization. The entire process is
dynamically changing balance between pro-angiogenic signals and factors tha
induce vessel quiescence.

Hypoxic Induction

When tissue metabolic demands exceed O_2 supply, cells trigger a number
of adaptive responses through the hypoxia-inducible transcription factors (HIF
[37]. The master regulator of, HIF-1, is a ubiquitously expressed
heterodimer consisting of an O_2-regulated HIF-1α subunit and a stable HIF-1β
subunit[38]. In well-oxygenated environments, HIF-1α activity is controlled by two
oxygen-dependent hydroxylases. Prolyl-4-hydroxylase (PHD) hydroxylates HI
1α at two proline residues[39], marking it for proteasomal destruction by an E3
ubiquitin ligase, the von Hippel-Lindau protein (VHL) complex[40]. A second
hydroxylase, factor-inhibiting HIF-1 (FIH), modifies asparginine residues
HIF-1α and prevents its binding to transcriptional co-activators CBP and p300[41].
Hypoxic conditions inhibit the activity of prolyl and aspara
lead to HIF-1α accumulation in the cytoplasm. HIF-1α and β dimerize in the
nucleus, bind to hypoxia response elements (HREs), and di
transcription of a wide array of target genes, many of whic
angiogenic regulation[37,39,41].

Figure 2. Hypoxia Inducible Factors

In well-oxygenated environments, hypoxia-inducible factor-1α (HIF) is hydroxylated by prolyl-4-hydroxylase (PHD), which marks the protein ubiquitination and proteasomal degradation by the von Hippel-Lindau (VHL) complex. A second hydroxylase, factor inhibiting HIF-1α (FIH), modifies asparginine residues and prevents HIF-1α binding to CBP and p300 transcriptional co-activators. Hypoxia inhibits the activity of PHD and FIH-1, leading to HIF-1α accumulation and nuclear translocation. HIF-1α dimerizes with HIF-1β in the nucleus, binds to hypoxia-responsive elements (HREs), and directs the transcription of a numerous target genes, many involved in angiogenesis.

Formation

Sprouting angiogenesis is initiated in response to vasc growth factor A (VEGFA), a secreted glycoprotein that is p under conditions of hypoxia. VEGFA isoforms signal throug tyrosine kinases-1 (VEGFR1) and -2 (VEGFR2), but can also bind to co receptors such as neuropilins (NRP1 and NRP2) and hepara proteoglycans (HSPGs).[42] VEGFR1 facilitates monocyte/macrophage ch and antagonizes VEGFR2 activity, which is the primary mediator of VEGFA induced vascular sprouting.[13] Sprouting requires modulation of vascu permeability, induction of EC migration and proliferation, a lumenization, and perfusion of vascular channels.

Vessel destabilization and permeability are induced by NO production, endothelial perforation, and EC/pericyte de VEGFA is a known activator of eNOS, which is required for vasodilation.[43] VEGFA also induces the formation of endothelial caveolae, tr pores, and fenestrae within the endothelium, which allow l and proteins into the extravascular space. Internalization o (VE)-cadherin in adherens junctions in response to VEGFA lo endothelial cell contacts and relieves periendothelial supp protein extravasation.[44] Together, vasodilation and vascular permeability allow ECs to emigrate from their resident sites and assemble int

Growing vascular sprouts are led by endothelial tip ce
selected and guided by extracellular VEGFA (Fig. 3b). Tip
number of phenotypic changes in response to VEGFR2 acti
reversal of apical-basal polarity, extension of filopodia, and adopti
invasive and motile behaviour.[13] These characteristics allow tip cells to
matrix-anchored VEGFA and NRP axon guidance molecules, wh
cell chemotaxis and polarized sprout elongation.[45,47] Tip cells are trailed by a
zone of proliferating and differentiating stalk cells, which
of pericyte-covered phalanx cells. VEGFA imposes differential b
these ECs through the Delta-like-4 (DLL4)/Notch signalling pathway, stifling
VEGFR2 expression in stalk and phalanx cells and limiting
to the tip cell.[48]

Upon encountering their targets (i.e. the tips of other
capillaries), tip cells suppress their motile behaviour and establish EC-EC
junctional contacts (Fig. 3c). Fusion of vascular sprouts ar
endothelial cells allow nascent vessels to increase their di
lumenization, which involves intracellular vacuole formation,[49] among other
mechanisms.[50] Generation of lumen and onset of blood flow help
vascular connections, but vessels must undergo further ma
become a functional vascular network.[13]

Figure 3. Angiogenic Sprout Formation

Mechanisms of angiogenic sprout formation. a, Vessel destabilization and permeability are induced by VEGFA-mediated nitric oxide (NO) production, endothelial perforation (calveolae), and EC/pericyte detach b, Growing vascular sprouts are led by endothelial tip cells, proliferating and differentiating stalk cells and pericytes. Tip cells are selected and guided by extracellular VEGFA, whic angiogenic capacity of neighbouring cells through the delta-like-4 (DLL4)-Notch signalling pathway. c, Fusion of vascular sprouts allows nascent ves increase their diameter and undergo lumenization, a proces and vacuole formation. Pericytes (yellow), quiescent endot activated endothelial cells (green), basement membrane (b

Stabilization

Immature vessels are stabilized by recruiting mural ce
ECM. At least three molecular pathways are involved in reg
(1) platelet-derived growth factor (PDGF) B and PDGF receptor (
-β; (2) the angiopoietin (Ang)-Tie system; and (3) transforming growth facto
-β signalling.

Tip cells of growing vascular sprouts generate a high c
gradient of PDGFB, which promotes the recruitment of peri
PDGFR-β.[51] Sphingosine-1-phosphate signalling through the endothelia
differentiation sphingolipid G-protein-coupled receptor-1 (EDG1), which is also
expressed by mural cells, augments this mural cell recruitment.[52] Together, these recruit
pathways ensure that the endothelium of nascent vessels b
supporting periendothelial cells.[13]

The Tie receptors and Ang ligands further stabilize ne
promoting endothelial-mural cell interactions and vessel quiescence
expression of Ang1 is upregulated during angiogenesis and
endothelial Tie2 activation. Signal transduction through the Tie2 pathway
positively regulates periendothelial cell recruitment by strengthening endothelial-mural
communications, reducing vascular permeability,[53] and promo
all of which contribute to vascular quiescence.

TGF-β signalling is involved in vessel maturation in a pleiotropic
context-dependent manner. TGF-β cytokines signal through various

transmembrane type I (TGFβRI) and type II (TGFβRII) recep
regulated by accessory receptors, endoglin and betaglycan
ALK1 and ALK5 are responsible for the majority of signal transd
and VSMCs. The endoglin-ALK1 pathway has been shown to stimulat
migration and proliferation,[54,55] however, activation of the betaglycan-ALK5
pathway inhibits endothelial proliferation and migration, in
differentiation, and stimulates ECM production, thereby so
interactions and promoting vessel stabilization.[54]

Branching, Remodelling, and Pruning

Maturation of the stabilized endothelial network involv
remodelling, and pruning of capillary-like vessels to match tissue metaboli
needs. The ECM plays an important role in this vascular pa
the proliferation, migration, differentiation, and survival of ECs and mu[34]
ECM regulation is largely mediated through the action of p
plasminogen activator (uPA), matrix metalloproteinases (M
chymases, tryptases, and cathepsins) and their inhibitors (
activator inhibitor-1 (PAI-1), tissue inhibitor of MMPs (TIMPs)).[24,56] Proteases
liberate matrix-bound angiogenic factors, alter the function of ECM structural
proteins, and modulate cell-matrix interactions. As such, their spatial a
temporal distribution plays an important role in determinin
persistence and/or regression.[36]

Specialization

To respond to physiological needs of the host tissue, E
number of specialized properties, including permeability co
regulation, adhesion molecule expression, transcellular tra
formation, and others. This further specialization is induced by cro
cells of the perivascular tissue, which produce growth and
capable of activating specific gene expression programs[13]. Key to this
specialization process is the establishment of cell–cell junctions and formation
of organ-specific capillary structures[36].

1.4.2.2 Diabetes, Microvascular Disease, and Angiogenesis

Hyperglycemia is known to have diametric effects on the
microvasculature, and can lead to excessive or insufficient
different vascular beds. Excessive angiogenesis is involve
[57] and nephropathy[58], whereas inhibited angiogenesis is a key featu
neuropathy[59], delayed wound healing[60], and impaired collateral vessel form
[61]. Common to each of these pathologies are early morpholo
microvascular regression. Key morphological changes inclu
mural cells and thickening of the capillary BM[62,63], which lead to increased
vessel permeability, protein extravasation, capillary microa
occlusion. Ultimately, non-perfused microvessels undergo cellular apo

vascular regression, a process which may or may not be accompanie
formation of new vessels.

Pathological neovascularisation in diabetes is site-specific and is a result
alterations in the pro- and anti-angiogenic factors. The most extens
studied angiogenic factor, VEGF, is variably increased and/or d
different vascular beds. Elevated VEGF levels have been c
diabetic retinopathy,[57,64] and hyperglycemia increases VEGF expression in renal
podocytes and glomerular capillaries.[65,66] By contrast, VEGF expression is
significantly reduced in human diabetic neuropathy[67] and wounds,[68] and
both increased and decreased levels of VEGF protein have
diabetic myocardium.[69,70] Such changes in VEGF concentration are dynamic and
context dependent, and may be accompanied by altered exp
angiogenic proteins.[71,72]

Hyperglycemia is thought to alter angiogenic protein e via the
four damaging pathways described above. Aldose reductase
repress RUNX2 DNA binding and transcriptional activity in
ECs, leading to impaired EC migration and proliferation un
hyperglycemia.[73] Glycosylation of intracellular transcription facto
expression of thrombospondin-1 (TSP-1)[74] and Ang-2[75] in human VSMCs and
kidney ECs, respectively. Glucose-induced PKC activation has also been
implicated as a mediator of VEGF mRNA and protein produc

VSMCs[76], and methylglyoxal-induced modification of the mSin3A co-repressor complex has been shown to increase Ang-2 promoter activity[75] in kidney ECs

1.4.3 ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease char accumulation within the walls of large muscular arteries[77]. It is the underlying cause of CVD and cerebrovascular disease, which account majority of mortality in people with DM.

1.4.3.1 Mechanisms of Atherosclerosis

Atherosclerosis is thought to be initiated in response Activated ECs express adhesion proteins (vascular cell adh (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin) and secrete chemotactic factors that actively recruit monocytes and T (Fig. 4). Increased endothelial permeability allows the tran monocytes, T cells, and lipoprotein particles into the intima. Here, monocytes differentiate into macrophages and express scavenger rece (SR-A and CD36) for oxidized low-density lipoprotein (oxLDL) particles. Scavenging engulf LDL-derived cholesterol esters and become foam cells, w to form fatty streaks. As lesion development progresses, a foam cells and T cells further stimulate the inflammatory p

cytokines that induce VSMC growth and migration to the in
and secrete collagen, glycoprotein, and elastin, which collectively f
fibrous cap and stabilize the lesion. However, VSMCs and
also secrete matrix-degrading proteases (ex. MMPs, collagenases, g
cathepsins) which lead to cap thinning and plaque instability. Foam cell deat
necrotic core expansion further contribute to lesion destab
rupture occurs, the blood comes into contact with lipids an
of the necrotic core, initiating platelet adhesion and thrombus formation. Most
MIs and strokes occur when the thrombus or its emboli occ
impair blood flow to the cardiac or cerebral tissue.[7,77,79]

Figure 4. Mechanisms of Atherosclerosis

Activated endothelial cells (green) express adhesion molec
proteins in response to vascular injury. Monocytes migrate
mature into macrophages, which express scavenger recep
oxidized low density lipoprotein (oxLDL). Scavenging macro
oxLDL particles and become foam cells, which secrete cyto
proliferation and migration of medial smooth muscle cells (
Here, smooth muscle cells secrete collagen, glycoprotein, and
the fibrous cap. Smooth muscle cells and foam cells also r
degrading proteases, which lead to cap thinning and plaque destabili
progresses, foam cells undergo apoptosis and contribute to necrotic
expansion.

Adapted from Glass and Witztum (2001)[79]

1.4.3.2 Diabetes, Macrovascular Disease, and Atheroscler

In people with DM, the atherogenic process is thought
identical series of events. However, diabetes increases the
endothelial injury, accelerates plaque growth and developm
plaque stability. Indeed, people with diabetes present with a signifi
number of diseased vessel segments[80] than those without dia
Atherosclerotic lesions isolated from diabetic patients also
core and inflammatory infiltrate, and a higher frequency of p
intracoronary thrombus formation.[81,82] Furthermore, diabetic plaques have
VSMC apoptosis, NF-κB activation, elevated MMP levels, and lesse
interstitial collagen content, making them less stable and r
and thrombus formation.[19]

Because CVD accounts for over 70% of diabetic mortal
hyperglycemia-induced tissue damage in the development and pro
diabetic atherosclerosis has been extensively studied. How
involvement of microvascular disease in this process is un

1.5 THE VASA VASORUM and ATHEROSCLEROSIS

1.5.1 Vasa Vasorum Structure and Function

1.5.1.1 Functional Geometry

The Vv consists of small arterioles, capillaries, and ve
the outer media and adventitia of large arteries.[83]

in the adventitia (vasa vasorum externa) or from the main vessel lumen (vasa vasorum interna)[84] (Fig. 5A). Arterial vasa are readily distinguishable from venous vasa, for they are smaller, straight in course, and branch infrec while venous vasa are larger, tortuous in course, and bran[84] Microvessels of the Vv are further defined by their branchi order vasa run longitudinally between the media and adventitia, second order branches originate from first order vasa and around the main vessel (Fig. 5B).[83] The spatial distribution of Vv perfusion and drainage depends on whether they exist as anatomic plexus vein,[85] or functional endarteries, as in the coronary arteries[86]

Figure 5. Structure of the Vasa Vasorum

a, Arterial vasa vasorum (Vv) originate from the main vesse[l] (vasa vasorum interna) or from adventitia (vasa vasorum externa). b, Micro vessels of the Vv are further defined by their branching order: first order vasa run longitudinally between the media and adventitia, while sma[ller] branches originate from first order vasa and wrap circumfe[rentially around the] main vessel

Adapted from Langhorne et al. The Lancet (2011)[87]

1.5.1.2 Physiologic Activity

Vessels with walls greater than 29-layers thick[88] or luminal diameter greater than[89] (0.4mm in[90] 0.5mm mice) require Vv microvessels to nourish cells within their outer wall layers. The Vv is a dynamic st regulate its tone and vascular perfusion[91], and can undergo expansion and remodelling in response to the local microenvironment[92]. Because it plays a significant role in vessel wall maintenance, facilitating the flux of cells both into and out of the perivascular tissue, the Vv has be number of large vessel diseases, including atherosclerosis

1.5.2 Neovascularisation in Atherosclerosis

A general correlation between Vv neovascularisation and atherosclerotic a plaque progression has been well established. In 1984, Ba Vv microvessels were present in human vessels with athero were absent in non-diseased vessels. He noticed that these microve through the media and into the thickened intima, and sugge neovascularisation might play a role in the pathophysiolog[93] In support of this hypothesis, numerous studies have since demonstrated that Vv density is increased in lesions with severe inflammatory bu haemorrhage, and[81] plaque rupture, the principal cause of acute coronary th in patients dying of myocardial infarction[94].

Experiments in murine models of atherosclerosis have
from human studies, further supporting the association bet
neovascularisation and plaque progression. Langheinrich e[t al. showed that ApoE-/-]
/LDL-/- double deficient mice developed Vv in association with le
and showed that adventitial vasa communicate with intrapla
Vv density was positively correlated with adventitial inflam
intraplaque haemorrhage in advanced atherosclerosis, and
local differences between fibrotic, calcified, and haemorrh[96]

1.5.3 Neovascularisation and Plaque Progression

Vv neovascularisation has been described as a double-edged sword in
the context of atherosclerosis[97], for it is the body's natural protective re
ischemic injury, but may ultimately contribute to disease p
Microvessels of the Vv may facilitate plaque progression b
mechanisms: (1) by altering the delivery and/or drainage o
inflammatory cells within the vessel wall, and (2) by predisposing the lesion to
intraplaque haemorrhage. Together, altered solute exchang
haemorrhage contribute to plaque destabilization and incre
rupture.

Microvessels of the Vv serve as a conduit for lipids and inflammatory
cells into the arterial wall, and may facilitate both leukocy
retention. Adventitial microvessels in human coronary plaq

fold more VCAM-1, ICAM-1, and E-selectin than the luminal endothelium[98,99]
and adventitial immune cell accumulation exceeds that of t
fold in ApoE-/- mice[100], thus confirming a role for Vv microvessels in leukocyte
trafficking. While arterial vasa account for only 30% of to
influx[101], aggregated LDL may accumulate in the extracellular
high density lipoprotein (HDL) efflux through Vv lymphatic arms[102].
Together, enhanced influx and impaired efflux likely contri
expansion and necrosis[103].

Intraplaque microvessels have poorly formed endothelial junctions a
lack mural pericytes, which make them leaky and prone to[104]. These
microvessels originate in the adventitia and penetrate the
breakpoints, directly below sites of early necrotic core formation[105]. By
contributing to cholesterol deposition, macrophage infiltrat
of the necrotic core, the accumulation of erythrocyte memb
atherosclerotic plaque may represent a potent atherogenic sti
necrotic core expansion directly correlates with intraplaqu
accumulation[106]. Vv neovascularisation may thus facilitate plaque destabilization
and rupture by contributing to expansion of the necrotic co
increased risk of acute coronary events.

1.5.4 Angiogenic Modulation and Atherosclerosis

The combined studies clearly demonstrate that adventi
atherosclerotic plaque acquire a significantly altered branc
theory is that thickening of the vessel wall and increased c
plaque constituents act as a hypoxic environment, which activates
processes in the adventitial Vv. Consistent with this hypot
treatment in ApoE-/-/ApoB100 mice significantly increased plaq
endothelial cell content and plaque size.[107] Moulton et al found that Vv densi
correlated more strongly with the number of inflammatory c
atheroma size in ApoE-deficient mice, and showed that Angiostatin
reduce plaque angiogenesis, macrophage content and atherosclerotic
progression.[108] Similarly, the antiproliferative drug, 2-chlorodeoxyadenosine, decreased V
density and lesion volume in the aortas of hypercholesterolemic ApoE/LDL
Thalidomide prevented Vv neovascularisation and early neo
hypercholesterolemic pigs.[110] Drinane showed that PAI-1-/- in LDLR-/-
/ApoB48-deficient mice reduced Vv density and actually promoted
outward remodelling and plaque regression in the innominat

While these studies do suggest a role for the Vv in ath
development, there is no conclusive evidence to indicate w
causative or merely reactive. In addition, since these observations hav
reported in non-diabetic, hypercholesterolemic models, little is known about the
the Vv in diabetic atherosclerosis.

1.6 BENFOTIAMINE and DIABETIC MICROVASCULAR DISEA

Benfotiamine is a lipid-soluble, orally available thiamine derivative tha has been shown to attenuate hyperglycaemia-induced microvascular complications in STZ-injected rodents and in patients with DM.[112] High-dose oral benfotiamine supplementation (70-80 mg/kg/day) prevents incipient diab nephropathy and retinopathy in STZ-induced hyperglycaemic rodents[112,113], and a number of placebo controlled trials have reported reduced diabetic patients treated with the supplement (300-600 mg/day).[114,115] Benfotiamine is believed to act as a coenzyme for transket phosphate pathway enzyme capable of shunting glucose me pathways associated with vascular complications, including the hexosam diacylglycerol-PKC, and AGE-production processes.[116,117]

While the effects of benfotiamine have been investigat diabetic retinopathy, nephropathy, and neuropathy, its potential eff vasorum in diabetes-accelerated atherosclerosis has not been exam

2.0 RATIONALE, HYPOTHESIS, AND OBJECTIVES

2.1 RATIONALE

Diabetes is a major risk factor for microvascular and macr long-term complications. Data from several independent stud microvascular abnormalities and cardiovascular outcomes a[8,11,118,119]; however, these studies also show a much stronger re glucose levels and microvascular versus cardiovascular ou[5,12,14,120]. One explanation for these differences is the possibility that mic abnormalities contribute to the pathogenesis of cardiovasc vasorum, a distinct microvascular network located within th vessels, undergoes neovascularisation in association with atheros progression in normoglycemic humans[93,95,105] and animal models; however, the mechanisms underlying this remain unknown. These studies su that hyperglycemia-induced damage to the Vv microvasculature cou to atherosclerotic plaque progression in the macrovessels

2.2 HYPOTHESIS

Accelerated atherosclerosis in hyperglycemic $ApoE^{-/-}$ mice, generally macrovascular complication of DM, results from hyperglycemia-induced damage to the microvessels of the vasa vasorum. In other words, d disease is a microvascular complication.

2.3 OBJECTIVES

Using an established mouse model of hyperglycemia-induced accelerated atherosclerosis, we intend to:

1) Examine the effects of chronic hyperglycemia on the va relative to the progression and development of atheros
   a. Verify that hyperglycemia alters the microvasculature in our mouse model by examining the retinal capillaries
   b. Quantify atherosclerotic plaque parameters and va density at the aortic root
   c. Examine the effects of glucose normalization on th and accelerated atherosclerosis
2) Investigate the effects of hyperglycemia on cellular hy angiogenic protein expression
3) Examine the effects of benfotiamine on the vasa vasor atherosclerosis

3.0 EXPERIMENTAL PROCEDURES

3.1 MATERIALS

3.1.1 Animals

Female homozygous apolipoprotein E-deficient mice (B6.129P2-ApoE^{tm1Unc}), female homozygous low density lipoprotein recepto deficient mice (B6.129S7-Ldlr^{tm1Her}), male heterozygous Ins2^{Akita} (C57BL/6-Ins2^{Akita}/J), and female C57Bl/6J mice (C57Bl/6J) were purchased from The Jackson Laboratory (Bar Harbor, ME).

3.1.2 Diets

Standard chow diet (TD-92078) and high-fat Western diet (TD97363) wer purchased from Harlan Laboratories (Madison, WI). Benfoti (BFT090707/QB) was purchased from Shanghai Pharmaceutical Chemical Co., Ltd. (China), and a custom benfotiamine die (TD10062) was produced by Harlan Laboratories (Madison,

3.1.3 Reagents

Polyclonal rabbit anti-human von Willebrand Factor (vWF) (A0082) was purchased from Dako (Burlington, ON). Hypoxyprobe™-1 Plus Kit was purchased from HPI, Inc. (Burlington, MA). Mouse monoclo -1 alpha (NB100-105) was purchased from Novus Biologicals (Oakville,

goat anti-mouse CD105 (AF1320) and rat anti-mouse VEGF-C monoclonal (MAB471) were purchased from R&D Systems (Minneapolis, rabbit anti-mouse VEGFR-2 (#2479) and polyclonal rabbit anti-mouse cleaved caspase-3 (#9661) were purchased from Cell Signaling Technol ON). Polyclonal rabbit anti-mouse VEGF-A (sc-152), normal rabbit IgG (sc-2027), normal goat IgG (sc-2028), and normal mouse IgG (sc-2025) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz (14131) was purchased from Sigma-Aldrich (Oakville, ON).

AlexaFluor® 488 goat anti-rabbit IgG (A11034), AlexaFluor® 594 goat anti-rabbit IgG (A11037), AlexaFluor® 488 goat anti-rat IgG (A11006), AlexaFluor® 488 goat anti-mouse IgG (A11029), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Burlington, ON). Biotinylated rabbit anti-goat IgG (BA-5000) and Vector® NovaRED peroxidase subs (SK-4800) were purchased from Vector Laboratories (Burling

India Ink (Rötring) was purchased from Grand & Toy Of The Microfil Kit (MV-122; yellow) was purchased from Flow Tech In MA). FluoSpheres® 0.04µm diameter, carboxylate-modified microspheres (F-8793) were purchased from Invitrogen (Burlington, ON).

3.2 ANIMAL MODELS

3.2.1 Streptozotocin-Induced Hyperglycemia

Five week old female ApoE$^{-/-}$ mice were placed on standard chow di randomized to receive multiple low dose (MLD) injections o streptozotocin (STZ) (n= 60) or citrate buffer (n= 45). Two sets of five intraperotineal (i.p.) injections (30-40 mg/kg) were administered over the co three weeks for each treatment group[25] (Fig 6a). Mice from each experimenta group were sacrificed at 10, 15, and 20 weeks of age (n= 10). A subset of STZ-treated mice (n= 15) received low dose (0.05 U/24 hours) insulin (LinShin Canada Inc.) or blank control implants (n= 5) and were sacrifice (n= 10) weeks of age (Fig 6b). An additional group of ApoE$^{-/-}$ mice (n= 10) was sacrificed at 5 weeks of age, prior to any injection. Plasma and tissu were collected from each mouse for further examination.

In a parallel experiment, 5 week old female C57Bl/6J m randomized to receive either MLD-STZ or citrate buffer injections. A subs mice from each treatment group were sacrificed at 10 week (n= 3).

3.2.2 Genetically Induced Hyperglycemia

Male heterozygous $Ins2^{Akita}$ mice (n= 3) were sacrificed at 10 weeks o age, and their plasma and tissues were collected for further examination.

3.2.3 Diet-Induced Dyslipidemia

Five week old female LDLR$^{-/-}$ mice were randomly assigned to standard chow or high-fat Western diet (n= 46). All mice had unrestricted

access to food and water throughout the study. Mice were s[...] at 15 weeks
of age, and plasma and tissue samples were collected for f

3.2.4 Benfotiamine Treatment in Hyperglycemic ApoE$^{-/-}$ Mice

Five week old female ApoE$^{-/-}$ mice were randomized to one of four
treatment groups: (i) MLD-STZ injection plus standard chow diet, (ii) ML
injection plus benfotiamine-supplemented diet, (iii) MLD-citrate injection plus
standard chow diet, or (iv) MLD-citrate injection plus benfotiamine-supplemented
diet (Fig 6c). All mice had unrestricted access to food and water throughout the
study. Mice were sacrificed at 15 weeks of age, and plasma
were collected for further examination. All animal procedur
the McMaster University Animal Research Ethics Board.

Figure 6. Injection timeline and experimental design

a, Five week old mice received multiple low dose injections
streptozotocin (STZ) or citrate buffer. Two sets of five intr
injections (40 mg/kg) were administered over three weeks (n=30) for
each treatment group. Mice were sacrificed at 5, 10, 15, or
asterisk), depending on the experiment. b, Five week old female ApoE$^{-/-}$ mice
were placed on standard chow diet and randomized to rece (n=60)
or citrate buffer (n=45) injection. A subset of STZ-treated mice received low
dose (0.05 U/24 hours) insulin pellet implants (LinShin Ca
control. c, Five week old female ApoE$^{-/-}$ mice were randomly assigned to rec
STZ or citrate buffer injection (n=6). After one week, half of the mice in
group were switched to control diet supplemented with 640
(CHOW+BNF).

3.3 PLASMA ANALYSIS

Whole blood glucose levels were measured prior to sac using a DEX glucometer (Bayer). Plasma lipid levels were analyzed usin diagnostic kits for total cholesterol and triglycerides purch DMA Inc.

3.4 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

3.4.1 Tissue Preparation

Mice were anaesthetized with isoflurane, injected wit 0.4mL of 200U/mL heparin, and flushed with 10mL of 1X PBS plus 20U/mL heparin through the left ventricle. After PBS, mice were fixed with 8-10mL of perfusion 10% neutral buffered formalin. Upon excision of the heart and ascending a were, the apex of the heart was cut transversely and the re embedded in paraffin. Using the valve leaflets as a point o sections (4µm in thickness) of the aortic root were collected onto pre-coated glass slides and used for lesion measurement or immunohistoche immunofluorescence (IF) staining.

3.4.2 Immunohistochemistry and Immunofluorescence

Tissues were deparaffinised in xylene and rehydrated t serial dilutions of ethanol. Heat induced epitope retrieval (HIER), quenching endogenous peroxidase, and blocking serum steps were per

application of the primary antibody. Unless otherwise spec
manufacturer, all primary antibodies were incubated overnight at 4°C. IHC
performed using biotinylated secondary antibodies, horsera
streptavidin, and Nova Red chromogenic substrates. Fluore
performed using secondary antibodies tagged with AlexaFlu
88 and AlexaFluor® 594, followed by DAPI nuclear counterstain. N
-specific staining was controlled for by incubating similar aortic sections wit
non-immune IgG normal serum absent of the primary antibody. All procedure
according to manufacturers' instructions.

3.5 AORTIC LESION ANALYSIS

To determine the extent of atherosclerosis, the serial cros
-sections of the aorta were stained with hematoxylin and eosin. Plaque cros
-sectional area was measured at the aortic root and every 40 µm until the end
of the aorta. Plaque volume was estimated by plotting serial measureme
-sectional area and calculating the area under the curve.[121,122] Necrotic core content was
asessed at the aortic root by measuring the total acellular
regions.

3.6 RETINAL CAPILLARY DENSITY

Normoglycemic and hyperglycemic ApoE$^{-/-}$ mice at 5, 6, 7, 8, 9, 10, and
15 weeks of age (n = 2 per group) were anesthetized with isoflurane, flushed with

with 8-10 mL of PBS-heparin solution (20U/mL), and perfused with 4-5 mL of India Ink. Mouse eyes were excised and retinal flatmounts density was estimated using a method outlined by Browning[123]. Briefly, a quantification template comprised of 64 sampling boxes ali centred upon the optic disk (Fig 7). The number of vessel i edges of each sampling box was tallied for central, mid, and peripheral regions of the vasculature. Regional and total capillary densities are number of vessel intersections per number of boxes analyz

Figure 7. Retinal capillary density template

A quantification template of 64 sampling boxes aligned along
was centred upon the optic disk. For each tangent (red box
of vessel intersections with the edges of each sampling bo
densities were estimated in central, mid, peripheral regions by
dividing the total number of vessel intersections by the nu

3.7 VASA VASORUM QUANTIFICATION

3.7.1 Vasa Vasorum Density

Cross sections of the aorta were immunostained with p
vWF antibody (1:250), a specific EC marker, followed by Al
anti-rabbit IgG (1:250) and DAPI nuclear counterstain (1:500
positive microvessels were counted if having: (1) a single-layer endothelium with
visible EC nuclei and vessel lumen, (2) a smooth muscle ce
lamellae thick, and (3) a lumen diameter less than 50µm.[81,124] Vv density was
defined as the total number of Vv microvessels residing wi
adventitia and/or peri-adventitia per aortic cross section.

3.7.2 Microfil Resin Casting

15 week old $ApoE^{-/-}$ mice were anaesthetized with isoflurane an
with 10-15 mL of PBS-heparin solution (20U/mL) via the left ventricle
gauge needle and PE50 polyethylene tube. Mice received 4-5mL of Microfil
through the left ventricle at a flow rate of 100µL/min.[125] Following Microfil
polymerization overnight at 4°C, the heart and aorta were
in serial dilutions of glycerol (40-100% in water).[126] Clarified specimens were
sent to the Mice Imaging Centre (MICe) at the University of Toronto for mi
computed tomography (micro-CT) imaging.

3.7.3 Fluorescent Microangiography (FMA)

Normoglycemic and hyperglycemic ApoE-/- mice (n = 3 per group) at 15 weeks of age were anaesthetized with isoflurane and flushe PBS-heparin solution (20U/mL) through the left ventricle. Us -gauge needle and PE50 polyethylene tube, mice were perfused wi bead solution containing 0.04µm-diameter fluorescently-labelled microspheres (FluoSpheres® Invitrogen, Burlington, ON)[127] at a flow rate o Animals were placed on ice overnight, and the heart and gel solidif aorta were harvested and embedded in 0.1% agarose gel bl used to section the gel blocks at 100µm-thick serial sections of the ascending aorta, aortic arch, and descending aorta were co glass slides for analysis.

3.8. HYPOXIA AND ANGIOGENIC PROTEIN EXPRESSION

3.8.1 Hypoxia Analysis

Two hours prior to sacrifice, 15 week old ApoE-/- mice were given an i.p. injection of 60mg/kg pimonidazole hydrochloride (Hypoxyprobe™, chemical Hypoxypr which forms stable, immunogenic protein adducts in hypoxic cells. Cross of the aorta were stained with FITC-conjugated MAb1 (1:100), a mouse monoclonal antibody raised against protein adducts of pimonidazole followed by peroxidase-conjugated anti-FITC secondary reagent (1:100), Nov Red substrate, and hematoxylin. Tissue sections IHC for hypoxic

conditions, aortic cross-sections were stained with a mouse monoclon
against HIF-1α antigen (1:25), followed by an anti-mouse IgG AlexaFluor® 488 goa
(1:200) and DAPI nuclear counterstain (1:5000). Staining w
assessed in the intima, media, adventitia, and peri-adventitia.

3.8.2 Endoglin Quantification

Hypoxia-induced endothelial cell activation was examined
root by immunostaining for hypoxia-regulated endoglin (Fig. 5), a TGF-β
receptor expressed by active endothelium.[128,129] Cross-sections of the aorta were
incubated in polyclonal goat CD105 (1:200), followed by biotinylated anti-goat
secondary antibody (1:200), streptavidin, Nova Red substra
Using the Vv criteria outlined in 3.4.5, the total number of positive
microvessels per cross-section was quantified.

3.8.3 VEGF, VEGF Receptors, and Cleaved Caspase-3 Expression

Serial cross-sections of the aorta were immunostained with p
rabbit VEGF-A (1:50), monoclonal rat VEGF-R1 (1:100), monoclonal rabbit
VEGF-R2 (1:200), and polyclonal rabbit cleaved caspase-3 (1:250). Primary
antibody binding was detected using AlexaFluor® 594 goat anti-rabbit IgG
(1:200) for VEGF-A, AlexaFluor® 488 goat anti-rat IgG (1:200) for VEGF-R1,
and AlexaFluor® 488 goat anti-rabbit IgG (1:200) for VEGF-R2 and cleaved
caspase-3. All sections were co-stained with DAPI nuclear counterstain (

VEGF-A and cleaved caspase-3 immunofluorescence was quantified
the intima, media, and adventitia by taking sequential image
each aortic cross-section. Magnification, lamp intensity, and camera ex
were standardized for each stain. Images were processed a
of interest (intima, media, or adventitia) was manually trac
internal clipboard, (2) copied into a 32-bit colour image and convert
thresholded, (3) upper and lower threshold limits were set
the total pixel area was measured. Data were normalized to
interest (intima, media, or adventitia).

3.9 IMAGING

Light microscopy images were captured with an Olympus DP7
camera (Olympus America Inc.) mounted atop a Leitz Labor
microscope (Leica Microsystems, Germany). Fluorescence i
with an Olympus DP71 digital camera (Olympus America Inc.) mounted on top of
an Olympus BX41 microscope (Olympus America Inc.). Slide
(Intelligent Imaging Innovations, Inc) was used to capture
images.

3.10 STATISTICAL ANALYSIS

Results are presented as the mean ± standard error (S

student t test was used to assess differences between experim

Probability values of less than 0.05 were considered statis

4.0 RESULTS

4.1 MICRO- AND MACRO-VASCULAR COMPLICATIONS IN HYPERGLYCEMIC APOLIPOPROTEIN E-DEFICIENT MICE

4.1.1 MLD-STZ injection induces chronic and progressive hyperg ApoE$^{-/-}$ mice

Female ApoE$^{-/-}$ mice treated with MLD-STZ developed significantly elevated blood glucose levels by 10 weeks of age (Table 1) MLD-STZ and 8.50 ± 0.87 mM for citrate buffer control. Hype remained persistent at 15 and 20 weeks of age, and could be normalized by 15 weeks by implanting 0.05 U/day insulin pellets (9.44 ± 0.76 triglyceride and cholesterol levels did not differ significan 15 and 20 weeks of age (Table 1), indicating that MLD-STZ induces hyperglycemia without altering plasma lipid levels.

Table 1. Metabolic parameters in ApoE$^{-/-}$ mice

	10 weeks		15 weeks			20 weeks	
Plasma	Contr	STZ-HG	Contr	STZ-HG	STZ+Ins	Contr	STZ-HG
Glucos (mM)	8.50±0	32.30± ***	7.85±0	22.96± ***	9.44±0	7.73±0	26.81± ***
Triglyce (mM)	0.58±0	1.40±0 *	0.49±0	0.64±0	1.74±0	0.63±0	1.00±0
Choles (mM)	4.80±0	10.44± **	6.74±0	7.00±1	9.65±2	5.37±1	6.33±1 *
Tissues							
Body Weight	19.08±	14.50± ***	20.19±	17.86± ***	16.70±	19.76±	17.66± *
Lesion (10^{-3} mm^2)	1.10±0	0.55±0	3.83±0	11.40± **	2.60±0	15.96±	16.88±
Vv Den	6.50±0	4.80±0 *	8.64±0	5.92 ± **	7.50±0	14.86±	8.29±0 **
N	6-12	6-12	6-12	6-12	5-10	6-12	6-12

STZ-HG, streptozotocin-induced hyperglycemic ApoE$^{-/-}$

STZ+Insulin, streptozotocin, insulin-supplemented ApoE$^{-/-}$

Vv Density, number of vasa vasorum microvessels per aortic

*P<0.05, **P<0.01, ***P<0.001 relative to age-matched control mice

P<0.05 relative to age-matched STZ-HG mice

4.1.2 Hyperglycemic ApoE$^{-/-}$ mice have accelerated atherosclerosis at aortic root

Plaque formation at the aortic root was assessed on se
sections to determine cross-sectional plaque area (Fig. 8d), plaque volume
8e) and necrotic content (Fig. 8f) in normoglycemic (Fig. 8
hyperglycemic (Fig. 8b) ApoE$^{-/-}$ mice. At 15 weeks of age, hyperglycemic
had 3-fold larger cross-sectional plaque area than normoglycemic contr
(0.114 ± 0.015 mm^2 for hyperglycemic and 0.038 ± 0.007 mm^2 for
normoglycemic). They also had significantly greater plaque volume (0.479 ±
0.076 mm^3 versus 0.199 ± 0.032 mm^3) and necrotic content (21.5 ± 2.34%
5.57 ± 2.34%). By 20 weeks of age, differences in plaque cross-sectional
parameters were no longer significant.

To determine whether plaque progression was due STZ injection
hyperglycemia, we examined cross-sectional plaque area in STZ ApoE$^{-/-}$
mice that received 0.05 U/day insulin pellet implants (STZ-
Insulin supplementation significantly reduced cross-sectional plaque area
to hyperglycemic ApoE$^{-/-}$ mice by 15 weeks of age (Fig. 8g; 0.026 ± 0
for STZ+Insulin and 0.114 ± 0.015 mm^2 for hyperglycemic), suggesting that
accelerated atherosclerosis is directly attributable to hype
artefact of STZ injection.

Figure 8. Hyperglycemia is associated with accelerated dev
atherosclerosis at the aortic root in ApoE$^{-/-}$ mice. A) Representative images of H&E-stained aortic cross-sections from 15 week old normoglycemic (N
STZ-induced hyperglycemic (HG-STZ), and STZ-treated, insulin supplemented (STZ+Insulin) ApoE$^{-/-}$ mice. Atherosclerotic lesions are indicated by black arrows, lesion thickness by black bars. B-D) Quantification of plaque cross-sectional area (B), plaque volume (C), and necrotic content (D) at the aortic root in 5, 10, 15 and 20 week NG and HG-STZ ApoE$^{-/-}$ mice, as indicated. E) Quantification of plaque cross-sectional area in 15 week old ApoE$^{-/-}$ mice supplemented with insulin. Statistical analy
mice per group (n=8-12 mice per group; mean ± SEM). * $p<0.05$ (Student's t-test).

4.1.3 Hyperglycemic ApoE-/- mice show indications of microvascular ch in the retina

To assess the effect of hyperglycemia on classic early microvascular abnormalities in our model, retinal microvessel density was normoglycemic and hyperglycemic ApoE-/- mice (Fig. 9). Mice were perfused with India Ink (Rötring), and retinal flatmounts were prepa as described by Browning[1,2,3]. Briefly, a quantification template comp 64 sampling boxes was centred on the optic disk, and the t intersections with each box was tabulated. Density was est the average number of vessel intersections per box analyzed. Relative to normoglycemic controls, hyperglycemic mice had significan microvessels immediately after the onset of hyperglycemia was particularly evident in the area immediately surroundin disk (Fig. 9a), and persisted from 6 to 8 weeks of age (Fig. 9c). By 1 hyperglycemic mice had a significantly denser retinal micro normoglycemic controls (Fig. 9b).

Figure 9: Hyperglycemia is associated with microvascular chan
retina. a, Retinal flatmounts from 6 week old normoglycemic an
ApoE$^{-/-}$ mice perfused with India Ink. Hyperglycemia is associa
microvessel deficiency, particularly in the region immediately surrounding
optic disk (boxed). Representative images taken at low (le
high (right, scale=100 µm) magnification. b, Images of the optic disk from 15
week old ApoE$^{-/-}$ mice perfused with fluorescein. Scale=100 µm. c,
Quantification of retinal microvessel density in normoglyce
hyperglycemic (STZ) ApoE$^{-/-}$ (HG) mice, from 5 to 15 weeks of age (n=4 per
group; mean ± SEM). (Student's t-test). $*p<0.05$.

4.2 EFFECTS OF HYPERGLYCEMIA ON THE VASA VASORUM

4.2.1 Hyperglycemic ApoE$^{-/-}$ mice have reduced vasa vasorum density aortic root

Vv density was quantified at the aortic root by immuno antigen, a specific endothelial marker (Fig. 10a). Microves within the intima, media, and adventitia were identified as having a single-layered endothelium, lumen diameter less than 50 µm, and SMC layer less than t are reported as the total number of vWF-positive microvessels per aortic cro section (Fig. 10b). In normoglycemic ApoE$^{-/-}$ mice, Vv density increased in a progressive and significant manner over the 15 week perio 0.66, and 14.86 ± 1.14 at 5-, 15- and 20-weeks, respectively). By contrast, hyperglycemic ApoE$^{-/-}$ mice had no significant increase in Vv density ov time, and had significantly fewer Vv microvessels than nor each time point examined (4.80 ± 0.39, 5.92 ± 0.61, and 8. and 20-weeks, respectively).

To determine whether Vv deficiency was a direct result of or a side effect of STZ injection, we analyzed Vv density in ApoE$^{-/-}$ STZ mice that received 0.05 U/day insulin pellet implants (STZ- Vv density in STZ-treated ApoE$^{-/-}$ mice provided with insulin was similar t of normoglycemic ApoE$^{-/-}$ controls, suggesting that decreased Vv dens specific effect of hyperglycemia, and not STZ-injection.

Figure 10. Hyperglycemia is associated with reduced vasa v at the aortic root in ApoE$^{-/-}$ mice. a, Cross-sections of the aortic root from 15 old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice, immunostained against vWF antigen. Vasa vasorum microvessels within the advent (arrows). Representative images taken at low (left, scale=100µm) and scale=50µm) magnification. b, Quantification of vasa vasorum density in 15, and 20 week old normoglycemic (NG) and hyperglycemic (HG) ApoE$^{-/-}$ mice. Vasa vasorum density defined as the total number of vasa vasorum microvessels residing within the intima, media and adventi section (n=8-10 mice per group; mean ± SEM). (*$p<0.01$; Student's t test). c and d, Quantification of vasa vasorum density at the aorti 10 week old NG, HG, STZ and STZ+Insulin ApoE$^{-/-}$ mice (panel c) and NG, HG, STZ and Ins2^{Akita} mice on C57Bl/6J background (panel d; n=8 mice per group; mean ± SEM); Student's t test.

4.2.2 Normolipidemic, hyperglycemic C57Bl/6J and Ins2^{Akita} mice have reduced vasa vasorum density at the aortic root

To control for ApoE-deficiency, Vv density was assessed in STZ-treated C57Bl/6J mice and hyperglycemic Ins2^{Akita} mice. Both STZ-treated C57Bl/6J mice and Ins2^{Akita} mice developed significantly elevated blood gluco levels by 10 weeks of age (22.30 ± 1.37 mM and 21.30 ± 2. 8.30 ± 0.26 mM for C57Bl/6J controls) (Table 2). Plasma tr cholesterol levels in C57Bl/6J and Ins2^{Akita} mice were significantly than those in age-matched ApoE$^{-/-}$ groups (Table 1), and neither C57Bl/6J nor Ins2^{Akita} mice developed atherosclerotic lesions at the aortic root. I hyperglycemic C57Bl/6J and Ins2^{Akita} mice had fewer Vv microvessels than normoglycemic C57Bl/6J controls (Fig. 10d). These data suggest th hyperglycemia-induced Vv deficiency occurs independently of dys impaired lipoprotein particle clearance, and atherosclerosi deficiency, and provide further evidence of a hyperglycemic-specific effect.

Table 2. Metabolic parameters in Ins2^{Akita} and C57Bl/6J mice

	C57Bl/6J		Ins2^{Akita}
Plasma	Contro	STZ-HG	HG
Glucos (mM)	8.30±0.	22.00±1.*	21.30±2.*
Triglycer (mM)	0.67±0.	1.41±0.2*	0.30±0.
Choleste (mM)	2.32±0.	3.77±0.	0.79±0.(*
Tissues			
Body We (g)	19.70±(	15.43±0.*	18.97±0
Lesion A (10^{-2} mm^2)	0	0	0
Vv Dens	7.00±1.	4.67±0.	4.33±0.
N	3	3	3

STZ-HG, streptozotocin-induced hyperglycemic C57Bl/6J

Vv Density, total number of vasa vasorum microvessels per aortic section

* P<0.05 relative to age-matched control mice

4.2.3 Fluorescent Microangiography (FMA) imaging confirm
deficiency in the aortic arch of hyperglycemic ApoE$^{-/-}$ mice

To further examine the effects of hyperglycemia on the Vv,
casts of the coronary vasculature were prepared (Fig. 11a)
Mouse Imaging Centre (MICe) at the University of Toronto
resolution micro-computed tomography (micro-CT); however, the degree of
resolution required for 3D analysis of the Vv could not be

The ascending aorta, aortic arch, and descending aorta
analyzed using fluorescent microangiography (FMA). 15 we
normoglycemic and hyperglycemic ApoE$^{-/-}$ mice were perfused with an agaros
bead solution containing fluorescently-labelled microspheres. Hearts were
embedded in agarose blocks and cut transversely at 100 μm
sections were collected and analyzed. In support of the reported histolo
findings, hyperglycemic ApoE$^{-/-}$ mice showed fewer Vv microvessels in th
arch compared to normoglycemic controls (Fig. 11c).

Figure 11. Microfil resin casting and Fluorescent microangiography (FMA) of the vasa vasorum in ApoE$^{-/-}$ mice. Coronary vasculature from a 15 week old normoglycemic ApoE$^{-/-}$ mouse perfused with Microfil casting compound, Micro-CT image of the coronary vasculature generated by the Mice Imaging Centre (MICe), University of Toronto. Sections of the aortic arch from 15 week old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice (n=3) perfused with 0.04 µm diameter fluorescently-labeled microspheres. Panels on the right (scale=50µm) represent higher magnification images of boxed regions on the left (scale=100µm). Aortic lumen (a) is indicated.

4.2.4 Accelerated atherosclerosis is associated with vasa v neovascularisation in normoglycemic, dyslipidemic $ApoE^{-/-}LDLR^{-/-}$ mice

To investigate the effects of accelerated atherogenesis hyperglycemic model, we measured lesion area and VV density i 15 week old $ApoE^{-/-}LDLR^{-/-}$ mice on high fat (Western) diet (Fig. 12a and b). In these models, we observed a relative increase in plasma significant change in blood glucose levels (Table 3). Furth mice showed a direct correlation between atherosclerotic p and VV expansion (Fig. 12b). These findings are consistent w by other groups in non-diabetic, normoglycemic models, and thus val quantification methods[90,95,106].

Table 3. Metabolic parameters in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice fed Control or high-fat Western diet

	ApoE$^{-/-}$		LDLR$^{-/-}$	
Plasma	Control	Western	Control	Western
Blood Glu (mM)	8.53 ± 0	9.05 ± 1	8.50 ± 0	8.80 ± 0.92
Triglyceri (mM)	0.40 ± 0	0.74 ± ***0.	0.28 ± 0	1.05 ± *0.
Choleste (mM)	4.69 ± 0	9.44 ± *1.	2.85 ± 0	11.47 ± *0
Tissues				
Body Wei (g)	21.68 ± (	23.18 ± 1	20.00 ± (	23.43 ± *0
Lesion Ar (10^{-3} mm^2)	5.86 ± 1	37.51 ± **2	0.34 ± 0	13.94 ± *2
Vv Densi	7.83 ± 1	15.83 ± **0	3.25 ± 0	8.33 ± *0.
N	4-6	4-6	3	3

Western, high-fat Western diet

Vv Density, total number of vasa vasorum micro-vessels per

*P<0.05, **P<0.01, ***P<0.001 relative to age-matched control mice

Figure 12: Western Fed ApoE$^{-/-}$ and LDLr$^{-/-}$ Mice have accelerated atherosclerosis accompanied by vasa vasorum proliferation. Quantification of lesion cross-sectional area at the aortic root in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice fed standard chow (control) or Western diet (n=4-6 mice per group; *$p<0.05$; **$p<0.01$ (Student t-test)). Quantification of vasa vasorum density at the aortic root in ApoE$^{-/-}$ and LDLr$^{-/-}$ mice fed standard chow (control) or Western diet (n=4-6 mice per group; *$p<0.05$; **$p<0.01$ (Student t-test)). Aortic cross section from a 15 week old Western-fed LDLr$^{-/-}$ mouse, stained with an antibody against vWF (red) and nuclear counterstain (blue). Atherosclerotic le (asterisk) and microvessel within the lesion cap (arrow) in

4.3 MARKERS OF ANGIOGENESIS AND APOPTOSIS IN HYPERGLYCEMIC APOLIPOPROTEIN E DEFICIENT MICE

4.3.1 Hyperglycemic ApoE$^{-/-}$ mice have elevated levels of hypoxia and HIF-1α at the aortic root

Regional hypoxia (Fig. 13a) and HIF-1α expression (Fig. 13b) were qualitatively assessed on serial aortic cross-sections from 15 week old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice. Compared to normoglycemic controls, hyperglycemic ApoE$^{-/-}$ mice had more hypoxic cells within the adventitia (Fig. 13a). Regions staining most intensely for h directly adjacent to atherosclerotic lesions, where the vascular wall was thickest. Hyperglycemic ApoE$^{-/-}$ mice also had increased HIF-1α staining (Fig. 13b) relative to normoglycemic mice, which is consistent w HIF-1α stabilization under conditions of hypoxia.

Figure 13. Hyperglycemia is associated with elevated levels of HIF-1α at the aortic root in 15 week old ApoE$^{-/-}$ mice. Aortic sections from 15 week old normoglycemic and hyperglycemic ApoE$^{-/-}$ mice stained with an antibody against pimonidazole (HypoxyProbe Inc.). Orang arrows indicate hypoxic cells, which are located specifical surrounding atherosclerotic plaque (boxed). Representative (left, scale=100µm) and high (right, scale=20µm) magnific sections of the aortic root from normoglycemic ApoE$^{-/-}$ mice and hyperglycemic immunostained with an antibody against HIF-1α. Elastic lamina (dashe line) and atherosclerotic lesion (asterisk) are marked. Sca

4.3.2 Hyperglycemic ApoE$^{-/-}$ mice have increased endothelial cell activ the aortic root.

Hypoxia-induced endothelial cell activation was examined root in normoglycemic and hyperglycemic ApoE$^{-/-}$ mice by immunostaining for endoglin (Fig. 14a-e), a hypoxia-regulated TGF receptor expressed by active endothelium.[128,129] Data are presented as the total number of positive endo microvessels residing within the intimia, media, and adventitia Relative to normoglycemic controls, hyperglycemic mice ha endoglin-positive or active Vv microvessels at 15 weeks of ag for hyperglycemic and 4.29 ± 1.19 for normoglycemic). Differences betwee groups were no longer significant by the 20 week time poin

Figure 14. Hyperglycemia is associated with increased end
at the aortic root. Aortic sections from 15 week old normoglycemic and
hyperglycemic ApoE-/- mice stained with an antibody against endogli
taken at low (left, scale=50µm) and high (right, scale=20µ
Endoglin-positive microvessels are indicated (arrows). Quantification of
endoglin-positive microvessels at the aortic root in 5, 10, 15, an
normoglycemic (NG) and hyperglycemic (HG) ApoE-/- (STZ) mice (n=7-9 mice
per group; mean±SEM; *$p<0.05$ (Student's t-test)).

4.3.3 Hyperglycemic ApoE-/- mice have a relative deficiency of VEGF-A and VEGF receptors at the aortic root

We assessed HIF-1α regulated expression of VEGF-A, VEGFR1, and VEGFR2 protein at the aortic root in 15 week old normoglyc hyperglycemic ApoE-/- mice using immunofluorescence (Fig. 15a). VEGF-A was expressed predominantly within the atherosclerotic plaques mice had significantly less VEGF-A than normoglycemic controls 15b). VEGFR1 protein levels, which are directly regulated by HIF-1α under hypoxic conditions[42], were also reduced in hyperglycemic mice rela controls (Fig. 15a). Similarly, VEGFR2 expression, which i A in an autocrine manner[42], was significantly reduced within the lesio hyperglycemic mice compared to controls (Fig. 15a). Collec observations indicate a hyperglycemia-associated impairment of HIF-1α regulated VEGF expression.

Figure 15. Hyperglycemia is associated with reduced VEGF-A and VEGF receptor expression at the aortic root. Sections of the aortic root from 15 week old normoglycemic and hyperglycemic ApoE-/- mice immunostained against VEGF-A, VEGFR1 and VEGFR2, as indicated. Internal elastic lamina (dashed line) and lesion (asterisk) are marked. Scale = 200 µm. Quantification of VEGF-A density within the lesion, media, and adventitia of normogl hyperglycemic (HG) STZ ApoE-/- mice at 15 weeks of age. n=6; mean ± (SEM). *p<0.05 (Student's t-test).

4.3.4 Hyperglycemic ApoE$^{-/-}$ mice have reduced expression of cleaved caspase-3 at the aortic root

Cellular apoptosis was assessed at the aortic root in n
hyperglycemic ApoE$^{-/-}$ mice by staining for cleaved (activated) caspa-3 (Fig.
16a). At 10 and 15 weeks of age, hyperglycemic mice had l-3
expression within their atherosclerotic lesions than normog
16b). By 20 weeks of age, differences between groups were no longer appar
However, 20 week old hyperglycemic mice had positive cas-3 expression in
medial smooth muscle cells directly adjacent to atheroscle
normoglycemic controls did not (Fig. 16a).

Figure 16. Cleaved caspase-3 expression within the lesions of ApoE-/- mice. a, Aortic cross sections from 15 and 20 week old normoglycemic an hyperglycemic ApoE-/- mice stained with an antibody against cleaved caspase-3. Internal elastic lamina (white) and atherosclerotic plaque (ye asterisk) indicated. Positive caspase-3 staining was also noted in medial SM 20 week old hyperglycemic mice (white arrows). b, Quantification of cleaved caspase-3 staining area within the lesions of normoglycemic (NG) and STZ-induced hyperglycemic (HG) ApoE-/- mice at 5, 10, 15, and 20 weeks of ag are normalized to total lesion area (n=4 mice per group). *P<0.05 (Student t-test)

4.4 EFFECT OF BENFOTIAMINE ON THE DEVELOPMENT OF MICRO- AND MACRO-VASCULAR COMPLICATIONS IN HYPERGLYCEMIC APOLIPOPROTEIN E DEFICIENT MICE

4.4.1 Benfotiamine supplementation reduces atherosclerosi normoglycemic and hyperglycemic ApoE$^{-/-}$ mice

To determine the effect of benfotiamine on lesion development, we measured plaque cross-sectional area and plaque volume at the aor normoglycemic and hyperglycemic ApoE$^{-/-}$ mice on standard chow or benfotiamine-supplemented diet. At 15 weeks of age, hyperglyce normoglycemic ApoE$^{-/-}$ mice on benfotiamine-supplemented diet had plaques t were smaller in cross-sectional area than control chow-fed mice (Fig 17a). In addition, benfotiamine supplementation reduced plaque vol standard chow in both hyperglycemic and normoglycemic mice (Fig. 17b).

Figure 17. Benfotiamine supplementation reduces atherosc
normoglycemic and hyperglycemic ApoE$^{-/-}$ mice. Plaque cross-sectional area
at the aortic root (0 µm) and every 40 µm thereafter in aortas from 15 week old
normoglycemic (NG) or hyperglycemic (HG) ApoE$^{-/-}$ mice fed control diet or
control diet supplemented with benfotiamine (+BNF). Statis
mice per group (n=3 mice per group; mean ± SEM). *$p<0.05$ relative to control
diet within same treatment group (t-test). Plaque volume in NG and
HG ApoE$^{-/-}$ mice fed control diet or control diet supplemented w
(+BNF). Statistical analysis of 3 mice per group (n=3 mice
SEM).

5.0 DISCUSSION

Atherosclerosis, a macrovascular complication of DM,
cause of CVD and the primary mediator of mortality in peop
Much effort has been focused on delineating the cellular a
mechanisms by which hyperglycemia promotes atherosclerosis, but the role
Vv in this relationship has not been explored.

Here, we show that chronic hyperglycemia in ApoE$^{-/-}$ mice is associated
with classical diabetic microvascular and macrovascular pa
demonstrate that Vv density is reduced at the aortic root in
and show that such deficiency directly correlates to accele
Altered expression of hypoxic markers and key angiogenic
with these morphological changes. Lastly, preliminary data
benfotiamine, a drug used to treat diabetic microvascular c
atherosclerosis in hyperglycemic mice.

5.1 Classical Micro and Macro Vascular Complications in H
ApoE$^{-/-}$ Mice

Consistent with previously reported findings[25,130,131], we show that
hyperglycemic ApoE$^{-/-}$ mice develop significantly larger atheroscler
the aortic root than normoglycemic controls by 15 weeks of
these lesions larger in cross-sectional area, but also in necrotic content

that hyperglycemic mice have more advanced lesions than control

These vascular changes appear to be directly attributable

hyperglycemia, because they occur prior to the onset of dy

attenuated when glucose levels are normalized by insulin. Plaque parameters in

both groups plateau at 20 weeks of age, likely because hig

within the aorta induce a physiological threshold on inwarc

and luminal narrowing.

In addition to changes in the macrovessels, hyperglycemic ApoE-/- mice

show indications of early-onset microvessel deficiency in the retina, f

significant capillary neovascularisation at later time points

also seen retinal capillary changes STZ-injection,[132] as early as one week a

and vasoregression is a well-known early clinical feature of proliferativ

retinopathy.[133] Capillary dropout leads to ischemia and increase

protein expression in the retina, which is thought to stimul

neovascularisation and proliferative diabetic retinopathy.[5,7] The temporal course

of vascular changes in our hyperglycemic ApoE-/- mice is consistent with these

reported clinical findings.

5.2 Hyperglycemia and Vasa Vasorum Deficiency

Using this model, we investigated the effects of chroni

the microvessels of the Vv. We found that hyperglycemic ApoE-/- mice had A

significant expansion of the Vv at any time point examined, des

increased lesion size. Hyperglycemic Ins2^{Akita} and STZ-treated C57Bl/6J
mice also had significant reductions in Vv density, indicati
microvascular changes are likely independent of and can occur in the
absence of atherogenesis. Normalization of blood glucose
restored Vv density, further supporting a direct role for hy
effect.

Progressive vasodegeneration and impaired vessel regrowth of m
recognized as major underlying factors in the initiation and
diabetic complications, including retinopathy, nephropathy
Diabetic retinopathy is characterized by pericyte loss and
capillary blood flow, followed by EC death and aberrant vessel[57,134]
Endothelial damage in the glomeruli of diabetic kidneys res
intrarenal capillaries, leading to peritubular capillary regression, tubulointe
hypoxia, and progressive renal fibrosis.[135,136] Furthermore, structural changes
the nerve microvasculature, which include EC and BM thickening, p
and vessel occlusion, lead to vasoregression, reduced nerv
endoneurial hypoxia.[137,138] Thus, early Vv deficiency in our hyperglyc
ApoE$^{-/-}$ mice may represent an evolutionary process in the course
diabetic microvascular disease. Given the temporal pattern
the retina, it is possible that this observed Vv deficiency is an earl
hyperglycemia, and may precede ischemia-induced neovascularisation at a later
time point.

5.3 Vasa Vasorum Deficiency and Accelerated Atherogenes

To validate our quantification techniques, we analyzed
aortic root in normoglycemic LDLR-/- mice on a high-fat Western diet.
We found that Vv expansion directly correlated to atherosc
dyslipidemic ApoE-/- and LDLR-/- mice. This observation is consistent wit
reported by other groups in humans and animal models of lipid-driven atherogenesis[81,93,95,109,124]. Vv neovascularisation in normoglycemic, dyslipiden
models is thought to facilitate plaque progression by delivering lipi
inflammatory cells to arterial wall. Newly formed microvess
prone to rupture, and may contribute to necrotic core expa
destabilization via intraplaque hemorrhage.

The fact that Vv density was negatively related to plaque progression
hyperglycemic ApoE-/- mice means that Vv neovascularisation is not
for accelerated atherosclerosis to occur in this model. It is
microvessel density could directly contribute to atherogenesi
removal via the reverse cholesterol transport (RCT) system
accumulation of lipids and inflammatory cells, accelerated
necrosis[139]. Indeed, the venous and lymphatic arms of the Vv ar
important for HDL efflux[102], and low Vv densities correlate with inflar
and subintimal thickening in the coronary arteries[140]. Reduced Vv blood
flow and ischemic injury also induce VSMC proliferation an
deposition, which may further exacerbate plaque growth[89,141]. In support of this,

Barker et al reported that the occlusion of adventitial Vv in
intimal hyperplasia and arterial wall hypoxia.[142] The observed correlation
between adventitial hypoxia and accelerated atherosclerosis in our hype
mice is consistent with this hypothesis.

5.4 Hypoxia-Induced Expression of Endoglin, VEGF-, and VEGF R 1 and 2

In our hyperglycemic ApoE$^{-/-}$ mice, we observed increased hypoxia,
elevated levels of HIF-1 protein, and increased hypoxia-induced endothelial cell
activation in the aortic root microvessels at 15 weeks of ag
consistent with an angiogenic response. However, VEGF-A, VEGF-R1, and VEGF-R2 levels were lower in hyperglycemic mice compared to
normoglycemic controls. This discrepancy between angioge
response likely explains the reduced Vv density seen in 15
hyperglycemic mice. These findings are consistent with tho
al (2009), who reported that diabetic fibroblasts and db/db m
defective in their capacity to upregulate VEGF in response
ischemia.[143] They attributed this impaired VEGF upregulation to
methylglyoxal-induced modification of the cysteine/histidine-rich (CH1) region
of transcriptional coactivator, p300, which is essential for HIF-1 transactivation
and HRE binding.[37] This mechanism could explain the decreased expression of
VEGF and its receptors in our hyperglycemic mice.

Although VEGF and endoglin are both hypoxia-inducible, and both contain the same HRE functional consensus sequence withi report differential expression in the context of hyperglycemia: VE suppressed, while endoglin is upregulated. The reason for expression is probably multifactorial. The endoglin and VE synergistically activated by hypoxia and TGF-β[144,145], endoglin's promoter is more sensitive to TGF stimulation, while hypoxia is a stron VEGF promoter. Furthermore, HIF-1α activity depends on the recruitment of CBP/p300 transcription co-activators[146]. CBP and p300 exhibit varying degrees of specificity for HIF target genes, and co regulation on the VEGF and endoglin promoters[147]. Lastly, while the CH1 domains of CBP and p300 are indispensable for HIF-1 transactivation, they are only required for an average of 35-50% of global HIF-responsive gene expression[148]. Additional studies are required to elucidate the relevan mechanisms that underlie this effect.

5.5 Benfotiamine and Atherosclerosis

Benfotiamine attenuates hyperglycemia-induced vascular complications in STZ-injected rodents and in patients with DM[112,113], but its effect on the Vv and atherosclerotic progression are unkno hyperglycemic ApoE$^{-/-}$ mouse model, we examined atherosclerosis at root in mice fed standard chow or benfotiamine-supplemented chow. Early

preliminary data indicate that benfotiamine reduces plaque hyperglycemic and normoglycemic animals, however, the ro effect has yet to be explored. The ability of benfotiamine to counter glucose toxicity in microvascular cells has been well documented[113,116,149], and it is possible that benfotiamine may have a protective effect on Vv in hyperglycemic mice. The fact that atherosclerosis wa normoglycemic $ApoE^{-/-}$ mice on benfotiamine diet suggests that the d affect atherogenesis independently of glycemic status. Such an e complicate the interpretation of these interventions.

5.6 Critical Appraisal and Significance of Work

Conclusions drawn from this work are subject to a num relating to the model itself and to the role of angiogenesis in Firstly, it is virtually impossible to separate the effects of hypoinsulinemia in animal models of hyperglycemia, which insulin to induce high blood glucose. As such, the effects that we h ascribed to hyperglycemia in $ApoE^{-/-}$; C57BL/6J $Ins2^{Akita}$ mice may be equally attributable to the lack of insulin in these models. association between Vv neovascularization and atherosclerosis has been well established, direct evidence supporting a causal role for the Vv in this effect is lacking. Lastly, the interpretation of data sup angiogenesis in atherosclerosis is complicated by our relat

understanding of the mechanisms underlying both of these
plieotropic nature of many angiogenic proteins, including V
complexity.[42]

Despite these concerns, the work presented here provides significant new
insights into the relationship between hyperglycemia and C
incidence of DM is steadily increasing, and the cardiovasc
DM account for the majority of morbidity and mortality in p
Thus, understanding how diabetes and hyperglycemia prom
critical to the identification of novel therapeutic targets ar
strategies.

5.6 Future Directions

The following experiments would extend the findings of this project and
provide further insight into the role of the vasa vasorum in
atherosclerosis:

A. Characterize structural and morphological changes in th
of hyperglycemic mice. Vv deficiency at the aortic root should be co
expanded upon using various Micro-CT analyses. Micro-CT could be used to
characterize Vv branching morphology, vessel tortuosity, v
endothelial surface exchange, among other outcome measu
capillary ultrastructure would shed further light on the integrity of ce

junctions, BM and pericyte abnormalities, and other marker
The presence of classical features of diabetic microvascula
presence of acellular capillaries, microaneurysms, and haemorrhages, would
further support the findings of this work.

B. Extend vasa vasorum analysis to longer time points and
beds. Early Vv deficiency in hyperglycemic mice may represen
evolutionary process in the progression of diabetic microvascular disea
may precede neovascularisation at later stages, as in the r
vessel growth, as in diabetic wounds. Carrying mice out to
would allow us to further examine the temporal course of Vv changes at the
root. Correlation of these findings to atherosclerotic progr
the fact that plaque parameters at the aortic root plateau a
such, temporal Vv studies may be extended to different vascular beds,
such as the coronary arteries or descending aorta, where p
not plateau as early.

C. Further examine the role of angiogenic factors and meth
vasorum deficiency. The current findings suggest that altered expression of
hypoxic markers and angiogenic proteins coincide with stru
aortic Vv. Further quantification of angiogenesis markers a
using multiple techniques (ex. IHC, in situ hybridization, plasma ELISA)

would strengthen this work. Investigation into the potentia
induced modification of p300 in impaired VEGF expression
would also provide further mechanistic insight into our resul
of angiogenesis using genetically controllable mouse strai
mice) or administration of anti-angiogenic proteins could clarify the
of Vv neovascularisation in diabetic atherosclerosis.

D. Extend vasa vasorum analysis in diabetic humans. The development of
effective therapeutic interventions relies on translating research be
the human disease state. Extending Vv studies into diabeti
enhance the clinical significance of our results, and would provide further insight
into the effects of glucose intolerance on Vv dysfunction and
atherosclerotic progression. The presence of co-existing metabolic abnormalities
in human forms of DM, including dyslipidemia and insulin r
complicate the interpretation of results.

6.0 CONCLUSIONS

Hyperglycemia significantly increases the risk of micro
vascular complications in DM, and microvascular dysfuncti
explain the relationship between blood glucose levels and diabetic CVD. Using an
in vivo model of STZ-induced hyperglycemia, we have demonstrated
chronically elevated blood glucose levels are associated w
aortic root. Accelerated development of atherosclerosis oc
mice despite this Vv deficiency, and impaired neovasculari
contribute to atherosclerotic necrosis and core expansion.
appear to be directly attributable to chronic hyperglycemia
independently of dyslipidemia, and can be attenuated when glu
normalized by insulin. A localized deficiency of VEGF and
aortic root may explain the neovascularisation defect seen
Lastly, preliminary data indicate that benfotiamine, a drug used to tre
microvascular disease, may reduce atherosclerosis in hype
knowledge, this is the first evidence to suggest a potential
neovascularisation in diabetic atherosclerosis. The next challenge lies in
determining whether temporal changes in Vv neovascularisa
actively contribute to the development and progression of

7.0 REFERENCES

1. World Health Organization. Diabetes. Fact Sheet N°312. http://www.who.int/mediacentre/factsheets/fs312/en/. Updated January 2011April 2011.
2. Unwin N, Whiting D, Gan D, Jacqmain O, Ghyoot G. International Diabe Federation. IDF Diabetes Atlas, Fourth Edition. . 2009
3. Canadian Diabetes Association Clinical Practice Guideli Canadian Diabetes Association 2008 clinical practice prevention and management of diabetes in Canada. Can J Diabetes. 2008;32(Supplement 1).
4. Shaw JE, Zimmet PZ, de Courten M, et al. Impaired fast impaired glucose tolerance. What best predicts future Mauritius? Diabetes Care. 1999;22(3):399-402.
5. The effect of intensive treatment of diabetes on the dev progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Tria N Engl J Med. 1993;329(14):977-986. doi: 10.1056/NEJM199309303291401.
6. Beckman JA, Creager MA, Libby P. Diabetes and atheros epidemiology, pathophysiology, and management. JAMA. 2002;287(19):2570-2581.

7. Miettinen H, Haffner SM, Lehto S, Ronnemaa T, Pyorala
Retinopathy predicts coronary heart disease events in
Diabetes Care. 1996;19(12):1445-1448.

8. van Hecke MV, Dekker JM, Stehouwer CD, et al. Diabeti
associated with mortality and cardiovascular disease
EURODIAB Prospective complications study. Diabetes Care.
2005;28(6):1383-1389.

9. Juutilainen A, Lehto S, Ronnemaa T, Pyorala K, Laakso
predicts cardiovascular mortality in type 2 diabetic m
Diabetes Care. 2007;30(2):292-299. doi:10.2337/dc06-1747.

10. Mogensen CE. Microalbuminuria predicts clinical protei
mortality in maturity-onset diabetes. N Engl J Med. 1984;310(6):356-
360. doi: 10.1056/NEJM198402093100605.

11. Dinneen SF, Gerstein HC. The association of microalbu
in non-insulin-dependent diabetes mellitus. A systematic ove
literature. Arch Intern Med. 1997;157(13):1413-1418.

12. Intensive blood-glucose control with sulphonylureas or insulin c
conventional treatment and risk of complications in patients with t
diabetes (UKPDS 33). UK Prospective Diabetes Study
Lancet. 1998;352(9131):837-853.

13. Adams RH, Alitalo K. Molecular regulation of angiogene
lymphangiogenesis. Nat Rev Mol Cell Biol. 2007;8(6):464-478. doi: 10.1038/nrm2183.

14. ADVANCE Collaborative Group, Patel A, MacMahon S, e
blood glucose control and vascular outcomes in patien
diabetes. N Engl J Med. 2008;358(24):2560-2572. doi: 10.1056/NEJMoa0802987.

15 Action to Control Cardiovascular Risk in Diabetes Study
Miller ME, et al. Effects of intensive glucose lowering
N Engl J Med. 2008;358(24):2545-2559. doi: 10.1056/NEJMoa080274

16. Report of the Expert Committee on the Diagnosis and Classification of
Diabetes Mellitus. Diabetes Care. 1997;20(7):1183-1197.

17. Norhammar A, Tenerz A, Nilsson G, et al. Glucose meta
with acute myocardial infarction and no previous diag
mellitus: a prospective study. Lancet. 2002;359(9324):2140-2144. doi: 10.1016/S0140-6736(02)09089-X.

18. Weir CJ, Murray GD, Dyker AG, Lees KR. Is hyperglyca
predictor of poor outcome after acute stroke? Results
follow up study. BMJ. 1997;314(7090):1303-1306.

19. D'Souza A, Hussain M, Howarth FC, Woods NM, Bidase
Pathogenesis and pathophysiology of accelerated athe

diabetic heart. Mol Cell Biochem. 2009;331(1-2):81-96. doi: 10.1007/s11010-009-0148-8.

20. Giacco F, Brownlee M. Oxidative stress and diabetic co Circ Res. 2010;107(9):1058-1070. doi: 10.1161/CIRCRESAHA.110.22354

21. Han I, Kudlow JE. Reduced O glycosylation of Sp1 is a increased proteasome susceptibility. Mol Cell Biol. 1997;17(5):2550-2558.

22. Han I, Oh ES, Kudlow JE. Responsiveness of the state of O-linked N-acetylglucosamine modification of nuclear pore protei extracellular glucose concentration. Biochem J. 2000;350 Pt 1:109-114.

23. Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownlee M. Hy inhibits endothelial nitric oxide synthase activity by p modification at the Akt site. J Clin Invest. 2001;108(9):1341-1348. doi: 10.1172/JCI11235.

24. Jackson C. Matrix metalloproteinases and angiogenesis. Curr Opin Nephrol Hypertens. 2002;11(3):295-299.

25. Werstuck GH, Khan MI, Femia G, et al. Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated a hyperglycemic mouse model. Diabetes. 2006;55(1):93-101.

26. Kim AJ, Shi Y, Austin RC, Werstuck GH. Valproate prot stress-induced lipid accumulation and apoptosis by inhibi

synthase-3 kinase. J Cell Sci. 2005;118(Pt 1):89-99. doi: 10.1242/jcs.01562.

27. Kuboki K, Jiang ZY, Takahara N, et al. Regulation of e
nitric oxide synthase gene expression in endothelial c
specific vascular action of insulin. Circulation. 2000;101(6):676-

28. Brownlee M. Biochemistry and molecular cell biology of
complications. Nature. 2001;414(6865):813-820. doi: 10.1038/414813a.

29. Punthakee Z, Werstuck GH, Gerstein HC. Diabetes and
disease: explaining the relationship. Rev Cardiovasc Med.
2007;8(3):145-153.

30. Uflacker R. Microcirculation of the Aorta. In: Uflacker
Vascular Anatomy: An Angiographic Approach. 2nd Edition ed.
Lippincott Williams & Wilkins; 2007.

31. Gallagher PJ, van der Wal AC. Blood Vessels. Chapter 9. In: Mills SE, ed.
Hisology for Pathologists. 3rd Edition ed. Lippincott Williams &
Wilkins; 2006.

32. Stary HC, Blankenhorn DH, Chandler AB, et al. A defin
human arteries and of atherosclerosis-prone regions. A report from th
Committee on Vascular Lesions of the Council on Arte
American Heart Association. Arterioscler Thromb. 1992;12(1):120-134.

33. Gingras M, Farand P, Safar ME, Plante GE. Adventitia:
conduit arteries. J Am Soc Hypertens. 2009;3(3):166-83. doi:
10.1016/j.jash.2009.03.002.

34. Carmeliet P. Mechanisms of angiogenesis and arteri Nat Med.
2000;6(4):389-95. doi: 10.1038/74651.

35. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9(6):653
doi: 10.1038/nm0603-653

36. Jain RK. Molecular regulation of vessel maturation. Nat Med. 2003;9(6):685-
693. doi: 10.1038/nm0603-685

37. Rey S, Semenza GL. Hypoxia-inducible factor-1-dependent mechanisms of
vascularization and vascular remodelling. Cardiovasc Res.
2010;86(2):236-42. doi: 10.1093/cvr/cvq045.

38. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a
basic-helix-loop-helix-PAS heterodimer regulated by cellular O2
Proc Natl Acad Sci U S A. 1995;92(12):5510-4.

39. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-alpha to the
Hippel-Lindau ubiquitylation complex by O2-regulated prolyl
hydroxylation. Science. 2001;292(5516):468-72. doi:
10.1126/science.1059796.

40. Maxwell PH, Wiesener MS, Chang GW, et al. The tumou
VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis.
Nature. 1999;399(6733):271-5. doi: 10.1038/20459.

41. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML
is an asparaginyl hydroxylase enzyme that regulates t
activity of hypoxia-inducible factor. Genes Dev. 2002;16(12):1466-1471.
doi: 10.1101/gad.991402.

42. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006;7(5):359-371. doi: 10.1038/nrm1911.

43. Kroll J, Waltenberger J. A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. Biochem Biophys Res Commun. 1999;265(3):636-639. doi: 10.1006/bbrc.1999.1729.

44. Bates DO, Hillman NJ, Williams B, Neal CR, Pocock TM
microvascular permeability by vascular endothelial gro
J Anat 2002;200(6):581-597.

45. Ruhrberg C, Gerhardt H, Golding M, et al. Spatially res
provided by heparin-binding VEGF-A control blood vessel branching
morphogenesis. Genes Dev. 2002;16(20):2684-2698. doi: 10.1101/gad.242002.

46. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, He
neuropilins: multifunctional semaphorin and VEGF rec
modulate axon guidance and angiogenesis. Trends Cardiovasc Med 2002;12(1):13-19.

47. Gerhardt H, Golding M, Fruttiger M, et al. VEGF guides
utilizing endothelial tip cells. J Cell Biol. 2003;161(6):1163-1177. doi: 10.1083/jcb.200302047.

48. Suchting S, Freitas C, le Noble F, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and
Proc Natl Acad Sci U S A. 2007;104(9):3225-3230. doi: 10.1073/pnas.0611177104.

49. Kamei M, Saunders WB, Bayless KJ, Dye L, Davis GE,
Endothelial tubes assemble from intracellular vacuole
Nature. 2006;442(7101):453-456. doi: 10.1038/nature04923.

50. Lubarsky B, Krasnow MA. Tube morphogenesis: making
biological tubes. Cell. 2003;112(1):19-28.

51. Hellstrom M, Gerhardt H, Kalen M, et al. Lack of pericy
endothelial hyperplasia and abnormal vascular morpho
J Cell Biol. 2001;153(3):543-553.

52. Kluk MJ, Hla T. Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. Biochim Biophys Acta. 2002;1582(1-3):72-80.

53. Augustin HG, Koh GY, Thurston G, Alitalo K. Control of
morphogenesis and homeostasis through the angiopoie
Tie system. Nat Rev Mol Cell Biol. 2009;10(3):165-177. doi: 10.1038/nrm2639.

54. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, S
Balancing the activation state of the endothelium via
beta type I receptors. EMBO J. 2002;21(7):1743-1753. doi:
10.1093/emboj/21.7.1743.

55. ten Dijke P, Goumans MJ, Pardali E. Endoglin in angiogene
diseases. Angiogenesis. 2008;11(1):79-89. doi: 10.1007/s10456-008-
9101-9.

56. Pepper MS. Extracellular proteolysis and angiogenesis. Thromb Haemost.
2001;86(1):346-355.

57. Frank RN. Diabetic retinopathy. N Engl J Med. 2004;350(1):48-58. doi:
10.1056/NEJMra021678.

58. Nyengaard JR, Rasch R. The impact of experimental dia
on glomerular capillary number and sizes. Diabetologia. 1993;36(3):189-
194.

59. Kennedy JM, Zochodne DW. Influence of experimental diabetes
microcirculation of injured peripheral nerve: functiona
morphological aspects. Diabetes. 2002;51(7):2233-2240.

60. Falanga V. Wound healing and its impairment in the dia
Lancet. 2005;366(9498):1736-1743. doi: 10.1016/S0140-6736(05)67700

61. Abaci A, Oguzhan A, Kahraman S, et al. Effect of diabe
formation of coronary collaterals. Circulation. 1999;99(17):2239-
2242.

62. Orasanu G, Plutzky J. The pathologic continuum of diabetic vascular diseas
J Am Coll Cardiol. 2009;53(5 Suppl):S35-42. doi: 10.1016/j.jacc.2008.09.055.

63. Roy S, Trudeau K, Roy S, Behl Y, Dhar S, Chronopoulo
into hyperglycemia-induced molecular changes in microvascula
J Dent Res. 2010;89(2):116-127. doi: 10.1177/0022034509355765.

64. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothe
ocular fluid of patients with diabetic retinopathy and
disorders. N Engl J Med. 1994;331(22):1480-1487. doi: 10.1056/NEJM199412013312203.

65. Brown LF, Berse B, Tognazzi K, et al. Vascular permea
and protein expression in human kidney. Kidney Int. 1992;42(6):1457-1461.

66. Cooper ME, Vranes D, Youssef S, et al. Increased rena
vascular endothelial growth factor (VEGF) and its rece
experimental diabetes. Diabetes. 1999;48(11):2229-2239.

67. Quattrini C, Jeziorska M, Boulton AJ, Malik RA. Reduce
endothelial growth factor expression and intra-epidermal nerve fibre loss
in human diabetic neuropathy. Diabetes Care. 2008;31(1):140-145. doi: 10.2337/dc07-1556.

68. Kampfer H, Pfeilschifter J, Frank S. Expressional regul
and -2 and the tie-1 and -2 receptor tyrosine kinases durin

wound healing: a comparative study of normal and imp Lab Invest. 2001;81(3):361-373.

69. Chou E, Suzuma I, Way KJ, et al. Decreased cardiac ex endothelial growth factor and its receptors in insulin-resistant diabetic States: a possible explanation for impaired c in cardiac tissues. Circulation. 2002;105(3):373-379.

70. Sasso FC, Torella D, Carbonara O, et al. Increased vas growth factor expression but impaired vascular endothelial growth factor receptor signaling in the myocardium of type 2 diabet chronic coronary heart disease. J Am Coll Cardiol. 2005;46(5):827-834. doi: 10.1016/j.jacc.2005.06.007.

71. Ziyadeh FN, Hoffman BB, Han DC, et al. Long-term prevention of renal insufficiency, excess matrix gene expression, and glo matrix expansion by treatment with monoclonal antitra factor-beta antibody in db/db diabetic mice. Proc Natl Acad Sci U S A. 2000;97(14):8015-8020. doi: 10.1073/pnas.120055097.

72. Kim BC, Kim HT, Park SH, et al. Fibroblasts from chron altered TGF-beta signaling and decreased TGF-beta Type II receptor expression. J Cell Physiol. 2003;195(3):331-336. doi: 10.1002/jcp.10301.

73. D'Souza DR, Salib MM, Bennett J, et al. Hyperglycemia regulat activation and cellular wound healing through the aldo

polyol pathway. J Biol Chem 2009;284(27):17947-17955. doi: 10.1074/jbc.M109.002378.

74. Raman P, Krukovets I, Marinic TE, Bornstein P, Stenina OI. Glycosylation mediates up-regulation of a potent antiangiogenic and proa protein, thrombospondin-1, by glucose in vascular smooth muscl J Biol Chem 2007;282(8):5704-5714. doi: 10.1074/jbc.M610965200.

75. Yao D, Taguchi T, Matsumura T, et al. High glucose increases a 2 transcription in microvascular endothelial cells throug modification of mSin3A. J Biol Chem 2007;282(42):31038-31045. doi: 10.1074/jbc.M704703200.

76. Williams B, Gallacher B, Patel H, Orme C. Glucose-induced protein kinase C activation regulates vascular permeability factor mRN peptide production by human vascular smooth muscle Diabetes 1997;46(9):1497-1503.

77. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340(2):115-126. doi: 10.1056/NEJM199901143400207.

78. Lusis AJ. Atherosclerosis. Nature 2000;407(6801):233-241. doi: 10.1038/35025203.

79. Glass CK, Witztum JL. Atherosclerosis. the road ahead Cell 2001;104(4):503-516.

80. Dortimer AC, Shenoy PN, Shiroff RA, et al. Diffuse cor in diabetic patients: fact or fiction? Circulation 1978;57(1):133-136.

81. Moreno PR, Purushothaman KR, Fuster V, et al. Plaque
increased in ruptured atherosclerotic lesions of human
for plaque vulnerability. Circulation 2004;110(14):2032-2038. doi:
10.1161/01.CIR.0000143233.87854.23.
82. Moreno PR, Murcia AM, Palacios IF, et al. Coronary co
macrophage infiltration in atherectomy specimens from p
diabetes mellitus. Circulation 2000;102(18):2180-2184.
83. Mulligan-Kehoe MJ. The Vasa Vasorum in Diseased and Non
Arteries. Am J Physiol Heart Circ Physiol 2009. doi:
10.1152/ajpheart.00884.2009.
84. LOWENBERG RI, SHUMACKER HB,Jr. Experimental stud
repair; morphologic observations of the vasa vaso Yale J Biol
Med 1948;20(4):395-401.
85. Short RHD. The vasa vasorum of the femoral vein. J Pathol Bacteriol
1940;50(3):419-430.
86. Gossl M, Malyar NM, Rosol M, Beighley PE, Ritman EL.
vasa vasorum functional structure on coronary vessel
distribution. Am J Physiol Heart Circ Physiol 2003;285(5):H2019-26.
doi: 10.1152/ajpheart.00399.2003.
87. Langheinrich AC, Kampschulte M, Buch T, Bohle RM. Va
atherosclerosis: Quo vadis? Thromb Haemost 2007;97(6):873-879.

88. Wolinsky H, Glagov S. Nature of species differences in distribution of aortic vasa vasorum in mammals. Circ Res 1967;20(4):409-421.

89. Heistad DD, Marcus ML. Role of vasa vasorum in nouris Blood Vessels 1979;16(5):225-238.

90. Langheinrich AC, Michniewicz A, Bohle RM, Ritman EL. neovascularization and lesion distribution among different vascular b in ApoE-/-/LDL-/- double knockout mice. Atherosclerosis 2007;191(1):73-81. doi: 10.1016/j.atherosclerosis.2006.05.0

91. Scotland RS, Vallance PJ, Ahluwalia A. Endogenous fa regulation of tone of arterial vasa vasorum: implications for co vessel physiology. Cardiovasc Res 2000;46(3):403-411.

92. Ritman EL, Lerman A. The dynamic vasa vasorum. Cardiovasc Res 2007;75(4):649-658. doi: 10.1016/j.cardiores.2007.06.020.

93. Barger AC, Beeuwkes R 3rd, Lainey LL, Silverman KJ. Hypothesis: vasorum and neovascularization of human coronary ar role in the pathophysiology of atherosclerosis. N Engl J Med 1984;310(3):175-177. doi: 10.1056/NEJM198401193100307.

94. Davies MJ, Thomas AC. Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. N Engl J Med 1984;310(18):1137-1140. doi: 10.1056/NEJM198405033101801.

95. Langheinrich AC, Michniewicz A, Sedding DG, et al. Co
vasorum neovascularization and plaque progression in aortas of
apolipoprotein E(-/-)/low-density lipoprotein(-/-) double knockout mice.
Arterioscler Thromb Vasc Biol. 2006;26(2):347-52. doi:
10.1161/01.ATV.0000196565.38679.6d.

96. Kampschulte M, Brinkmann A, Stieger P, et al. Quantitative CT imaging of
the spatio-temporal distribution patterns of vasa vasorum i
apoE-/-/LDL-/- double knockout mice. Atherosclerosis.
2010;212(2):444-50. doi: 10.1016/j.atherosclerosis.2010.07.

97. Ribatti D, Levi-Schaffer F, Kovanen PT. Inflammatory angiogenesis in
atherogenesis--a double-edged sword. Ann Med. 2008;40(8):606-21.
doi: 10.1080/07853890802186913.

98. O'Brien KD, Allen MD, McDonald TO, et al. Vascular ce
molecule-1 is expressed in human coronary atherosclerot
Implications for the mode of progression of advanced
atherosclerosis. J Clin Invest. 1993;92(2):945-51. doi:
10.1172/JCI116670.

99. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers C
expression of E-selectin, intercellular adhesion molecule-1, and vascular
cell adhesion molecule-1 in human atherosclerosis and their rela
intimal leukocyte content. Circulation. 1996;93(4):672-82.

100. Moos MP, John N, Grabner R, et al. The lamina adven
of immune cell accumulation in standard chow-fed apolipoprotein E
deficient mice. Arterioscler Thromb Vasc Biol 2005;25(11):2386-91.
doi: 10.1161/01.ATV.0000187470.31662.fe.

101. Bratzler RL, Chisolm GM, Colton CK, Smith KA, Lees
of labeled low-density lipoproteins across the rabbit thoraci
vivo. Atherosclerosis 1977;28(3):289-307.

102. Nordestgaard BG, Hjelms E, Stender S, Kjeldsen K. D
pathways for high and low density lipoproteins from p
intima. Arteriosclerosis 1990;10(3):477-85.

103. Guyton JR, Klemp KF. Transitional features in human
Intimal thickening, cholesterol clefts, and cell loss in
streaks. Am J Pathol 1993;143(5):1444-57.

104. Sluimer JC, Kolodgie FD, Bijnens AP, et al. Thin-walled microvessels in
human coronary atherosclerotic plaques show incompl
junctions relevance of compromised structural integrit
microvascular leakage. J Am Coll Cardiol 2009;53(17):1517-27. doi:
10.1016/j.jacc.2008.12.056.

105. Virmani R, Kolodgie FD, Burke AP, et al. Atheroscler
progression and vulnerability to rupture: angiogenesis
intraplaque hemorrhage. Arterioscler Thromb Vasc Biol
2005;25(10):2054-61. doi: 10.1161/01.ATV.0000178991.716

106. Kolodgie FD, Gold HK, Burke AP, et al. Intraplaque he
progression of coronary atheroma. N Engl J Med. 2003;349(24):2316-2325. doi: 10.1056/NEJMoa035655.

107. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR,
Vascular endothelial growth factor enhances atherosc
progression. Nat Med. 2001;7(4):425-429. doi: 10.1038/86490.

108. Moulton KS, Vakili K, Zurakowski D, et al. Inhibition o
neovascularization reduces macrophage accumulation
advanced atherosclerosis. Proc Natl Acad Sci U S A. 2003;100(8):4736-4741. doi: 10.1073/pnas.0730843100.

109. Langheinrich AC, Sedding DG, Kampschulte M, et al. 3-Deazaadenosine
inhibits vasa vasorum neovascularization in aortas of ApoE(-/-)/LDL(-/-)
double knockout mice. Atherosclerosis. 2009;202(1):103-110. doi: 10.1016/j.atherosclerosis.2008.04.008.

110. Gossl M, Herrmann J, Tang H, et al. Prevention of vas
neovascularization attenuates early neointima formation in exp
hypercholesterolemia. Basic Res Cardiol. 2009;104(6):695-706. doi: 10.1007/s00395-009-0036-0.

111. Drinane M, Mollmark J, Zagorchev L, et al. The antian
rPAI-1(23) inhibits vasa vasorum and growth of atherosclerotic pla
Circ Res. 2009;104(3):337-345. doi: 10.1161/CIRCRESAHA.108.184622.

112. Babaei-Jadidi R, Karachalias N, Ahmed N, Battah S, Thorna
Prevention of incipient diabetic nephropathy by high-dose thiamine and high
benfotiamine. Diabetes. 2003;52(8):2110-2120.

113. Hammes HP, Du X, Edelstein D, et al. Benfotiamine bl
pathways of hyperglycemic damage and prevents expe
retinopathy. Nat Med. 2003;9(3):294-299. doi: 10.1038/nm834.

114. Haupt E, Ledermann H, Kopcke W. Benfotiamine in the tre
diabetic polyneuropathy--a three-week randomized, controlled pilot
study (BEDIP study). Int J Clin Pharmacol Ther. 2005;43(2):71-77.

115. Stracke H, Gaus W, Achenbach U, Federlin K, Bretzel
diabetic polyneuropathy (BENDIP): results of a random
blind, placebo-controlled clinical study. Exp Clin Endocrinol Diabetes.
2008;116(10):600-605. doi: 10.1055/s-2008-1065351.

116. Beltramo E, Nizheradze K, Berrone E, Tarallo S, Porta M. Thiamine and
benfotiamine prevent apoptosis induced by high gluco
extracellular matrix in human retinal pericytes. Diabetes Metab Res Rev.
2009;25(7):647-656. doi: 10.1002/dmrr.1008.

117. Beltramo E, Berrone E, Tarallo S, Porta M. Effects of thiamine and
benfotiamine on intracellular glucose metabolism and
prevention of diabetic complications. Acta Diabetol. 2008;45(3):131-
141. doi: 10.1007/s00592-008-0042-y.

118. Targher G, Bertolini L, Zenari L, et al. Diabetic retinopathy is associated with an increased incidence of cardiovascular events
patients. Diabet Med. 2008;25(1):45-50. doi: 10.1111/j.1464-5491.2007.02327.x.

119. Elliott J, Tesfaye S, Chaturvedi N, et al. Large-fiber dysfunction in diabetic peripheral neuropathy is predicted by cardiovascular
Diabetes Care. 2009;32(10):1896-1900. doi: 10.2337/dc09

120. Action to Control Cardiovascular Risk in Diabetes Stu
HC, Miller ME, et al. Effects of intensive glucose lowering in type 2 diabetes. N Engl J Med. 2008;358(24):2545-2559. doi: 10.1056/NEJMoa0802743.

121. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams
assessment of atherosclerotic lesions in mice. Atherosclerosis. 1987;68(3):231-240.

122. Paigen B, Ishida BY, Verstuyft J, Winters RB, Albee D
susceptibility differences among progenitors of recom
strains of mice. Arteriosclerosis. 1990;10(2):316-323.

123. Browning J, Wylie CK, Gole G. Quantification of oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci. 1997;38(6):1168-1174.

124. Moulton KS, Heller E, Konerding MA, Flynn E, Palinsk
Angiogenesis inhibitors endostatin or TNP-470 reduce intimal

neovascularization and plaque growth in apolipoprotein E-deficient mice. Circulation. 1999;99(13):1726-32.

125. Operschall C, Falivene L, Clozel JP, Roux S. A new m
cardiac ischemia in rabbits. J Appl Physiol. 2000;88(4):1438-45.

126. Enis DR, Shepherd BR, Wang Y, et al. Induction, differentiation, and
remodeling of blood vessels after transplantation of B
endothelial cells. Proc Natl Acad Sci U S A. 2005;102(2):425-430. doi: 10.1073/pnas.0408357102.

127. Dutly AE, Kugathasan L, Trogadis JE, Keshavjee SH, Stewart DJ, Courtma
DW. Fluorescent microangiography (FMA): an improve
visualize the pulmonary microvasculature. Lab Invest. 2006;86(4):409-416. doi: 10.1038/labinvest.3700399.

128. El-Gohary YM, Silverman JF, Olson PR, et al. Endoglin (CD105) and vascular endothelial growth factor as prognostic mark
adenocarcinoma. Am J Clin Pathol. 2007;127(4):572-9. doi: 10.1309/X6NXYE57DLUE2NQ8.

129. Luque A, Slevin M, Turu MM, Juan-Babot O, Badimon L, Krupinski J. CD105 positive neovessels are prevalent in early stag
and correlate with the grade in more advanced carotid
plaques. J Angiogenes Res. 2009;1:6. doi: 10.1186/2040-2384-1-6.

130. Kunjathoor VV, Wilson DL, LeBoeuf RC. Increased ath
streptozotocin-induced diabetic mice. J Clin Invest. 1996;97(7):1767-1773. doi: 10.1172/JCI118604.

131. Bowes AJ, Khan MI, Shi Y, Robertson L, Werstuck GH.
attenuates accelerated atherosclerosis in -deficient hyperglycem
mice: evidence in support of a role for endoplasmic re
glycogen synthase kinase-3 in lesion development and hepatic ste
Am J Pathol. 2009;174(1):330-342. doi: 10.2353/ajpath.2009.080385.

132. Xu Q, Qaum T, Adamis AP. Sensitive blood-retinal barrier breakdown quantitation using Evans blue. Invest Ophthalmol Vis Sci. 2001;42(3):789-794.

133. Hammes HP, Feng Y, Pfister F, Brownlee M. Diabetic
vasoregression. Diabetes. 2011;60(1):9-16. doi: 10.2337/db10-0454.

134. KUWABARA T, COGAN DG. Retinal vascular patterns.
the retinal capillaries. Arch Ophthalmol. 1963;69:492-502.

135. Temm C, Dominguez JH. Microcirculation: nexus of co
diabetes. Am J Physiol Renal Physiol. 2007;293(2):F486. doi: 10.1152/ajprenal.00503.2006.

136. Singh DK, Winocour P, Farrington K. Mechanisms of d
tubular hypothesis of diabetic nephropathy. Nat Clin Pract Nephrol. 2008;4(4):216-226. doi: 10.1038/ncpneph0757.

137. Giannini C, Dyck PJ. Basement membrane reduplicatio degeneration precede development of diabetic polyneu associated with its severity. Ann Neurol 1995;37(4):498-504. doi: 10.1002/ana.410370412.

138. Cameron NE, Eaton SE, Cotter MA, Tesfaye S. Vascul metabolic interactions in the pathogenesis of diabetic Diabetologia 2001;44(11):1973-88. doi: 10.1007/s001250100001.

139. Sviridov D, Nestel P. Dynamics of reverse cholesterol against atherosclerosis. Atherosclerosis 2002;161(2):245-54.

140. Gossl M, Versari D, Lerman LO, et al. Low vasa vasor with inflammation and subintimal thickening: potential determination of atherogenic lesions. Atherosclerosis 2009;206(2):362-8. doi: 10.1016/j.atherosclerosis.2009.03.010.

141. Nakata Y, Shionoya S. Vascular lesions due to obst vasorum. Nature 1966;212(5067):1258-9.

142. Barker SG, Talbert A, Cottam S, Baskerville PA, Marti hyperplasia after occlusion of the adventitial vasa vas Arterioscler. Thromb 1993;13(1):70-7.

143. Thangarajah H, Yao D, Chang EI, et al. The molecular hypoxia-induced VEGF expression in diabetic tissues. Proc Natl Acad Sci U.S.A 2009;106(32):13505-10. doi: 10.1073/pnas.0906670

144. Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, Attisano L, Bernabeu
Synergistic cooperation between hypoxia and transfor
factor-beta pathways on human vascular endothelial grow
expression. J Biol Chem. 2001;276(42):38527-35. doi: 10.1074/jbc.M104536200.

145. Sanchez-Elsner T, Botella LM, Velasco B, Langa C, Bernabe
expression is regulated by transcriptional cooperation
hypoxia and transforming growth factor-beta pathways. J Biol Chem. 2002;277(46):43799-808. doi: 10.1074/jbc.M207160

146. Arany Z, Huang LE, Eckner R, et al. An essential role
cellular response to hypoxia. Proc Natl Acad Sci U S A. 1996;93(23):12969-73.

147. Wang F, Zhang R, Wu X, Hankinson O. Roles of coacti
induction of erythropoietin gene. PLoS One. 2010;5(4):e10002. doi: 10.1371/journal.pone.0010002.

148. Kasper LH, Boussouar F, Boyd K, et al. Two transacti
cooperate for the bulk of HIF-1-responsive gene expression. EMBO J. 2005;24(22):3846-58. doi: 10.1038/sj.emboj.7600846.

149. Schmid U, Stopper H, Heidland A, Schupp N. Benfotia
antioxidative capacity and prevents induction of DNA
Diabetes Metab Res Rev. 2008;24(5):371-7. doi: 10.1002/dmrr.860.