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Mechanisms and consequences of alterations in vascular function in combined type 2 diabetes and sickle cell trait

Sarah Skinner

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**Mécanismes et conséquences des
altérations de la fonction vasculaire
dans le diabète de type 2 associé au
trait drépanocytaire**

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Abbreviations

A

ADA: American Diabetes Association
ADMA: Asymmetric dimethylarginine
AGEs: Advanced Glycation Endproducts
ANGII: Angiotensin II
ATP: Adenosine triphosphate
ADPKD: Autosomal dominant polycystic kidney disease

B

BH₄: Tetrahydrobiopterin
BMP: Bone morphogenetic proteins

C

cAMP: Cyclic adenosine monophosphate levels
cGMP: Guanosine-3,5-monophosphate-mediated
CKD: Chronic kidney disease
COX-1: Cyclooxygenase-1
COX-2: Cyclooxygenase-2
CRP: C-reactive protein
CSF: Colony stimulating factors

D

DAG: Diacylglycerol
DDAH: Dimethylarginine dimethylaminohydrolase
DNA: Deoxyribonucleic acid

E

ECM: Extra cellular matrix
EDHF: Endothelium derived hyperpolarizing factor
eGFR: Estimated glomerular filtration rate
eNOS: Endothelial nitric oxide synthase
ERK: Extracellular signal-regulated kinase
ERSD: Exercise-related sudden death
ESRD: End stage renal disease
ET: Endothelin
ETC: Electron transport chain
ET_AR: Endothelin receptor type A
ET_BR: Endothelin receptor type B

F

FFA: Free fatty acid

G

GLUT-4: Glucose transporter type 4
GPX: Glutathione peroxidase
GR: Glutathione reductase
GSH: Reduced glutathione
GSSG: Oxidized glutathione

H

HAEC: Human aortic endothelial cells
Hb: Hemoglobin
HbA1c: Hemoglobin A1c
HbA: Hemoglobin A
HbS: Hemoglobin S
HFHS: High fat high sucrose
HMGB1-protein: High mobility group box 1 protein
HO[•]: Hydroxyl radical
H₂O₂: Hydrogen peroxide

I

ICAM: Intercellular adhesion molecule
IDF: International Diabetes Foundation
IFN: Interferon
IGF-1: Insulin-like growth factor-1
IL: Interleukin
iNOS: inducible nitric oxide synthase

J

JNK: C-jun N-terminal kinase
JAK-STAT: Janus kinase-Signal Transducer and Activator of Transcription proteins

M

MAdCAM-1: Mucosal addressin cell adhesion molecule-1
MAPK: Mitogen-activated protein kinase
MCP: Monocyte chemoattractant proteins
MCT-1: Monocarboxylate transporter 1
MIP: Macrophage inflammatory proteins

N

NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS: Neural nitric oxide synthase
NO: Nitric oxide
NOS: Nitric oxide synthase
NOX: NADPH oxidase

O

O₂: Molecular oxygen
O₂⁻: Superoxide anion
ONOO⁻: Peroxynitrite

P

PAI-1: Plasminogen activator inhibitor-1
PARP: Poly ADP ribose polymerase
PECAM-1: Platelet endothelial cell adhesion molecule-1
PF-4/CXCL4: Platelet factor-4
PGE₂: Prostaglandin E₂
PGD₂: Prostaglandin D₂
PGF₂α: Prostaglandin F₂α
PGH₂: Prostaglandin H₂
PGI₂: Prostacyclin
PI3K: Phosphoinositide 3-kinase
PIV: Pressure induced vasodilation
PKC: Protein kinase C
PCR: Polymerase chain reaction
PMN: Polymorphonuclear lymphocytes
PPARG: Peroxisome proliferator-activated receptor gamma
PSGL-1: P-selectin glycoprotein ligand-1

R

ROS: Reactive oxygen species

S

SCA: Sickle cell anemia
SCD: Sickle Cell Disease
SCT: Sickle Cell Trait
sICAM: Soluble intercellular adhesion molecule
SOD: Superoxide dismutase
Smad: Sma- and maad-related proteins
SNP: Sodium nitroprusside
STAT: Signal transducers and activators of transcription
sVCAM: Soluble vascular adhesion molecule

T

T2D: Type 2 diabetes
TGF: Transforming growth factor
TNF: Tumor necrosis factor
TXA₂: Thromboxane A₂

V

VTE: venous thromboembolism

VCAM-1: vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

VSCM: Vascular smooth muscle cells

W

WHO: World Health organization

X

XO: xanthine oxidase

Résumé en français

Résumé en Français

Le diabète est l'une des principales urgences mondiales du 21^{ème} siècle en matière de santé. Les estimations actuelles montrent que 425 millions d'individus dans le monde sont atteints d'un diabète dont la plupart de type 2 (DT2) (Cho, Mooney, and Cho 2008). Le DT2 se caractérise par une inflammation chronique, résultant des altérations métaboliques de la maladie, en particulier l'hyperglycémie, l'hyperinsulinémie, l'insulinoresistance, et la dyslipidémie (Sena, Pereira, and Seica 2013). Ces altérations augmentent la production d'espèces réactives de l'oxygène (ROS), à l'origine d'une diminution de la biodisponibilité du monoxyde d'azote (NO). En outre, l'élévation chronique de la glycémie conduit à une production exagérée des produits avancés de la glycation (AGE) qui contribuent aussi à une surproduction de ROS et à une inflammation chronique (Brownlee 1995; Nowotny et al. 2015). Par ailleurs, le DT2 est caractérisé par des anomalies hémorhéologiques telles qu'une viscosité plasmatique élevée, une augmentation de l'agrégation érythrocytaire, une réduction de la déformabilité érythrocytaire, et une hyperviscosité sanguine (Cho, Mooney, and Cho 2008). Toutes ces anomalies biologiques participent à l'émergence d'une dysfonction vasculaire chronique qui est à l'origine du risque accru de complications micro- et macrovasculaires chez les patients diabétiques de type 2.

L'Afrique est la région où la prévalence du diabète devrait augmenter le plus rapidement dans le monde pendant le prochain quart de siècle, et cette tendance est amplifiée dans l'Afrique sub-saharienne (Cho et al. 2018). De plus, aux États-Unis et dans les Caraïbes, les personnes d'origines africaines ont un risque deux fois plus élevé de développer le DT2 par comparaison aux personnes d'origines européennes (Hennis et al. 2002; Menke et al. 2015). Après l'Afrique, les régions qui devraient

connaître la hausse la plus importante du taux de DT2 au cours des 25 prochaines années sont le Moyen Orient, l'Asie du Sud-Est, et l'Amérique du sud et centrale (Cho et al. 2018). Chacune de ces régions fera face aux complications spécifiques vis à vis du dépistage et du diagnostique du diabète.

Ces régions sont également marquées par une forte prévalence du trait drépanocytaire (TD) (Piel et al. 2013). Le trait drépanocytaire (TD) est la forme hétérozygote de la drépanocytose, une maladie héréditaire qui se caractérise par la présence dans les globules rouges d'une hémoglobine mutée (HbS). L'HbS a la particularité de polymériser lorsqu'elle est désoxygénée, engendrant alors une falciformation des globules rouges. Ces globules rouges sont très fragiles et rigides. Ainsi, la maladie se manifeste par une anémie chronique, la survenue fréquente de crises vaso-occlusives et de complications chroniques dégénératives (Rees, Williams, and Gladwin 2010). Contrairement aux drépanocytaires homozygotes, les porteurs du TD sont considérés comme asymptomatiques due à la présence à la fois d'hémoglobine normale et mutée dans leurs érythrocytes. Néanmoins, plusieurs études ont rapporté des perturbations hémorhéologiques modérées (Monchanin et al. 2005), ainsi qu'un risque élevé de complications rénales et thromboemboliques dans cette population (Key, Connes, and Derebail 2015). De plus, il a été montré que les porteurs du TD présentaient un niveau de stress oxydant plus élevé et un métabolisme du NO légèrement perturbé en réponse à un exercice physique intense (Faës et al. 2012). L'ensemble de ces résultats suggère que les porteurs du TD pourraient avoir une fonction vasculaire altérée.

Dans ce contexte, certaines zones géographiques et populations dans le monde devraient faire face à une prévalence élevée de cette double condition. Des travaux récents de notre laboratoire ont rapporté une dysfonction vasculaire plus

importante chez les patients DT2 porteurs du TD par rapport à des patients DT2 non-porteurs du TD (Diaw et al. 2015). De ce fait, il est possible que le TD puisse augmenter le risque de complications vasculaires. La meilleure façon d'éviter ou retarder les complications vasculaires liées au diabète est de diagnostiquer la maladie le plus tôt possible (Utumatwishima et al. 2018). Néanmoins, 50% des adultes dans le monde, et presque 70% des adultes vivant en Afrique, atteints de diabète ne sont pas diagnostiqués (IDF 8th édition). Ce problème pourrait être partialement être attribué à l'accès limité aux soins médicaux. Cependant, dans certaines populations, il est aussi possible que la fiabilité des méthodes de dépistage ne soit pas optimale (Utumatwishima et al. 2018). En effet, une étude menée par Lacy et al a montré que la mesure de l'hémoglobine A1c (HbA1c), qui est souvent utilisée pour le diagnostic et le suivi du diabète, pourrait sous-estimer les niveaux de glycémie chez les personnes porteuses du TD (Lacy et al. 2017). Pour cette raison, il a été suggéré que d'autres mesures de glycémie, comme l'albumine glyquée et la fructosamine, seules ou combinées avec l'HbA1c pourraient éventuellement être utilisées comme solution alternative pour un dépistage plus fiable du diabète chez les porteurs du TD (Danese et al. 2015; Selvin and Sacks 2017).

Au regard de toutes ces informations, les deux objectifs principaux de ce travail de thèse étaient d'évaluer les mécanismes et conséquences de la dysfonction vasculaire amplifiée chez les porteurs du TD, et d'étudier les difficultés liées au dépistage et au suivi du DT2 chez les porteurs du TD . Pour répondre à ces objectifs des études ont été réalisées chez l'homme et l'animal.

La première partie de cette thèse est composée d'une étude clinique et une étude méthodologique, menées au Sénégal, et un bref article de synthèse. Une étude de cohorte a été réalisée afin de comparer la prévalence des complications

vasculaires entre les sujets porteurs du TD atteints d'un DT2 (DT2-TD), les sujets atteints d'un DT2 (DT2), les porteurs du TD (TD), et des contrôles. Ensuite, afin d'explorer les mécanismes qui pourraient éventuellement contribuer à la dysfonction vasculaire et au risque accru de certaines complications vasculaires chez les DT2-TD, nous avons évalué la rigidité artérielle ainsi que différents biomarqueurs. Les résultats de cette étude ont démontré que les prévalences d'hypertension, de rétinopathie et de néphropathie étaient plus élevées chez les sujets DT2-TD que chez les sujets DT2. Les résultats ont aussi montré une rigidité artérielle, une viscosité sanguine et une concentration des AGEs plus importante chez les DT2-TD. Une analyse multivariée a montré que la concentration en AGE était significativement associée à la rigidité artérielle. Nous avons ensuite réalisé des expérimentations *in-vitro* qui consistaient à incuber des cellules endothéliales HEAC avec le plasma des patients des 4 groupes de sujets. Nous avons ainsi démontré que l'utilisation du plasma du groupe DT2-TD conduisait à une expression accrue d'E-selectine, et qu'un inhibiteur d'AGEs supprimait cet effet. L'ensemble de ces résultats indique que le TD accentue la rigidité artérielle, la viscosité sanguine, et les concentrations des AGEs habituellement observées chez les patients atteints d'un DT2, et ces anomalies pourraient contribuer à un risque accru de complications vasculaires chez ces patients DT2-TD. Les résultats de cette étude sont décrits dans un article sous presse dans *Diabetes Care*.

Cette étude de cohorte nous a également emmené à répondre à une autre question concernant les difficultés liées au dépistage du DT2 chez les porteurs du TD. J'ai dans un premier temps discuté cette difficulté de diagnostic du DT2 dans les populations de porteurs du TD dans une lettre de synthèse publiée dans *The Lancet Diabetes & Endocrinology*. Nous avons expliqué que différents facteurs dont la

méthode d'analyse utilisée pour mesurer l'HbA1c, l'origine ethnique du patient et la présence d'une hémoglobinopathie pouvait gêner le diagnostic du DT2. L'article souligne la nécessité d'études supplémentaires pour mieux comprendre si l'HbA1c doit être utilisé comme méthode de diagnostic du DT2 chez les porteurs du TD, ou s'il serait mieux d'utiliser d'autres méthodes alternatives comme la fructosamine. Ainsi, les données collectées lors de l'étude de cohorte nous ont permis de comparer deux méthodes souvent utilisées pour dépister le diabète (l'HbA1c et la glycémie à jeun) et une mesure atypique de la glycémie (la fructosamine) chez des adultes sénégalais non-porteurs et porteurs du TD. Les résultats ont démontré une disparité entre ces mesures qui était plus marquée chez les porteurs du TD, et que les trois mesures ne pouvaient pas être considérées comme interchangeables. De plus, il est possible que l'utilisation d'une de ces mesures seule puisse entraîner un diagnostic tardif du DT2 chez les porteurs du TD. Ces résultats ont été présentés dans un article soumis dans *PLOS One*.

La deuxième partie de ce travail de thèse a consisté en deux études chez l'animal. L'objectif du première étude était de tester, de manière plus directe que chez l'homme, l'hypothèse que le TD amplifie la dysfonction vasculaire habituellement observée dans le DT2. Des souris Townes (un modèle transgénique murin de la drépanocytose), non-porteurs (AA) et porteurs du trait drépanocytaire (TD), ont été utilisées pour ces études. Un régime riche en lipides et sucre était utilisé pour induire un DT2 et différencier les quatre groupes de souris : les souris TD atteintes d'un DT2 (DT2-TD), les souris AA-DT2, les souris TD, et les contrôles, ces 2 derniers groupe étant sous régime standard. La rhéologie du sang et la fonction microvasculaire *in-vivo* étaient évaluées. Nos résultats ont montré que la viscosité sanguine, l'hématocrite, la déformabilité des érythrocytes, et la fonction

microvasculaire n'étaient pas différentes entre les groupes contrôle et DT2, ou entre les groupes contrôle et TD. Cependant, la viscosité sanguine était plus élevée et la déformabilité des globules rouges était diminuée chez les souris DT2-TD par rapport aux contrôles. De plus, la vasodilatation en réponse à la pression cutanée (PIV) était significativement réduite chez des souris DT2-TD, tandis que la réponse au sodium nitroprusside n'était pas différente entre les groupes. La vasodilatation en réponse au PIV est dépendante sur la production endothéliale du NO. Ainsi, ces résultats suggèrent premièrement que la vasodilatation endothélium-dépendante est significativement réduite chez des souris DT2-TD, et deuxièmement qu'il est possible que cette vasodilatation diminuée soit due à une biodisponibilité réduite en NO. Ces résultats ont fait l'objet d'un article soumis dans *Microvascular Research*

Dans un deuxième temps, nos résultats ont aussi montré une vasodilatation *in-vivo* induite par l'iontophorèse d'acétylcholine plus importante chez les souris DT2-TD par comparaison aux contrôles. Ensuite, nous avons montré que cette vasodilatation amplifiée chez les souris DT2-TD était dépendante de l'activité de COX-2. Ainsi, dans la suite de cette étude, il est prévu d'étudier le rôle de l'inflammation dans le phénomène de vasodilatation accrue à l'acétylcholine observée chez les souris DT2-TD

Ainsi, de manière générale, ce travail approfondit notre compréhension des difficultés liées au dépistage et traitement du DT2 chez les porteurs du TD. Il sera cependant nécessaire de mener des études supplémentaires dont les objectifs ultime seront de développer des recommandations permettant aux cliniciens de mieux prendre en charge des patients DT2 qui sont aussi porteurs du TD, afin de retarder le plus possible la survenue de complications cardiovasculaires.

PART 1 – Foreword

Diabetes is one of the greatest global public health challenges of the 21st century. Current estimates show that almost half a billion people around the world have diabetes, of which the vast majority has type 2 diabetes (T2D) (Cho et al. 2018). The metabolic abnormalities associated with T2D, which include hyperglycemia, insulin resistance, hyperinsulinemia, and hyperlipidemia, result in increased oxidative stress, low-grade inflammation, and platelet hyperactivity (Sena, Pereira, and Seica 2013). All of these factors contribute to the development of vascular dysfunction, and increase the risk of developing T2D-related microvascular and macrovascular complications (Paneni et al. 2013).

The worldwide prevalence of T2D is expected to increase 48% by 2045, leading the International Diabetes Foundation (IDF) to declare the T2D pandemic a global emergency (Cho et al. 2018). Epidemiological data shows that this crisis is amplified in certain populations around the world. Indeed, in the next twenty-five years the prevalence of diabetes is expected to increase more rapidly in Africa than anywhere else on the globe, with rates rising particularly rapidly in Sub-Saharan Africa (Cho et al. 2018). Additionally, in the Caribbean and the USA, individuals whose ancestors come from Africa are two times more likely to have T2D than people of European descent (Hennis et al. 2002; Menke et al. 2015). Following Africa, the three areas where rates of T2D are anticipated to increase the most in the next quarter century are the Middle East, South East Asia, and South and Central America (Cho et al. 2018). Each of these populations faces specific challenges for diabetes diagnosis and management. However, one issue that affects them all is sickle cell trait (SCT), the heterozygous form of sickle cell anemia.

People with SCT inherit one regular β^A -globin gene and one mutated β^S -globin gene, and thus produce both normal hemoglobin A (HbA) and abnormal

hemoglobin S (HbS) (Rees, Williams, and Gladwin 2010). The presence of HbA in the red blood cells of individuals with SCT prevents them from experiencing the severe complications associated with sickle cell anemia (Key, Connes, and Derebail 2015). Therefore, SCT has generally been considered to be benign. However, recent research shows that carriers of SCT may have increased blood viscosity and slightly decreased red blood cell deformability, as well as elevated oxidative stress and reduced nitric oxide (NO) bioavailability compared to controls during exercise (Chirico et al. 2012; Connes et al. 2008; Connes et al. 2005). Furthermore, studies have concluded that carriers of SCT may have an increased risk of chronic kidney disease, venous thromboembolism, end stage renal disease, and possibly stroke (Caughey et al. 2014; Derebail et al. 2010; Little et al. 2017; Naik et al. 2017). Together, these findings suggest that individuals with SCT could have impaired vascular function. This is significant as a recent study conducted by Diaw et al showed that individuals with both T2D and SCT have more severely impaired endothelium-dependent vasodilation and increased arterial stiffness compared to individuals with T2D alone (Diaw et al. 2015). These findings indicate that SCT could potentially exaggerate vascular dysfunction in T2D, and thereby increase the risk of developing diabetes-related vascular complications.

Early diagnosis and effective monitoring of T2D are the most effective ways to prevent the development of T2D-related complications (Utumatwishima et al. 2018). However, an estimated 50% of adults worldwide, and 70% of adults in Africa with T2D are undiagnosed (Cho et al. 2018). This problem could be attributed to inadequate access to sufficient health care in many areas of the world. However, it is also possible that, in certain populations, the sub-optimal performance of tests used for the diagnosis of diabetes may also play an important role in this predicament

(Utumatwishima et al. 2018). Some evidence suggests that this could be the case among carriers of SCT, in whom hemoglobin A1c (HbA1c), a frequently used measure of glycemia, could potentially perform inadequately (English et al. 2015; Lacy, Wellenius, and Wu 2017). This has led to the suggestion that alternative measures of glycemia, such as glycated albumin and fructosamine, could be used alone or in combination with HbA1c for T2D screening and followup (Utumatwishima et al. 2018).

In light of all of this information, the primary objectives of this thesis are 1) to study the challenges related to diagnosing and monitoring T2D in individuals with SCT and 2) to evaluate the mechanisms and consequences of the amplified vascular dysfunction observed in combined T2D and SCT. The first published article of this thesis is a “comment”, written to simultaneously draw attention to and summarize the challenges related to diagnosing and monitoring T2D in individuals with SCT. The first original article of the thesis also addresses this issue by evaluating the agreement between fructosamine, an alternative measure of glycemia, and HbA1c and fasting glucose, two standard measures of glucose control, in a cohort of Senegalese adults with and without SCT. The second article of this thesis was conducted using the same cohort of adults from Senegal, and included four groups: individuals with combined T2D and SCT, T2D alone, SCT alone, or neither T2D nor SCT (controls). The primary aim of the study was to determine if T2D-related complications are more prevalent among individuals with both T2D and SCT compared to those with T2D alone. The secondary objective was to evaluate factors that could play a role in increasing the risk of vascular complications in combined T2D and SCT, including arterial stiffness, rheological parameters, plasma advanced glycation end product (AGE) and cytokine concentrations, and the expression of

certain adhesion molecules in human aortic endothelial cells incubated with patients' plasma. Finally, studies three and four used a mouse model of combined T2D and SCT to study changes in blood rheology and microvascular function in combined T2D and SCT, as well as the mechanisms of these alterations.

PART 2 – Review of the literature

I. VASCULAR FUNCTION AND DYSFUNCTION

The cardiovascular system relies on functional blood vessels to transport nutrients, gases, and waste around the body. In a healthy cardiovascular system, arteries are highly compliant. This vascular compliance enables the propagation of the pressure wave along the arterial tree, which permits the maintenance of continuous blood flow across the capillaries (Glasser et al. 1997). Additionally, in a healthy cardiovascular system, vascular endothelial and smooth muscle cells function effectively to regulate vascular tone and blood flow (Nyberg, Gliemann, and Hellsten 2015). Vascular endothelial cells regulate vascular tone by producing vasoactive substances, while the vascular smooth muscle cells (VSMC) respond to the vascular dilating or constricting signals in order to achieve the correct contractile state (Nyberg, Gliemann, and Hellsten 2015). Vascular dysfunction, on the other hand, is characterized by a loss of vascular compliance (i.e. elevated arterial stiffness), and altered vascular tone, largely due to endothelial dysfunction (van Sloten 2017).

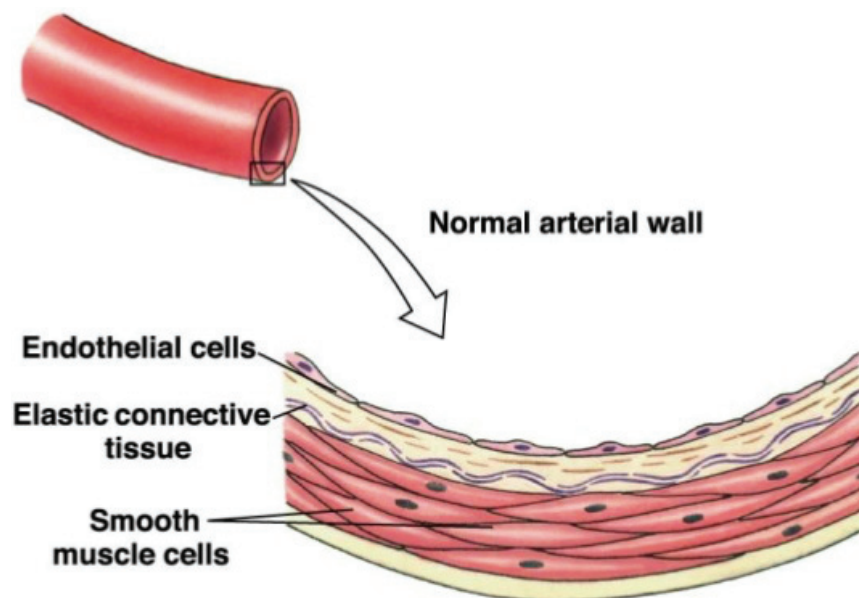


Figure 1: A normal arterial wall with a single layer of endothelial cells.
Image from www.heartsense.in

A. THE ENDOTHELIUM IN NORMAL VASCULAR HOMEOSTASIS

The vascular endothelium is composed of a single layer of endothelial cells that lines the inner surface of the entire vasculature (Feletou, Huang, and Vanhoutte 2011) (Figure 1). In general, the primary functions of endothelial cells include the regulation of vascular tone, cellular adhesion, thromboresistance, VSMC proliferation, and vessel wall inflammation (Deanfield, Halcox, and Rabelink 2007). However, endothelial cells are heterogeneous, and their morphology and function varies widely depending on the type and location of the blood vessel within the vascular tree (de la Paz and D'Amore 2009). For example, leukocyte trafficking, which includes the attraction, rolling, firm adhesion, and infiltration of circulating leukocytes into the vascular tissue, takes place predominantly in post-capillary venules, although these steps can also be observed in large veins, capillaries, and arterioles (de la Paz and D'Amore 2009; Feletou, Huang, and Vanhoutte 2011). On the other hand, the regulation of vascular tone is an arterial function that primarily takes place at the level of the arterioles, as well as in the arteries (de la Paz and D'Amore 2009). Vascular tone refers to the level of vessel constriction in comparison to the maximal dilated state of the vessel, and is controlled by vasoactive compounds that stimulate the VSMCs to relax (vasodilators) or constrict (vasoconstrictors) the blood vessels (Sena, Pereira, and Seica 2013).

1. Vasodilators

a. Nitric oxide

In 1980 Furchgott and Zawadzki demonstrated for the first time that the endothelium was required for acetylcholine-mediated relaxation of isolated arteries (Furchgott and Zawadzki 1980). This seminal study led to the discovery of the most well characterized endothelium-derived vasodilator, nitric oxide (NO). NO is

generated by nitric oxide synthases (NOS) located in endothelial tissue (eNOS) or neural tissue (nNOS), and from an inducible form of NOS referred to as iNOS.

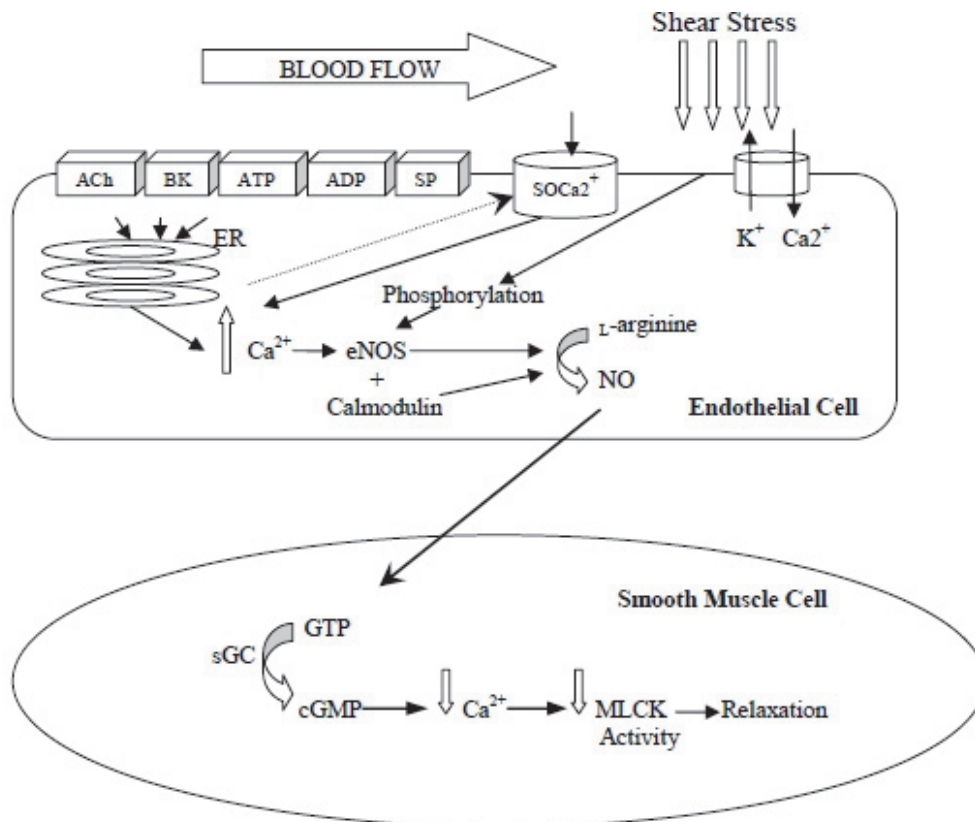


Figure 2: Endothelial nitric oxide production, and its actions in the vascular smooth muscle cell. ACh= acetylcholine; BK= bradykinin; ATP= adenosine triphosphate; ADP= adenosine diphosphate; SP= substance P; SOCa²⁺= store-operated Ca²⁺ channel; ER= endoplasmic reticulum; NO= nitric oxide; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5-monophosphate; MLCK= myosin light chain kinase *Figure from Sandoo 2010 Open Cardiovascular Med J*

In endothelial cells eNOS produces NO during the conversion of L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS) in the presence of co-factors, such as tetrahydrobiopterin (BH₄). When NO diffuses to the VSMC it activates soluble guanylate cyclase, resulting in cyclic guanosine-3,5-monophosphate (cGMP)-mediated relaxation of the VSMC (Deanfield, Halcox, and Rabelink 2007) (Figure 2). The key activator of eNOS in the vasculature is shear stress, defined as the tangential force of flowing blood acting on the surface of the endothelium of the blood vessel (Baskurt and Meiselman 2003; Corson et al. 1996). Other activators of

eNOS include signaling molecules like bradykinin, adenosine, vascular endothelial growth factor and serotonin (Govers and Rabelink 2001). In addition to its role as a vasodilator, NO also exerts an anti-atherogenic effect by opposing leukocyte adhesion and migration, smooth muscle cell proliferation, platelet adhesion and aggregation, apoptosis, and inflammation (Sena, Pereira, and Seica 2013).

b. Vasodilatory prostaglandins

Prostaglandins are prostanoids derived from arachidonic acid, a 20-carbon unsaturated fatty acid, and generated by cyclooxygenase enzymes (Ricciotti and FitzGerald 2011). Two main isoforms of cyclooxygenase have been identified: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, and generally helps to preserve homeostasis (Dubois et al. 1998). COX-2, on the other hand, is induced by inflammatory stimuli, and thus plays a more important role in prostaglandin formation during inflammation and in diseases, like cancer and hypertension (Dubois et al. 1998; Wong et al. 2010). Both COX-1 and COX-2 are expressed by endothelial cells, and to a lesser extent by VSMC (Feletou, Huang, and Vanhoutte 2011). The four principal bioactive prostaglandins generated *in vivo* are prostaglandin (PG) E₂ (PGE₂), prostacyclin (PGI₂), prostaglandin D₂ (PGD₂) and prostaglandin F₂α (PGF₂α). These molecules are involved in a multitude of physiological and pathological processes in almost all of the tissues in the body via the interaction with prostanoid receptors, which are classified into five subtypes (DP, EP, FP, IP and TP receptors) (Dubois et al. 1998; Feletou, Huang, and Vanhoutte 2011).

PGI₂ is the major metabolite of arachadonic acid produced by the successive action of cyclooxygenase (COX-1 and COX-2 isoforms) and prostacyclin synthase in endothelial cells (Moncada and Vane 1978). Prostacyclin produces vasodilation by

activating prostacyclin receptors on vascular smooth muscle, resulting in activation of adenylate cyclase, which increases cyclic adenosine monophosphate (cAMP) levels. This stimulates protein kinase A, ultimately resulting in VSMC relaxation (Lai et al. 2014). Prostacyclin production is stimulated by bradykinin, histamine, thrombin, serotonin, and shear stress (Shireman and Pearce 1996). Typically, the vasodilatory effects of prostacyclin are masked by other endothelium-derived vasodilators, and can only be observed when other pathways leading to endothelium-dependent vasodilation are inhibited (Feletou, Huang, and Vanhoutte 2011). However, prostacyclin appears to play an important role in maintaining vascular function in environments in which NO is limited in both animals and humans (Chataigneau et al. 1999; Feletou, Huang, and Vanhoutte 2011; Sun et al. 1999). For example, in people with cardiovascular diseases, COX-2 derived prostaglandin-mediated vasodilation can compensate for decreased NO bioavailability (Bulut et al. 2003; Szerafin et al. 2006). In addition to inducing vasodilation, prostacyclin also prevents platelet aggregation, and has anti-inflammatory and anti-thrombotic effects (Lai et al. 2014).

PGE₂ is one of the most abundant prostaglandins, and exerts diverse effects on the body. For example PGE₂ can produce both relaxation and contraction of vascular smooth muscle depending on the receptor with which it interacts (Feletou, Huang, and Vanhoutte 2011). PGE₂ plays a critical role as a mediator of a variety of biological activities, including immune responses, gastrointestinal integrity, fertility, and blood pressure (Ricciotti and FitzGerald 2011). During inflammation, PGE₂ contributes to the development of the classic signs of inflammation: redness, swelling, and pain (Funk 2001). Indeed, redness and swelling are a result of PGE₂-mediated vasodilation, resulting in increased blood flow into inflamed tissues (Funk 2001). Furthermore, macrophages are known to generate PGE₂, and their

production of PGE₂ increases in states of inflammation (Ricciotti and FitzGerald 2011). Additionally, endothelial cell-derived PGE₂ can act as a potent vasodilator (Feletou, Huang, and Vanhoutte 2011). Although there is little evidence that PGE₂ plays a role in endothelium-dependent vasodilation under normal conditions, some studies suggest that it could mediate vasodilation in inflammatory states (Feletou, Huang, and Vanhoutte 2011; Nguyen-Tu et al. 2018; Pelletier et al. 2012).

Endothelium-dependent vasodilation that is not mediated by NO or PGI₂ is generally attributed to Endothelium derived hyperpolarizing factor (EDHF) (Feletou, Huang, and Vanhoutte 2011). The vasodilatory effect of EDHF becomes more important as vessel size decreases; therefore EDHF activity is most predominant in resistance vessels (Coats et al. 2001). Furthermore, EDHF-mediated vasodilation helps compensate for the loss of NO- and prostaglandin-mediated vasodilation in states in which NO is reduced, such as in aging and diabetes (Brandes et al. 2000; Gaubert et al. 2007; Scotland et al. 2005).

2. Vasoconstrictors

The endothelium also produces substances that cause vasoconstriction, such as endothelin, angiotensin II (ANGII), and vaso-constricting prostanoids like prostaglandin H₂ (PGH₂), PGF₂ α , and Thromboxane A₂ (TXA₂). Endothelin was originally identified as a potent endogenous vasoconstrictor, but is now known to exert a diverse set of actions in the vasculature. Three endothelin isoforms exist (ET-1, ET-2, and ET-3), of which ET-1 is the most frequently expressed (Rodriguez-Pascual et al. 2011; Shireman and Pearce 1996). The release of ET-1 can result in the activation of two different G-protein coupled receptors: endothelin receptor type A (ET_AR) and endothelin receptor type B (ET_BR) (Haynes and Webb 1998). The ET_AR and ET_BR expressed on VSMC mediate the sustained vasoconstriction

response that is characteristic of the endothelins (Haynes and Webb 1998). On the other hand, activation of ET_BR expressed on the endothelium mediates the release of endothelium-derived vasodilators, including NO, PGI₂, and EDHF, as well as the rapid uptake of ET-1 (Haynes and Webb 1998). Therefore, the actions of the endothelial ET_BR oppose the contracting vascular effects of the ET_ARs and ET_BRs expressed on the VSMC. Acute blockade of ET_AR receptor produces a small or no effect on mean arterial pressure, whereas blockade of ET_BR results in increased mean arterial pressure (Pollock 2001; Pollock and Opgenorth 1993; Pollock and Pollock 2001). Therefore, evidence suggests that in healthy individuals ET_BR plays a more important role in controlling basal blood pressure and vascular tone by protecting against the contracting effects of the endothelins.

In addition to its effects on vascular tone, endothelin can also act as a regulator of vascular remodeling, angiogenesis, and extracellular matrix synthesis (Rodriguez-Pascual et al. 2011). VSMC, cardiomyocytes, fibroblasts, and most notably endothelial cells express endothelin (Rodriguez-Pascual et al. 2011). The expression of endothelin is upregulated by TGF- β , TNF- α , the interleukins, insulin, and ANGII, and downregulated by NO, PGI₂, hypoxia, and shear stress (Thorin and Webb 2010).

The cleavage of angiotensinogen via renin produces angiotensin I, which can be subsequently cleaved by angiotensin converting enzyme to produce ANGII. Two main ANGII receptors, AT₁ and AT₂, exist. The predominate vasoconstriction action of ANGII is mediated by AT₁ expressed by the VSMC. This effect can be countered by the AT₂ receptor, which causes vasodilation (Hernandez Schulman, Zhou, and Raj 2007).

TXA₂ is mainly produced by platelets, but also by the endothelium, and causes both platelet aggregation and vasoconstriction (Oates et al. 1988). PGH₂ is the precursor of TXA₂, and exerts its effects on the vascular wall via the same receptors (Davidge 2001). PGF₂α isoforms are synthesized in the vascular wall by PGH₂, PGD₂, and PGE₂, and act as potent vasoconstrictors.

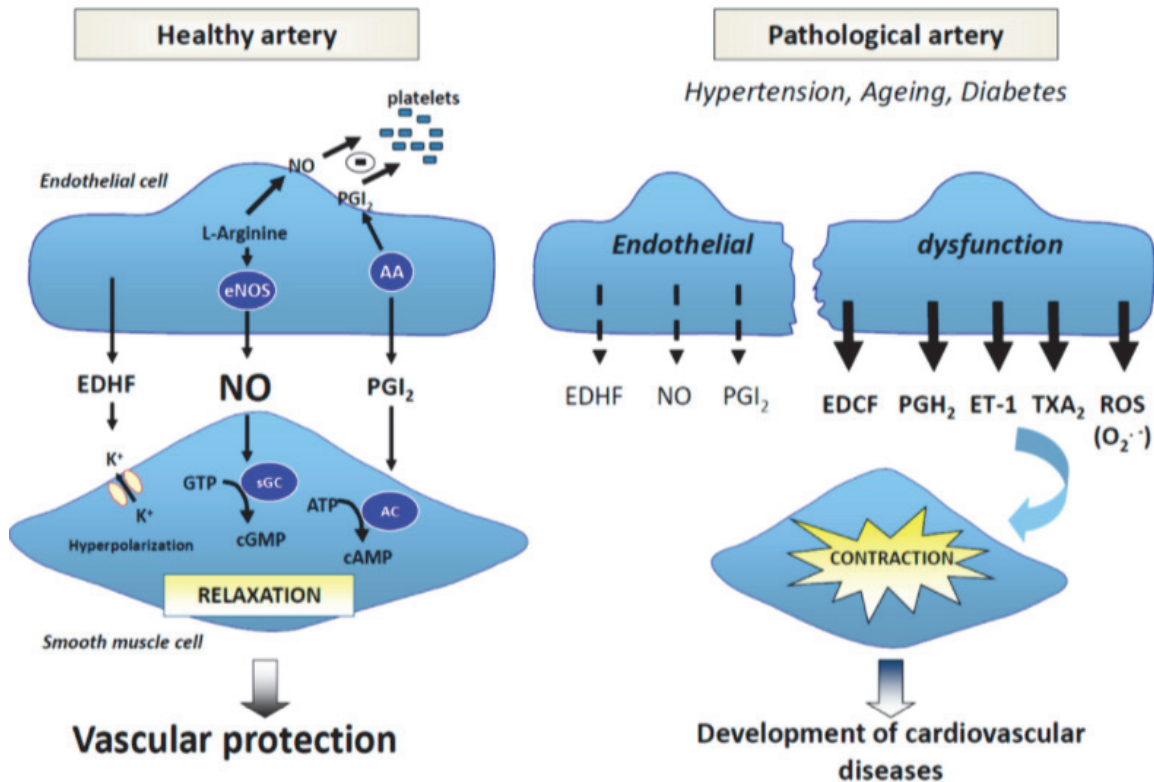


Figure 3: Vasoactive molecules in healthy and pathological arteries. In a healthy artery, vasodilators factors such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI₂) play a key role in homeostasis. In a pathological artery, contracting factors such as prostaglandin (PGH₂), endothelin-1 (ET-1), and thromboxane A₂ (TXA₂) contribute to the pathogenesis of cardiovascular disease in the presence of oxidative stress and superoxide anions (O₂^{·-}). AA: arachidonic acid, eNOS: endothelial nitric oxide synthase, sGC: soluble guanylate cyclase; AC: adenylate cyclase; K⁺: potassium. *Figure and text from Dal and Sigrist 2016 Diseases*

B. ENDOTHELIAL DYSFUNCTION: THE ROLE OF OXIDATIVE STRESS

The endothelium plays a crucial role in maintaining vascular homeostasis. Therefore, damage to the endothelium can result in an increased expression of

chemokines, cytokines, and adhesion molecules (Deanfield, Halcox, and Rabelink 2007). This leads to endothelial dysfunction, characterized by altered anti-coagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, dysregulation of vascular remodeling, and impaired endothelium-dependent vasodilation (Cai and Harrison 2000; Hansson 2005). One of the most fundamental changes that contributes to endothelial dysfunction is reduced NO bioavailability due to increased oxidative stress (Deanfield, Halcox, and Rabelink 2007; Xu and Zou 2009).

1. Oxidative stress

Reactive oxygen species (ROS) are a family of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen (Turrens 2003). Many ROS including, superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (HO^{\cdot}), NO (also denoted as NO^{\cdot}), and lipid radicals, are considered to be free radicals, as they possess unpaired electrons (Cai and Harrison 2000). Other ROS, such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^{\cdot}$), are not considered to be free radicals, but have oxidizing effects, and therefore contribute to oxidative stress. Furthermore, the production of one ROS, can lead to the production of several other ROS (Cai and Harrison 2000).

In normal physiological conditions, ROS are produced during aerobic metabolism, and are balanced by anti-oxidant systems in order to maintain homeostasis (Sies 1997). However, in pathophysiological conditions, excessive production of ROS can overwhelm anti-oxidant defense mechanisms, leading to the oxidative damage of DNA, proteins, carbohydrates, and lipids (Cai and Harrison 2000). This imbalance between ROS and antioxidants in favor of ROS is referred to as oxidative stress (Sies 1997).

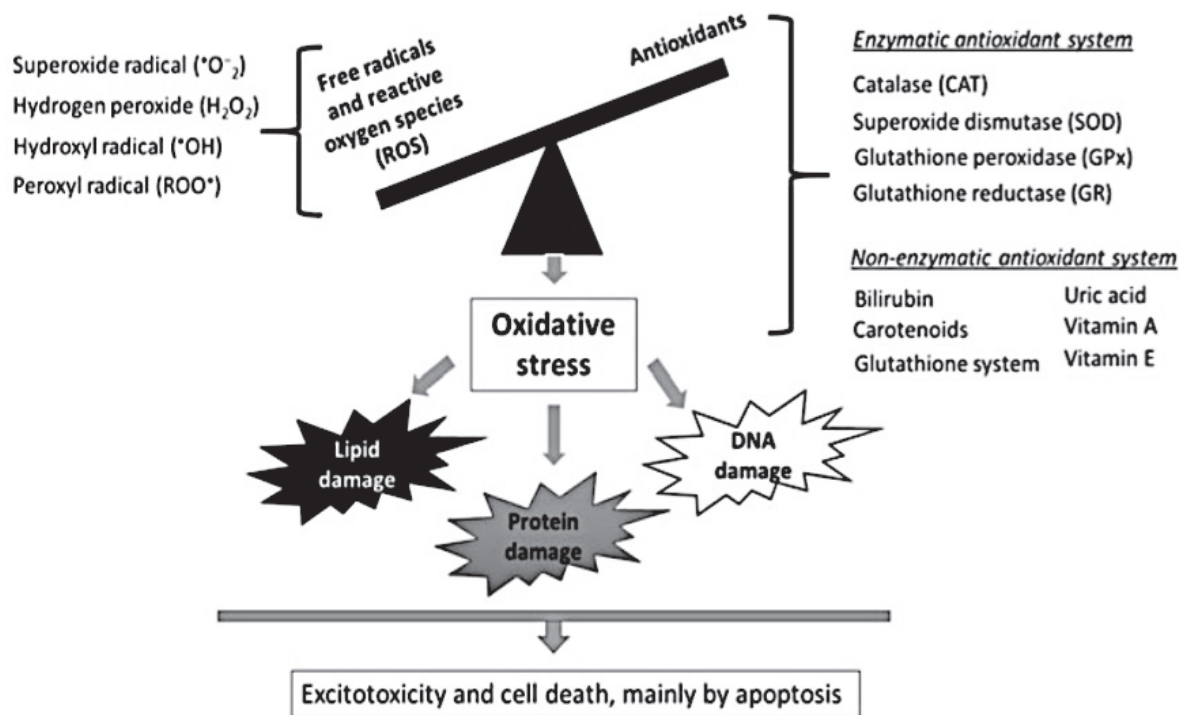


Figure 4: Depiction of reactive oxygen species (ROS). An excessive production of ROS can overwhelm the anti-oxidant system, resulting in oxidative stress. Oxidative stress results in oxidative damage of DNA, lipids, and proteins (*Image from Porres-Martinez 2017*).

a. Major sources of ROS in the vasculature

The most important sources of ROS in the vasculature are the mitochondrial electron transport chain (ETC), NADPH oxidases (NOX), and xanthine oxidase (XO) (Sena, Pereira, and Seica 2013). Other sources of ROS include uncoupled eNOS, and other enzymes such as lipoxygenase, cyclooxygenase, cytochrome P450s, the peroxidases, and other hemoproteins (Cai and Harrison 2000).

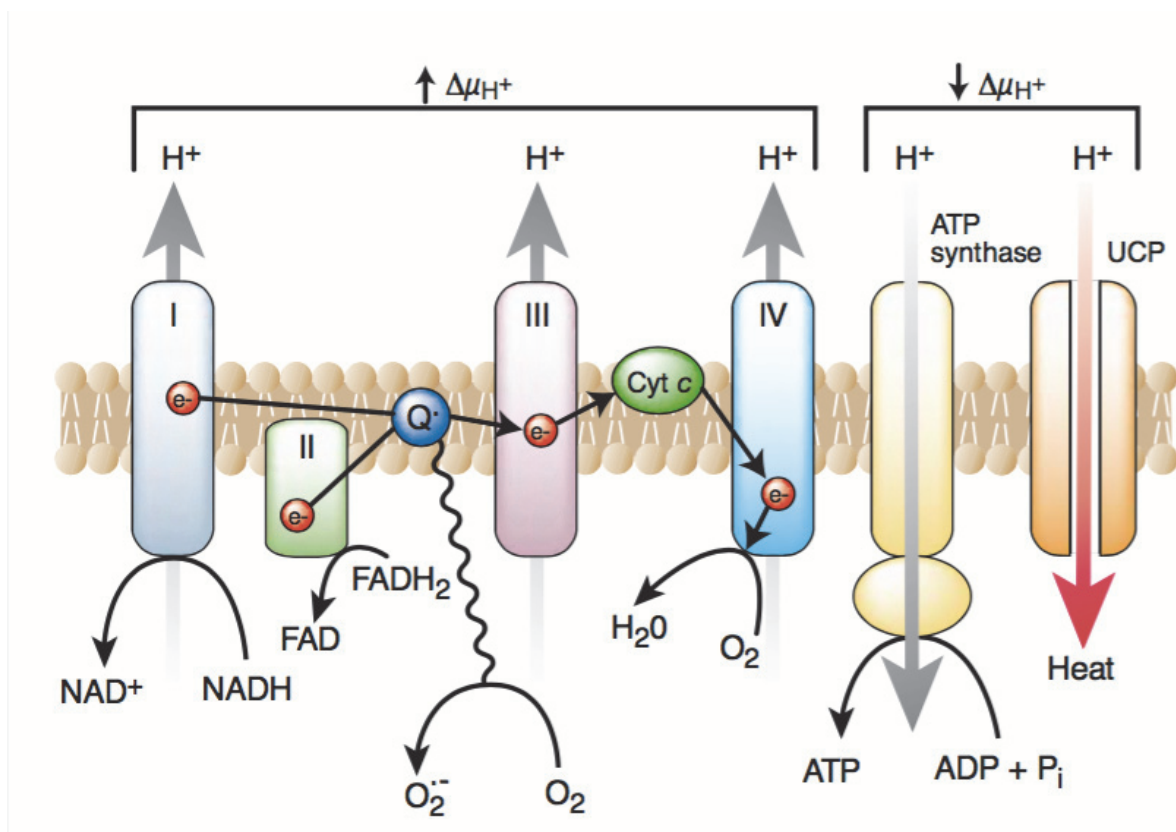


Figure 5: Production of superoxide by the mitochondrial electron-transport chain. Increased hyperglycemia-derived electron donors from the TCA cycle (NADH and FADH₂) generate a high mitochondrial membrane potential ($\Delta\mu_{H^+}$) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce O₂ to superoxide *Figure and text from Brownlee 2001*

- **The mitochondrial electron transport chain:** The mitochondrial ETC is composed of five multi-subunit protein complexes situated in the inner mitochondrial membrane. The transfer of electrons along the ETC creates a proton gradient that provides the potential energy needed for ATP synthase to generate ATP (Ballinger 2005; Murphy 2009). ROS is produced as a byproduct of this process, but under normal physiological conditions anti-oxidant systems are able to effectively protect against ROS damage of mitochondrial proteins, lipids, and nucleic acids (Murphy 2009). However, in conditions of oxidative stress, anti-oxidant mechanisms are

overwhelmed, and as a result excess ROS production exerts harmful effects that can potentially alter mitochondrial function (Lowell and Shulman 2005) (Figure 5).

- **NADPH oxidases:** NOX are a family of multi-subunit enzymes that catalyze the production of $O_2^{\cdot-}$ via the reduction of O_2 using NADPH or NADH ($2O_2 + NAD(P)H \rightarrow 2\cdot O_2^{\cdot-} + NAD(P)^+ + H^+$) (Paravicini and Touyz 2008). In total, seven NOX isoforms exist, four of which (NOX1, NOX2, NOX4, NOX5) are expressed in blood vessels (Schramm et al. 2012). Both NOX1 and NOX2 appear to contribute to vascular pathology, and promote atherosclerosis. NOX4, on the other hand, appears to protect blood vessels against damage and disease (Lassegue and Griendling 2010). NOX5 also contributes to vascular damage as it generates ROS in endothelial cells and promotes endothelial proliferation (BelAiba et al. 2007)

- **Xanthine oxidase:** XO is a form of xanthine oxidoreductase, an enzyme that catalyzes the oxidation of hypoxanthine to xanthine, and then to uric acid in the last steps of purine degradation (Battelli, Bolognesi, and Polito 2014). This process requires molecular oxygen as the oxidant of the reaction, and produces $O_2^{\cdot-}$ and H_2O_2 , in addition to uric acid (Hancock, Desikan, and Neill 2001). XO plays a major role in ischemia/reperfusion injury because xanthine dehydrogenase, which uses NAD^+ as a substrate, is converted to XO, which uses oxygen as an electron acceptor, under hypoxic conditions. Additionally, during ischemia ATP is successively degraded to adenosine diphosphate, adenosine monophosphate, and ultimately to hypoxanthine and xanthine. Upon reperfusion, when oxygen-rich blood flow returns, the accumulated hypoxanthine and xanthine in the tissue are metabolized by XO, yielding $O_2^{\cdot-}$ and H_2O_2 (Szocs 2004).

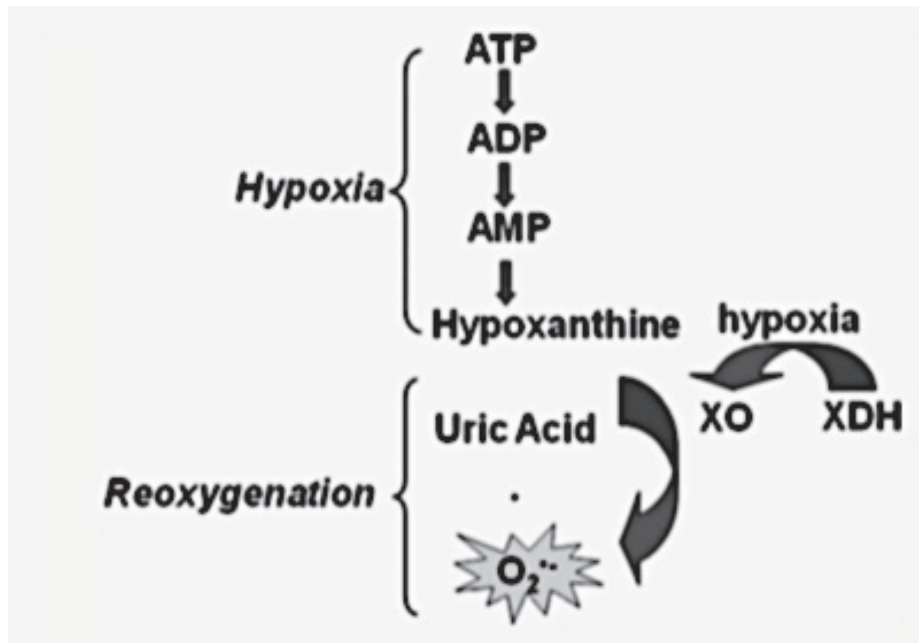


Figure 6: Superoxide anion production following ischemia and reperfusion. Hypoxanthine and xanthine oxidase are generated during hypoxia. Upon reoxygenation, xanthine oxidase catabolizes hypoxanthine to uric acid and superoxide anion. *Figure from Chirico 2012*

b. Antioxidants

Antioxidants are defined as, “any substance that delays, prevents, or removes oxidative damage to a target molecule” (Gutteridge and Halliwell 2010). Both enzymatic (Figure 7) and non-enzymatic mechanisms exist to combat the deleterious effects of ROS.

i. Non-enzymatic antioxidants

The principal non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and glutathione (Carocho and Ferreira 2013). Vitamin C is a water-soluble molecule that scavenges ROS, and protects vitamin E and glutathione against oxidation in cell membranes. Vitamin E is considered to be the most important antioxidant in cell membranes, and functions as a chain-breaking lipid scavenger (Li and Shah 2004). Glutathione is an endogenous tripeptide that exists as reduced glutathione (GSH) and oxidized glutathione (GSSG) in the body (Clarkson and Thompson 2000). GSH can act as an antioxidant by exchanging

electrons and reducing organic peroxides and H_2O_2 . GSH is converted to GSSG through this process (Clarkson and Thompson 2000). Other important non-enzymatic anti-oxidants include bilirubin, carotenoids, and vitamin A (Carocho and Ferreira 2013).

ii. Enzymatic anti-oxidants

- **Superoxide dismutase (SOD):** SOD converts $\text{O}_2^{\bullet-}$ to H_2O_2 , and is considered to be the primary and most important defense against ROS (Sena, Pereira, and Seica 2013). Three isoforms of SOD exist, SOD1 or CuZnSOD, located in the cytoplasm, SOD2 or MnSOD, which exists exclusively in the mitochondrial space, and SOD3 or EC-SOD, which exists as a copper and zinc containing tetramer (Zelko and Folz 2004).

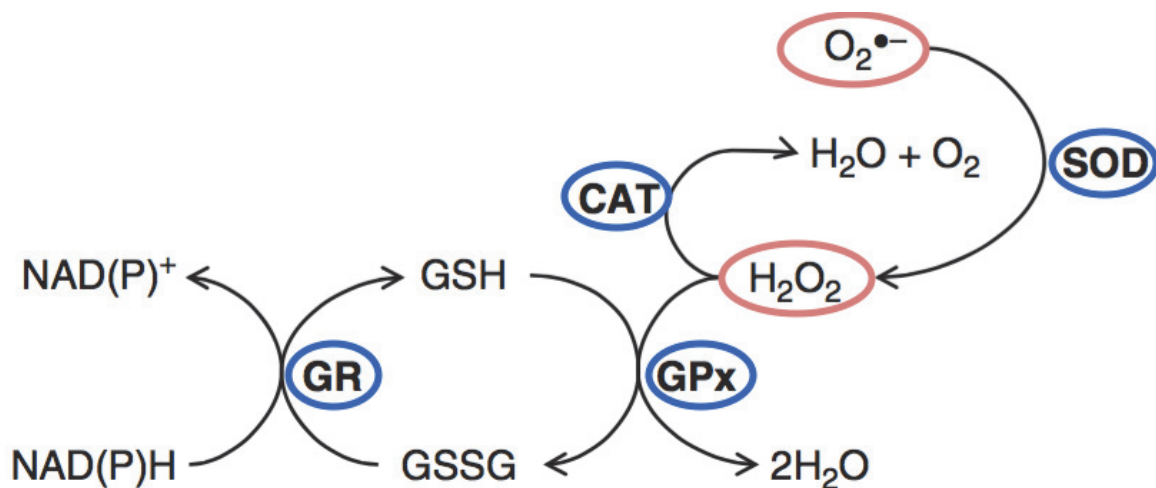


Figure 7: Synergy of antioxidant enzymes in the removal of reactive oxygen species. $\text{O}_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2 , hydrogen peroxide. *Image from: Funnekotter et al., 2016*

- **Catalase:** Catalase is a heme-containing enzyme that can remove high concentrations of H_2O_2 produced by SOD (Li and Shah 2004; Zelko and Folz 2004).

Catalase uses H_2O_2 as a substrate to produce water and molecular oxygen (Kohen and Nyska 2002).

- **Glutathione peroxidases (GPxs):** GPxs are a family of enzymes that play an important role in balancing ROS. Notably, GPxs reduce H_2O_2 to water with the simultaneous oxidation of GSH (Li and Shah 2004). GPx also play an important role in reducing lipid peroxides (Sena, Pereira, and Seica 2013).

- **Glutathione reductase (GR):** GR is a homodimeric enzyme mainly located in the cytosol. The main role of this enzyme is to reduce GSSG using NADPH as a cofactor (Clarkson and Thompson 2000).

2. ROS and reduced NO bioavailability

One of the main ways that ROS contributes to endothelial dysfunction is by reducing the availability of NO, via the degradation of NO to ONOO^- , and through the uncoupling of eNOS.

a. NO degradation to peroxynitrite

Accelerated degradation of NO by ROS is one of the primary mechanisms leading to diminished NO bioavailability, and therefore impaired vascular function (Xu and Zou 2009). The diffusion-limited reaction of $\text{O}_2^{\cdot-}$ with NO forms ONOO^- , a powerful and highly potent oxidant (Szabo, Ischiropoulos, and Radi 2007). The reaction of NO with $\text{O}_2^{\cdot-}$ is over three times faster than the dismutation of $\text{O}_2^{\cdot-}$ by SOD (Szabo, Ischiropoulos, and Radi 2007). Therefore, in conditions of oxidative stress, when anti-oxidant defenses are saturated, $\text{O}_2^{\cdot-}$ reacts with NO, forming ONOO^- , instead of being converted to H_2O_2 . At a physiological pH, ONOO^- can form HO^\bullet and nitrogen dioxide, two other potent oxidants (Beckman et al. 1994). Therefore, the damaging effect of the reaction of $\text{O}_2^{\cdot-}$ with NO is twofold in that it not only decreases the concentration of NO, by also generates more ROS.

b. Endothelial Nitric Oxide Synthase Uncoupling

Under normal conditions, eNOS produces NO and L-citrulline from L-arginine. This reaction requires molecular oxygen and reduced NADPH as co-substrates, and flavin adenine dinucleotide, flavin mononucleotide, BH₄, and calmodulin as cofactors (Moncada and Higgs 2006). However, under certain conditions, eNOS can produce O₂^{•-} instead of NO. This phenomenon, known as eNOS uncoupling, takes place when NOS is not coupled with its cofactor or substrate, as can occur when concentrations of BH₄ are reduced, or the enzyme substrate L-arginine is depleted (Antoniades et al. 2009; Luo et al. 2014). Increased ROS can cause eNOS uncoupling via the oxidative depletion of BH₄ and the oxidative disruption of the dimeric eNOS complex (Antoniades et al. 2009; Luo et al. 2014).

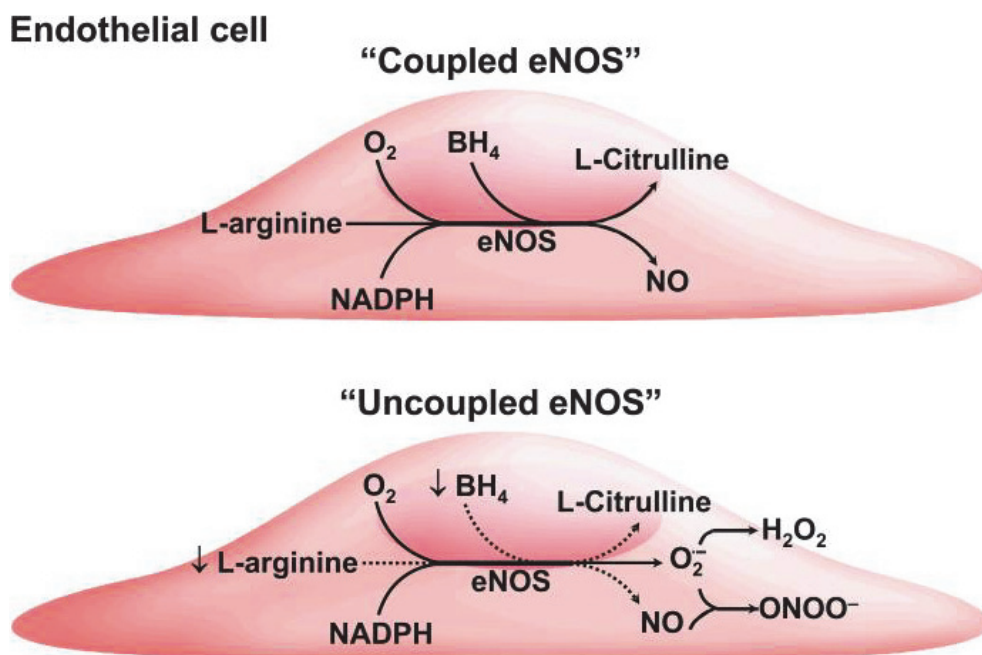


Figure 8: Endothelial nitric oxide synthase (eNOS) uncoupling. eNOS uncoupling occurs when concentrations of BH₄ are reduced, or the enzyme substrate L-arginine is depleted. As a result, eNOS produces superoxide instead of NO. The subsequent reaction between O₂^{•-} and NO generates peroxynitrite anion (ONOO⁻). *Image from Katusic ZS et al Trends Pharmacol Sci. 2009*

C. INFLAMMATION AND VASCULAR DYSFUNCTION

Inflammation is a defense mechanism that is triggered in response to tissue damage and pathogens. Stimuli that instigate inflammation include infection, mechanical factors, oxygen radicals, immune complexes, ANGII, inflammasomes, heat shock proteins, cellular microparticles, adipokines, platelet products, and coagulation factors (Sprague and Khalil 2009). Acute inflammation is a transient, adaptive response generally resulting in the elimination of harmful pathogens or the restoration of damaged cells (Medzhitov 2008). In the blood vessels, acute inflammation results in increased blood flow, vascular permeability, leukocyte concentration, and localized cytokine secretion (Medzhitov 2008). Many elements play an important role in the vascular response to inflammation, including circulating inflammatory cells (neutrophils, lymphocytes, monocytes, and macrophages), endothelial cells, VSMC, connective tissue cells, and extracellular matrix (ECM) (Sprague and Khalil 2009). Acute inflammation is generally terminated when the inflammatory stimulus is removed, and all of the inflammatory mediators are dissipated (Sprague and Khalil 2009).

Chronic inflammation, on the other hand, is characterized by increased activity of inflammatory mediators, resulting in elevated levels of low-grade systemic inflammation (Stevens et al. 2005). Increased levels of inflammation have been shown to impair endothelium-dependent relaxation in conductance and resistance vessels in humans and animals (Hingorani et al. 2000; Nakamura et al. 2000; Rask-Madsen et al. 2003). Furthermore, epidemiological studies have found increased vascular risk is associated with increased basal levels of cytokines, including IL-6, TNF- α , circulating cell adhesion molecules (including ICAM-1, P-selectin, E-selectin),

and down-stream acute-phase reactants, such as C-reactive protein (CRP), fibrinogen, and serum amyloid A (*Ridker et al. 2000*).

1. The inflammatory response in the vasculature

Damage to the endothelial cell lining of the blood vessels initiates a complex inflammatory process that results in adhesion and infiltration of leukocytes to the site of damage or infection. The initial steps of leukocyte recruitment during an inflammatory response are leukocyte capture, or tethering, and rolling. These steps are dependent on the release of cytokines and chemokines, and the subsequent endothelial activation (*Wadley, Veldhuijzen van Zanten, and Aldred 2013*). Leukocyte recruitment begins when activated endothelial cells expressing P-selectin draw in white blood cells expressing L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) towards the endothelium (*Mayrovitz, Wiedman, and Tuma 1977*).

Next, cellular adhesion molecules and immunoglobulins facilitate the rolling of the neutrophils on the endothelial surface. The endothelium expresses P-selectin, peripheral node addressin, MAdCAM-1, and E-selectin, which interact with L-selectin and PSGL-1 expressed by the leukocytes. Additionally the leukocytes express integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$, which interact with endothelial MAdCAM-1 and VCAM-1, respectively. These interactions result in leukocyte activation, which leads to the subsequent steps of the inflammatory process: firm adhesion and trans-endothelial migration (*Granger and Senchenkova 2010*). The firm adhesion of leukocytes to the endothelium is largely mediated by integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$, expressed by the activated leukocytes, which form high affinity bonds with VCAM-1 and MAdCAM-1, expressed by the endothelium. Finally, the interaction of integrins CD11/CD18 and PECAM-1, expressed on the leukocytes, with endothelial ICAM-1, ICAM-2, and PECAM-1 mediates both firm adhesion and subsequent leukocyte emigration,

thereby enabling leukocytes to migrate to the interior of the inflamed tissue (Granger and Senchenkova 2010).

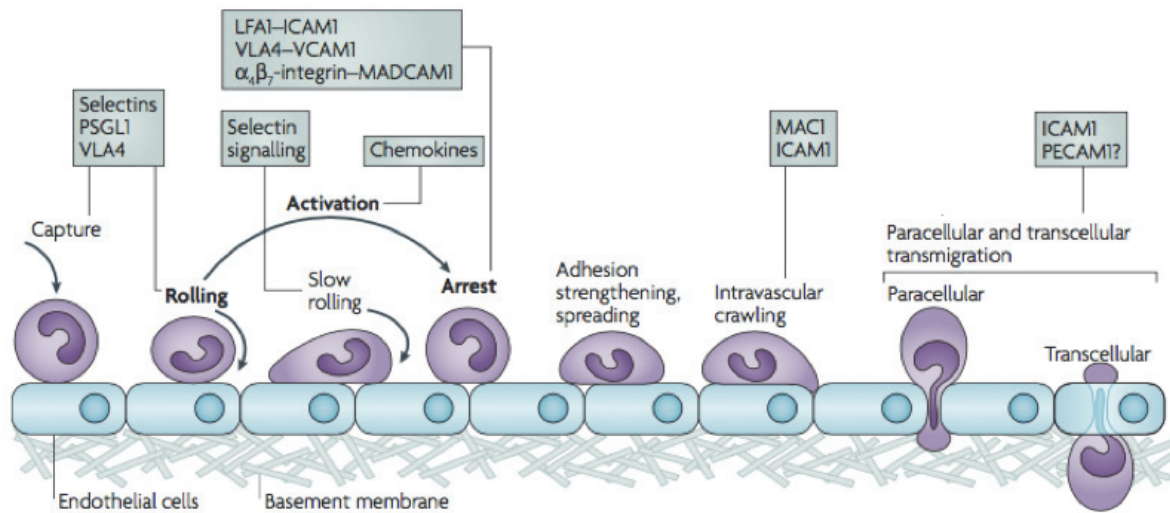


Figure 9: Steps of leukocyte adhesion and integration. The leukocyte is “captured” by endothelial cells expressing adhesion factors (the selectins), and begins rolling along the endothelial surface. Interactions between integrins expressed by the leukocyte and additional adhesion factors expressed by the endothelium then mediate the firm adhesion of the leukocyte to the endothelium. Finally, the leukocyte migrates to the interior of the inflamed tissue. ICAM1, intercellular adhesion molecule 1; LFA1, lymphocyte function-associated antigen 1 (also known as $\alpha_4\beta_2$ -integrin); MAC1, macrophage antigen 1; MADCAM1, mucosal vascular addressin cell-adhesion molecule 1; PSGL1, P-selectin glycoprotein ligand 1; PECAM1, platelet/endothelial-cell adhesion molecule 1; vascular cell-adhesion molecule 1; VLA4, very late antigen 4 (also known as $\alpha_4\beta_1$ -integrin). (Image from Ley et al., 2014)

In acute inflammation the infectious agent is destroyed, or the damaged tissue is repaired. The inflammation is then gradually resolved due to the progressive elimination of activated inflammatory cells via apoptosis, and the reduction of circulating leukocytes to a normal level (Maskrey et al. 2011). However, in the case of chronic inflammation, the persistent activation and accumulation of leukocytes contributes to endothelial dysfunction.

2. Cytokines: major mediators of the immune response

Cytokines are a large and diverse family of small cell-signaling proteins, peptides, and glycoproteins used extensively in intercellular communication, and

produced by nearly all nucleated cells. Different cell types can secrete the same cytokine, and one cytokine can have a variety of effects (both paracrine and autocrine), depending on the timing and location of its secretion. Additionally, different cytokines can share similar functions and can act together synergistically, or have antagonistic effects (Sprague and Khalil 2009). In states of vascular inflammation cytokines stimulate immune cell proliferation, induce vascular cell growth and migration, induce apoptosis, promote the adhesion of immune cells to endothelial cells, and increase vascular permeability. Table 1 summarizes the sources, receptors, target cells, and functions of several key inflammatory cytokines that play an important role in mediating vascular inflammation (Sprague and Khalil 2009).

Cytokines are generally classified into the following categories according to their main actions (Sprague and Khalil 2009):

- Tumor necrosis factors (TNFs) – play an important role in inducing cell death
- Interleukins (ILs) – cytokines that are both produced by leukocytes, and act on other leukocytes
- Lymphokines – originally thought to be produced only by lymphocytes
- Monokines – initially considered to be generated exclusively by monocytes
- Interferons (IFNs) – Play a role in anti-viral responses, activate natural killer cells and macrophages, include IFN- α , - β , and - γ
- Colony stimulating factors (CSF) – thought to support cell growth in semi-solid media.

Cytokine	Producing Cell	Receptor	Target cells/functions
IL-1	Monocytes Macrophages T Cells B cells DC, ECs, VSMC	IL-1R type1, IL-1R type2	- Co-stimulation and pro-inflammatory effect on Th cells - Maturation and proliferation of B cells - Activation of NK cells - EC and VSMC activation, migration, proliferation - Inflammation, acute phase response, fever *
IL-4	Th2 cells, mast cells	IL-4R α , IL-4R γ	- Proliferation and differentiation of B cells (Ig switching to IgG1 and IgE) and Th2 cells (anti-inflammatory); stimulates VCAM-1. *
IL-6	Monocytes Macrophages Th2 cells ECs, VSMCs Fibroblasts Stromal cells	IL-6R α IL-6R β	- Antibody secretion - Acute phase response - Proliferation of VSMC, EC, T cells, activated B cells *
IL-8	Macrophages ECs Monocytes, T cells	IL-8R	- Chemotaxis, proinflammatory, promotes leukocyte arrest (Targets: Neutrophils T cells, monocytes). *
IL-10	Macrophages, Th2 cells, Treg and B cells	IL-10R-1 IL-10R-2	- Inhibit proinflammatory cytokine production (targets Macrophages, T & B cells. **)
IL-13	Th2 cells	IL-4R α	- Activation of Ig transcription (Targets B cells)
IL-18	Macrophages, ECs, VSMCs	IL-18R α IL-18R β	- Remodelling, proinflammatory, induces IFN- γ and other Th1 cytokines, promotes Th1 development and NK activity (ECs, T cells; NK cells; myeloid, monocytic, erythroid, and megakaryocytic cells). *

Table 1: Sources, receptors, target cells, and functions of several key inflammatory cytokines. Adapted from Sprague 2009; DC (Dendritic Cells), ECs (Endothelial cells), VSMC (Vascular Smooth Muscle Cells), NK cells (Natural Killer), Th cells (T helper cells)

*pro-atherogenic effect

**Anti-atherogenic effect

- Transforming growth factors (TGFs) - include TGF- β 1, 2, 3, bone morphogenetic proteins (BMPs), activins, and inhibins
- Chemokines – cause chemotaxis, or the movement of cells. Their primary role is to locate or attract cells to the site of damage. Include IL-8 (CXCL8), platelet factor-4 (PF-4/CXCL4), macrophage inflammatory proteins (MIP)-

1 α (CCL3) and 1 β (CCL4), RANTES (regulated on activation, normal T-expressed and secreted, CCL5), and monocyte chemoattractant proteins (MCP)-1 (CCL2), -2(CCL8), -3, and -4 (Luster 1998).

Cytokines can also be classified as either pro-inflammatory or anti-inflammatory. Anti-inflammatory cytokines mediate the down-regulation of inflammation and include IL-4, IL-10, IL-13, IFN- α , and TGF- β . Inflammatory cytokines, on the other hand, up-regulate inflammation by mediating several processes including: the secretion of endogenous pyrogens, the up-regulation of inflammatory reactions, and the stimulation of acute phase reactants and chemoattractants. The most important pro-inflammatory cytokines are TNF- α , IL-1, and IL-6 (Vila and Salaiques 2005).

The production of TNF- α by leukocyte and endothelial cells is stimulated by other pro-inflammatory cytokines, like IL-1. TNF- α plays a crucial role in micro- and macro-vascular function by inducing the gene expression of various inflammatory cytokines and chemokines, either dependently or independently of the activation of transcription factors, like NF- κ B (Zhang et al. 2009). TNF- α mediated signaling contributes to impaired vascular function by initiating and accelerating atherogenesis, thrombosis, vascular remodeling, vascular inflammation, endothelium apoptosis, vascular oxidative stress, and impaired NO bioavailability, which contribute to impaired vascular function (Zhang et al. 2009).

IL-1 is secreted by monocytes, macrophages, T cells, C cells, endothelial cells, VSMC, and dendritic cells (Sprague and Khalil 2009). Several different types of IL-1 exist, of which IL-1 α and IL-1 β are the best characterized. The interaction of IL-1 with its receptor IL-1R induces the expression of a wide variety of cytokines, chemokines, and cellular adhesion molecules, mostly on the endothelial cells

(Kleemann, Zadelaar, and Kooistra 2008). Furthermore, IL-1 can mediate vascular dysfunction by causing endothelial cell and VSMC activation, migration, and cell proliferation (Sprague and Khalil 2009). Overall, IL-1 and TNF- α interact synergistically to promote inflammation (Dinarello 2000).

IL-6 has a pleiotropic effect on inflammation, immune response and hematopoiesis. It induces synthesis of acute phase proteins like CRP, serum amyloid A, fibrinogen, and hepcidin in hepatocytes. IL-6 plays an important role in acquired immune response by stimulating antibody production and effector T-cell development. Furthermore, IL-6 also promotes proliferation and differentiation of non-immune cells (Tanaka, Narazaki, and Kishimoto 2014). A variety of cells, including monocytes, macrophages, Th2 cells, VSMCs, endothelial cells, fibroblasts, and stromal cells produce IL-6 (Sprague and Khalil 2009).

3. Cytokine induced signaling and vascular reactivity

Although a large number of different cytokines exist, most of their effects are mediated via the actions of the same signaling pathways. Cytokines activate intracellular signal transduction by interacting with specific receptors of target cells. Stimulation of these intra-cellular signaling cascades results in the activation transcription factors such as NF- κ B, as well as signal transducers and activators of transcription (STAT) and sma- and maad-related proteins (Smad) (De Martin et al. 2000).

Two key cytokine-induced signaling pathways that play an important role in vascular dysfunction are the NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways (Figure 10). These pathways are activated by pro-inflammatory cytokines, such as the IL-1 family and the TNF family. The NF- κ B signaling pathway plays an intricate role in promoting inflammation by up-regulating genes coding for

pro-inflammatory cytokines, cellular adhesion molecules, chemokines, growth factors, and inducible enzymes like COX2 and iNOS (Sprague and Khalil 2009). Data from animal and cell culture experiments indicate that NF- κ B plays an important role in promoting endothelial dysfunction (Brand et al. 1996; Csiszar et al. 2006; Hajra et al. 2000; Ungvari et al. 2007). Additionally, a study conducted in overweight and obese humans aged 52-68 showed that NF- κ B, in part via stimulation by oxidative stress, plays a key role in mediating vascular endothelial dysfunction (Pierce et al. 2009).

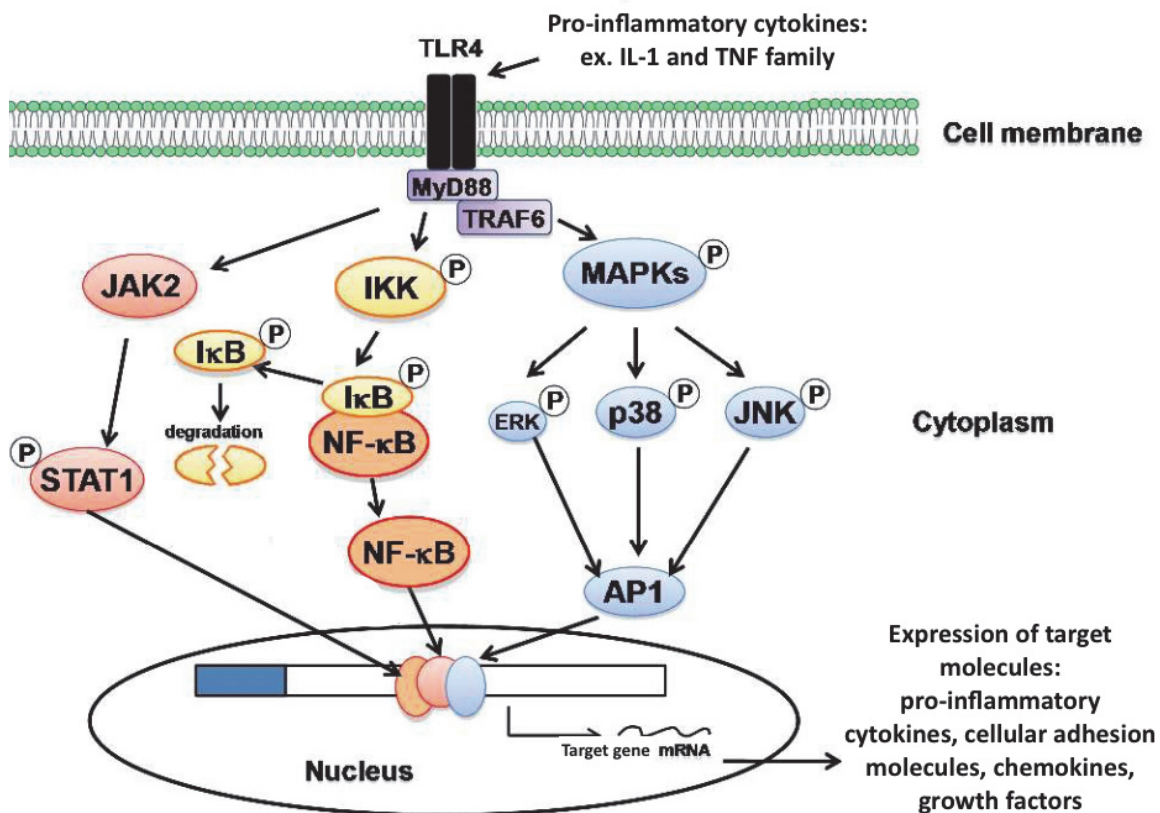


Figure 10: Signaling pathways activated by pro-inflammatory cytokines.

Interaction of proinflammatory cytokines (such as IL-1 and the TNF family) with receptors on target cells results in the activation of signaling pathways that promote inflammation by up-regulating genes coding for pro-inflammatory cytokines, cellular adhesion molecules, chemokines, growth factors, and inducible enzymes like COX2 and iNOS via the activation of mitogen-activated protein kinases MAPKs (ERK, p38 and JNK), the nuclear factor NF- κ B as well as a Janus tyrosine kinase (JAK) and signal transducers and activators of transcription (STATs). Other shown protein components that participate in inflammatory signaling are: ERK (extracellular-regulated kinase), JNK (NH₂-terminal kinase), AP-1 (activating protein-1), MyD88 (myeloid differentiation primary response gene 88), IKK (I κ B kinase) and I κ B (NF- κ B inhibitor protein). *Figure and text adapted from Hegazy et al. 2015*

The MAPK signaling cascades regulate a wide variety of cell processes including proliferation, differentiation, apoptosis, and stress response (Plotnikov et al. 2011). The three most well characterized MAPK signaling pathways are the extracellular signal-regulated kinase (ERK), C-jun N-terminal kinase (JNK), and p38 MAPK pathways. All three have been shown to play important roles in the progression of cardiovascular disease (Muslin 2008). Other important cytokine-induced signaling pathways include the Janus kinase (JAK)-STAT pathway, through which most ILs, CSFs, and IFNs mediate their effects, and the Smad Pathway, which is activated by TGF- β (Sprague and Khalil 2009).

Through signaling cascades, cytokines alter vascular function by modulating the expression of several mediators, including prostanoids, leukotrienes, NO, ET-1, bradykinin, and reactive oxygen species (Vila and Salaices 2005). Indeed, animal and human studies show that TNF- α , IL-6, and IL-1 can induce vasodilation and vasoconstriction *in-vitro* and *in-vivo* during acute inflammation (Iversen et al. 1999; Klemm et al. 1995; Minghini, Britt, and Hill 1998; Nakamura et al. 2000). Cytokines also appear to contribute to endothelial dysfunction in states of chronic inflammation, as multiple studies have shown that cytokine-mediated chronic inflammation impairs endothelium-dependent relaxation in conductance and resistance vessels in humans and animals (Hingorani et al. 2000), (Nakamura et al. 2000; Vila and Salaices 2005; Rask-Madsen et al. 2003). Additionally elevated levels of inflammatory cytokines have been observed in several cardiovascular diseases including atherosclerosis, diabetes, and hypertension (Cooper et al. 2001; Drexler and Hornig 1999; Viridis and Schiffrin 2003).

4. Oxidative stress and inflammation: sources of vascular dysfunction

Inflammation and ROS appear to function together as part of a vicious cycle in which oxidative stress is a direct stimulus for inflammation and vice versa (Figure 11) (Wadley, Veldhuijzen van Zanten, and Aldred 2013). Indeed, in response to infection and inflammation, leukocytes can directly produce $O_2^{\cdot-}$ via the activation of NOX (Babior, Kipnes, and Curnutte 1973). Furthermore, inflammatory cytokines, including TNF- α , IL-1 β and IFN- γ have been shown to increase mitochondrial and NOX-generated ROS *in-vitro* (Yang et al. 2007).

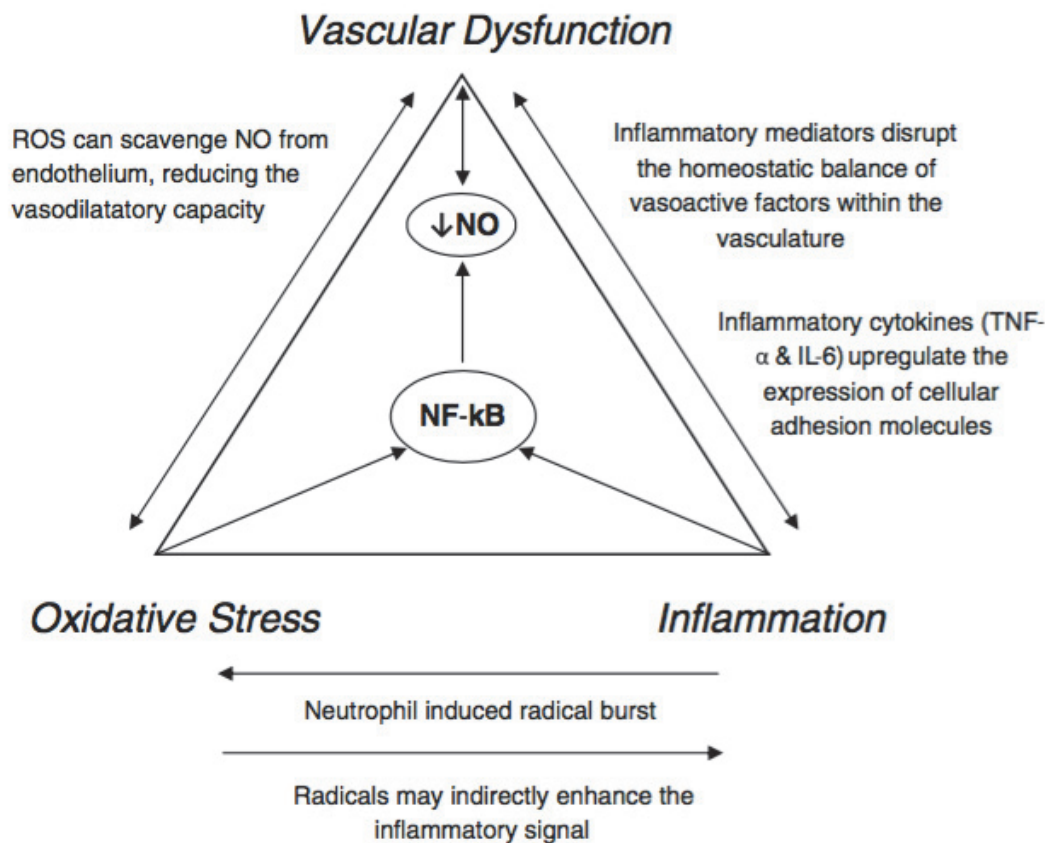


Figure 11: The Vascular Health Triad. The relationships between oxidative stress, inflammation and vascular health, adapted from *Wadley et al 2013*

On the other hand, ROS can also modulate inflammation. One of the main mechanisms by which ROS regulates inflammation is via the constitutive expression of NOX (De Keulenaer et al. 2000). The $O_2^{\cdot-}$ produced by these enzymes can

regulate adhesion molecule expression on endothelial and inflammatory cells (I.B.1.a). Furthermore, ROS, especially $O_2^{\cdot-}$, can act as signaling molecules to trigger cytokine production (Bulua et al. 2011; Luster 1998).

Overall, inflammation contributes to vascular dysfunction by causing an imbalance in mediators of vasoconstriction and vasodilation, as well as by altering the expression of vascular adhesion molecules (I.C.3). ROS also causes vascular dysfunction by decreasing NO availability (I.B.2). Additionally, both ROS and inflammatory cytokines can activate NF- κ B, a key transcription factor that plays a major role in vascular dysfunction (I.C.3).

II. TYPE 2 DIABETES

A. DIABETES: A GLOBAL HEALTH PROBLEM

1. Diabetes mellitus: a brief definition

Diabetes mellitus, often referred to simply as diabetes, is a serious chronic disease characterized by elevated levels of blood glucose due to either the body's inability to create insulin, or the body's inability to effectively use insulin (WHO 2016). Several different types of diabetes exist, and although the categorization system has gone through numerous changes, there is now a general consensus that diabetes can be classified into the following general categories (Hauk 2018):

- Type 1 diabetes: caused by autoimmune destruction of pancreatic β -cells, generally resulting in absolute insulin deficiency
- Type 2 diabetes (T2D): due to a progressive decrease in β -cell function, frequently linked to insulin resistance
- Gestational diabetes mellitus: diabetes diagnosed in the second or third trimester of pregnancy, when there was no overt diabetes prior to gestation

- Specific types of diabetes due to other causes: monogenic diabetes syndromes (including neonatal diabetes and maturity onset diabetes of the young), diseases of the exocrine pancreas (i.e. cystic fibrosis and pancreatitis) and drug- or chemical-induced diabetes

The diagnostic criteria for diabetes are controversial, and have been debated and modified over time. The current diagnostic criteria published by the World Health Organization (WHO) and the American Diabetes Association (ADA) state that diabetes mellitus is diagnosed when one or more of the following indications of elevated blood glucose are met: fasting plasma glucose of ≥ 7.0 mmol/L (126 mg/dL), or two-hour plasma glucose ≥ 11.1 mmol/L (200 mg/dL) following a 75g oral glucose load, or a random plasma glucose ≥ 11.1 mmol/L (200 mg/dL), or HbA1c ≥ 48 mmol/mol (6.5%) (Hauk 2018; WHO 2016).

2. The global burden of diabetes mellitus

Diabetes mellitus is a significant global public health problem. Current epidemiological data show that an estimated 424.9 million people across the globe have diabetes mellitus. Furthermore, rates of diabetes are increasing at alarming rates. If current trends continue, the number of people with diabetes will increase 628.6 million by 2045, which translates to a 48% increase in less than thirty years (Cho et al. 2018).

T2D is by far the most common form of diabetes, accounting for an estimated 90% of all diabetes mellitus cases (Cho et al. 2018). T2D is currently most prevalent in North America and the Caribbean, however African, Middle Eastern, and South-East Asian regions are expected to face the highest increase in prevalence of T2D in the next 30 years (Cho et al. 2018). No interventions currently exist to prevent type

1 diabetes. However, many modifiable risk factors, including unhealthy eating habits and lack of physical activity, contribute to the development of T2D (section II.B.1). Therefore, efforts to decrease the global burden of diabetes are focused on T2D prevention (Cho et al. 2018).

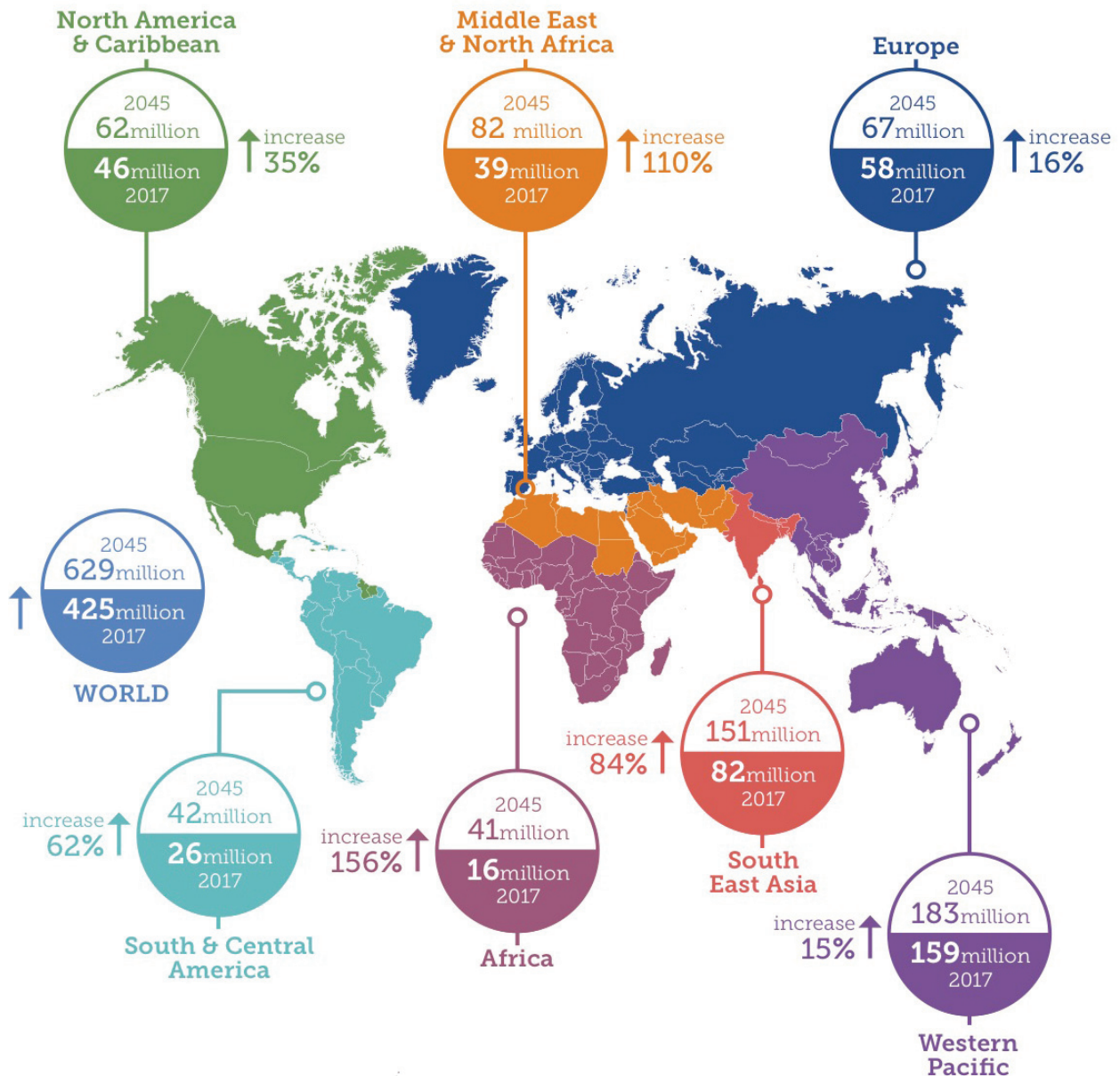


Figure 12: Number of people with diabetes worldwide and per region in 2017 and 2045 (20-79 years). Figure from *IDF Diabetes Atlas 8th Edition*

B. PATHOPHYSIOLOGY OF T2D

1. The interplay of genes and the environment in the development of T2D

Both genes and the environment play integral roles in the pathogenesis of T2D. Twin studies provided the earliest convincing evidence that genes contribute to the development of T2D (Barnett et al. 1981; Medici et al. 1999). Advances in technology enabled the identification of specific genes linked to T2D, such as the peroxisome proliferator-activated receptor gamma (PPARG) gene. PPARG, which codes for a nuclear receptor that regulates adipocyte differentiation, was the first gene identified as playing a role in the pathogenesis of T2D (Deeb et al. 1998). Since this initial discovery, many gene loci associated with T2D, insulin concentrations, glucose concentrations, and especially β -cell function have been identified (Kahn, Cooper, and Del Prato 2014).

Large-scale epidemiological studies have identified several key modifiable risk factors linked to the development of T2D, including diet, physical activity, smoking, overweight/obesity, and alcohol consumption (Hu et al. 2001; Mozaffarian et al. 2009). Results from randomized controlled trials of intensive lifestyle interventions have established the effectiveness of lifestyle modification, including physical activity programs, diets, or both, in preventing or slowing the progression of T2D (Gillies et al. 2007; Howells et al. 2016). Specific dietary factors such as nutrient composition, particularly fat content, and high consumption of sugar-sweetened beverages, have also been shown to increase the risk of developing T2D (Hu, van Dam, and Liu 2001; Imamura et al. 2015).

A growing body of evidence shows that the in-utero environment plays an important role in modulating T2D risk by modifying epigenetic gene expression (Barres and Zierath 2011; Guenard et al. 2013). Epigenetic alterations are heritable alterations in gene expression that are not due to gene sequence, but are dependent on DNA methylation and histone modification. A number of factors, including fetal under-nutrition, over-nutrition, and exposure to hyperglycemia, have been linked to an increased risk of developing obesity and T2D later in life (Chen, Magliano, and Zimmet 2011).

2. Insulin resistance

Insulin is an anabolic peptide hormone synthesized by the β -cells of the pancreas. Under normal conditions, insulin is stored in vacuoles and then secreted postprandially, primarily in response to increased levels of blood glucose (Pessin and Saltiel 2000). Insulin regulates glucose homeostasis by acting on receptors located on target cells throughout the body. The main targets of insulin action are the liver, where insulin promotes glucose storage in the form of glycogen and reduces hepatic glucose output, and the skeletal muscle and adipose tissues, where it increases uptake of glucose, amino acids, and fatty acids. In addition, insulin also exerts pleiotropic effects on β -cells, brain cells, and many other cells (De Meyts 2000).

Insulin-stimulated glucose uptake into the cells occurs when insulin binds to its receptor, located on the membrane of target cells, thereby stimulating the translocation of the glucose transporter GLUT-4 isoform to the cell surface. Insulin also plays a major role in regulating lipid metabolism by decreasing fatty acid release from triglycerides in fat and muscle, and increasing lipid synthesis in liver and fat

cells. Insulin resistance occurs when normal concentrations of insulin are no longer sufficient to effectively regulate these mechanisms (Pessin and Saltiel 2000).

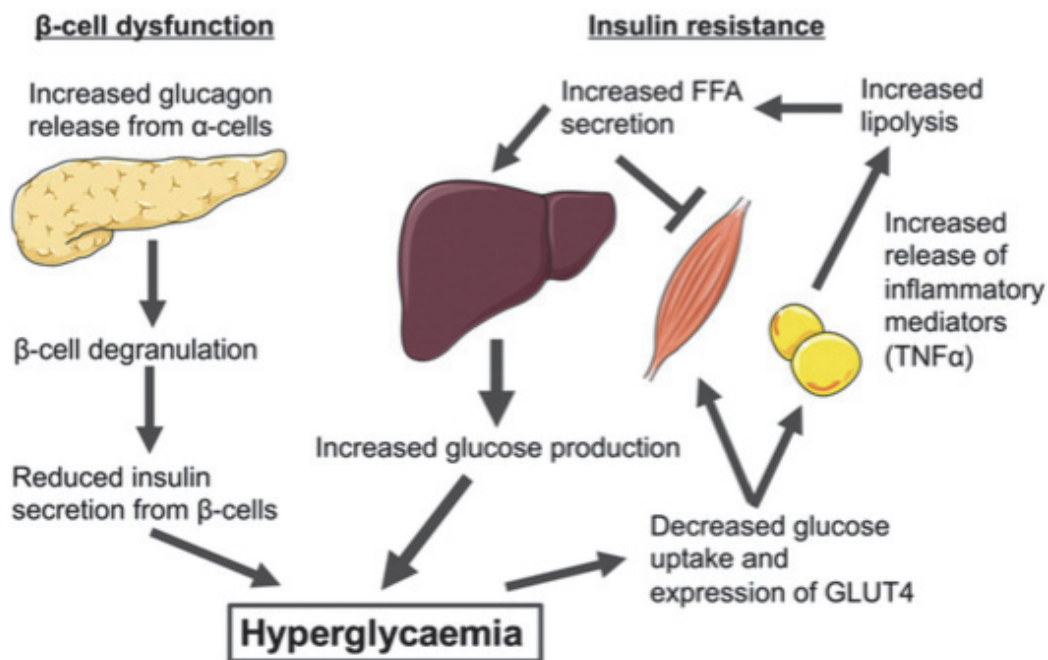


Figure 13: Pathology of T2DM. The amount of insulin released to maintain normal concentrations of glucose is determined by prevailing insulin sensitivity. Initially β -cells compensate for hyperglycemia by increasing insulin release; however, over time this compensatory mechanism fails and reduction in β -cell mass is evident. The reduced plasma insulin results in an increase in glycemia. Furthermore, insulin resistance in the muscle and adipose tissues result in decreased glucose uptake, increased lipolysis, and increased release of inflammatory mediators. Persistent glucose release by the insulin resistant liver preserves the hyperglycemic environment, leading ultimately to T2DM. *Figure from Riddy et al 2017*

Muscle and the liver insulin resistance are core pathologic features of T2D development (DeFronzo 2009). In the liver, insulin resistance results in an overproduction of glucose during the basal state, despite fasting hyperinsulinemia, and impaired attenuation of hepatic glucose production in response to insulin (DeFronzo 2009; DeFronzo, Ferrannini, and Simonson 1989; Groop et al. 1989). Insulin resistance in the muscle results in impaired glucose uptake following a meal, and therefore postprandial hyperglycemia (Ferrannini et al. 1988).

Insulin resistance also has a detrimental effect on adipose tissue. Indeed, in insulin resistant states fat cells are resistant to the antilipolytic effect of insulin, resulting in continued lipolysis and chronically elevated plasma free fatty acid (FFA) concentrations (DeFronzo 2009; Groop et al. 1989; Groop et al. 1991).

3. β -cell dysfunction

β -cells play a crucial role in the maintenance of glucose homeostasis. A feedback loop, relying on crosstalk between β -cells and insulin sensitive tissues, functions to maintain glucose concentrations within a narrow range. Therefore, if insulin resistance is present, β -cells will increase insulin secretion to maintain normoglycemia (Kahn, Cooper, and Del Prato 2014). However, the ability of β -cells to maintain normoglycemia does not indicate that β -cells are functioning normally. Indeed, individuals with impaired glucose tolerance are not only severely insulin resistant, but they have also generally lost over 80% of their β -cell function and approximately half of their β -cell volume (DeFronzo 2009). Many factors contribute to the progression of β -cell dysfunction, including age, genetics, cytokine-induced inflammation, obesity, insulin resistance, lipotoxicity, and glucotoxicity (Cerf 2013). As β -cell function deteriorates, postprandial, and then fasting blood glucose levels rise, eventually leading to the manifestation of overt T2D (DeFronzo 2009).

4. Adipose tissue and inflammation

Obesity has been identified as the major cause of the global T2D epidemic. Mechanisms by which obesity contributes to the development of T2D include increased free fatty acid (FFA) release and altered secretion of adipokines from adipose tissue (Smith and Kahn 2016). Under normal physiological conditions, adipose tissue stores energy as fat and releases lipids and FFA in response to energy needs and fuel availability (Smith and Kahn 2016). In insulin resistant states,

like obesity, adipose cells do not properly respond to the antilipolytic effects of insulin (II.B.2). Consequently, circulating FFA levels are chronically elevated, and thus stimulate gluconeogenesis, cause insulin resistance in the muscle and liver, and contribute to impaired insulin secretion (DeFronzo 2009). These detrimental effects of elevated plasma FFA are referred to as lipotoxicity (DeFronzo 2009).

Lipotoxicity results in the increased production of pro-inflammatory and atherogenic adipokines, which promote insulin resistance, along with the decreased secretion of insulin-sensitizing adipokines by fat cells (Bays, Mandarino, and DeFronzo 2004; Bays et al. 2008). One of the first studies to demonstrate a link between adiposity, inflammation, and insulin resistance was published by Hotamisligil et al in 1993 (Hotamisligil, Shargill, and Spiegelman 1993). In this study, Hotamisligil and colleagues showed that adipocytes constitutively express TNF- α , and that adipocyte TNF- α expression was increased in obese and diabetic animals. However, when TNF- α was neutralized insulin resistance in these animals was improved (Hotamisligil, Shargill, and Spiegelman 1993). Further work from cross-sectional and prospective studies confirmed that obesity is a state of chronic inflammation, associated with elevated plasma concentrations of TNF- α (Dandona et al. 1998; Kern et al. 1995; Mantzoros et al. 1997) as well as CRP (Yudkin et al. 1999), IL-6 (Mohamed-Ali et al. 1997), and PAI-1 (Lundgren et al. 1996). The release of these pro-inflammatory factors can promote inflammation in other tissues, including the islets, and contribute to insulin resistance (Dandona, Aljada, and Bandyopadhyay 2004). Adipose tissue can also contribute to the pathogenesis of T2D when the capacity of adipocytes to store lipids is exceeded. Indeed in obesity, adipose cells are “stored” in the muscle, liver, and β -cells, thereby contributing to

muscle and hepatic insulin resistance as well as impaired insulin secretion (Bays, Mandarino, and DeFronzo 2004; Bays et al. 2008).

C. DIABETES MELLITUS AS A VASCULAR DISEASE

1. Evidence of vascular dysfunction in type 2 diabetes

The metabolic abnormalities associated with diabetes mellitus cause vascular injury, and eventually lead to the development of vascular complications (Prenner and Chirinos 2015; Xu and Zou 2009). Therefore, T2D is not only considered to be a metabolic disorder, but also a cause of vascular disease (Beckman and Creager 2016). The development of vascular dysfunction is an integral step leading to the pathogenesis of vascular complications in individuals with T2D (Beckman and Creager 2016) (Prenner and Chirinos 2015). Indeed, an enormous body of research demonstrates that diabetes is associated with impaired vascular reactivity in both animals and humans, as well as with increased arterial stiffness (De Vriese et al. 2000; Prenner and Chirinos 2015; Xu and Zou 2009).

a. Human studies

A large number of studies have shown that both endothelium-dependent and -independent vasodilation is impaired in individuals with T2D (Montero et al. 2013). Common non-invasive techniques used to assess endothelium-dependent vascular function include laser Doppler flowmetry paired with iontophoresis of vasoactive molecules, plethysmography, and flow-mediated dilation (FMD) using Doppler ultrasound (Jansson 2007).

Iontophoresis is based on the principle that charged molecules of drugs in a solution will migrate across the skin when under the influence of an applied electric current (Chien and Banga 1989). Acetylcholine is the standard drug used to evaluate endothelium-dependent vasodilation, although other drugs, including bradykinin,

methacholine, and substance P, can also be used (Morris, Shore, and Tooke 1995). Acetylcholine mediates vasodilation via the endothelium-dependent production of NO and/or prostanoids, and possibly EDHF (Turner, Belch, and Khan 2008). Sodium nitroprusside is an NO donor that stimulates VSMC relaxation, and is commonly used to measure endothelium-independent vasodilation (Turner, Belch, and Khan 2008). Studies using laser Doppler flowmetry to measure changes in skin blood flow have observed attenuated responses to iontophoretic delivery of acetylcholine and sodium nitroprusside in individuals with T2D, indicating that both endothelium-dependent and -independent vasodilation are impaired in the microvasculature of humans with T2D (Brooks et al. 2008; Morris, Shore, and Tooke 1995; Nitenberg et al. 1993).

Plethysmography is commonly used in combination with local brachial infusion of vasoactive drugs, like acetylcholine or other muscarinic receptor agonists, as well as with sodium nitroprusside, to assess endothelium-dependent and -independent vasodilator responses in forearm resistance vessels (Flammer et al. 2012; Jansson 2007). Studies conducted in individuals with T2D using plethysmography have consistently shown that endothelium-dependent vasodilation is impaired, and have tended to show that VSMC function is impaired, in resistance vessel of individuals with T2D (McVeigh et al. 1992; van de Ree et al. 2001; Williams et al. 1996).

FMD is a simple, non-invasive technique that reflects endothelium-dependent vasodilation by measuring changes in conduit artery diameter caused by the shear stress-induced production of endothelial-derived vasodilators (predominantly NO) following arterial occlusion (Raitakari and Celermajer 2000). Multiple studies using FMD and either sodium nitroprusside or nitrate-mediated dilation of the brachial

artery have observed impaired endothelial and VSMC function in individuals with T2D (Bruno et al. 2012; Doupis et al. 2011; Henry et al. 2004; Karabag et al. 2007; Montero et al. 2013). Several factors could possibly contribute to the impaired endothelium-independent vasodilation observed in individuals with T2D. For example, the reduced vasodilation could be related to changes in baseline vasomotor tone (Schachinger and Zeiher 1995). Additionally, functional changes in the VSMC could result in decreased activity of intracellular guanylate cyclase, cyclic GMP, or calcium-dependent relaxation (Adams et al. 1998).

Elevated arterial stiffness has been shown to be associated with cardiovascular risk in middle aged and older people (Mitchell et al. 2010). Arterial stiffness, or the loss of vascular compliance, occurs as a consequence of pathophysiological changes in endothelial cells, VSMC, the ECM, and other elements of the vascular wall (Jia et al. 2015). Three general non-invasive approaches can be used to measure arterial stiffness: analysis of pulse transit time, evaluation of wave contour of arterial pulse, and direct measure of arterial geometry and pressure (Pannier et al. 2002). The gold standard for non-invasive measurement of arterial stiffness is pulse wave velocity (PWV), which reflects the velocity with which the pulse wave travels through the arterial tree (Mancia et al. 2014). Although PWV can be measured in any segment of the vasculature, both the American Heart Association and a European expert consensus recommend using carotid-femoral PWV (Laurent et al. 2006; Townsend et al. 2015).

Multiple studies have observed elevated PWV in individuals with T2D, compared to controls, in different segments of the vasculature, including the carotid-femoral, carotid-radial, aortic-finger, heart-brachial, heart-femoral, and femoral-ankle segments (Cameron et al. 2003; Cruickshank et al. 2002; Kimoto et al. 2006; Safar,

Czernichow, and Blacher 2006). Additionally, studies show that deteriorating glucose tolerance and fasting glucose are associated with central and peripheral PWV in individuals with and without T2D (Ohnishi et al. 2003; Schram et al. 2004). Evidence suggests that elevated AGE concentrations (Semba et al. 2009) and altered NO bioavailability (Brillante, O'Sullivan, and Howes 2009) likely contribute to arterial stiffness in T2D.

It is important to recognize that not all studies show an independent association between T2D and arterial stiffness. Indeed, a systematic review conducted by Cecelja et al. showed that carotid-femoral PWV was independently associated with diabetes in only 52% of studies (Cecelja and Chowienczyk 2009). Thus it appears that the close association between T2D and hypertension could possibly obscure the relationship between T2D and arterial stiffness (Prenner and Chirinos 2015). Furthermore, one study has shown that T2D is associated with greater arterial stiffness in women, but not necessarily in men, and this difference was not explained by hypertension (De Angelis et al. 2004). Therefore, it is possible that the relationship between T2D and arterial stiffness may vary between sexes.

b. Animal Studies

The large majority of studies conducted in rodent models of insulin dependent diabetes mellitus have observed impaired endothelium-dependent vasodilation *ex-vivo* in isolated conduit vessels and isolated organs (Bourgoin et al. 2008; De Vriese et al. 2000; Sallam et al. 2011). Most animal studies that have evaluated microvascular function *in-vivo* have been conducted in animals treated with streptozotocin or alloxan, and have observed impaired acetylcholine-mediated endothelium-dependent vasodilation, with preserved sodium nitroprusside-mediated endothelium-independent vasodilation (Sigaud-Roussel et al. 2004; Demiot et al. 2006), (De

Vriese et al. 2000)). It may be important to consider that both streptozotocin and alloxan cause increased oxidative stress, altered cellular metabolism, and tissue damage, which could potentially contribute to the endothelial dysfunction in these animal models (Nahdi, John, and Raza 2017; Pately, Srivastava, and Kohli 2013).

Interestingly, a recent study conducted by Nguyen-Tu and colleagues observed impaired acetylcholine-mediated vasodilation *in-vivo* in the microvasculature of mice fed a high calorie diet for two or four weeks (after the development of obesity, but before the development of hyperglycemia) (Nguyen-Tu et al. 2018). However, after 12-weeks on a high calorie diet, the mice had developed obesity-related T2D and demonstrated an enhanced, compensatory vasodilatory response to iontophoresis of acetylcholine that was dependent on COX-2. Therefore, the authors suggested that these obese T2D mice had developed an adaptive vasodilatory response involving a sequential switch from NO-dependent to COX-2 dependent mechanisms (Nguyen-Tu et al. 2018).

2. Microvascular complications

The microvascular dysfunction associated with T2D contributes to the development of microvascular disease (Sena, Pereira, and Seica 2013). The three major manifestations of microvascular disease in diabetes are diabetic retinopathy, nephropathy, and neuropathy (Beckman and Creager 2016). A study by Brooks et al measured vascular function in individuals with T2D with and without microvascular complications using iontophoresis of acetylcholine and sodium nitroprusside. The results showed that abnormalities in vascular blood flow are evident in individuals with T2D, and they become more marked as microvascular complications develop (Brooks et al. 2008). Additionally, a review of the literature on arterial stiffness in individuals with T2D showed that large artery stiffness is correlated with the

development of microvascular organ damage, including retinopathy, nephropathy, and neuropathy (Prenner and Chirinos 2015).

a. Retinopathy

Diabetic retinopathy affects an estimated one third of individuals with diabetes, and is the leading cause of vision loss in adults between the ages of 20-65 (Cho et al. 2018). The pathogenesis of diabetic retinopathy is linked to both endothelial dysfunction and arterial stiffness (Targher et al. 2008; Malecki et al. 2008; Juutilainen et al. 2007). The earliest manifestation of diabetic retinopathy is the loss of the pericyte cells that wrap around arterial and capillary endothelial cells, and serve to protect against oxidative stress, maintain permeability, and regulate growth (Beckman and Creager 2016). This initial step is then followed by basement membrane thickening, increased endothelial permeability, and the formation of micro-aneurysms (Beckman and Creager 2016; Cho, Mooney, and Cho 2008). Diabetic retinopathy is generally classified as either non-proliferative retinopathy, or proliferative retinopathy, which is characterized by retinal neovascularization, possible vitreous hemorrhage, and potential vision loss (Nentwich and Ulbig 2015).

The United Kingdom Prospective Diabetes Study confirmed the importance of hyperglycemia in the development and progression of diabetic retinopathy in individuals with T2D (Stratton et al. 2001). Numerous possible inter-related biological pathways have been proposed as possible links between hyperglycemia and the development of diabetic retinopathy including polyol pathway flux, activation of the diacylglycerol (DAG)-Protein kinase C (PKC) pathway, increased expression of growth factors like vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), hemodynamic changes, accelerated formation of AGE, oxidative

stress, activation of the renin-angiotensin-aldosterone system, and inflammation (Tarr et al. 2013).

b. Nephropathy

Diabetic nephropathy affects 20-40% of people with diabetes, and is the leading cause of end-stage renal disease (Gallagher and Suckling 2016). Diabetic kidney disease is characterized by progressive kidney damage, reflected by albuminuria (>300 mg/24 h, or > 300 mg/g creatinine) and reduced renal function (decline in glomerular filtration rate). These symptoms are accompanied by elevated blood pressure and increased morbidity and mortality due to cardiovascular complications (Rossing, Persson, and Frimodt-Moller 2018). A study by Schmiedel et al showed that microalbuminuria in T2D is associated with impaired endothelium-dependent microvascular function, and therefore suggested that endothelial dysfunction could contribute to the progression of diabetic nephropathy (Schmiedel, Schroeter, and Harvey 2007). Furthermore, a number of studies show that arterial stiffness is associated with kidney damage. Indeed, a study by Smith et al showed higher carotid-femoral PWV in patients with T2D and elevated albumin to creatinine ratio and/or lower glomerular filtration rate compared to subjects with T2D and normal renal function (Smith et al. 2005). Additionally, Ishimura and colleagues demonstrated a direct relationship between PWV and albuminuria, even after adjusting for blood pressure (Ishimura et al. 2007). Another study by Aoun et al showed that decreased GFR over time is associated with increased aortic stiffness (Aoun et al. 2001).

The pathophysiology of diabetic nephropathy is similar to that of diabetic retinopathy as it includes basement membrane thickening and the development of micro-aneurysms. Furthermore, glomerular hyperfiltration is associated with

expansion of the ECM, and the progression of tubular and glomerular sclerosis (Beckman and Creager 2016). The pathogenesis of diabetic nephropathy is complex, and attributed to the interaction of altered metabolic pathways (including increased AGE accumulation and activation of the polyol pathway) and hemodynamic pathways (including the renin-aldosterone system). These changes result in increased ROS generation, modified gene regulation, the activation of transcription factors, and the up-regulation of growth factors and inflammatory cytokines. These alterations cause the structural changes (altered permeability, filtration, and reabsorption) and functional changes (including podocytopathy, extracellular protein production, and glomerulosclerosis) that are characteristic of kidney damage (Cao and Cooper 2011).

c. Neuropathy

Diabetic neuropathy, one of the most common chronic complications associated with diabetes, is a heterogeneous group of conditions that affect different parts of the nervous system (Pop-Busui et al. 2017). Out of the different forms of diabetic neuropathy, distal symmetric polyneuropathy and diabetic autonomic neuropathies are the best characterized (Pop-Busui et al. 2017). The pathogenesis of diabetic nephropathy is due to both vascular and nonvascular abnormalities. As in retinopathy and nephropathy, the development of neuropathy is associated with basement membrane thickening and pericyte loss (Beckman and Creager 2016). These changes are coupled with decreased capillary blood flow to c-fibers, resulting in impaired nerve perfusion and endoneurial hypoxia, with subsequent axonal thickening and eventual nerve loss (Beckman and Creager 2016). Evidence suggests that impaired microvascular function could be associated with diabetic peripheral neuropathy (Sun 2013 *Diab Vasc Dis Res*). Furthermore, PWV has also

been associated with peripheral neuropathy in subjects with T2D (Ha et al. 2012; Yokoyama et al. 2007).

3. Macrovascular complications

Diabetes increases the risk of cardiovascular morbidity and mortality (Resnick et al. 2001). Indeed, compared to the general population, people with diabetes have a two-fold higher risk of having cardiovascular disease, a group of disorders of the heart and blood vessels that includes coronary heart disease, cerebrovascular disease, peripheral arterial disease, deep vein thrombosis, and pulmonary embolism (Sarwar et al. 2010). Results of the Hoorn study, a prospective population-based cohort study, showed that individuals with T2D are particularly sensitive to the adverse effects of endothelial dysfunction, and that this could explain the elevated cardiovascular risk seen in T2D (van Sloten et al. 2014).

Atherosclerosis is the primary mechanism involved in the pathogenesis of macrovascular disease in individuals with diabetes (Fowler 2008). The arteries of individuals with diabetes are prone to atherosclerosis due to the chronic hyperglycemia, dyslipidemia, and insulin resistance associated with the disease. These metabolic abnormalities cause oxidative stress and inflammation, with subsequent endothelial cell dysfunction, VSMC dysfunction, impaired platelet function, and abnormal coagulation, which all contribute to an increased risk of cardiovascular disease (Beckman and Creager 2016).

Arterial stiffness, or loss of arterial elasticity, is a strong predictor of cardiovascular risk (Shirwany and Zou 2010; Vlachopoulos, Aznaouridis, and Stefanadis 2010). Multiple studies have shown that PWV is elevated in individuals with T2D compared to controls without T2D, indicating that accelerated arterial

stiffening could contribute to the increased cardiovascular disease risk in T2D (Cruickshank et al. 2002; Kimoto et al. 2006).

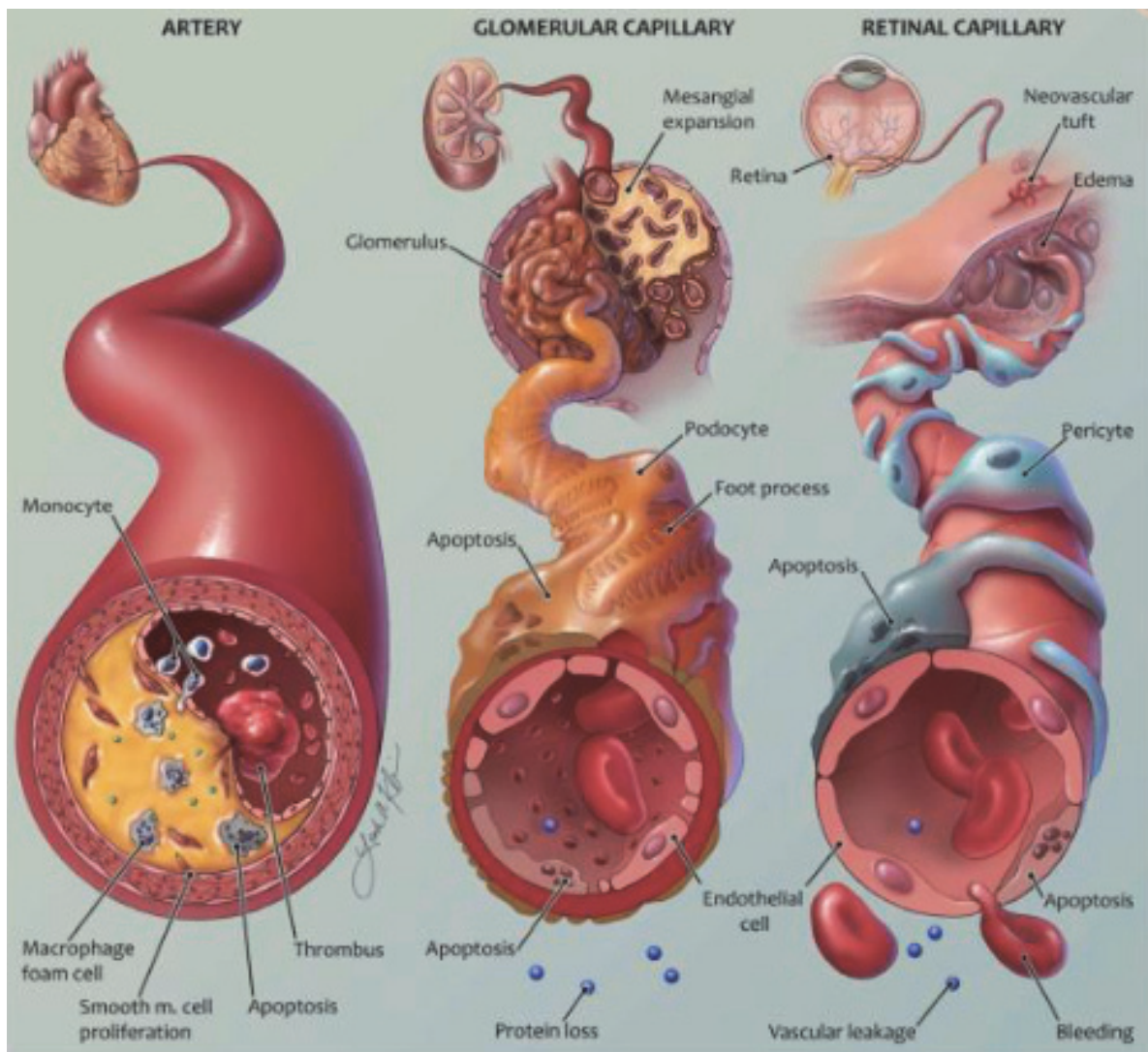


Figure 14: Pathological changes related to the development of vascular T2D-related vascular complications. Pathological changes in a coronary artery (left) and in glomerular and retinal capillaries (middle and right, respectively) in diabetes. Some of the main features are accumulation of lipid-laden macrophages in the atherosclerotic plaque and subsequent macrophage apoptosis (left); podocyte apoptosis, thickening of the glomerular basement membrane, and breakdown of the filtration barrier in the renal glomeruli (middle); and pericyte and endothelial cell apoptosis, vascular leakage, and hemorrhage in the retina (right). *Figure and text from Rask-Madsen 2013 Cell Metab - Artwork by Leah A. Klein.*

D. CAUSES OF VASCULAR DYSFUNCTION IN T2D

1. Hyperglycemia and vascular dysfunction

One of the primary causes of vascular dysfunction in T2D is hyperglycemia. Both chronic and acute hyperglycemia have been shown to impair endothelium-dependent vasodilation in macro- and micro-vascular beds in studies conducted in animal models and in human subjects (Brouwers et al. 2010; Han et al. 2011; Kito et al. 2018; Williams et al. 1998). Furthermore, results from randomized control trials show that intensive glycemic control can reduce the risk of microvascular complications in individuals with T2D (1998; 1998; Ohkubo et al. 1995). One of the most notable studies to confirm the relationship between hyperglycemia and microvascular disease was the UK Prospective Diabetes Study, which showed that intensive glycemic control (median HbA1c of 7%) reduced the overall microvascular complication rate by 25% compared to conventional treatment (median HbA1c of 7.9%) (King, Peacock, and Donnelly 1999). Interestingly, two large cohort studies, the ADVANCE (Patel et al. 2008) and the VA Diabetes Trials (Duckworth et al. 2009), have shown that intensive glycemic control results in no significant reduction in cardiovascular risk, while a third, the ACCORD study, showed increased mortality and cardiovascular death in the intensive glycemic control arm compared to the standard glycemic control arm (Gerstein et al. 2008) (Skyler et al. 2009). One possible explanation for the findings of the ACCORD study is that intensive insulin therapy in highly insulin resistant individuals can over-stimulate the insulin pathways that are not affected by insulin resistance, resulting in the activation of pro-atherogenic pathways (II.D.2) (Giacco and Brownlee 2010).

Increased mitochondrial $O_2^{\cdot -}$ production in endothelial cells has been identified as a mechanism by which hyperglycemia causes vascular damage

(Brownlee 2001). Indeed, elevated levels of circulating glucose result in increased production of $O_2^{\cdot-}$ by the mitochondrial electron transport chain. This hyperglycemia-induced $O_2^{\cdot-}$ overproduction causes DNA damage and the activation of the enzyme poly ADP ribose polymerase (PARP). Consequently, the production of polymers of ADP-ribose increases, thereby reducing glyceraldehyde 3-phosphate dehydrogenase activity. Ultimately, levels of all upstream glycolytic intermediates increase, resulting in the activation of five damaging pathways that contribute to vascular injury by increasing oxidative stress and promoting inflammation, vascular permeability, and apoptosis (Figure 15) (Brownlee 2001):

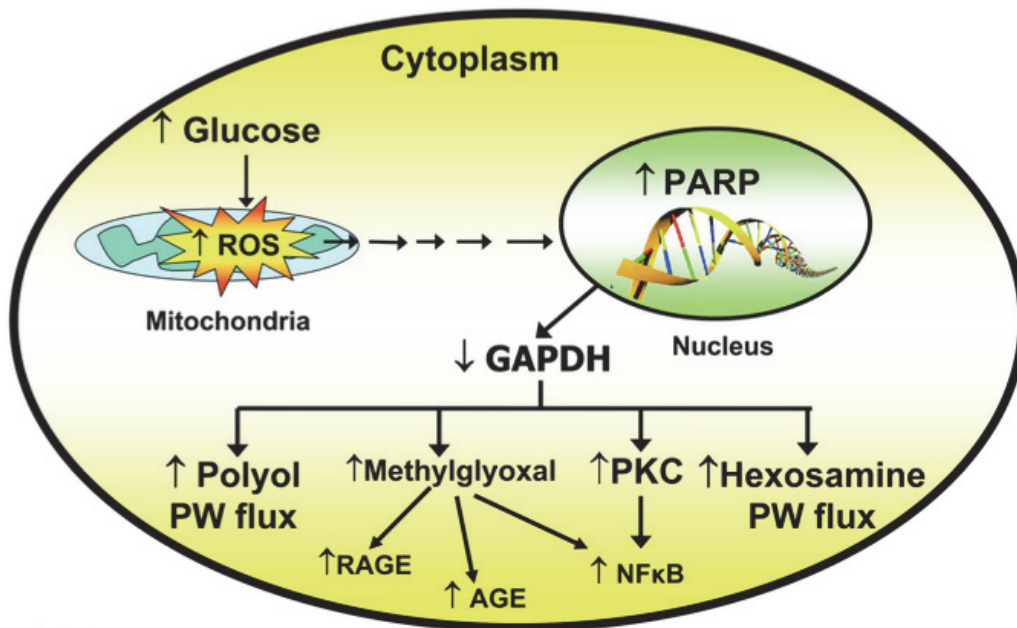


Figure 15: A schematic representation of the hyperglycemia-induced pathways that contribute to vascular injury in type 2 diabetes. *Figure from Brownlee 2010*

1) Increased activation of the polyol pathway: The polyol pathway reduces glucose to sorbitol, but in the process uses NADPH, which is a cofactor required to regenerate GSH. Therefore, increased polyol pathway flux can lead to increased redox stress due to the consumption of NADPH, and the consequent inability to regenerate GSH (Giacco and Brownlee 2010).

2) Increased advanced glycation end-product (AGE) production: AGEs are products of non-enzymatic reactions between the amino groups of proteins and carbonyl groups of sugars, formed via the Maillard process (Yamagishi 2008). This process begins with the conversion of reversible Schiff base adducts to more stable, Amadori products that are covalently bound. These Amadori products then undergo further rearrangements and reactions over the course of days and weeks to form irreversible AGE (Yamagishi 2008). AGE production is a normal process that occurs with ageing, but these reactions are accelerated in T2D, and contribute to the pathogenesis of vascular complications (Brownlee 1988 *N Engl J Med*). Increased AGE production can cause vascular damage in several ways. First, AGE can modify the intracellular proteins involved in the regulation of gene transcription. Additionally, AGE can modify ECM proteins, which can increase arterial stiffness and alter signaling between the matrix and the cell, causing cellular dysfunction. Finally, AGE can bind to and activate RAGE. (Schaffer, Jong, and Mozaffari 2012; Yamagishi et al. 2012)

3) Increased expression of the receptor for AGE (RAGE): RAGE is a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules. Although originally identified as a receptor for AGE, it is now recognized that RAGE also binds with S100/calgranulins, HMGB1-proteins, amyloid- β peptides, and β -sheet fibrils (Bierhaus et al. 2005). RAGE activation results in sustained dysfunction and tissue destruction (Bierhaus et al. 2005). Engagement of RAGE produces ROS, which subsequently up-regulates the activation of transcription factors, such as NF- κ B, which

promotes the expression of adhesion molecules and growth factors (Yamagishi et al. 2012).

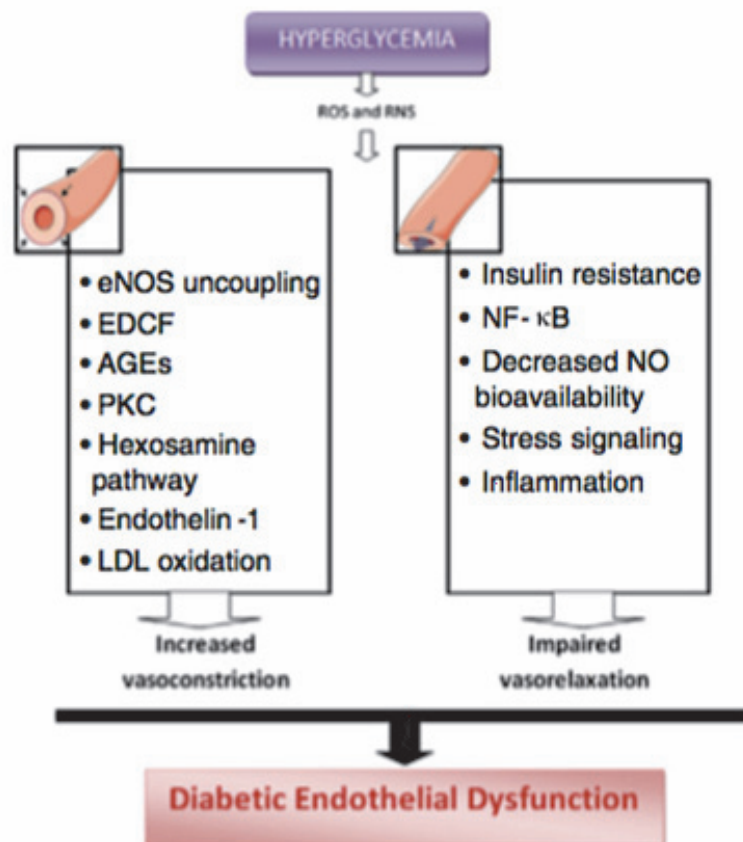


Figure 16: Endothelial dysfunction in diabetes. Prolonged exposure to hyperglycemia results in oxidative stress, which stimulates a variety of mechanisms resulting in increased vasoconstriction and impaired vasorelaxation, ultimately leading to endothelium dysfunction. AGEs, advanced glycation end products; EDCF, endothelium-derived contracting factors; eNOS, endothelial nitric oxide synthase; FFAs, free fatty acids; PKC, protein kinase C; PGIS, prostacyclin synthase; NF-κB, nuclear factor-kappa B; NO, nitric oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species. *Figure from Sena 2013*

4) Activation of protein kinase C (PKC) isoforms: PKC are a family of eleven isoforms that are expressed in a wide variety of tissues. Nine of the eleven PKC isoforms are activated by DAG, a lipid second messenger that is increased in hyperglycemia (Giacco and Brownlee 2010). An important effect of PKC activation is increased ROS production, particularly increased $O_2^{\bullet-}$ generation via NOX. PKC-mediated ROS production results in alterations in

cellular membrane permeability, vascular inflammation, angiogenesis, cell growth, ECM expansion, and apoptosis. PKC also decreases eNOS activity, increases ET-1, upregulates the production of COX-2, and reduces production of prostacyclin (Paneni et al. 2013).

5) Over-activity of the hexosamine pathway: Increased activity of the hexosamine pathway can up-regulate the expression of genes that code for proteins that cause vascular damage, such as growth factor- β 1 and PAI-1. Hexosamine can also inhibit eNOS activity (Giacco and Brownlee 2010).

2. Insulin resistance

Insulin signaling is dependent on two major pathways. The PI3K pathway mediates the metabolic and hemodynamic effects of insulin, whereas the MAPK pathway regulates gene expression, cell growth, and differentiation. Under normal conditions, insulin participates in the maintenance of normal endothelial function by stimulating NO production via activation of the PI3K pathway, which results in the serine phosphorylation of eNOS (Giacco and Brownlee 2010). However, the PI3K pathway is impaired in insulin resistant states, and therefore NO production is decreased (Xu and Zou 2009). On the other hand, the MAPK pathway is unaffected by insulin resistance. Thus, the increasing levels of insulin, secreted to overcome hyperglycemia, result in an overdrive of the non-insulin resistant MAPK pathway. In endothelial cells, activation of MAPK results in ET-1 production and the overexpression of PAI-1 and adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin. Therefore, insulin resistance promotes a dysfunctional vascular state in which NO production is decreased, while the generation of the ET-1 and cell adhesion molecules are increased. Furthermore, insulin resistance causes excessive

release of FFA from adipose tissues, which contributes to vascular damage via multiple mechanisms (Muniyappa and Sowers 2013).

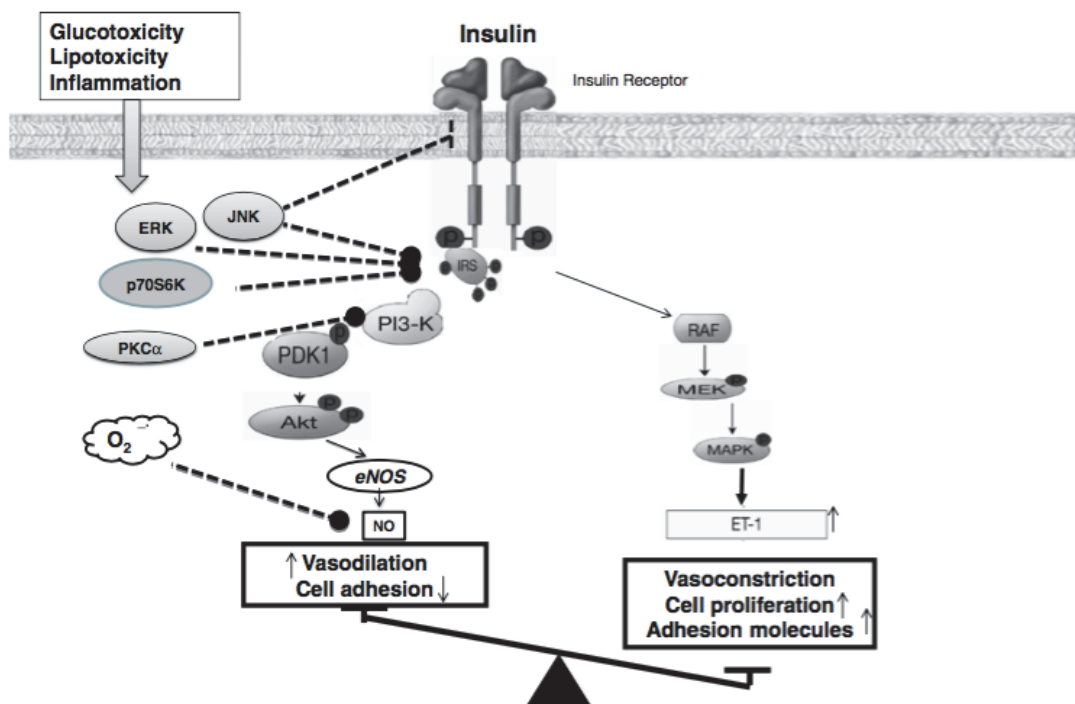


Figure 17: Pathway-specific impairment of insulin signaling pathway and endothelial dysfunction. PI3-kinase branch of insulin signaling regulates NO production and vasodilation in vascular endothelium. MAP-kinase branch of insulin signaling controls secretion of endothelin-1 (ET-1) and adhesion molecule expression in vascular endothelium. Glucotoxicity, lipotoxicity, and various cytokines activate signaling molecules that inhibit PI3K/Akt signaling. eNOS, endothelial nitric oxide synthase; IRS, insulin receptor substrate; MEK, MAPK kinase; PDK, phosphoinositide-dependent protein kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; JNK, C-Jun N-terminal kinase; p70S6K, p70 ribosomal S6 kinase; AP-1, activator protein-1; NO, nitric oxide; and ET-1, endothelin-1. *Figure and text from Muniyappa 2013*

3. Free fatty acids

Elevated levels of circulating FFAs in T2D cause lipotoxicity (II.B.4), which contributes to vascular dysfunction. Indeed, acute infusion of FFAs results in reduced endothelium-dependent vasodilation (Boden et al. 2005; Steinberg et al. 1997). One of the primary ways by which FFAs cause vascular damage is via ROS production. FFAs increase O₂^{•-} production through mitochondrial ETC uncoupling, and by up-regulating the expression and protein content of NOX (Giacco and

Brownlee 2010), (Inoguchi et al. 2000). This increased ROS production activates the same five detrimental pathways as hyperglycemia-induced ROS, including increased activation of the polyol pathway, activation of PKC, stimulation of the hexosamine pathway, AGE formation, and RAGE expression (II.D.1) (Giacco and Brownlee 2010), (Inoguchi et al. 2000).

The FFA-induced $O_2^{\cdot-}$ overproduction can contribute to vascular dysfunction in several other ways. For example, the ROS produced by FFA can inactivate prostacyclin synthase and eNOS, two anti-atherogenic enzymes (Sena, Pereira, and Seica 2013). Additionally, FFA-induced ROS can stimulate the generation of pro-inflammatory cytokines via the activation of NF- κ B (Boden et al. 2005; Jove et al. 2006). Overall, FFAs contribute to vascular dysfunction by increasing ROS production, endothelial and monocyte activation, and inflammation, as well as reducing NO availability, and causing endothelial cell apoptosis (Sena, Pereira, and Seica 2013).

E. BLOOD RHEOLOGY IN TYPE 2 DIABETES

1. Blood rheology and its parameters: a brief summary

Whole blood is a two-phase liquid, composed of cellular elements (including red blood cells, leukocytes, and platelets) suspended in plasma, an aqueous solution containing organic molecules, proteins, and salts (Baskurt and Meiselman 2003). Red blood cells are the most important constituents of the cellular phase of blood. White blood cells and platelets can affect blood rheology, but under normal conditions they have a much smaller influence than red blood cells (Pop et al. 2002).

a. Whole blood viscosity

The resistance of a fluid to flow is referred to as viscosity. The viscosity of a fluid is calculated by dividing shear stress by shear rate (Baskurt and Meiselman

2003; Pop et al. 2002). Blood is considered a non-Newtonian fluid because its viscosity is dependent on variations in shear forces (Somer and Meiselman 1993; Baskurt and Meiselman 2003). Indeed, whole blood viscosity decreases as shear rate increases (Baskurt and Meiselman 2003; Pop et al. 2002). This shear-thinning characteristic of blood is attributed to the response of red blood cells to changes in shear (Baskurt and Meiselman 2003; Pop et al. 2002). At low shear rates, red blood cells aggregate, resulting in increased blood viscosity (Baskurt and Meiselman 2003; Pop et al. 2002). However, when shear rate increases red blood cell aggregates disperse. Therefore, at low shear rate whole blood viscosity is highly dependent on red blood cell aggregation, but as shear rate increases erythrocyte deformability plays a more important role (Schmid-Schonbein, Wells, and Goldstone 1969).

In addition to red blood cell deformability and aggregation, hematocrit and plasma viscosity also affect whole blood viscosity (Baskurt and Meiselman 2003). The viscosity of plasma is dependent on the concentration of plasma proteins, including fibrinogen, globulin, albumin, and lipoprotein (Baskurt and Meiselman 2003). Therefore, as the concentration of plasma proteins rises, whole blood viscosity increases. Furthermore, whole blood viscosity is dependent on the number of erythrocytes in the blood, and is thus linearly related to hematocrit (Chien et al. 1975). At high shear rate, a rise of hematocrit of one point increases blood viscosity by 4% (Baskurt, Hardeman, and Rampling 2007).

b. Red blood cell deformability

Under normal physiological conditions erythrocytes are biconcave, highly deformable disks (Baskurt and Meiselman 2003). The average erythrocyte has a diameter of 8 μ m, which exceeds the diameter of the smallest capillaries (Cho, Mooney, and Cho 2008). Therefore, erythrocyte deformability is crucial to facilitating

effective blood flow through the capillaries (Cho, Mooney, and Cho 2008). The foundations of our understanding of red blood cell deformability are based on observations of red blood cells that were carried out in the 1970s and 1980s. These experiments observed red blood cells in microtubes (Goldsmith, Marlow, and MacIntosh 1972) and rheoscopes (Fischer, Stohr-Lissen, and Schmid-Schonbein 1978), and found that at low shear stresses (less than 0.05 Pa) individual red blood cells behave like rigid disks and “flip like a coin” (Goldsmith, Marlow, and MacIntosh 1972; Fischer, Stohr-Lissen, and Schmid-Schonbein 1978). Then, as shear stress increases, the red blood cells align with the direction of flow by deforming into an elliptical shape via a “tank tread-like” motion of the cell membrane around the cytoplasm, thereby enabling smooth flow (Schmid-Schonbein, Wells, and Goldstone 1969; Goldsmith, Marlow, and MacIntosh 1972; Fischer, Stohr-Lissen, and Schmid-Schonbein 1978). In these fundamental experiments red blood cells were suspended in a solution, such as dextrose, that has a higher viscosity than the internal viscosity of a red blood cell (Goldsmith, Marlow, and MacIntosh 1972; Fischer, Stohr-Lissen, and Schmid-Schonbein 1978). However, *in-vivo* (when the blood cell is flowing in plasma), plasma viscosity is lower than the viscosity of the erythrocyte’s cytosol. This is important because recent experiments reveal that the “tank-treading” behavior of erythrocytes does not occur when red blood cells are in dilute suspensions with lower viscosities that are similar to plasma (Dupire, Socol, and Viallat 2012). Instead, observations by Lanotte et al show that red blood cells display a wide variety of cell shapes for any given flow condition. Indeed, erythrocytes in dilute suspensions behave like rigid oblate ellipsoids at low shear rates ($<1 \text{ s}^{-1}$). Then, as shear rates increase, the erythrocytes successively tumble, roll, deform into stomatocytes, and eventually adopt highly deformed poly-lobed shapes (Lanotte et al. 2016). The

findings of Lanotte and colleagues suggest that the pathological alterations of multiple parameters, including plasma composition, erythrocyte cytosol viscosity, and/or membrane mechanical properties could contribute to pathological blood rheology and flow (Lanotte et al. 2016).

Red blood cell deformability is dependent on the viscosity of the cytoplasm (largely determined by the concentration of hemoglobin), the rheological properties of the membrane (membrane shear modulus), and the maintenance of the cell's bio-concave discoid geometry (i.e. the surface to volume ratio) (Chien 1987), (Baskurt and Meiselman 2003). Over or under hydration of the cell alters the surface to volume ratio of the red blood cell, thereby decreasing its deformability (Baskurt and Meiselman 2003). Furthermore, membrane protein cross linkages caused by oxidative alterations of the cell wall can increase erythrocyte rigidity (Baskurt and Meiselman 2003; Cho, Mooney, and Cho 2008). Decreased red blood cell deformability impairs blood flow in the microcirculation, and causes a rise of whole blood viscosity in large arteries (Baskurt and Meiselman 2003).

c. Red blood cell aggregation

Red blood cell aggregation is the reversible formation of stacks of erythrocytes, known as rouleaux, which takes place at low shear rates (Figure 18) (Chien and Sung 1987). Erythrocyte aggregates disperse when shear rate increases, but reform when shear forces are removed (Chien and Sung 1987). For this reason, red blood cell aggregates form most readily in areas where blood moves slowly, such as in the veins, or in bifurcations (Chien and Sung 1987). Although blood flows slowly through capillaries, red blood cell aggregates do not generally form because capillary diameters are only large enough to allow single cells to flow through.

However, red blood cell clusters can form in capillaries as a result of both hydrodynamic and macromolecule-induced interactions (Claveria et al. 2016).

Red blood cell aggregation is largely dependent on fibrous plasma proteins, of which fibrinogen plays the most important role (Rampling 1988). Therefore, red blood cell aggregation is increased in pathological states in which fibrinogen and other acute phase proteins, including CRP, serum amyloid A, haptoglobin, and ceruloplasmin, are elevated (Baskurt and Meiselman 2003). Elevations in red blood cell aggregation result in a rise in blood viscosity at low shear rates, as aggregation is the main determinant of low shear blood viscosity (Baskurt and Meiselman 2013).

This phenomenon would lead to the prediction that increased red blood cell aggregation results in decreased tissue blood flow, however this is not necessarily the case. Indeed, red blood cell aggregation exerts an important effect on multiple hemodynamic mechanisms, such as plasma skimming, the Fahraeus Effect, and microvascular hematocrit, and can thus actually improve blood flow under certain conditions (Baskurt and Meiselman 2008, 2013). Furthermore, a study by Yalcin et al showed that elevated erythrocyte aggregation related to alterations in red blood cell properties, but not changes in plasma composition decreased the production of NO metabolites and reduced eNOS phosphorylation (Yalcin et al. 2008). On the other hand elevated erythrocyte aggregation coupled with increased plasma viscosity did not alter NO production, and only insignificantly decreased eNOS phosphorylation. These results suggest that different mechanisms of alterations in erythrocyte aggregation could have different effects on shear stress-mediated NO production by endothelial cells (Yalcin et al. 2008). Ultimately, whether or not enhanced red blood cell aggregation increases or decreases tissue blood flow depends on a large variety of factors including, but not limited to, plasma viscosity, the disaggregation energy at

the capillary entrance, the axial accumulation of the erythrocytes, and platelet and leukocyte margination (Baskurt and Meiselman 2013).



Figure 18: Representation of Red blood cell aggregates.
Figure from Baskurt 2003

2. Alterations in blood rheology related to type 2 diabetes

In 1966 Scovborg et al published the first study showing that whole blood viscosity is significantly elevated in individuals with diabetes (Skovborg et al. 1966). Since this initial report, numerous other studies have confirmed their findings (McMillan and Gion 1981; Memeh and Reid 1988). The increased blood viscosity observed in individuals with T2D is largely attributed to changes in red blood cell deformability and aggregation (Schmid-Schonbein et al. 1976; Babu and Singh 2004; Cho, Mooney, and Cho 2008). In T2D, concentrations of glucose are elevated, which can result in increased non-enzymatic glycation of hemoglobin and membrane proteins of red blood cells, and these modifications are associated with decreased membrane fluidity (Watala 1993). Evidence suggests that increased glycosylation of spectrin, a major protein of the inner membrane surface, may play a particularly important role in decreasing red blood cell deformability in T2D (McMillan, Utterback, and La Puma 1978). Furthermore, the erythrocytes of individuals with T2D have

different shape parameters than those of healthy individuals (Babu and Singh 2004). This suggests that the surface-volume ratio may be altered, thereby decreasing the deformability of the T2D erythrocytes.

Increased erythrocyte aggregation is another factor that contributes to the increased whole blood viscosity in T2D. Numerous studies have shown that erythrocyte aggregation is enhanced in T2D (Budak et al. 2004; Foresto et al. 2000; Le Devehat et al. 1990; Martinez et al. 1998), and red blood cell hyper-aggregation becomes more severe as glucose control worsens (Babu and Singh 2004). Red blood cell aggregation is highly dependent on fibrinogen concentration (Cho, Mooney, and Cho 2008), (Barazzoni et al. 2003). Therefore, increased insulin-induced fibrinogen production likely plays a major role in the increased red blood cell aggregation observed in T2D (Cho, Mooney, and Cho 2008), (Barazzoni et al. 2003). Other factors that may increase red blood cell aggregation in T2D include reductions in the anionic charge of the cell membranes (Budak et al. 2004), and increased $O_2^{•-}$ release from polymorphonuclear leukocytes, which increases red blood cell aggregability (Baskurt and Meiselman 1998).

In addition to red blood cell deformability and aggregation, elevations in hematocrit and plasma viscosity contribute to the increased whole blood viscosity in T2D. As blood sugar levels increase, blood osmolarity also increases, resulting in augmented capillary permeability, which subsequently elevates hematocrit, and consequently whole blood viscosity (Meiselman et al. 1967). Indeed, hematocrit, red blood cell count, hemoglobin, and plasma iron have all been shown to correlate with insulin resistance (Barbieri et al. 2001; Facchini et al. 1998). Additionally, as plasma protein levels increase in T2D, plasma viscosity increases, thereby contributing to the elevated whole blood viscosity (Irace, Carallo, Scavelli, De Franceschi, et al.

2014). Changes in blood lipids in T2D also contribute to the increased plasma viscosity, but to a lesser extent (Irace, Carallo, Scavelli, Esposito, et al. 2014).

Whole blood viscosity has been shown to correlate with blood glucose and with insulin resistance in non-diabetic subjects, indicating that these changes in blood rheology actually precede the development of T2D (Perez-Martin et al. 2001). Interestingly, several studies have suggested that increased whole blood viscosity could actually contribute to the development of T2D (Hoieggen et al. 1998; Tamariz et al. 2008). As increased viscosity results in decreased blood flow, the delivery of crucial substrates, such as glucose, insulin, and oxygen, to target tissues is reduced (Hoieggen et al. 1998; Tamariz et al. 2008). Initially, blood vessels will vasodilate in order to increase blood flow, and consequently augment substrate delivery. However, when compensational vasodilatory mechanisms to increase blood flow are maximized, concentrations of glucose and insulin will increase, potentially contributing to the development of T2D (Tamariz et al. 2008).

III. SICKLE CELL DISEASE AND SICKLE CELL TRAIT

A. SICKLE CELL DISEASE

1. Sickle cell disease and the hemoglobin molecule

Sickle cell disease (SCD) is a term that encompasses a group of hereditary blood disorders (including sickle cell anemia (SCA), HbSC, and HbS β -thalassemia) that are all characterized by mutations of the gene that codes for hemoglobin β . Hemoglobin is a tetrameric protein that is expressed by red blood cells, and made up of combinations of four globin subunits. Each globin subunit is associated with a heme complex that contains an iron molecule that is capable of carrying a molecule of oxygen (Kato et al. 2018).

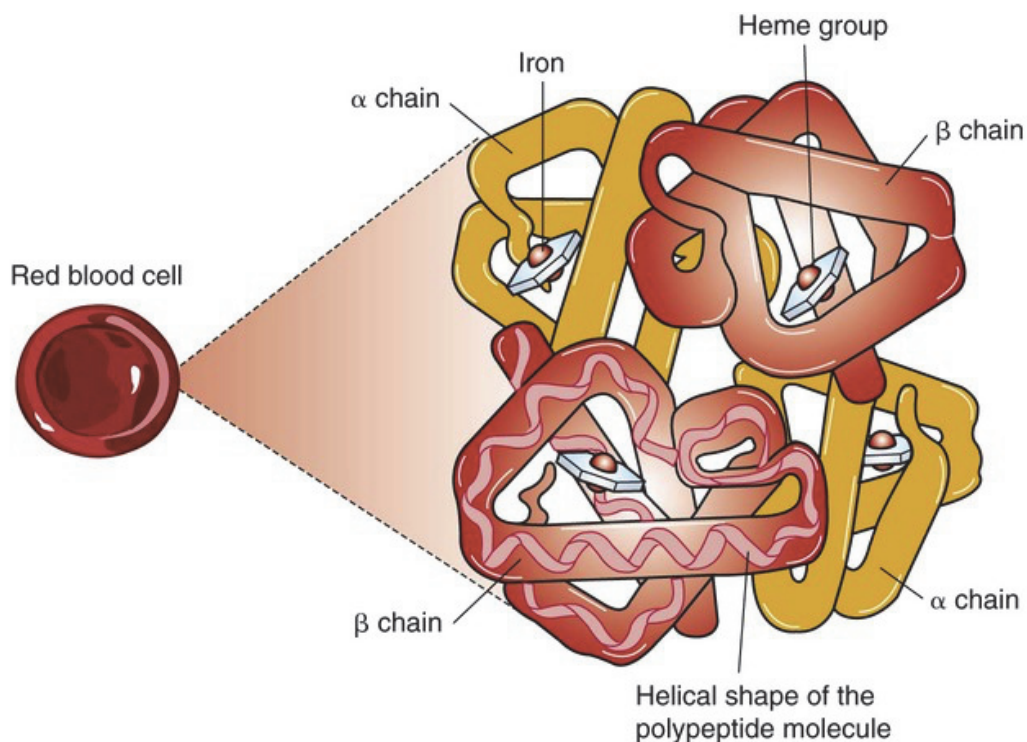


Figure 19: Structure of the hemoglobin A molecule showing the two pairs of globin subunits. Within each globin subunit is a heme, four in all. The ferrous iron in the center of each heme provides the binding site for molecular oxygen. (From Mader SS: *Inquiry into life*, ed 8, New York, 1997, McGraw-Hill.)

Several globin gene variants exist and code for different types of globin proteins, including α -, γ -, δ -, ϵ -, and ζ -globin, which are expressed at specific times during human development (Ashley-Koch, Yang, and Olney 2000). The most

predominant form of hemoglobin found in adults is HbA (>90%), which is composed of two α -globin protein chains, two β -globin protein chains and a heme (Figure 19). A single mutation at the sixth amino acid of the β -globin gene, caused by the substitution of valine for glutamic acid, results in the production of the sickle hemoglobin allele β^s , which generates an abnormal sickle β -globin subunit (Rees, Williams, and Gladwin 2010). Hemoglobin tetramers composed of two α -globin protein chains and two sickle β -globin protein chains are referred to as HbS. Under deoxygenated conditions HbS polymerizes, resulting in the formation of the distinctive sickle-shaped erythrocytes originally identified by Herrick in 1910 (Herrick 2001) (Figure 20).

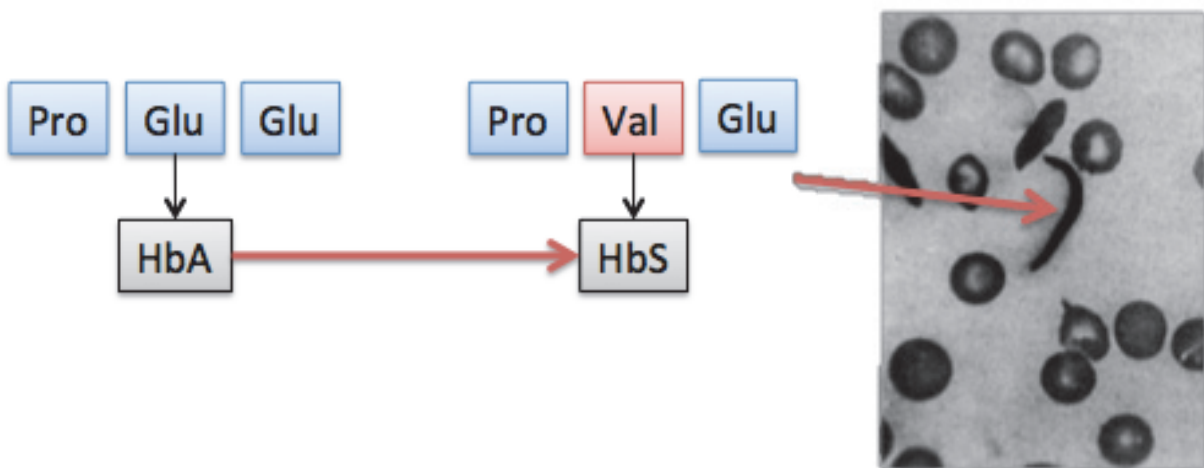


Figure 20: Hemoglobin S mutation and sickled red blood cell. A) Representation of the single point mutation that results in the production of an abnormal sickle β -globin subunit. B) Photomicrographs showing the peculiar elongated forms of sickled red blood cells observed by Herrick in 1910 (Herrick 2001)

2. Sickle cell anemia

SCA is an autosomal recessive genetic disorder. Therefore, individuals who are heterozygote for the β^s allele are carriers of the mutation, and have sickle cell trait (SCT). Individuals who are homozygous for the β^s allele have SCA, the most common form of SCD. SCA is characterized by chronic hemolytic anemia and

frequent vaso-occlusive crises that contribute to episodes of pain, as well as severe acute and chronic clinical complications that can affect a variety of organs, including the lungs, spleen, kidneys, liver, heart, eyes, reproductive organs, and skin (Kato et al. 2018; Rees, Williams, and Gladwin 2010). The severity of SCA disease complications as well as the life expectancy of individuals with SCA varies widely (Steinberg and Sebastiani 2012). The main determinant of disease severity is thought to be the rate and extent of HbS polymerization (Rees, Williams, and Gladwin 2010). Therefore the coinheritance of factors that alter intracellular HbS or fetal hemoglobin concentrations can modulate the disease severity. For example, coinheritance of α -thalassemia (caused by mutation of the α -globin gene) or the hereditary persistence of fetal hemoglobin both result in lower concentrations of HbS, and therefore less severe SCA phenotypes. However, these factors do not explain all of the variability associated with the disease (Rees, Williams, and Gladwin 2010), (Kato et al. 2018).

3. Distribution and prevalence of the HbS mutation

Five different region-specific haplotypes of the sickle mutation have been identified. Four of these haplotypes originated in Africa (the Senegal, Bantu, Benin, and Cameroon haplotypes), and one in Asia (the Arab-India haplotype) (Gabriel and Przybylski 2010). As *P. falciparum malaria* is endemic in all of these areas, researchers in the 1950s developed a hypothesis that the geographic distribution of the sickle mutation could be attributed to a protective effect of SCT against the severe consequences of malaria (Allison 1954). Today, conclusive evidence that SCT protects against severe malaria has been provided. Indeed people with SCT have been shown to be 90% less likely to present with severe malaria compared to individuals with normal HbA (Piel et al. 2010). This explains why the highest

frequencies of the β^S allele are observed in Sub-Saharan Africa, as well as in parts of the Mediterranean, the Middle East, and India, where malaria is prevalent (Piel et al. 2010). Population movements, including the slave trade, have resulted in a larger distribution of the β^S allele, particularly in the Americas and Western Europe (Piel et al. 2013) (Figure 21).



Figure 21: Global predicted distribution of HbS. *Figure from Piel 2013*

Global and country specific estimates of SCA and SCT prevalence are difficult to calculate due to a lack of national screening programs for SCD in many countries (Naik and Haywood 2015). However, in a review published in 2013 in *The Lancet*, Piel et al showed that an estimated 5,476,000 neonates were affected by SCT and 312,000 neonates were affected by SCA in 2010 (Piel et al. 2013). Furthermore, the study showed that high frequencies of HbS (>10%) were mostly observed in Sub-Saharan Africa, with the highest frequencies (between 7.5-12.5%) predicted in large areas spanning from southern Senegal to northern Liberia, and from southern Ghana to northern Zambia. Other smaller, isolated areas with similar frequencies were

predicted across Sub-Saharan Africa, in Greece, Madagascar, southern Turkey, southwestern Syria, Saudi Arabia, and India (Piel et al. 2013). In the Americas, the highest predicted frequency (5%) was observed on the northeastern coast of South America (Piel et al. 2013). A review of data acquired from national neonatal screening programs in Brazil published in 2011 found that the incidence of SCD in Brazil ranged from 0.8 to 60 per 100,000 live births, while the prevalence of SCT ranged between 1.1-9.8% (Lervolino et al. 2011). In the United States in 2010, the incidence of SCD was 15.5 per 1,000 newborns overall, although the incidence varied greatly between states, with the highest incidence in Mississippi (34.1 per 1,000 newborns) and the lowest incidence observed in Montana (0.8 per 1,000 newborns) (Ojodu, Hulihan, Pope, and Grant 2014). Overall, an estimated 1.5% of infants born in the United States have SCT (Ojodu, Hulihan, Pope, and Grant 2014).

B. PATHOPHYSIOLOGY OF SCA

1. HbS polymerization

HbS polymerization is essential to the pathophysiology of SCA. Under conditions of low-oxygen saturation, as can occur in the microcirculation for example, the tetrameric subunits of HbS interact and form rigid polymers. Aggregation of the large hemoglobin polymers results in the formation of sickled red blood cells, which are rigid, highly adherent, and prone to hemolysis (Rees, Williams, and Gladwin 2010). The two main determinants of HbS polymerizations are the concentration of HbS in the erythrocyte, which correlates exponentially with HbS polymerization, and the affinity of HbS for oxygen (Noguchi and Schechter 1985; Seakins et al. 1973). The reduced HbS oxygen affinity increases the polymerization of HbS, which then further decreases HbS oxygen affinity (Seakins et al. 1973). Other factors, including temperature, pH, 2,3 diphosphoglycerate concentration, and the presence of

different hemoglobin molecules can also lower HbS oxygen affinity, and thus increased HbS polymerization (Bookchin, Balazs, and Landau 1976; Briehl and Ewert 1973; Eaton and Hofrichter 1990).

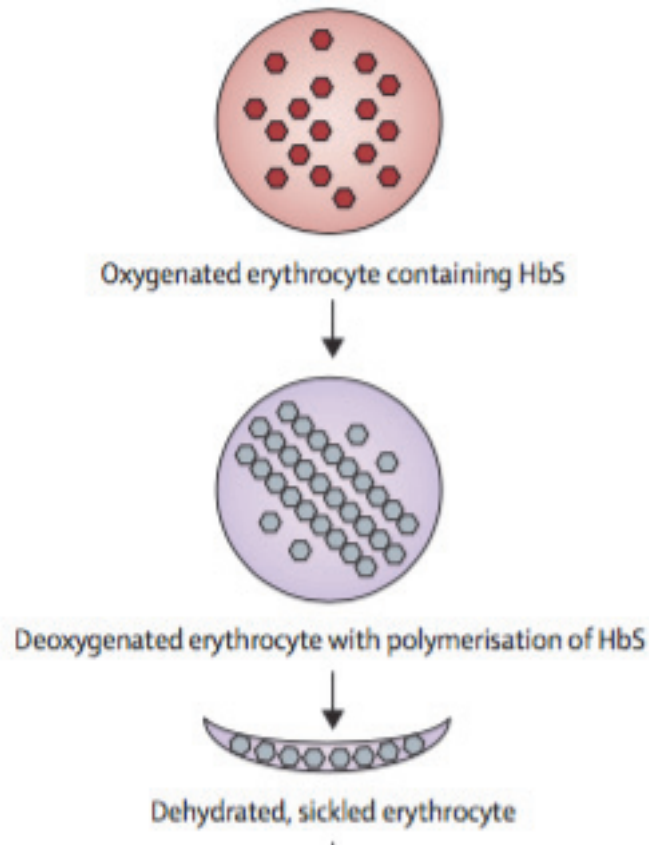


Figure 22: Representation of HbS polymerization under deoxygenated conditions *Figure from Rees 2010*

HbS polymerization is reversible, and therefore SCA red blood cells generally regain their normal shape when they return to an environment with a high concentration of oxygen. However, repeated cycles of HbS polymerization, and erythrocyte sickling and un-sickling can lead to severe alterations in the red blood cell membrane structure and function (Kato et al. 2018). Severe membrane deformation and red blood cell dehydration lead to irreversible red blood cell sickling, when red blood cells can no longer return to their normal shape. (Nash, Johnson, and Meiselman 1988; Smith, Krivit, and White 1982). The modifications of the lipid

bilayer and proteins of the red blood cell membrane during sickling can result in reduced cellular hydration, increased hemolysis, and early apoptosis (Fadok et al. 2001; Kuypers 2014).

2. Hemolytic Anemia

Anemia is a condition in which the concentration of hemoglobin in the blood is abnormally low. The chronic anemia associated with SCA is attributed to high rates of hemolysis. Indeed, sickle red blood cells have extremely short life spans of only 15-17 days, compared to a lifespan of 120 days in a normal red blood cell (Hebbel 2011). As previously stated, repetitive episodes of sickling and un-sickling disrupts the structure of the red blood cell membrane and results in the formation of highly unstable, dehydrated, sickled red blood cells (Fadok et al. 2001; Kuypers 2014). One of the major changes that occurs in sickled red blood cells is phosphatidylserine exposure on the erythrocyte membrane (Lubin et al. 1981). The exposure of phosphatidylserine is a sign of eryptosis, and therefore a signal for removal of the cells by the macrophage system. The extravascular phagocytosis of sickle erythrocytes by macrophages accounts for two-thirds of the total hemolysis in SCA, while the remaining one-third is attributed to intravascular hemolysis (Bensinger and Gillette 1974). The lysis of red blood cells in the vascular environment liberates the interior contents of the red blood cell into the circulation. When hemoglobin is released into the plasma it autoxidizes, producing free heme, iron, and ROS, which cause eNOS uncoupling, and the activation of pro-inflammatory signaling (Kato, Steinberg, and Gladwin 2017; Voskou et al. 2015). These alterations can contribute to the onset of vaso-occlusive crises and the development of chronic organ damage (Hebbel, Osarogiagbon, and Kaul 2004).

3. Vaso-occlusive crises

Painful vaso-occlusive crises are the number one cause of hospitalizations of individuals with SCA. The pain experienced during vaso-occlusive crises occurs when erythrocytes and leukocytes become trapped in the microcirculation, resulting in vascular obstruction and ischemia (Rees, Williams, and Gladwin 2010). Vaso-occlusion in SCA is a complex process that is triggered by inflammation, and is dependent on a dynamic interaction between red blood cells, leukocytes, activated endothelial cells, and platelets (Figure 23) (Rees, Williams, and Gladwin 2010), (Kato et al. 2018).

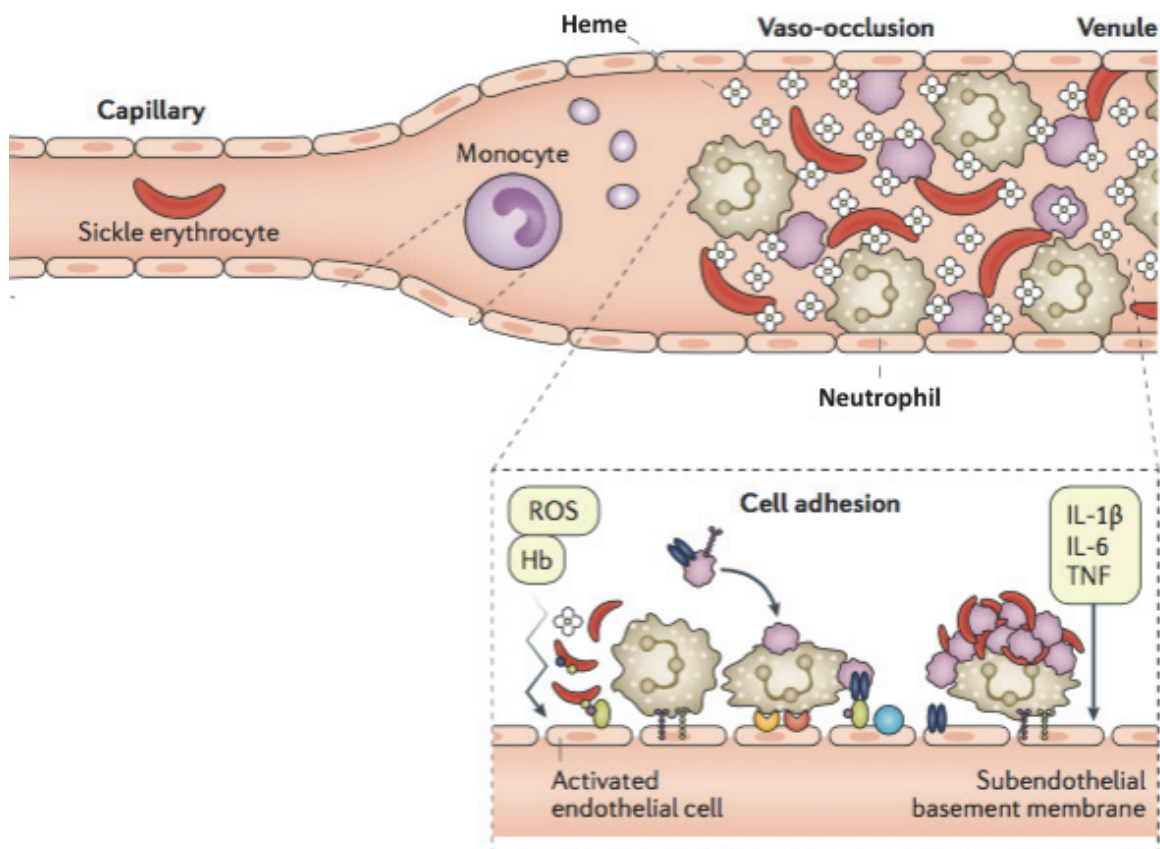


Figure 23: Vaso-occlusion in SCA. As a result of inflammation, circulating blood cells adhere to each other and to the activated endothelium, which expresses adhesion molecules. ICAM1, intercellular adhesion molecule 1; IL-6, Interleukin 6; TNF, tumour necrosis factor; VCAM1, vascular cell adhesion protein 1. *Figure from Kato 2018*

Vascular endothelial cells in SCA are activated as a result of inflammation. Therefore, the endothelial expression of adhesion molecules such as VCAM, P-selectin, and E-selectin is upregulated. Consequently, the adhesion of erythrocytes and polymorphonuclear lymphocytes (PMNs) to the endothelium is increased, mainly in the post-capillary venules (Hebbel, Osarogiagbon, and Kaul 2004). Furthermore, in individuals with SCA, sickled erythrocytes, erythrocytes that have not yet sickled, and reticulocytes are all more adhesive than normal erythrocytes (Murphy et al. 2005; Sugihara et al. 1992). Additionally, platelets are activated in SCA. Therefore, the adherent erythrocytes and activated platelets adhere to circulating neutrophils and neutrophils bound to the endothelium (Figure 23) (Kato et al. 2018). These phenomena can cause partial vessel occlusion. As a result, blood flow through the microcirculation slows substantially, and the transit time of erythrocytes through this oxygen poor area rises. As result, HbS polymerization increases, and the subsequent formation of rigid sickled cells contributes to vessel obstruction. This initial blockage can alter hemodynamics, oxygen tension, and pH, thereby exacerbating erythrocyte sickling, and leading to the onset of painful vaso-occlusive crises (Kaul and Nagel 1993). The ischemia caused by vaso-occlusive crises is followed by reperfusion, which causes oxidative stress (I.B.1.a), activation of vascular oxidases, inflammatory stress, increased expression of endothelial cell adhesion molecules, and increased synthesis of inflammatory cytokines (Kato et al. 2018).

4. Blood rheological profile in SCA

Under normal physiological conditions and at native hematocrit, individuals with SCA have low whole blood viscosity compared to people with normal hemoglobin, due to the severe, chronic anemia associated with SCA (Tripette et al.

2009). However, when blood is adjusted to regular hematocrit (40-45%), individuals with SCA have higher blood viscosity than people with normal hemoglobin, due to the presence of irreversibly sickled red blood cells. Additionally, when SCA blood is deoxygenated, red blood cell sickling causes blood viscosity to increase even more (Chien, Usami, and Bertles 1970).

Several factors likely contribute to the elevated whole blood viscosity of SCA blood adjusted to native hematocrit. First, red blood cell deformability is reduced in individuals with SCA (Chien, Usami, and Bertles 1970; Nash, Johnson, and Meiselman 1988; Tripette et al. 2009). Second, red blood cell aggregation is altered in people with SCA. In fact, several studies have shown that although red blood cell aggregation is decreased in SCA, the strength of red blood cell aggregates is higher in individuals with SCA compared to those with normal hemoglobin (Tripette et al. 2009; Waltz et al. 2012). Finally, plasma viscosity is slightly elevated in individuals with SCA at steady state, and this is probably due to elevated levels of circulating plasma proteins, such as fibrinogen (Connes et al. 2016).

The characteristics of SCA red blood cells appear to change over the course of vaso-occlusive crises, resulting in alterations in blood rheology. For example, in many patients, extensive hemolysis precedes vaso-occlusive crises. Additionally, the percentage of sickled red blood cells (both reversible and irreversible) increases during the one to three days that precede the onset of the clinical symptoms of the vaso-occlusive crisis. As a result, the bulk red blood cell deformability decreases prior to the onset of the vaso-occlusive crisis. As the individual recovers from the vaso-occlusive crisis, the percentage of sickled cells decreases, and therefore the bulk red blood cell deformability increases (Ballas and Smith 1992; Clark, Mohandas, and Shohet 1980; Connes et al. 2016; Kenny et al. 1981).

C. OXIDATIVE STRESS IN SCA

Elevated oxidative stress is both a cause and an effect of hemolysis and vaso-occlusive crises in SCA. Several phenomena contribute to the elevated oxidative stress in SCA, including decreased anti-oxidant defenses, and activation of non-enzymatic and enzymatic (mainly XO and NOX) sources of ROS (Wood et al. 2007).

1. HbS auto-oxidation

Although oxygenated hemoglobin is generally considered to be a stable molecule, under normal physiological conditions hemoglobin can auto-oxidize to methemoglobin at a rate of 0.5-3% per day (Voskou et al. 2015). This process, in which hemoglobin transfers electrons from the iron molecule associated with heme to an oxygen molecule ($\text{Hb-Fe}^{2+} + \text{O}_2 \rightarrow \text{MetHb-Fe}^{3+} + \text{O}_2^{\cdot-}$), is the primary source of ROS inside the red blood cell (van Zwieten, Verhoeven, and Roos 2014). The $\text{O}_2^{\cdot-}$ ions that are produced are quickly dismutated into H_2O_2 (Rifkind, Mohanty, and Nagababu 2014). Rates of auto-oxidation increase in hypoxic conditions; therefore auto-oxidation is more pronounced in the microcirculation (Rifkind, Mohanty, and Nagababu 2014). Hebbel and colleagues first described that HbS has an increased rate of auto-oxidation compared with normal HbA in 1988 (Hebbel et al. 1988). HbS autoxidation is linked to the elevated levels of $\text{O}_2^{\cdot-}$ and hydroxyl radicals observed in the erythrocytes of individuals with SCA (Hebbel et al. 1988).

2. Free plasma hemoglobin, heme, and iron

Elevated intravascular hemolysis results in high concentrations of extracellular hemoglobin and heme in the plasma of individuals with SCA, and these factors promote oxidative stress, particularly in blood vessels and blood cells (Alayash 2018). Extracellular hemoglobin auto-oxidizes, producing methemoglobin and $\text{O}_2^{\cdot-}$,

which is converted into H_2O_2 (Alayash 2018). Methemoglobin has a lower affinity for heme than oxyhemoglobin, and as a consequence heme dissociation is increased (Bunn and Jandl 1968). Heme is a hydrophobic molecule that can interact with the lipids and proteins in endothelial cell plasma membranes, resulting in oxidative reactions and the liberation of iron (Balla et al. 1993; Voskou et al. 2015). The iron can participate in the Haber-Weiss cycle as a Fenton reagent, and thereby generate the highly potent hydroxyl radical from H_2O_2 (Nur et al. 2011).

3. Vaso-occlusive ischemia-reperfusion: activation of XO

Vaso-occlusive crises cause an interruption in blood flow to the tissues, resulting in a hypoxic environment. When the vaso-occlusion is resolved, the oxygen-rich blood that returns to the vessel causes tissue damage, notably due to the increased activity of XO (I.B.1.a) (Voskou et al. 2015). Additionally, ischemia and reperfusion appears to increase the expression of NOX, an important source of $O_2^{\cdot-}$, in PMNs, monocytes, and endothelial cells (Voskou et al. 2015).

4. Inflammation

The elevated rates of hemolysis and vaso-occlusive crises that occur in SCA result in chronic inflammation, which is associated with an increased concentration of leukocytes (Wood and Granger 2007). NOX is the major source of $O_2^{\cdot-}$ production in leukocytes, and is therefore an important source of ROS production in SCA (Wood and Granger 2007). However, NOX-mediated $O_2^{\cdot-}$ production is not only upregulated in leukocytes, but also in red blood cells in SCA (George et al. 2013). Indeed, George and colleagues showed that elevated levels of $TGF\beta-1$ and ET-1 upregulate PKC, Rac GTPase, and Ca^{2+} signaling inside the SCA erythrocyte, thereby stimulating NOX activity (George et al. 2013). Therefore, in SCA there is a vicious

cycle in which increased ROS promotes pro-inflammatory cytokine expression, resulting in NOX-mediated $O_2^{\cdot-}$ production.

5. NO availability in SCD

a. Peroxynitrite formation

As explained in section I.B.2.a, $O_2^{\cdot-}$ reacts three times more rapidly with NO than with SOD (Szabo, Ischiropoulos, and Radi 2007). In individuals with SCA, increased $O_2^{\cdot-}$ production from uncoupled eNOS, XO, and NOX overwhelms the anti-oxidant defense systems. Therefore, the excess $O_2^{\cdot-}$ readily binds to NO, generating the strong oxidant $ONOO^-$, which consequently both decreases NO bioavailability and increases ROS (Voskou et al. 2015). Furthermore, $ONOO^-$ readily oxidizes BH_4 , a cofactor of eNOS, thereby contributing to eNOS uncoupling (Wood and Granger 2007).

b. Hemolysis and NO bioavailability

Hemolysis results in increased plasma concentrations of free hemoglobin, the enzyme arginase, and asymmetric dimethylarginine (ADMA) (Kato et al. 2018; Wood and Granger 2007). Free hemoglobin scavenges NO ~1,000 times more rapidly than cytoplasmic hemoglobin (Reiter et al. 2002). Free deoxyhemoglobin in the plasma forms $Hb(Fe^{2+})-NO$ complex, while oxyhemoglobin reacts with NO to form methemoglobin and NO_3^- (Herold, Exner, and Nauser 2001; Voskou et al. 2015).

Arginase-1 consumes L-arginine in a reaction that produces ornithine, a substrate that fuels the synthesis of polyamines, which facilitate cell proliferation (Kato et al. 2018). Therefore, arginase-1 competes with NOS for L-arginine, and thus contributes to eNOS uncoupling (Wood and Granger 2007). ADMA, an L-arginine analogue, is a naturally occurring product of normal protein turnover that is abundant in erythrocytes, and is released during hemolysis (Antoniades et al. 2009). ADMA is

an endogenous competitive inhibitor of NO synthase, and is thought to increase $O_2^{\cdot-}$ production by contributing to eNOS uncoupling (Antoniades et al. 2009). Under normal conditions, ADMA is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). However, the activity of DDAH is impaired by oxidation. Therefore ADMA accumulates when in conditions when oxidative stress is elevated, like in SCA (Ito et al. 1999).

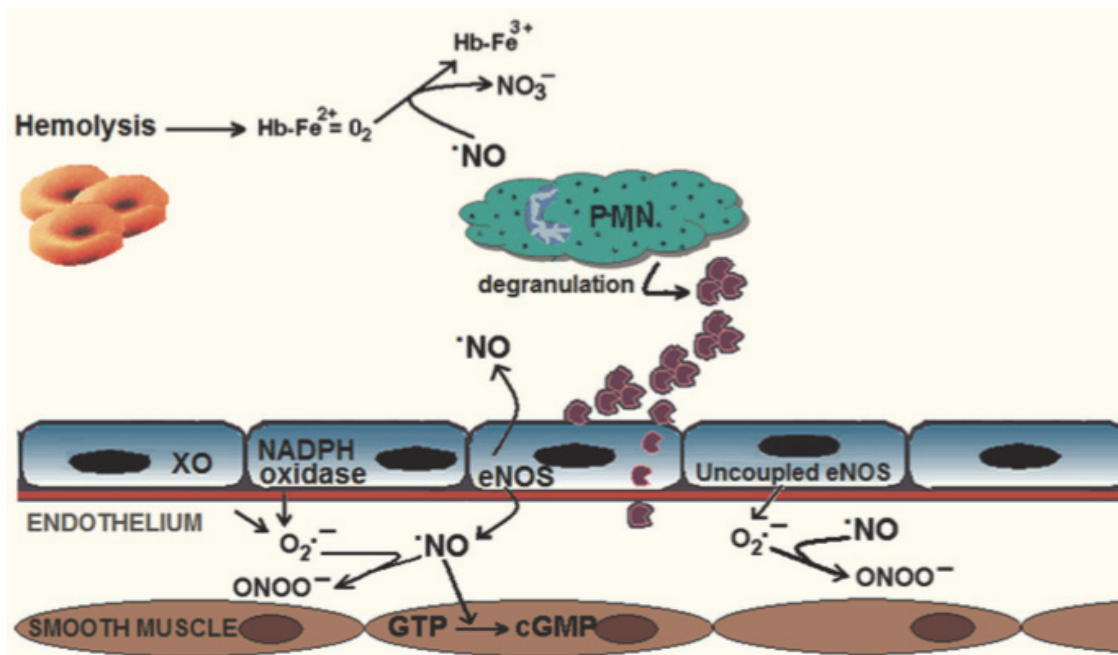


Figure 24: Scheme of intravascular nitric oxide consumption in sickle cell disease. Superoxide ($O_2^{\cdot-}$) generated by uncoupled eNOS, xanthine oxidase, and NADPH oxidase reacts with NO to form peroxynitrite ($ONOO^-$). Nitric oxide is also consumed by plasma free hemoglobin, released by intravascular hemolysis *Figure and text from Voskou 2015*

D. SICKLE CELL TRAIT

Individuals with SCT, the heterozygous form of SCA, inherit a β^a allele from one parent and a sickle β^s allele from the other parent. Therefore, the red blood cells of individuals with SCT contain approximately 60-70% HbA and 30-40% HbS (McCurdy 1969). The concentration of HbS in individuals with SCT can vary widely due to interaction with alpha-thalassemia. Indeed, a study by Steinberg and colleagues showed that in African American individuals with SCT and a deletion of

either one or two alpha genes, HbS levels were around 37% and 29%, respectively (Steinberg and Embury 1986).

As discussed in section III.A.3, heterozygosity for the sickle mutation confers protection against the severe effects of *Plasmodium falciparum* malaria. Therefore SCT is most prevalent in areas of the world where malaria is highly endemic, including Sub-Saharan Africa, the Middle East, and India (III.A.3). Although the exact mechanism of resistance remains unknown, a recent study conducted by Archer and colleagues demonstrated that the polymerization of HbS stops the growth of *P. falciparum* parasites inside of the red blood cell. The study ultimately suggests that the protective effect of SCT could be due to the effective sequestration of infected red blood cells into the hypoxic microcirculation (Archer et al. 2018). Other possible mechanisms that have been proposed include sickling of infected red blood cells, (Miller, Neel, and Livingstone 1956), increased splenic phagocytosis (Luzzatto, Nwachuku-Jarrett, and Reddy 1970), premature hemolysis and parasite death (Roth 1978 *Science*), impaired hemoglobin digestion (Pasvol 1980; Pasvol, Weatherall, and Wilson 1978), weakened cytoadherence (Cholera et al. 2008; Cyrklaff et al. 2011), acquired host immunity (Williams et al. 2005), translocation of HbS-specific parasite growth inhibiting micro-RNAs (LaMonte et al. 2012), and induction of heme oxygenase-1 (Ferreira et al. 2011). Although SCT has traditionally been considered to be a benign carrier state, accumulating evidence shows that SCT is associated with slight blood rheological abnormalities, hypercoagulation, and increased inflammation and oxidative stress following exercise, as well as with an increased risk of several complications.

1. Blood rheological abnormalities in SCT

Several studies have shown that whole blood viscosity is increased in individuals with SCT (Connes et al. 2008; Connes et al. 2005) (Diaw et al. 2013). The elevated whole blood viscosity in SCT is associated with higher red blood cell aggregation and reduced deformability (Monchanin et al. 2005; Obiefuna 1991; Reid and Obi 1982; Brandao et al. 2003; Connes et al. 2005). A study by Obiefuna et al found that SCT red blood cells showed more rouleaux formation than red blood cells with regular hemoglobin in native plasma and in a dextran solution at a pH of 6.5 (Obiefun 1991). These findings indicates that individuals with SCT may have an increased risk of stasis in the microvasculature (Obiefuna 1991).

Several studies have also observed decreased erythrocyte deformability in individuals with SCT (Brandao et al. 2003; Connes et al. 2005) (Monchanin et al. 2005). Connes and colleagues showed that increased red blood cell rigidity in SCT was accompanied by increased monocarboxylate transporter-1 (MCT-1) activity (Connes et al. 2005). The authors suggest that the increased MCT-1 activity would lead to increased lactate and hydrogen ion concentrations in the red blood cell, resulting in increased red blood cell rigidity (Connes et al. 2005). Additionally, this study found that whole blood viscosity was increased in individuals with SCT compared to controls. However, no differences in plasma viscosity or hematocrit between the two groups were found, indicating that the elevated erythrocyte deformability likely plays a crucial role in the increased whole blood viscosity observed in individuals with SCT (Connes et al. 2005).

2. Coagulation

Coagulation may be altered in individuals with SCT. Indeed, studies have shown that individuals with SCT have elevated levels of D-dimers, thrombin-

antithrombin complexes, and pro-thrombin fragment 1.2 compared to race matched controls (Westerman et al. 2002), (Amin et al. 2015). Westerman et al hypothesized that hypercoagulability in individuals with SCT could be attributed to increased monocyte levels and a loss of phospholipid asymmetry with exposure of phosphatidylserine on the membrane surface of SCT red blood cells that have been deoxygenated (Westerman et al. 2002). Furthermore, a study conducted by Raffield et al showed that the gene that encodes the sickle cell mutation (HBB rs334) was significantly associated with D-Dimer levels in African Americans in the Jackson Heart Study cohort, and could therefore contribute to the higher risk of venous thromboembolism (VTE) in this population (Raffield et al. 2017).

3. Inflammation in SCT

The results of studies examining levels of inflammatory markers in individuals with SCT have been contradictory. Amin and colleagues found no differences in levels of CRP, TNF α , IL-1 α , IL-1 β , IL-6, or IL-8 between subjects with SCT and controls (Amin et al. 2015). These results coincide with the findings of Duits et al, which showed no differences in levels of IL-6, granulocyte-macrophage colony stimulating factor, or soluble adhesion factors (E-selectin, P-selectin, ICAM-1, VCAM-1) between SCT and control subjects (Duits et al. 1996).

A study by Tripette et al examining the effect of submaximal exercise on adhesion factors in individuals with SCT observed no differences in sVCAM-1 or sICAM-1 at any time point between individuals with SCT and controls (Tripette, Connes, et al. 2010). However, the same study observed increased levels of P-selectin before exercise, after exercise, and during recovery, as well as increased levels of L-selectin during recovery, suggesting greater platelet and leukocyte activation in the SCT subjects (Tripette, Connes, et al. 2010). These results

contradict the findings of another study by Monchanin et al, which evaluated the effects of exercise on sVCAM-1 and sICAM-1 concentrations in athletes with SCT. The study observed increased levels of sVCAM-1 in subjects with SCT at rest, although no differences in levels of sICAM-1 or TNF- α were found (Monchanin et al. 2007). Furthermore, exercise resulted in increased sVCAM-1 levels in all subjects, although these levels only remained elevated in the SCT subjects one and two hours following exercise (Monchanin et al. 2007).

4. Oxidative stress in SCT

Whether or not oxidative stress is elevated in individuals with SCT remains unclear. A study examining oxidative stress in the Townes transgenic mouse model of SCT found that advanced oxidation protein products were elevated in the kidneys of SCT mice compared to controls (Charrin et al. 2016). However, no differences in other markers of oxidative stress were observed between the control and SCT mice (Charrin et al. 2016).

The majority of human studies that have been conducted to examine oxidative stress in individuals with SCT have done so in the context of exercise. One of the first studies to do so was conducted by Das and colleagues, and found that although there were few differences between the control and SCT subjects at baseline, continuous graded treadmill exercise resulted in an increased susceptibility to oxidation in SCT red blood cells, which was associated with both an excessive production of H₂O₂ and an inefficient breakdown of H₂O₂ by anti-oxidant enzymes (Das et al. 1993). Another study conducted by Tripette et al did not observe any differences in NO metabolites between individuals with SCT and controls following sub-maximal exercise (Tripette, Connes, et al. 2010). Similarly, Faës and colleagues observed no differences in SOD and catalase activity, or NO metabolism between

SCT and control individuals after a maximal exercise test (Faës et al. 2012). However, this study did observe elevated advanced oxidation protein products, and a decreased activity of GPX and two NADPH-generating enzymes in SCT compared to control subjects (Faës et al. 2012). Finally, a study by Chirico et al found higher levels of oxidative stress in individuals with SCT compared to controls, however exercise training appeared to reverse markers of oxidative stress in the carriers of SCT (Chirico et al. 2012).

5. Complications associated with SCT

SCT has historically been viewed as a benign condition, exhibiting no significant impact on life expectancy (Ashcroft and Desai 1976). However, an accumulating body of evidence shows that SCT is associated with exercise-related sudden death (ERSD), various renal complications, and VTE (Key, Connes, and Derebail 2015). Furthermore, some studies suggest that SCT could possibly be associated with a variety of other complications, including stroke and retinopathy, although the evidence remains inconsistent (Golomb 2005), (Caughey et al. 2014; Tsaras et al. 2009).

a. Exercise-related Deaths

One of the earliest SCT-related complications to be identified was the increased risk of ERSD observed in African American military recruits and athletes in the United States (Harmon et al. 2012; Kark et al. 1987). Exertional deaths may be caused by heat stroke, exertional rhabdomyolysis, acute renal failure, disseminated intravascular coagulation, and/or cardiac arrhythmia (Caughey et al. 2014; Tsaras et al. 2009). In individuals with SCT exercise has been shown to result in increases in oxidative stress (Faes et al. 2014), inflammation (Monchanin et al. 2005; Tripette, Connes, et al. 2010), red blood cell rigidity (Connes et al. 2006; Tripette, Connes, et

al. 2010), and whole blood viscosity (Connes et al. 2006), (Diaw et al. 2013), (Tripette, Connes, et al. 2010). All of these alterations are also present in people without SCT, but they are amplified in individuals with SCT, and could potentially contribute to ERSD in this population. Blood rheological abnormalities in individuals with SCT could play a particularly important role in the pathogenesis of ERSD (Connes et al. 2006; Connes et al. 2008; Key, Connes, and Derebail 2015). Elevated whole blood viscosity can increase vascular resistance, potentially impairing blood flow in the microvasculature, and increasing the risk of microvasculature complications (Key, Connes, and Derebail 2015). Furthermore, red blood cells with low deformability cannot pass through small capillaries, and this could lead to tissue ischemia (Connes et al. 2006). Current recommendations to prevent ERSD in individuals with SCT include maintaining adequate hydration, particularly in hot weather and at high altitude, as proper hydration can normalize blood viscosity during exercise in individuals with SCT (Tripette, Loko, et al. 2010), (Diaw et al. 2014), (O'Connor et al. 2012).

b. Renal complications

Renal abnormalities are possibly the most well established complications associated with SCT. For example, cases of a renal medullary carcinoma, a rare and aggressive tumor of the kidney, are seen almost exclusively in young individuals with SCT (Davis, Mostofi, and Sesterhenn 1995), (Watanabe et al. 2007). Additionally, the most common complication associated with SCT is hematuria (Heller et al. 1979), (Kiryluk et al. 2007). Many cases of hematuria in individuals with SCT are associated with renal papillary necrosis, which results from local microinfarctions in the renal medulla (Eckert, Jonutis, and Davidson 1974); (Zadeii and Lohr 1997) (Li and Carroll 2014). Another complication associated with recurrent microinfarctions in

the renal medulla is hyposthenuria, or impaired urine concentrating ability. A study by Gupta and colleagues showed that SCT is associated with hyposthenuria (Gupta et al. 1991). Studies showing increased rates of hematuria and hyposthenuria in SCT led to efforts to try to establish whether there is a link between SCT and kidney disease. One of the first studies to do so was conducted by Yium and colleagues in a cohort of hemodialysis patients (Yium et al. 1994). The results of this study showed that there was a higher rate of SCT in African Americans with autosomal dominant polycystic kidney disease compared with those with other types of end stage renal disease (ESRD), and that African Americans with SCT had an earlier onset of ESRD compared to those without SCT (Yium et al. 1994). More recent studies have established a clear link between SCT and chronic kidney disease (CKD), decline in estimated glomerular filtration rate, and albuminuria (Bucknor, Goo, and Coppolino 2014; Naik et al. 2014). Furthermore, SCT was recently shown to be an important genetic risk factor for the development of ESRD in blacks (Naik et al. 2017).

The mechanism by which SCT could contribute to the development and progression of CKD is currently unknown. However, the hematuria and hyposthenuria associated with SCT suggest damage to the renal medulla (Naik and Haywood 2015). The high osmolality, high acidity, and low partial pressure of oxygen in the kidney create a favorable environment for HbS polymerization and erythrocyte sickling (Noguchi, Torchia, and Schechter 1981). The damage caused by ischemia following silent episodes of sickling could contribute to renal injury in SCT (Naik and Haywood 2015; Tsaras et al. 2009). Furthermore, microradioangiographic studies of kidneys of individuals with SCA and SCT revealed gross lesions of the vessels of the renal medulla with almost a complete obliteration of the vasa recta in SCA, and a decreased number of vasa recta and a loss of normal bundle architecture in SCT

(Stadius van Eps et al. 1970). Although the damage was less severe in SCT compared to SCA kidneys, these observations suggest a common pathway of hypoxia, ischemic injury, and kidney damage in SCA and SCT (Stadius van Eps et al. 1970) (Naik and Haywood 2015). Finally, other factors such as inflammation, oxidative stress, endothelial injury, and hypercoagulation likely contribute to kidney damage in SCT, as they do in SCD (Key, Connes, and Derebail 2015).

c. Venous Thromboembolism

SCT has been established as a risk factor for VTE in multiple studies (Austin et al. 2007; Bucknor, Goo, and Coppolino 2014; Folsom et al. 2015; Heller et al. 1979). Interestingly, evidence suggests that SCT is specifically associated with pulmonary embolism, but not with deep vein thrombosis, although the reason for this remains unknown (Austin et al. 2007; Bucknor, Goo, and Coppolino 2014; Folsom et al. 2015). The increased levels of d-dimers, thrombin-antithrombin complexes, and prothrombin fragment 1.2 observed in SCT (Amin et al. 2015; Westerman et al. 2002) could increase coagulation, and consequently increase the risk of VTE (Key, Connes, and Derebail 2015). Furthermore, elevated levels of whole blood viscosity could also be related to the increased risk of VTE in SCT (Tripette et al. 2013).

d. Stroke

A large population-based study of African Americans in the Atherosclerosis Risk in Communities study observed an increased risk of stroke in African Americans with SCT compared to African Americans without SCT (Caughey et al. 2014). This finding is in accordance with previously published evidence suggesting that SCT could possibly be a risk factor for stroke (Golomb 2005; Radhakrishnan et al. 1990). However, a meta-analysis of four large prospective population-based studies concluded that African Americans with SCT do not have a higher risk of stroke

compared to African Americans with SCT (Hyacinth et al. 2018). Overall, whether or not SCT is associated with stroke remains controversial.

e. Retinopathy

There is mixed evidence as to whether or not retinopathy is associated with SCT in the absence of other diseases or complications. Numerous case studies have reported on individuals with SCT who present with retinopathy (Pandey 2015; Reynolds, Besada, and Winter-Corella 2007). However, these articles describe retinopathy developing after blunt ocular trauma (Jackson et al. 1995; Mehta, Whittaker, and Tsaloumas 2001; Pandey 2015), or in association with a systemic disease such as diabetes, syphilis, tuberculosis, or sarcoidosis (Jackson et al. 1995; Nagpal et al. 1977).

A study conducted in Ivory Coast carried out complete ophthalmologic examinations on 70 individuals with SCT, and found retinal lesions in 70% of subjects (Fany et al. 2004). However, no control subjects were included in the study. Furthermore, another study carried out in Canada conducted complete ophthalmologic examinations in 32 individuals. The study found no cases of SCT among the 32 individuals, and thus concluded that there is no increased risk of retinopathy in healthy people with SCT (Nia et al. 2003). Overall, more studies need to be done to confirm whether or not SCT is associated with retinopathy.

IV. COMBINED TYPE 2 DIABETES AND SICKLE CELL TRAIT

Rates of SCT are highest among populations living in Sub-Saharan Africa, and parts of the Mediterranean, the Middle East, and India, as well as among individuals whose ancestors come from these areas, such as people of African descent living in Europe or the Americas (III.A.3). Concomitantly, rates of T2D are either already high or on the rise in each of these populations. For example, the IDF estimated that more than 15.9 million people in Africa currently have T2D, and the prevalence is expected to increase 162% by 2045, which is more rapidly than anywhere else in the world (Cho et al. 2018). This presents a significant public health challenge, as a recent review determined that many health care systems in sub-Saharan African countries are currently unable to provide adequate medical care for the treatment of diabetes and its related complications (Atun et al. 2017).

Following Africa, the areas of the world where rates of T2D are expected to increase the most rapidly in the next 30 years are the Middle East and North Africa (110%), South East Asia (84%), and South and Central America (62%) (Cho et al. 2018). Furthermore, North America and the Caribbean are currently the areas of the world with the highest rates of T2D among individuals between the ages of 20 and 79 (Cho et al. 2018). In the United States, where an estimated 9.3% of the overall population has T2D, African Americans are both 77% more likely to have T2D and 2.3 times more likely to die from diabetes-related complications than non-Hispanic whites (Golden et al. 2012). Overall, these data indicate that there is a large and increasing population of individuals with both SCT and T2D. For this reason, multiple studies have been conducted to determine whether SCT may potentiate T2D and whether SCT may affect HbA1c measurement. A small number of studies have also

been carried out to determine whether SCT may exacerbate the vascular dysfunction present in T2D, and increase the risk of T2D related vascular complications.

A. SCT and metabolic control

The fact that rates of T2D are increasing rapidly in Africa, where SCT is also prevalent, raises the question as to whether SCT could possibly be a potentiating factor for the development of T2D (IDF 2015; Ojodu, Hulihan, Pope, Grant, et al. 2014; WHO 2010, 2012). Furthermore, studies have shown that carriers of SCT have an increased risk of rhabdomyolysis and ERSD, as well as abnormal responses to cardiopulmonary exercise testing (Hedreville et al. 2008; Key, Connes, and Derebail 2015; O'Connor et al. 2012; Sara et al. 2006; Thoreson et al. 2015; Triplette, Connes, et al. 2010). For these reasons, Liem et al sought to determine whether SCT might reduce cardiopulmonary fitness, thereby increasing the likelihood of developing T2D, hypertension, or metabolic syndrome (Liem et al. 2017). This multicenter longitudinal study included 2631 African Americans, of whom 6.8% were carriers of SCT (Liem et al. 2017). The results revealed that SCT is not associated with baseline fitness, or changes in fitness overtime, and is not an independent risk factor for T2D, hypertension, or metabolic syndrome (Liem et al. 2017). These findings were supported by another study that compared the prevalence of SCT in a population of 73 T2D patients in Cameroon to the prevalence of SCT in the general population of Cameroon (Ama et al. 2012). The rate of SCT among the subjects was 19%, with no sex differences, and therefore similar to the frequency in the general population of Cameroon (Ama et al. 2012). Furthermore, no differences in rates of hypertension, blood pressure, heart rate, or serum albumin were found between the groups, suggesting that SCT does not affect metabolic control in T2D (Ama et al. 2012).

These studies suggest that although there is a growing population of individuals with combined T2D and SCT, it is unlikely that SCT is a potentiating factor for the development of T2D. However, both of these studies have important limitations. The small cohort used in Ama et al included patients with long-standing T2D from a single diabetes referral center in Cameroon, raising questions as to whether the findings can be applied to other populations (Ama et al. 2012). Additionally, although Liem et al used a large sample size, the percentage of SCT carriers was relatively small (Liem et al. 2017). Furthermore, the study was not powered to answer the authors' specific research questions as participants were drawn from CARDIA (Friedman et al. 1988), a longitudinal study conducted to measure risk factors of coronary artery disease (Liem et al. 2017). Therefore, more properly powered studies with large sample sizes need to be conducted in order to determine whether SCT increases the risk of developing T2D.

B. SCT and type 2 diabetes diagnosis

HbA1c assay results are frequently used for diabetes diagnosis and follow up (Little and Roberts, 2009; (Bleyer and Aloï 2017). Indeed, in 2009 an international committee of experts composed of the ADA, the European Association for the Study of Diabetes, and the IDF recommended that HbA1c be used for diabetes diagnosis (International Expert 2009). However, evidence suggests that the HbS mutation may interfere with HbA1c measurement and the interpretation of assay results. HbA1c measurement is dependent on normal erythrocyte lifespans; therefore the use of HbA1c for estimation of glucose control cannot be used in individuals who are homozygous for HbS, HbC, or who have HbSC disease, as these conditions alter erythrocyte survival (Little and Roberts 2009). In individuals who are heterozygous carriers of these abnormal hemoglobins, erythrocyte survival is normal so HbA1c can

be used (Little and Roberts 2009). However, some methods of immunoassay and cation exchange ratio chromatographic assays are affected by the HbS in individuals with SCT, leading to an artificial increase or decrease in HbA1c (Little and Roberts 2009; Bry, Chen, and Sacks 2001). In 2010, Zhu et al reported that two different methods (cation-exchange high performance liquid chromatography assay and turbidimetric immune-inhibition method) resulted in significantly different estimated average glucose results in individuals with and without SCT, but the difference was more significant in those with SCT (Zhu, Williams, and Horne 2010). Furthermore, Mongia et al evaluated the effects of HbS on 14 different HbA1c measurement methods, including boronate affinity, enzymatic immunoassay, and ion exchange methods (Mongia et al. 2008). Several methods resulted in statistically significant differences, and one immunoassay method exhibited clinically significant differences (Mongia et al. 2008). In light of the evidence, it is imperative that labs confirm whether their HbA1c assay methods are affected by the presence of HbS. To aid in this endeavor the National Glycohemoglobin Standardization Program has published a list of the test methods that accurately measure HbA1c in patients with HbS (NGSP 2016). Furthermore, in order to ensure accurate diabetes diagnosis and follow up, HbA1c measurement can be used in combination with traditional glucose criteria (Shima et al. 1989; Sumner, Duong, Aldana, et al. 2016).

Multiple studies have been conducted to determine the relationship between HbA1c and serum glucose concentration in individuals with SCT compared to those without SCT. Three studies have shown that SCT has no impact on the relationship between HbA1c and serum glucose, however all three of these studies were conducted using fairly small sample sizes of people who were heterozygous for HbS (Ama et al. 2012; Bleyer, Vidya, et al. 2010; Golden et al. 2012; Sumner et al. 2015).

Interestingly, a multivariable repeated effects regression study conducted by Bleyer et al showed that although the relationship between HbA1c and serum glucose did not differ between African Americans with and without SCT, there was a difference between African Americans and European Americans (Bleyer, Vidya, et al. 2010). On the other hand, a recent study by Lacy et al that used 9062 concurrent measures of fasting glucose and HbA1c and 2001 concurrent measures of 2-hour postprandial glucose and HbA1c showed that, for any given fasting or 2-hour postprandial glucose concentration, African Americans with SCT had lower HbA1c values than those without SCT (Lacy et al. 2017). The study concluded that an HbA1c of 7.5% in blacks without SCT corresponded to an HbA1c of 7% in those with SCT with similar fasting glucose levels, indicating that HbA1c may systematically underestimate past glucose control in African American carriers of SCT (Lacy et al. 2017). However, in this study HbA1c was measured using two versions of the Tosoh ion-exchange high-performance liquid chromatography assays, which have been shown to result in non-statistically different results in persons with versus without SCT. Therefore, it is difficult to determine whether the findings were due to biological differences between the two groups, or assay interference caused by SCT (Rohlfing, Hanson, and Little 2017; Lacy, Wellenius, and Wu 2017). Nonetheless, Lacy et al reported a significant glucose-independent difference in HbA1c between African American carriers of SCT versus those without SCT that resulted in the diagnosis of 40% fewer cases of prediabetes and 48% fewer cases of diabetes in those with SCT (Lacy, Wellenius, and Wu 2017). Overall, these results suggest that SCT could affect HbA1c measurement for physiological and/or technological reasons, thereby complicating diabetes diagnosis. For this reason, alternative non-fasting measures of glycemia, such as glycated albumin and fructosamine, have been suggested as

potential alternatives to measure glycemia in populations where SCT is prevalent (Utumatwishima et al. 2018).

Fructosamine is a measure of glycosylated plasma protein, while glycosylated albumin is a subfraction of fructosamine, expressed as a percentage of total albumin (Danese et al. 2015). Neither measure requires an overnight fast, and neither one is affected by conditions that interfere with the interpretation of HbA1c, such as anemia or iron deficiency (Danese et al. 2015). Fructosamine is significantly associated with HbA1c and fasting glucose, and has been shown to independently predict T2D incidence and microvascular complications in populations including both black and white individuals, with and without T2D (Selvin et al. 2014), (Juraschek et al. 2012; Juraschek, Steffes, and Selvin 2012). The assay for glycosylated albumin is better standardized than that for fructosamine (Danese et al. 2015), and several studies have concluded that glycosylated albumin may perform better than fructosamine as a diagnostic test for hyperglycemia (Chan et al. 2016; Shima et al. 1989), (Sumner, Duong, Aldana, et al. 2016). On the other hand, glycosylated albumin performs poorly in conditions characterized by hypoalbuminuria (Danese et al. 2015). Furthermore, HbA1c has been shown to perform better in obese individuals, while glycosylated albumin performs better in non-obese individuals (Sumner, Duong, Bingham, et al. 2016). Overall, it seems that glycosylated proteins could be used in combination with HbA1c as a complementary measure of glycemia. However, whether or not these alternative measures of glycemia perform comparably to traditional measures of glycemia, including HbA1c and fasting glucose, in individuals with SCT remains unknown.

C. SCT and T2D-related complications

As discussed in section II, T2D is characterized by metabolic abnormalities including hyperglycemia, hyperlipidemia, and hyperinsulinemia, which leads to increased oxidative stress, inflammation, and platelet activity, resulting in decreased NO bioavailability and endothelial dysfunction (Sena, Pereira, and Seica 2013); (Paneni et al. 2013). Moreover, T2D patients have elevated plasma viscosity, augmented erythrocyte aggregation, decreased red blood cell deformability, and increased blood viscosity (MacRury and Lowe 1990). Together, these biological anomalies lead to chronic vascular dysfunction and the development of microvascular and macrovascular complications (Donnelly et al. 2000; Paneni et al. 2013; Sena, Pereira, and Seica 2013).

On the other hand, as discussed in section IV, SCT is associated with blood rheological abnormalities, hypercoagulation, and increased inflammation and oxidative stress following exercise, as well as an increased risk of several complications (Caughey et al. 2014; Derebail et al. 2010; Little et al. 2017; Naik et al. 2017). Additionally, recent findings from our laboratory suggest that SCT may impair flow-mediated dilation (Diaw et al. 2015). Thus, these studies indicate that SCT could potentially increase vascular dysfunction in T2D by exacerbating alterations in blood rheology, increasing coagulation activity, augmenting oxidative stress, and decreasing NO bioavailability, thereby increasing the risk of developing vascular complications (Figure 25).

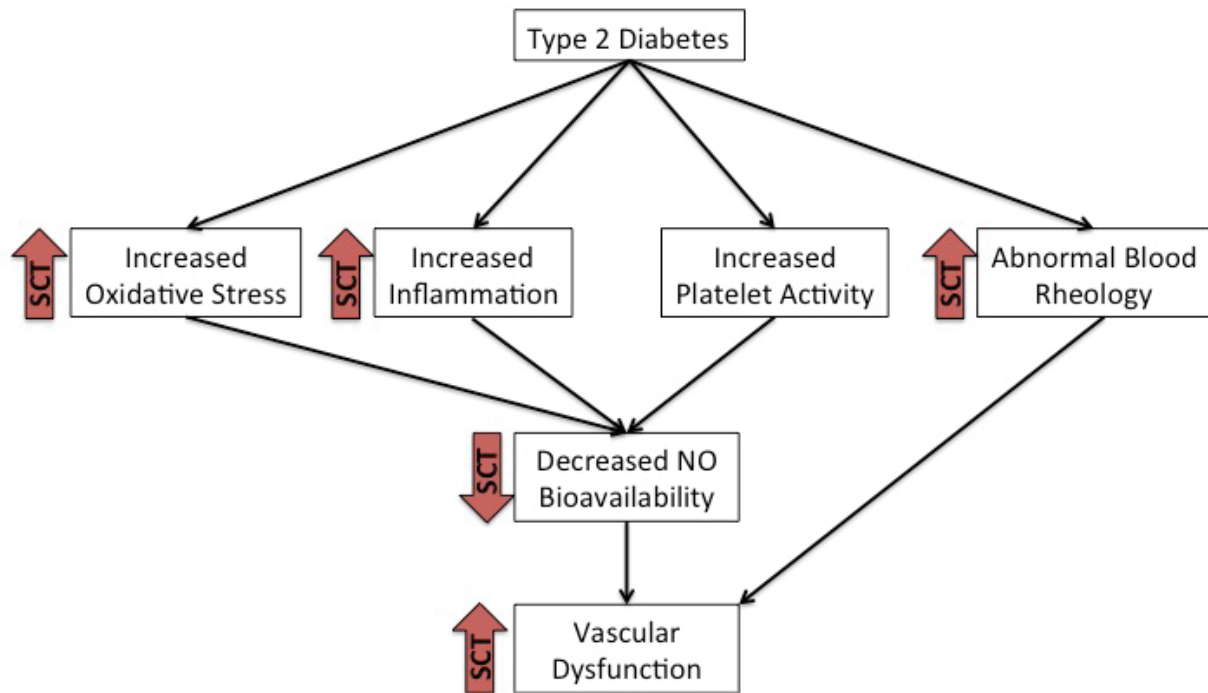


Figure 25: Possible sources of vascular dysfunction in combined T2D and SCT. In T2D hyperglycemia, hyperlipidemia, and hyperinsulinemia lead to increased oxidative stress, inflammation, and platelet activity, resulting in decreased NO bioavailability and vascular dysfunction. T2D is also characterized by alterations in blood rheology, including elevated plasma viscosity, augmented erythrocyte aggregation, decreased red blood cell deformability, and increased blood viscosity, which may contribute to vascular dysfunction. In the figure, the red arrows represent how SCT could exacerbate vascular dysfunction in T2D by intensifying alterations in blood rheology, increasing inflammation, augmenting oxidative stress, and decreasing NO bioavailability, thereby increasing the risk of developing vascular complications.

Although SCT could potentially increase vascular dysfunction in T2D, few studies have been conducted to determine whether heterozygosity for HbS increases the risk of diabetic vascular complications. The limited research that has been published has mainly concentrated on the effects of SCT on diabetic retinopathy and kidney complications, and has reported inconsistent results. The first study conducted to explore the association between SCT and diabetic complications was published by Page et al in 1979, and focused on retinopathy (Page, MacKay, and Paterson 1979). The study included 124 West Indian diabetic patients of African heritage, but only 18 of the participants had SCT (Page, MacKay, and Paterson

1979). Subjects were evaluated and classified into one of three groups: no retinopathy (SCT, n=6; Control, n=42), non-proliferative retinopathy (SCT, n=8; Control, n=41), or proliferative retinopathy (SCT, n=4; Control, n=23). Results of a chi-square test showed no differences between the proportions of SCT subjects in the proliferative retinopathy, non-proliferative retinopathy, and no retinopathy groups. Therefore, the authors concluded that SCT did not appear to have an adverse effect on the incidence or severity of diabetic retinopathy (Page, MacKay, and Paterson 1979). However this study included several major limitations, including the very small sample size of individuals with SCT, and the lack of information about subjects' medications. Furthermore, subjects were all patients at an ophthalmic center, thereby increasing the risk of referral bias.

Two recent studies have supported the findings of Page et al. In 2010, a study by Bleyer et al, including 376 African American patients with T2D, 110 of which were carriers of SCT, reported that 36.6% of African American patients without SCT and 22.7% of the African Americans with SCT had retinopathy, peripheral vascular disease, or end-stage kidney disease ($p < 0.01$) (Bleyer, Reddy, et al. 2010). Furthermore, no differences in mean estimated glomerular filtration rate (eGFR), or protein excretion were found between the groups (Bleyer, Reddy, et al. 2010). However, the controls were both older, and more likely to be on insulin than the SCT subjects. Therefore it is possible that age and/or diabetes severity could have affected the results. Finally, a major flaw of the study was the lack of information regarding subjects' use of medications, like angiotensin converting enzyme inhibitors, which prevent the development of microvascular complications (Bleyer, Reddy, et al. 2010). Similarly, a single center retrospective case control study of 100 patients with T2D and 100 patients with T2D and SCT by Al Harbi and colleagues

found that the prevalence of diabetic macular edema and/or proliferative diabetic retinopathy was significantly lower in the combined SCT and T2D group (58%) than the control T2D group (95%) ($p < 0.001$), suggesting a possible protective effect of SCT against the development and progression of retinopathy (Al Harbi et al. 2016). The study also found no significant differences in rates of nephropathy, hypertension, or hyperlipidemia between the two groups (Al Harbi et al. 2016). However, this study also had noteworthy flaws. For example, the SCT group had a significantly shorter duration of T2D compared to the controls (median [25% quartile] 15 [8.3] years versus 20 [14.7] years, respectively) ($p < 0.001$), and significantly better metabolic control than the control group (mean difference 1.6 mmol/l, (95% confidence interval [CI], 0.1– 3.3; $p = 0.03$). Additionally, random blood glucose was the only measure used to assess metabolic control in the study. Therefore, one cannot rule out the possibility that the between group differences observed in the study were actually due to differences in diabetes duration, or metabolic control. Finally, all of the subjects in the study were recruited from patients admitted to an ophthalmic care center, therefore there is a risk of referral bias (Al Harbi et al. 2016).

Two additional studies have found that SCT was associated with an increased risk of diabetic complications, although not significantly. One study conducted in a population of Afro-Caribbean patients (34 subjects with T2D and SCT, 147 subjects with T2D alone) calculated an odds ratio of 1.19 (CI 0.53-2.68; $p = 0.58$) for microalbuminuria and 1.57 (CI 0.56-4.49; $p = 1.57$) for all grade retinopathy in combined SCT and T2D compared to T2D alone (Oli et al. 2004). Although the differences between the experimental groups were not statistically significant the authors concluded that the increased odds ratio for retinopathy was still remarkable, as retinopathy in individuals with SCT is relatively rare (Oli et al. 2004). The second

study, by Ama et al, was conducted to determine the effect of SCT on metabolic control in individuals with T2D. The study's primary finding was that SCT does not affect metabolic control in T2D, but the study also reported a trend for decreased mean eGFR in the combined T2D and SCT group ($p=0.08$) (Ama et al. 2012). Both of these studies included significant shortcomings, including small sample sizes, and a lack of information about subjects' medications.

Three additional studies have reported significantly increased rates of T2D complications in carriers of SCT, thereby indicating that SCT could increase the risk of developing diabetes complications. First, a study conducted on a group of 39 Nigerian adults with SCT and T2D, and a group of 39 adults with T2D and normal hemoglobin concluded that diabetic patients with SCT may have an increased risk of developing proteinuria compared to diabetic patients with normal hemoglobin (Oli and Reid 1985). The two groups were matched for sex, age, duration of diabetes, and metabolic control (Oli and Reid 1985). In agreement with these findings, Ajayi et al reported that males with combined T2D and SCT ($n=8$) had a higher risk ratio (RR 1.6, CI 1.58-1.62; $p<0.02$) for complications (retinopathy, proteinuria, urinary albumin, neuropathy, and leg/foot ulcers) than males with T2D and normal hemoglobin ($n=31$). However no differences were observed between the groups of females (SCT $n=8$; control $n=13$) (Ajayi and Kolawole 2004). The authors argued that their findings were in accordance with past studies showing more end organ damage in male compared with female patients with T2D (Ajayi and Kolawole 2004). Finally, a recent study by Diaw et al measured the prevalence of diabetes complications as well as vascular function, blood viscosity, biomarkers of oxidative stress, inflammation, and nitric oxide metabolism in patients with combined T2D and SCT ($n=14$), T2D alone ($n=14$), SCT alone ($n=18$), or neither T2D nor SCT ($n=14$)

(Diaw et al. 2015). Patients with SCT and T2D had an increased frequency of arterial hypertension and microalbuminuria compared to the other groups, as well as a higher carotid-femoral PWV and reduced FMD, suggesting increased arterial stiffness and impaired endothelium dependent vasodilation (Diaw et al. 2015). The study also found that individuals with both SCT and T2D had increased plasma inflammation and oxidative stress (Malondialdehyde, Advanced Oxidation Protein Products, Nitrotyrosine), increased plasma AGEs, and increased blood viscosity, all factors that could play a role in exacerbating the vascular dysfunction present in T2D (Diaw et al. 2015). The major limitation of all three of these studies was the small sample sizes used.

In conclusion, the few prior studies that have evaluated whether SCT could increase the risk of developing diabetes-related vascular complications have reported conflicting results. These contradictory findings could be attributed to a variety of factors. First, the racial and ethnic backgrounds of the studies' populations, as well as the proportion of male and female participants varied widely between the studies. Furthermore, all of the studies had very small overall sample sizes and/or limited numbers of participants with combined T2D and SCT. Finally, the methods used to evaluate the presence of vascular complications varied between studies. For example, some studies evaluated renal function by measuring proteinuria, while others used estimated glomerular filtration rate. Overall, it remains unclear whether SCT could amplify the risk of developing vascular complications in people with T2D. For this reason, more studies need to be conducted to determine whether SCT could alter vascular function and increase the risk of vascular complications in people who have T2D. These studies are necessary to create guidelines for medical professionals about whether carriers of SCT with T2D should be screened more

frequently for vascular complications, especially in light of current predictions that the number of individuals with both SCT and T2D will increase in the future.

PART 3 – Personal Contribution

OBJECTIVES AND HYPOTHESES

Considering the large and growing number of individuals with both T2D and SCT, appropriate guidelines need to be created in order to effectively diagnose and treat individuals with both T2D and SCT. For this reason, the primary objectives of this thesis are to study: (1) the challenges associated with diagnosing T2D and measuring glycemia in individuals with SCT (2) the mechanisms and consequences of the amplified T2D-related vascular dysfunction associated with SCT.

In order to address the primary objectives of this thesis, this section includes one “comment”, and four original studies. The comment and the first study, which was conducted using a cohort of Senegalese adults with and without SCT, focus on the complications related to diagnosing T2D and measuring glycemia in people with SCT. The second study compares rates of vascular complications in the same cohort of adults from Senegal with SCT, T2D, combined T2D and SCT, or controls without SCT or T2D. Lastly, studies three and four were conducted to study the mechanisms of alterations in vascular function associated with combined T2D and SCT using a transgenic mouse model.

➤ **Comment:** The primary objective of this short report was to raise awareness of the many challenges associated with the diagnosis and management of T2D in individuals with SCT. This comment addresses: the interference of HbS with HbA1c measurement methods, the challenges faced by physicians who are unaware of patients’ SCT status, and the roles of race and inter-individual differences in HbA1c measurement.

➤ **Study 1:** The objective of this study was to evaluate the relationships and agreement between three measures of glycemia (HbA1c, fasting glucose, and

fructosamine) in Senegalese adults with and without SCT. HbA1c, fasting glucose, and fructosamine were measured in 103 people with SCT (SCT group) and 100 without SCT (control group). The associations among the measures of glycemia were calculated in each group using Pearson's correlations. The agreement between the markers was calculated using Bland Altman analyses in each group. Finally, a clinical cut point for hyperglycemia was set for each marker of glycemia, and the number of subjects over the clinical cut point for each marker was calculated in both groups.

➤ **Study 2:** The primary focus of study 2 was to compare the rates of vascular complications among individuals with combined T2D and SCT, T2D, SCT, or neither T2D nor SCT (controls). The secondary aim of the study was to study factors that could contribute to the development of vascular complications in people with combined T2D and SCT. Rates of hypertension, retinopathy, neuropathy, impaired renal function, and peripheral artery disease were determined for each group. Additionally, arterial stiffness, blood rheological parameters, and plasma concentrations of AGEs and cytokines were measured in all subjects. Furthermore, plasma from subjects in each group was incubated with human aortic endothelial cells to measure the expression of inflammatory and adhesion factors. Finally, the effects of AGE or oxidative stress inhibition on human aortic endothelial expression of E-selectin following incubation with subjects' plasma were measured.

➤ **Study 3:** The purpose of this study was to determine whether alterations in blood viscosity and vascular function are amplified in a mouse model of combined T2D and SCT. The study used Townes transgenic mice fed either a standard diet or a high fat high sucrose diet for 12 weeks in order to create mouse models of combined T2D and SCT, T2D, SCT, and controls. Endothelium independent

vasodilation was measured using laser Doppler flowmetry paired with iontophoretic delivery of sodium nitroprusside. Endothelium dependent vasodilation was measured using laser Doppler flowmetry and pressure-induced vasodilation. Blood rheological parameters, including hematocrit, whole blood viscosity, and red blood cell deformability, were also measured in all of the mice.

➤ **Study 4:** This study was carried out to study microvascular function in a mouse model of combined T2D and SCT. The same protocol used in study 3 was used to create mouse models of combined T2D and SCT, T2D, SCT, and controls. Laser Doppler flowmetry was used to measure skin blood flow during iontophoresis of acetylcholine and sodium nitroprusside, to measure endothelium-dependent and endothelium-independent vasodilation, respectively. The role of COX-2 in modulating acetylcholine-induced vasodilation was measured using a specific inhibitor of COX-2.

COMMENT: Sickle-cell trait and diagnosis of type 2 diabetes

Sarah Skinner, Vincent Pialoux, Bérengère Fromy, Dominique Sigaud-Roussel, Philippe Connes

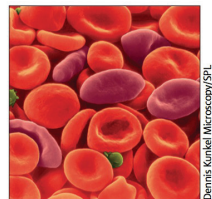
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As explained in the review of the literature in Part 2 of this thesis, the number of individuals with both T2D and SCT is on the rise in many populations around the world. Thus, it seems imperative that medical professionals be made aware of the specific challenges associated with diagnosing and treating patients with combined T2D and SCT. For this reason, this comment was written in order to summarize the wide variety of factors that complicate diabetes diagnosis and management in people with SCT. For instance, the accuracy of HbA1c as a measurement of glucose control could be affected by the measurement method used, the presence of SCT or other hemoglobinopathies, variations in race, and other inter-individual differences. Ultimately, this article draws attention to the fact that, until the reasons for the variability in HbA1c performance are better understood, use of HbA1c alone may not be ideal for diagnosing diabetes in individuals with SCT.

Sickle-cell trait and diagnosis of type 2 diabetes



Dennis Kunkel Microscopy/SHL

Sickle-cell trait is the heterozygous form of sickle-cell disease, a hereditary blood disorder caused by a single mutation of the β -globin gene, resulting in the production of abnormal haemoglobin called haemoglobin S (HbS).¹ Although sickle-cell disease results in a range of painful complications, sickle-cell trait is generally asymptomatic and confers a protective advantage against *Plasmodium falciparum* malaria.¹ Consequently, sickle-cell trait is prevalent in malarial regions, including sub-Saharan Africa, the Middle East, and India, as well as in certain populations of western Europe and the Americas, because of migrations from Africa.² Concurrently, estimates from the International Diabetes Foundation show that rates of type 2 diabetes are alarmingly high, and rapidly increasing, in each of these populations.³ Overall, these epidemiological trends suggest that there is probably a large and growing number of people with both type 2 diabetes and sickle-cell trait. This possibility is alarming because although sickle-cell trait has historically been regarded as benign, research in the past 15 years shows that sickle-cell trait could increase the risk of stroke, venous thromboembolism, chronic kidney disease, and end-stage renal disease.¹ Furthermore, whether the association of type 2 diabetes and sickle-cell trait could further increase the risk of diabetes-related complications remains an open question. To facilitate further research and ensure accurate diagnosis of type 2 diabetes in the sickle-cell trait population, guidelines for reliable diabetes screening in individuals with sickle-cell trait need to be established.

HbA_{1c} is frequently used for diabetes diagnosis, as recommended by an international committee of experts in 2009.^{4,5} However, this recommendation might not be appropriate for individuals with sickle-cell trait because HbS can interfere with HbA_{1c} measurement.⁴ HbA_{1c} is formed when glucose binds specifically to the N-terminal valine of the haemoglobin β chain, and its measurement relies on a normal erythrocyte lifespan.⁴ Sickle-cell-trait erythrocytes contain 60–70% HbA and 30–40% HbS, and are thought to have a normal lifespan.⁶ Therefore HbA_{1c} can be used in individuals with sickle-cell trait, unless the presence of HbS interferes with the measurement method used.⁴

The basic methods of HbA_{1c} measurement are immunoassay, ion-exchange high-performance liquid chromatography (HPLC), boronate affinity HPLC, enzymatic assays, and capillary electrophoresis (table).^{4,8} HbS has been shown to interfere with immunoassay and HPLC methods for several reasons (table), whereas enzymatic assays and capillary electrophoresis seem to reliably measure HbA_{1c} in individuals with sickle-cell trait.^{4,8} To help medical professionals and researchers to accurately measure HbA_{1c} in people with haemoglobin variants, the National Glycohemoglobin Standardization Program (NGSP) has published a table listing the most frequently used HbA_{1c} methods, and whether the presence of HbS, or other haemoglobinopathies, results in a clinically significant interference of HbA_{1c} measurement.⁷ However, in a study reported in 2017,

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	Mechanism	Effect of haemoglobin S
Immunoassay	Measures HbA _{1c} specifically using antibodies that recognise the structure of the N-terminal glycosylated amino acid of the β chain ⁴	Can be affected by the presence of haemoglobin S because the mutation is very close to the N-terminus of the β chain ⁴
Ion-exchange HPLC	Separates haemoglobin species on the basis of differences in charge between HbA _{1c} and other haemoglobin ⁴	Haemoglobin variants alter the ion charge of the haemoglobin, which could interfere with ion-exchange methods, but most HPLC ions are unaffected by sickle-cell trait ^{4,7}
Boronate affinity HPLC	m-aminophenylboronic acid reacts specifically with the cis-diol groups of glucose-bound haemoglobin, enabling the separation of total glycosylated haemoglobin from non-glycosylated haemoglobin, irrespective of species ⁴	Very low interference caused by the presence of haemoglobin S because this method separates total glycosylated haemoglobin from non-glycosylated haemoglobin, irrespective of the species ⁴
Enzymatic assays	Measures HbA _{1c} using an enzyme that specifically cleaves at the N-terminal valine ⁴	Has not been shown to be affected by the presence of haemoglobin S ⁴
Capillary electrophoresis	Separates haemoglobin fractions by electrophoresis ⁸	Has been shown to reliably measure HbA _{1c} in individuals with haemoglobin S; this method can reliably identify the presence of haemoglobin S ⁹

HPLC=high-performance liquid chromatography. *According to the National Glycohemoglobin Standardization Program.

Table: Basic methods of HbA_{1c} measurement

Lacy and colleagues⁶ compared HbA_{1c} between African Americans with and without sickle-cell trait using two versions of the Tosoh ion-exchange HPLC assay, which are classified by NGSP as not being subject to clinically significant interference from HbS. The results showed that African Americans with sickle-cell trait had significantly lower HbA_{1c} than African Americans without sickle-cell trait at the same 2 h fasting glucose classification.⁶ Moreover, the prevalence of prediabetes and type 2 diabetes was significantly lower in participants with sickle-cell trait compared with participants without when defined using HbA_{1c} values, but was similar when defined using glucose-based methods.⁶ These results suggest that HbA_{1c} methods that are not subject to clinically significant interference from HbS under the NGSP's standards can still result in a delayed diagnosis of diabetes in individuals with sickle-cell trait.

Overall, the evidence suggests that special care should be taken when choosing a method for HbA_{1c} assessment in individuals with sickle-cell trait. To ensure accurate diabetes diagnosis, HbA_{1c} can be used in combination with traditional glucose criteria. Additionally, alternative measures of glucose control such as fructosamine, glycated albumin, or 1,5-anhydroglucitol, could be used because they are not affected by HbS.⁹ However, these non-traditional markers do have other limitations, and diagnostic guidelines for their use are still under development, although glycated albumin is frequently used in China, Japan, and South Korea.⁹

Ideally, the method used for diabetes diagnosis should be decided on a case-by-case basis.¹⁰ However, choosing the appropriate method to use for individuals with sickle-cell trait might prove challenging for several reasons. First, many individuals are unaware that they have sickle-cell trait, and are therefore unable to report their sickle-cell-trait status to their doctor. National neonate screening programmes are rare, especially in areas where HbS is prevalent.² Even in the USA, where all states and Washington DC have implemented newborn screening programmes for sickle-cell disease and sickle-cell trait, only 37% of parents report having received notification of the sickle-cell-trait status of their child.¹¹ To further complicate matters, individuals with sickle-cell trait can also have other factors that interfere with HbA_{1c} measurement, such as thalassaemia syndromes, or high haemoglobin F.^{4,10}

Notably, studies have shown that abnormal results obtained from some routine HbA_{1c} methods (eg, HPLC and capillary electrophoresis) can be used to accurately (and inexpensively) identify some haemoglobin variants and haemoglobinopathies.^{8,12} However, these methods are not yet approved as diagnostic tools for this purpose.

Another factor that complicates the interpretation of HbA_{1c} in sickle-cell trait is race. Sickle-cell trait is highly prevalent among Africans and people of African descent.^{1,2} HbA_{1c} values are higher in African Americans than in white individuals, even after adjustment for fasting and 2 h glucose concentrations and covariates such as socioeconomic status, health-care access, diabetes treatment, and obesity.^{10,13} However, emerging research suggests that interindividual differences, caused by factors such as erythrocyte survival, extracellular-intracellular glucose balance, and genetic determinants of haemoglobin glycation, could have a more important role than inter-racial differences when measuring HbA_{1c}.^{10,13} Therefore, additional research needs to be done to determine whether type 2 diabetes can be accurately diagnosed in all individuals using HbA_{1c} alone, or whether additional tests for haemoglobinopathies, haemolytic anaemia, iron deficiency, and liver and renal impairment should be required to avoid delayed diagnosis of type 2 diabetes.¹⁰

Until the reasons for variations in HbA_{1c} measurements are better understood, HbA_{1c} might not be the ideal method to use for diagnosis of type 2 diabetes in individuals with sickle-cell trait, or in populations in which sickle-cell trait is common. As rates of diabetes continue to climb in these populations, it is crucial that more research be done to develop reliable guidelines for accurate diabetes diagnosis in individuals with sickle-cell trait. Use of HbA_{1c} as a sole measure of glucose control could provide researchers and medical professionals with inaccurate information. HbA_{1c} measurements that do not correctly reflect glucose control could result in delayed diabetes diagnoses, increasing patients' risks of developing diabetes-related complications. Furthermore, missed diabetes diagnoses could lead to an underestimation of the severity of the diabetes epidemic in these populations, and to flawed research about the relation between sickle-cell trait and diabetes-related complications.

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We declare no competing interests.

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STUDY 1: Evaluation of Agreement Between HbA1c, Fasting Glucose, and Fructosamine in Senegalese Individuals with and without Sickle-cell Trait

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*Equivalent position

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Timely diagnosis and effective monitoring of T2D is crucial to preventing the onset of severe T2D-related vascular complications. The oral glucose tolerance test is the gold standard for diagnosing diabetes; however the test requires an overnight fast and a substantial time commitment from patients and clinicians. For this reason, HbA1c and fasting glucose are the tests most commonly used to screen for and monitor diabetes. Yet, as explained in the comment, HbA1c could perform sub-optimally in people with SCT. Furthermore, fasting glucose has been shown to have a sensitivity of less than 50% in individuals of African descent (Utumatwishima et al. 2018). Therefore, alternative markers of glycemia, such as fructosamine, have been suggested as options to measure glycemia in people of African descent (Parrinello et al. 2016). However, it remains unknown whether HbA1c, fructosamine, and fasting glucose perform comparably in Africans with and without SCT. Therefore, the objective of this study was to evaluate the relationships and agreement between HbA1c, fructosamine, and fasting glucose in Senegalese adults with and without SCT.

This study was conducted in Senegal. Men and women with and without SCT (mean age 51.4, [SD, 7.6] years) were recruited as participants and sorted into either the SCT group (n=103), or the control group (n=100) (without SCT). Individuals with normoglycemia and T2D were included in the study. Following an overnight fast, blood was sampled from all participants, and fasting glucose, HbA1c, and fructosamine were measured. In order to compare the three measures of glycemic control, a “clinical cut point” for hyperglycemia was assigned for each marker of glycemia. Next, all values were normalized and expressed as percentages of the “clinical cut point”. Bland-Altman analyses were conducted to compare the agreement between the measures in both groups. The percentage of subjects above

the “clinical cut point” for diabetes was calculated for each measure of glycemia in both groups, and then compared within the groups. Finally the associations between HbA1c, fructosamine, and fasting glucose were investigated using Pearson’s correlations.

Positive, significant correlations were found between all three measures of glycemia in both the control and SCT groups. Considerable disparities between HbA1c, fasting glucose, and fructosamine were observed in both groups. However, these differences were exaggerated in the SCT group. Additionally, out of the three measures of glycemia tested in the study, using HbA1c resulted in the lowest percentage of subjects classified as over the “clinical cut point” for hyperglycemia in both groups. These results indicate that using only one of these measures of glycemia could potentially result in inaccurate assessments of individuals’ glycemic control, and delayed diagnoses of T2D, especially in people with SCT.

RESEARCH ARTICLE

Evaluation of agreement between hemoglobin A1c, fasting glucose, and fructosamine in Senegalese individuals with and without sickle-cell trait

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Data Availability Statement: The French data protection authority (Commission Nationale de l'Informatique et des Libertés) strictly forbids the provision of a full database with individual data, because the large number of variables used for this analysis could collectively be used to re-identify the persons participating in this study from a few key characteristics, making it possible to gain access to other personal data. However, we may provide stratified data on the patient studied on requests to the corresponding author: i.e., Dr Philippe Connes.

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Abstract

Fasting glucose (FG) and glycated hemoglobin A1c (HbA1c) perform sub-optimally in people of African origin, especially in individuals with sickle-cell trait (SCT). The purpose of this study was to compare the relationships between HbA1c, FG, and fructosamine in individuals from Senegal with and without SCT. HbA1c, FG, and fructosamine were measured in 203 adults from Senegal (100 control: 45 with type 2 diabetes (T2D); 103 SCT: 51 with T2D). Significant, positive correlations were observed between HbA1c and FG, fructosamine and FG, and fructosamine and HbA1c in both groups. The limits of agreement were inappropriately large in both groups for the Bland-Altman plots of HbA1c and FG (control: -95.97 to 83.97%; SCT: -115.9 to 91.52%), fructosamine and FG (control: -100.6 to 99.89%; SCT: -105.6 to 100.6%), and fructosamine and HbA1c (control: -52.03 to 38.98%; SCT: -88.04 to 71.41%). In both groups, the greatest proportion of subjects were considered above the clinical cut-point for hyperglycemia when fructosamine was used as the criterion (control: 33%; SCT: 44.6%), and the lowest percentage of subjects were classified as over the clinical cut-point when HbA1c was used as the criterion (control: 21%; SCT: 27.7%). Substantial disparities between HbA1c, FG, and fructosamine were observed in both groups, and these differences were exaggerated in the SCT group. Therefore, these three biomarkers should not be considered to be interchangeable measures of glycemic control. These biomarkers should be used thoughtfully, and special care should be taken when using them in individuals with SCT.

In addition, request can be sent to the Ethical committee: Dr Alioune Dieye; comite.ethique@ucad.edu.sn The French data protection authority does not allow the identification (i.e., name and surname) of patients participating in the study.

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Introduction

Sickle-cell trait (SCT), the heterozygous form of sickle-cell disease, is highly prevalent in Africa, especially in Central and Western Africa where between 10–30% of the population are carriers of the hemoglobin S (HbS) gene [1]. In 2017, the International Diabetes Federation (IDF) estimated that more than 15.9 million people in Africa had type 2 diabetes (T2D), and the prevalence is expected to increase 162% by 2045 [2]. This data indicates that there is likely a large, and growing, population of individuals in Africa who have both T2D and SCT.

Early diagnosis and effective monitoring of T2D are essential to decreasing the risk of developing T2D-related complications [3]. However, 70% of people in Africa who have T2D do not know that they have the disease, a higher percentage than in any other region of the world [2]. Although this problem is often attributed to a lack of access to adequate health care, inadequate performance of diagnostic tests may also contribute to this issue [3].

The oral glucose tolerance test (OGTT) is the gold standard for T2D diagnosis [3, 4]. However, the OGTT is expensive, requires patients to arrive in a fasted state, and demands a substantial time commitment from patients and medical professionals [3, 5]. For these reasons, fasting glucose (FG) and hemoglobin A1c (HbA1c) are the tests most commonly used by clinicians and epidemiologists around the globe to deliver patient care and generate public health statistics [3].

Measurement of HbA1c, a glycated form of hemoglobin A, does not require an overnight fast and reflects glucose control over a 2–3 month period [5, 6]. HbA1c has been widely used as a standard measure of glycemic control since the 1980s, and was recently recommended for use as a diagnostic test for diabetes by a committee of international experts [3, 5, 7]. However, HbA1c performs sub-optimally in people with SCT, likely due to the presence of HbS in SCT erythrocytes, thereby complicating T2D diagnosis in these individuals [8, 9]. Furthermore, FG measurement, which requires an overnight fast, has been shown to have a sensitivity of less than 50% in individuals of African descent [3]. The disadvantages associated with traditional measures of glucose tolerance have led to an increased interest in alternative markers of hyperglycemia, like glycated proteins [6, 10].

Fructosamine is a measure of total glycated serum protein that does not require an overnight fast, is cheaper and easier to perform than HbA1c assays, and could overcome the limitations of HbA1c in certain patients with conditions that affect the reliability of HbA1c, such as SCT [5, 6]. This biomarker has a half-life of 14–21 days, and thus measures glucose control over a 2–3 week period [6]. Fructosamine is significantly associated with HbA1c and FG, and has been shown to independently predict T2D incidence and microvascular complications in populations including both black and white individuals with and without T2D [10–12]. However, the relationships between HbA1c, FG, and fructosamine have not been studied in Africans with SCT. Therefore, the objective of this study was to compare the relationships between HbA1c, FG, and fructosamine in individuals with and without SCT (with normal glycemic control and T2D) living in Senegal.

Materials and methods

Setting and participants

The study was conducted at the Cheikh Anta Diop University in Dakar, Senegal. A total of 203 subjects were recruited between August and October of 2017 from the Anti-Diabetic Centre (Marc Sanakalé) at the Hospital Abass Ndao in Dakar, from the National Center of Blood Transfusion (CNTS) of Dakar, and from the general population of Dakar. The study population included individuals with (n = 103; SCT) and without (n = 100; control) SCT. 45% of the

control group and 49.5% of the SCT group had previously been diagnosed with T2D using the American Diabetes Association (ADA) Standards of Medical Care guidelines [4]. The large majority of the subjects with T2D (84.4% control; 84.3% SCT) in the study were being treated with metformin, sulfonylureas, or a combination of the two. Biological analyses were conducted to confirm whether or not subjects were carriers of SCT (blood was screened using iso-electric focusing, and results were confirmed using citrate agar electrophoresis, hemoglobin fraction quantification using high-performance liquid chromatography, and a solubility test confirming the presence of HbS). Hemoglobin concentrations and hematocrit were also measured in all patients. The protocol was conducted in accordance with the guidelines set by the Declaration of Helsinki and was approved by the Ethics Committee of Cheikh Anta Diop University (reference: 0221/2016/CER/UCAD). All subjects gave informed, written consent.

Blood samples and glycemic markers

All biomarkers of hyperglycemia were measured using blood samples drawn at 8:00 am following an overnight fast. Blood was drawn into fluoride tubes for glucose measurement and EDTA tubes for HbA1c and fructosamine analyses. FG was measured using an enzymatic glucosidase-peroxydase method (Urit Medical Electronic Co., Guilin, China). HbA1c was measured using capillary electrophoresis on a Capillary 3 Tera device (Sebia, France). Fructosamine was measured in plasma using a colorimetric assay with nitroterazolium blue (ABX Pentra Fructosamine kit, Horiba, Montpellier, France).

Statistical analysis

Results are presented as means \pm Standard Deviation (SD). Independent Samples t-tests were performed to determine between group differences for hemoglobin concentrations, hematocrit, age, body mass index (BMI), HbA1c, FG, and fructosamine. A χ^2 test was used to compare the sex distributions. Independent Samples t-tests were used to test for significant differences in average HbA1c, FG, and fructosamine between male and female subjects in the overall study population, and within the control and SCT groups. Pearson correlations were used to test the associations between HbA1c and FG, fructosamine and FG, and HbA1c and Fructosamine in the control and SCT groups. Additional Pearson correlations were also conducted to evaluate the associations between all of the measures of glycemic control in the subjects with T2D in the control and SCT groups, independently. Differences between the correlation coefficients of the two groups were then analyzed for statistical significance using Fisher's r to z transformation, followed by a calculation of the observed z -test statistic.

The agreement between HbA1c and FG, fructosamine and FG, and HbA1c and fructosamine for each group was determined using Bland-Altman Analyses [13]. In order to compare HbA1c, FG, and fructosamine, we normalized the values by expressing them as percentages of a clinical-cut point for the diagnosis of T2D. For HbA1c and FG, we used the guidelines established by the ADA for diabetes diagnosis (HbA1c \geq 6.5% and FG \geq 126 mg/dL, respectively) [4]. However, clinical guidelines for T2D diagnosis using fructosamine have yet to be established. Nevertheless, a recent study conducted by Parrinello et al determined that a fructosamine value of 260 μ mol/L was equivalent to a FG value of 126 mg/dL in a population of black adults [14]. Furthermore, a study conducted by Malström and colleagues concluded that it is possible to identify subjects with T2D with reasonable sensitivity and specificity using fructosamine values in the range of 240–260 μ mol/L [15]. Therefore, we chose to fix the clinical cut-point for fructosamine at 260 μ mol/L. For each plot, the bias was calculated as the mean difference between the two measures. To test whether the bias of each plot was significantly different from zero, a one-sample t-test was performed. The upper and lower limits of agreement

(LOA) were defined as ± 1.96 SD of the mean difference. Finally a linear regression line was drawn on the Bland-Altman plot to assess possible changes in bias with decreasing glucose control.

The percentage of subjects in each group classified as over the clinical cut-point for each biomarker was determined and then compared. Chi-Square Tests were conducted to test whether the percentages of subjects classified as above the clinical cut-point for each biomarker were significantly different within each group. Furthermore, an additional analysis was carried out in which subjects with borderline HbA1c values, defined using the ADA definition of prediabetes (HbA1c between 5.7–6.4%), were identified [4]. Average HbA1c, FG, and fructosamine were then compared between the subjects identified as having prediabetes and the subjects identified as over the cut-point when HbA1c was used as the criterion.

Significance level was defined as $p < 0.05$. All statistical analyses were conducted using SPSS (v.24, IBM SPSS Statistics, Chicago, IL). Bland-Altman Plots were drawn using GraphPad Prism version 6 (GraphPad, Sand Diego, CA).

Results

Comparisons between control and SCT groups

The descriptive characteristics of the subjects are shown in Table 1. The average FG, HbA1c, and fructosamine values for each group as well as the average percent cut-points of each value are shown in Table 2. There were no significant differences between groups for hemoglobin concentration, hematocrit age, body mass index, FG, HbA1c, or fructosamine. One subject was determined to be anemic, and was therefore excluded from the analyses. The percentage of male and female subjects did not vary significantly between the two groups. Average FG, HbA1c, and fructosamine did not vary between the male and female subjects. Additionally, analyses of the control and SCT groups individually showed that neither average HbA1c nor average fructosamine varied significantly between the male and female subjects in the control and SCT groups. Average FG was higher in the female subjects (132.61 ± 68.80 mg/dL) compared to the male subjects (104.36 ± 35.58 mg/dL) in the control group only ($p = 0.01$).

Correlations

HbA1c and FG, and fructosamine and FG were weakly correlated in the control group ($r = 0.32$; $p = 0.002$ and $r = 0.31$; $p = 0.002$, respectively) and moderately correlated in the SCT group ($r = 0.53$; $p = < 0.0001$ and $r = 0.58$; $p < 0.0001$, respectively). The correlations between

Table 1. Participant characteristics.

	Control	SCT
Total (n =)	100	103
Sex (M/F)	25/75	16/87
Hemoglobin concentration (g/dL) (M/F)	$12.87 \pm 1.52/12.63 \pm 1.83$	$12.42 \pm 2.22/12.59 \pm 1.38$
Hematocrit (%)	40.06 ± 2.4	40.05 ± 2.4
T2D	45 (45.00%)	51 (49.51%)
Age (years)	51.90 ± 7.64	50.79 ± 7.60
BMI (kg/m ²)	24.81 ± 4.27	24.65 ± 4.09

T2D = Type 2 diabetes

BMI = body mass index; HbS = hemoglobin S

Results are presented as means \pm SD. No significant differences were found between groups.

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Table 2. Biomarkers of hyperglycemia by group.

	Control		SCT	
	Original value	% Cut-point (%)	Original value	% Cut-point (%)
FG	125.69 ± 62.86 mg/dL	99.75 ± 49.89	139.73 ± 77.79 mg/dL	110.9 ± 61.73
HbA1c	6.03 ± 1.55%	89.15 ± 29.72	6.54 ± 2.31%	95.81 ± 40.92
Fructosamine	259.38 ± 86.80 μmol/L	96.76 ± 37.05	283.09 ± 128.05 μmol/L	107.00 ± 51.24

Average values of each biomarker are expressed as whole values and as normalized values, which were calculated as percentages of a clinical cut-point. Clinical cut-points were set at 126 mg/dL for FG, 6.5% for HbA1c, and 260 μmol for fructosamine.

FG = Fasting Glucose; HbA1c = Glycated Hemoglobin A

Results are presented as means ± SD. No significant differences were found between groups.

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fructosamine and HbA1c were fairly strong in both the control and SCT groups ($r = 0.71$; $p < 0.0001$ and $r = 0.61$; $p < 0.0001$, respectively) (Fig 1). The correlation between fructosamine and FG was significantly stronger in the SCT group compared to the control group ($p < 0.05$). The strength of the correlations did not differ between the groups for the correlations between HbA1c and FG ($p = 0.07$) or fructosamine and HbA1c ($p = 0.21$).

When subjects with T2D in the control and SCT groups were considered independently, HbA1c and fructosamine were significantly correlated in the subjects with T2D in the control and SCT groups ($r = 0.68$; $p < 0.0001$ and $r = 0.48$; $p < 0.0001$, respectively). Fructosamine and FG were significantly correlated in the subjects with T2D in the SCT group ($r = 0.39$; $p < 0.01$), but not in the control group. HbA1c and FG were not significantly correlated in the subjects with T2D in the control or SCT groups.

Bland-Altman analyses

The results of the Bland-Altman analyses are displayed in Fig 2.

HbA1c and FG. The mean biases revealed that the FG values were on average $5.54 \pm 45.67\%$ (95% LOA from -95.97 to 83.97%) and $12.21 \pm 52.92\%$ (95% LOA from -115.9 to 91.52%) greater than the HbA1c values in the control and SCT groups, respectively. One-sample t-tests showed that the bias was significantly different from zero in the SCT group ($p = 0.02$), but not in the control group ($p = 0.24$). The percentage of dots outside the LOA was relatively low in both groups (control: 6.2%; SCT: 6.1%). However, the LOA were unacceptably large for both groups. Thus, the two measures cannot be considered to be interchangeable. Furthermore, both plots show a moderate tendency for the bias to become negative, and more variable, with increasing glycemia in both groups (Control: $r = -0.61$; $R^2 = 0.37$; $p < 0.0001$ and SCT: $r = -0.57$; $R^2 = 0.33$; $p < 0.0001$).

Fructosamine and FG. The mean biases showed that the FG values were on average $0.37 \pm 51.16\%$ (95% LOA from -100.6 to 99.89%) and $2.50 \pm 52.59\%$ (95% LOA from -105.6 to 100.6%) greater than the fructosamine values for the control and SCT groups, respectively. One-sample t-tests revealed that these biases were not significantly different from zero for either group (control: $p = 0.94$; SCT: $p = 0.63$). A relatively small percentage of the dots (5.1% of the control group and 6.8% of the SCT group) were outside of the LOA for both groups. However, the LOA were unacceptably large for both groups, indicating that the two measures are not interchangeable. Finally, the significant negative linear regressions show a moderate tendency for the bias to decrease with increasing glycemia in the control ($r = -0.41$; $R^2 = 0.169$; $p < 0.0001$) and SCT ($r = -0.24$; $R^2 = 0.061$; $p = 0.012$) groups. Both plots showed that variability tended to increase in both groups as glycemia increased.

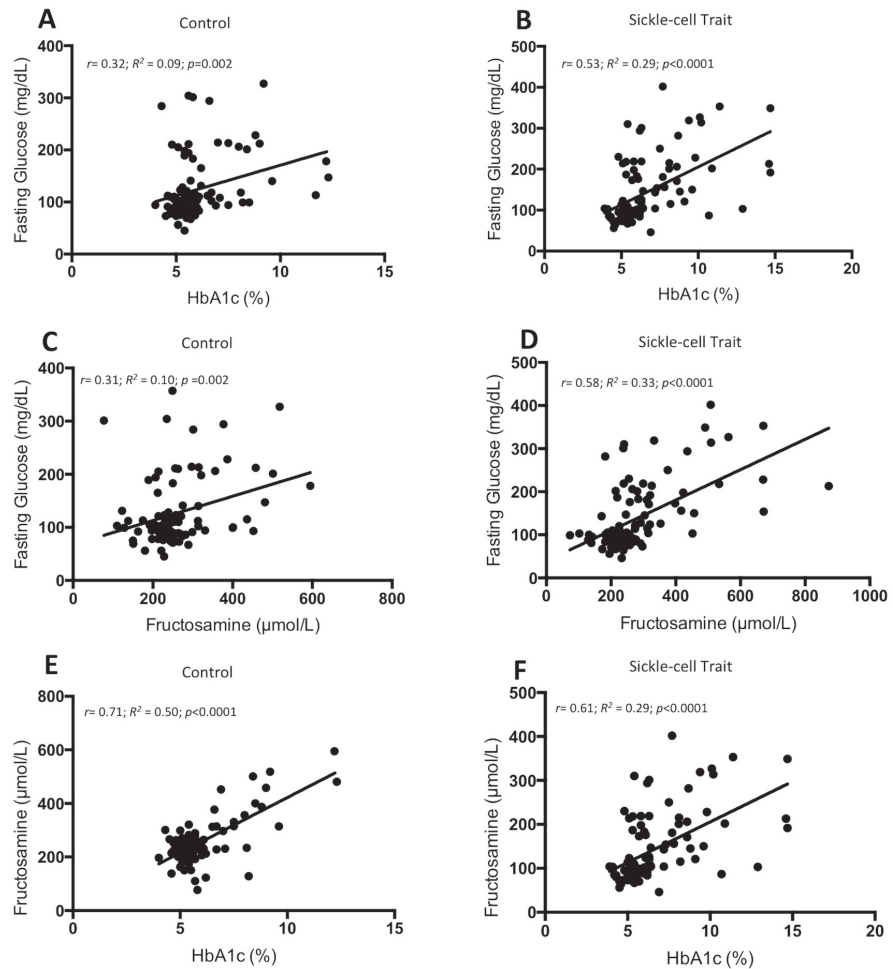


Fig 1. Scatterplots of glycated hemoglobin A1c (HbA1c) versus fasting glucose (FG), fructosamine versus FG, and HbA1c versus fructosamine for control and SCT groups. The best-fit model (solid line) is shown for each plot along with the coefficient of determination (R^2), the Pearson correlation coefficient, and the p-value of the correlation.

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Fructosamine and HbA1c. The mean biases showed that the fructosamine values were on average $6.52 \pm 23.22\%$ (95% LOA from -52.03 to 38.98%) and $8.31 \pm 40.68\%$ (95% LOA from -88.04 to 71.41%) larger than the HbA1c values for the control and SCT groups, respectively. The one-sample t-tests demonstrated that the biases for the two groups were significantly different from zero (control: $p = 0.008$; SCT: $p = 0.046$). The percentage of dots outside of the LOA was fairly small and similar between the two groups (7.5% of the control group and 8.2% of the SCT group). The LOA were unacceptably large for both groups, but they were much larger in the SCT than in the control group. This suggests that HbA1c and fructosamine are not interchangeable, especially not for carriers of SCT. Additionally, the negative linear

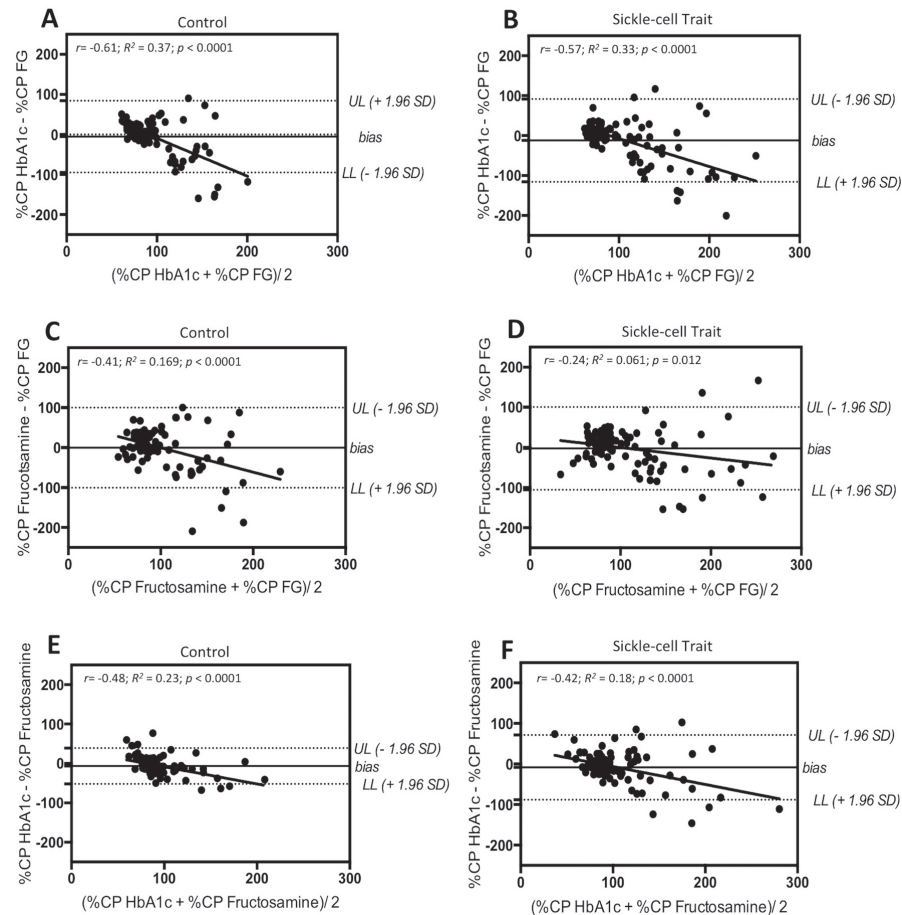


Fig 2. Bland-Altman plots showing the agreement between glycated hemoglobin A1c (HbA1c) and fasting glucose (FG) (plots A and B), fructosamine and FG (plots C and D), and HbA1c and fructosamine (plots E and F) for the control (plots A, C, and E) and sickle-cell trait groups (plots B, D, F). Each plot represents the mean of the two methods (x-axis) against their differences. The mean difference between the two methods (bias) is shown, as are the Upper (UL) and Lower limits (LL) of agreement. %CP HbA1c = percent of the clinical cut-point of HbA1c. %CP FG = percent of the clinical cut-point of fasting glucose. %CP Fructosamine = percent of the clinical cut-point of fructosamine.

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regressions show a moderate tendency for the bias to become more negative and more variable as glycemia increases in both groups (Control: $r = -0.48$; $R^2 = 0.23$; $p < 0.0001$ and SCT: $r = -0.42$; $R^2 = 0.18$; $p < 0.0001$).

Classification as over the clinical cut-point and chi-square analyses

10% more control subjects (four with T2D) and 12.1% more SCT subjects (eleven with T2D) were considered over the clinical cut-point when classified using FG as the criterion versus

HbA1c. The chi-square analyses showed that the number of subjects classified as over the clinical cut-point was significantly greater when using FG as compared to HbA1c in both the control ($p = 0.016$) and SCT ($p < 0.0001$) groups.

2% more control (neither diagnosed with T2D) and 4.8% more SCT (none with T2D) subjects were classified as above the clinical cut-point when using fructosamine rather than FG as the glycemic parameter. The chi-square analyses showed that the proportion of subjects classified as above the clinical cut-point when using fructosamine as the criterion was significantly greater than when using FG in both the control ($p = 0.003$) and SCT ($p < 0.0001$) groups.

12% more control (four with T2D) and 16.9% more SCT subjects (eight with T2D) were considered above the clinical cut-point when classified using fructosamine versus HbA1c. The results of the chi-square analyses indicated that these differences were significantly different in both the control ($p < 0.0001$) and SCT ($p < 0.0001$) groups (Table 3).

Average HbA1c and fructosamine values were significantly lower in individuals classified as having prediabetes compared to those classified as over the cut-point in both the control and SCT groups. Fasting glucose tended to be lower ($p = 0.06$) in individuals with prediabetes compared to those classified as over the cut-point in the control group, and was significantly lower in individuals with prediabetes compared to those above the clinical cut-point in the SCT group (Table 4).

Discussion

The primary finding of the study is that although HbA1c, FG, and fructosamine were significantly and positively correlated in both the control and SCT groups, analyses of agreement revealed considerable disparity between the three biomarkers of hyperglycemia. Furthermore, these differences were particularly evident in the SCT group. These results suggest that these biomarkers are not interchangeable, and should be interpreted carefully, especially in individuals with SCT.

The correlational analyses showed moderate associations between HbA1c and FG and between fructosamine and FG, as well as fairly strong associations between HbA1c and fructosamine in both groups. Additional analyses showed that HbA1c and fructosamine were significantly associated in individuals with diabetes in both groups. These findings correspond with multiple studies that have found significant, positive associations between HbA1c, fructosamine, and FG in individuals with and without diabetes [5, 15–18]. However, our findings that fructosamine and FG were associated in people with diabetes in the SCT group only, while HbA1c and FG were not associated in subjects with diabetes in either group, conflict with these previous findings [5, 15–18].

Furthermore, our results coincide with the findings of a study by Jurascheck et al, which also observed a stronger correlation between fructosamine and HbA1c than fructosamine and FG or HbA1c and FG [12]. In addition, as has been observed in other studies, none of the biomarkers of glycemia were very strongly correlated in the current study [5]. This could be due to the physiological differences between the three biomarkers (such as the differences in half

Table 3. Number of subjects classified as over the clinical cut-point for each biomarker of hyperglycemia.

	FG	HbA1c	Fructosamine
Control	31 (31.0%)	21 (21.0%)	33 (33%)
SCT	41 (39.8%)	28 (27.7%)	46 (44.6%)

Clinical cut-points were set at 126 mg/dL for FG, 6.5% for HbA1c, and 260 μ mol for fructosamine.

FG = Fasting Glucose; HbA1c = Glycated Hemoglobin

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Table 4. Average fasting glucose, HbA1c, and fructosamine in subjects classified as having prediabetes and subjects classified as over the clinical cut point in the control and SCT groups.

	Control		SCT	
	Prediabetes	Over the cut-point	Prediabetes	Over the cut-point
n =	22	21	23	28
FG mg/dL	125.55 ± 56.86	163.20 ± 70.40	141.43 ± 67.88	204.30 ± 92.43*
HbA1c %	5.90 ± 0.19	8.21 ± 1.58*	6.07 ± 0.25	9.33 ± 2.12*
Fructosamine μmol/L	223.76 ± 55.64	355.21 ± 114.84*	263.35 ± 83.81	375.92 ± 152.12*

Prediabetes defined as a HbA1c between 5.7–6.4%. The clinical cut-point for HbA1c (6.5%) was used.

FG = Fasting Glucose; HbA1c = Glycated Hemoglobin A

Results are presented as means ± SD

*Significantly different from prediabetes ($p < 0.05$)

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life of HbA1c and fructosamine), as well as other sources of biological and analytical variability [5, 6].

Correlation analysis evaluates the linear correlation between two measures, and does not automatically imply good agreement. For this reason, Bland-Altman plots were used to interpret the bias, data scatter, and agreement between HbA1c and FG, fructosamine and FG, and HbA1c and fructosamine for both groups. The Bland-Altman analyses revealed that the LOA between all of the measures were unacceptably large in both groups. Interestingly, the LOA tended to be larger in the SCT group compared to the control group. This phenomenon was particularly evident in the comparisons of HbA1c and fructosamine. Overall, the analyses of agreement revealed considerable disparities between the three biomarkers of hyperglycemia, and these differences were heightened in the SCT group. These disparities suggest that these biomarkers should not be considered to be interchangeable measures of hyperglycemia. Use of only one of these biomarkers could possibly result in an erroneous assessment of blood glucose control, potentially leading to missed diagnoses or inappropriate follow-up of T2D. Furthermore, there was a moderate tendency for the bias to become more negative, and more variable, as glycemic control became worse. These results indicate that the disparities between the three measures may be the most pronounced in the patients who have the poorest glycemic control.

Variability between HbA1c and other markers of glycemia has been widely reported, especially in populations of African descent [19–21]. For example, a study conducted by Bergenstal and colleagues in 2017 concluded that HbA1c overestimates average glucose in black individuals versus white individuals, likely because of racial differences in hemoglobin glycation [19]. However, accumulating evidence suggests that hemoglobin glycation could be more heavily influenced by inter-individual variability than by race or ethnicity, due to a variety of factors like differences in extra- and intra-cellular glucose balance, non-glycemic genetic determinants of hemoglobin glycation, and red blood cell survival [20, 22]. Additionally, evidence shows that HbA1c can be affected by sex, however no differences in average HbA1c were observed between the male and female subjects in the control or SCT groups in this study [23].

Disparities between HbA1c and fructosamine have been observed in several other studies [16–18, 24]. Indeed, Macdonald et al compared measured HbA1c to estimated HbA1c from fructosamine values in 1744 patients and found a substantial difference between the two measures, leading to the conclusion that HbA1c may not accurately reflect glucose control [24]. Similarly, Narbonne et al found that estimating HbA1c from fructosamine values did not provide an accurate enough measure of glucose control to evaluate the efficacy of diabetic treatments [17]. Furthermore, studies conducted by Nayak et al and Cohen et al have shown that

the discordance between HbA1c and fructosamine, referred to as the glycation gap, remains consistent within subjects and is positively associated with nephropathy, a common diabetes-related complication [18]. These studies suggest that the consistent disparities between HbA1c (a measure of intracellular glycation) and fructosamine (a measure of extra-cellular glycaemic control) could be relevant to the pathophysiology of diabetes-related vascular complications [16, 18]. A growing body of research shows that SCT increases the risk of chronic kidney disease and end stage renal disease [25, 26]. Additionally, a recent study by Diaw et al found that subjects with SCT and T2D had a higher frequency of microalbuminuria compared to control subjects and subjects with SCT or T2D [27]. More studies should be done to determine whether the exaggerated discrepancies between HbA1c and fructosamine observed in the SCT group could be linked to an increased risk of nephropathy.

The lack of agreement between the three biomarkers of hyperglycemia, which was more pronounced in the SCT group, was reflected by the inconsistency in the number of subjects classified as above the clinical cut-point depending on the criterion used. For example, when HbA1c was used as the criterion, fewer subjects were classified as above the clinical cut-point than when FG or fructosamine were used, and the differences were larger in the SCT group. These results are in accordance with a study by Guo et al, which concluded that the accuracy of HbA1c varied among subjects, resulting in many missed diagnoses when used as a sole diagnostic criterion [21]. Multiple studies have suggested that using multiple biomarkers of hyperglycemia may be the best way to improve the accuracy of blood glucose monitoring and T2D diagnosis [5, 6, 22, 28, 29]. More research should be performed to understand the potential benefits of using multiple biomarkers to measure glycaemic control in people with SCT.

The inclusion of individuals with T2D was a strength of the present study as it enabled us to evaluate the relationships between the three biomarkers over a large range of glucose control. Another strength of the study was that HbA1c was measured using the Sebia Capillary 3 Tera device, a method that shows no interference from HbS, or other common hemoglobin variants according to the National Glycohemoglobin Standardization Program (NGSP) [30]. However, it is worth recognizing that the findings of Lacy et al suggest that HbA1c may consistently underestimate glycaemic control in black individuals with SCT [31]. Additionally, a recent study reported that the number of functional alpha-genes in individuals with alpha-thalassemia could affect HbA1c values [32]. In this study, subjects were not tested for alpha-thalassemia. Therefore, future studies could be conducted to study the performance of HbA1c, fructosamine, and FG in individuals with SCT and alpha-thalassemia.

Conclusions

Overall, this study showed that HbA1c, FG, and fructosamine were positively correlated in Senegalese individuals with and without SCT. However, the disparities between the three biomarkers were substantial, and were especially pronounced in the SCT group. These results suggest that the three biomarkers should not be used interchangeably to measure glycaemic control, especially not in individuals with SCT. Future studies should be conducted in African individuals with and without SCT to compare the prognostic sensitivity of these biomarkers against the gold standard for diabetes diagnosis, the OGTT. Additionally, more research should be done to determine whether using multiple biomarkers could help provide complementary information about the risk of developing T2D complications, or improve the accuracy of assessments of glycaemic control, thereby enabling better patient care in Africans with and without SCT.

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STUDY 2: Increased prevalence of type 2 diabetes-related complications in combined type 2 diabetes and sickle cell trait

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T2D is a metabolic disease. However, when not well managed, the hyperglycemia, hyperinsulinemia, and dyslipidemia associated with T2D lead to the development of vascular dysfunction, blood rheological alterations, and the subsequent onset of microvascular and macrovascular complications (Part 2, section II.C). Additionally, a study conducted by our team found that individuals with both SCT and T2D have more severe vascular dysfunction than people with T2D alone. This suggests that people with combined T2D and SCT could be more at risk of developing T2D vascular complications. However, previous studies conducted to determine whether SCT could increase the risk of developing T2D complications have reported contradictory results (Part 2, section IV.C). Therefore, the primary objective of this study was to determine whether T2D-related complications are more prevalent in individuals with combined T2D and SCT than in individuals with T2D alone. Our secondary objective was to evaluate possible mechanisms that could be involved in the development of vascular complications in individuals with combined T2D and SCT.

The study took place in Dakar, Senegal. Participants were recruited into four groups: combined T2D and SCT (T2D-SCT) (n=60; mean age 50.8 ± 8.8), T2D (n=52; mean age 53.1 ± 8.4), SCT (n=53; mean age 49.3 ± 5.7), and the control group, which had neither T2D nor SCT (n=56; mean age 50.0 ± 6.5). Subject arrived on the day of the study following an overnight fast. Blood samples were taken in order to measure HbA1c, blood rheology, and plasma concentrations of AGEs and cytokines. Subjects were then screened for peripheral artery disease, hypertension, retinopathy, peripheral neuropathy, and impaired renal function. Arterial stiffness was measured using PWV. The blood rheological parameters measured included hematocrit, plasma viscosity, and whole blood viscosity, measured using a cone

plate viscometer. Additionally, whole blood viscosity measured at several shear rates was used to calculate a thixotropic index, a measure that reflects the influences of red blood cell aggregation and deformability on blood viscosity. Plasma AGEs and IL-1 β concentrations were measured using ELISA kits. Plasma concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, Granulocyte-macrophage colony-stimulating factor, IFN-gamma, and TNF- α were measured using a multiplex assay system. *In-vitro* experiments were conducted in which human aortic endothelial cells (HAEC) were incubated with subjects' plasma and TNF- α . Flow cytometry was then used to measure the HAEC expression of E-selectin and VCAM-1. Western blot was used to measure phospho- and total p38 MAPK expression, and PCR was used to measure ZO-1 and NF- κ B mRNA expression in HAEC. In order to determine the effects of oxidative stress and AGEs on HAEC expression of E-selectin, a second round of *in-vitro* experiments was performed. HAEC were incubated with TNF- α , plasma from control or T2D-SCT subjects, and either tiron, an O $_2^{\cdot-}$ scavenger and antioxidant, or aminoguanidine hydrochloride, an AGE inhibitor.

Our results showed that hypertension, retinopathy, and reduced renal function were more prevalent in the T2D-SCT group than in the other three groups. Additionally, arterial stiffness was elevated in the T2D-SCT group compared to the other three groups. Whole blood viscosity and thixotropic index were both higher in the T2D-SCT group than in the other three groups. No differences in plasma concentrations of cytokines were observed between the groups. However, plasma AGE concentrations were higher in the T2D-SCT subjects compared to the other three groups. Furthermore, multivariable analysis showed that plasma AGE concentration was significantly and independently associated with arterial stiffness. The results of the *in-vitro* experiments showed that HAEC incubated with plasma

from T2D-SCT subjects expressed more E-selectin than HAEC incubated with plasma from subjects in the other three groups. AGE inhibition with aminoguanidine hydrochloride significantly reduced HAEC expression in the T2D-SCT group, but not in the control group, indicating that AGEs likely contribute to the up-regulation of E-selectin expression in the T2D-SCT subjects.



Increased Prevalence of Type 2 Diabetes–Related Complications in Combined Type 2 Diabetes and Sickle-Cell Trait

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OBJECTIVE

The prevalence of type 2 diabetes (T2D) is rapidly increasing in Sub-Saharan Africa, where sickle-cell trait (SCT) is also frequent. Although SCT is generally considered a benign condition, evidence suggests that SCT could exaggerate vascular dysfunction in T2D. However, it remains unclear whether SCT could increase the risk of the development of T2D complications. Therefore, this study was conducted to determine whether T2D complications were more prevalent among Senegalese individuals with SCT and T2D than among those with T2D only.

RESEARCH DESIGN AND METHODS

Rates of hypertension, retinopathy, peripheral neuropathy, peripheral artery disease, and impaired renal function as well as arterial stiffness, blood rheology, and concentrations of plasma advanced glycation end products (AGEs) and cytokines were compared between groups of Senegalese individuals with combined SCT and T2D (T2D-SCT) ($n = 60$), T2D ($n = 52$), SCT ($n = 53$), and neither T2D nor SCT (control) ($n = 56$). Human aortic endothelial cell (HAEC) expression of inflammatory and adhesion factors were measured after treatment with tumor necrosis factor- α and subjects' plasma. Effects of AGE inhibition or tiron on HAEC expression of E-selectin was measured.

RESULTS

Retinopathy, hypertension, and reduced renal function were more prevalent, and arterial stiffness, blood viscosity at high shear rates, and thixotropic index were higher in the SCT group compared with the other groups. Multivariable analysis showed that plasma AGE concentration was significantly associated with arterial stiffness. E-selectin expression was elevated in HAECs treated with T2D-SCT plasma compared with the other groups, but AGE inhibition reversed this.

CONCLUSIONS

SCT could potentially augment the risk of the development of T2D-related complications, including retinopathy, nephropathy, and hypertension.

Type 2 diabetes (T2D) is an insidious disease characterized by progressive insulin resistance, resulting in elevated levels of blood glucose (1). Hyperglycemia results in increased levels of advanced glycation end products (AGEs), oxidative stress, chronic low-grade inflammation, and platelet hyperactivity (2). These biological alterations lead to impaired endothelial function, increased vasoconstriction, abnormal

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blood rheology, and a prothrombotic state, thereby augmenting the risk of microvascular and macrovascular complications (2). An estimated 425 million people around the globe have T2D, and current projections indicate that this number will increase by 48% by 2045 (3). The prevalence of T2D is increasing particularly rapidly in Africa, where urbanization, lifestyle changes, and an aging population contribute to an expected 162% increase in the disease by 2045 (3).

Sickle-cell trait (SCT), the heterozygous form of sickle-cell anemia, is highly frequent in Sub-Saharan African countries, with an estimated prevalence of 6–10% in Senegal (4). Sickle-cell anemia is caused by the mutation of the sixth amino acid of the β -globin gene, and results in the production of abnormal hemoglobin, called hemoglobin S (HbS) (5). Under deoxygenated conditions, HbS polymerizes and rigidifies, leading to severe acute and chronic complications (5). Unlike sickle-cell anemia, SCT is generally considered to be benign (6). However, accumulating evidence shows that individuals with SCT have altered blood rheology and increased coagulation activity (6–8). Moreover, studies show that SCT may increase the risk of stroke, venous thromboembolism, chronic kidney disease, and end-stage renal disease (9).

The high and increasing rates of T2D in Sub-Saharan Africa indicate that there is likely a large and growing population of individuals with both SCT and T2D. This could become problematic as a recent study suggests that SCT could exaggerate vascular dysfunction and arterial stiffness in T2D, and also could amplify blood rheological alterations, oxidative stress, AGE, and inflammation (10). However, it remains unclear whether SCT could increase the risk of the development of diabetes-related vascular complications (11–14).

Therefore, the primary objective of this study was to determine whether T2D complications are more prevalent among individuals with combined T2D and SCT (T2D-SCT) than among individuals with T2D alone. The secondary objective was to explore the possible mechanisms implicated in the pathogenesis of vascular complications in individuals with both SCT and T2D.

RESEARCH DESIGN AND METHODS

Setting and Participants

This study was performed at the Cheikh Anta Diop University in Dakar, Senegal. A total of 221 subjects were recruited from the Diabetic Centre at the Hospital Abass Ndao, from the National Center of Blood Transfusion, and from the general population in Dakar. Subjects were screened for T2D and SCT, and then assigned to one of the following four groups: subjects with T2D-SCT ($n = 60$); subjects with T2D ($n = 52$); subjects with SCT ($n = 53$); and subjects with neither SCT nor T2D (control group; $n = 56$). Calculations conducted prior to subject recruitment demonstrated that a minimum total sample size (all groups combined) of 57 participants would be required to address the main objective of the study ($\alpha = 0.05$, power = 90%). T2D was defined using the American Diabetes Association *Standards of Medical Care in Diabetes* guidelines (15). Biological analyses were conducted to confirm whether subjects were carriers of the HbS gene. Briefly, blood was screened using isoelectric focusing. Results were then confirmed using citrate agar electrophoresis, hemoglobin fraction quantification using high-performance liquid chromatography, and a solubility test to confirm the presence of HbS. The high-performance liquid chromatography results were also used to confirm that subjects had no other hemoglobinopathies.

Individuals who smoked as well as women who were pregnant or using oral contraception were excluded from the study. The majority of subjects in the T2D (71.2%) and T2D-SCT (71.7%) groups were treated with metformin, sulfonylureas, insulin, or a combination of these. The other T2D and T2D-SCT subjects were not taking any other medications to treat T2D. All participants were Senegalese. The study protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Cheikh Anta Diop University (reference: 0221/2016/CER/UCAD). All subjects gave informed, written consent.

Clinical Examination

Blood Sampling

Subjects arrived at the Laboratory of Medical Physiology (Cheikh Anta Diop University) at 8:00 A.M., after an overnight fast. All of the subjects were instructed to

refrain from physical activity for 24 h before the visit. Blood was drawn into heparin tubes for lipid measurement, fluoride tubes for glucose measurement, and EDTA tubes for analyses of hemoglobin A_{1c} (HbA_{1c}), blood rheology, AGEs, and cytokines. The blood samples used to measure AGEs and the cytokines were immediately centrifuged, and the plasma was then stored at -80°C until analyses were conducted.

Blood Pressure and Pulse Wave Velocity

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in the left arm using a manual sphygmomanometer (Omron M3; Intellisense, Kyoto, Japan), while the subject remained in a seated position. The measurements were taken three times after a 30-min period of rest. Mean arterial pressure (MAP) was calculated as $\text{DBP} + (\text{SBP} - \text{DBP})/3$. The carotid-femoral pulse wave velocity (PWV) was measured using an automated system (Pulse Pen; DiaTecne, Milan, Italy). The carotid and femoral waveforms were measured simultaneously using two pressure-sensitive transducers. The transit time of the pulse wave was calculated by the system software. The distance between the carotid and femoral measurement sites was measured over the body surface using a tape measure. The PWVs were calculated as the distance between the two measurement sites divided by the transit time (in meters per second). In order to cover the entire respiratory cycle, at least 12 readings were performed successively on each subject. The mean of three consecutive measures was calculated and used as the PWV value. The same trained individual performed all PWV measurements.

T2D-Associated Complications

All subjects were screened for hypertension, retinopathy, peripheral neuropathy, peripheral artery disease, and impaired renal function. Hypertension was defined as $\text{SBP} > 130$ mmHg or $\text{DBP} > 80$ mmHg, or the use of antihypertensive drugs (16). Indirect ophthalmoscopy with a noncontact slip lens lamp was used to assess the presence of retinopathy. The subjects were screened for diabetic peripheral neuropathy using the Semmes-Weinstein Monofilament Examination on three test sites (the great toe, the third metatarsal, and the fifth metatarsal) (17). Ankle brachial pressure

was calculated using a Doppler probe to confirm the presence or absence of peripheral artery disease (ankle brachial systolic pressures index <0.90).

Urinary albumin concentration (UAC) was measured using early morning spot urine (HemoCue Albumin 201 System). Serum creatinine level was measured using the standard Jaffe method. The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiological Equation, and the appropriate corrections for race and sex were applied (18). Subjects with an eGFR <60 mL/min/1.73 m² were classified as having reduced renal function (moderately or severely decreased eGFR) (18).

Laboratory Analyses

Biochemical Parameters

Fasting glucose was measured using an enzymatic glucosidase-peroxidase method (Urit Medical Electronic Co., Guilin, People's Republic of China). HbA_{1c} was measured using capillary electrophoresis on a Capillary 3 Tera device (Sebia, Lisses, France). Plasma lipids (triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol) were evaluated using standard enzymatic methods.

Hemorheological Parameters

Hematocrit was measured after blood microcentrifugation. Plasma viscosity was measured at 37°C using a cone-plate viscometer at a shear rate of 375 s⁻¹. Oxygenated whole blood at native hematocrit was used to measure whole-blood viscosity at varying shear rates (5.62, 11.25, 22.5, 45, 90, 225, and 375 s⁻¹), at 37°C, using a cone-plate viscometer (Pro DV-II+, with CPE40 spindle; Brookfield, Middleboro, MA). Furthermore, a "loop protocol" was used to characterize the blood thixotropic index, a measure that reflects the impact of altered red blood cell aggregation, and deformability (to a lesser extent), on blood viscosity (19). For this method, a cone-plate viscometer is used to measure blood viscosity because the shear rate is increased every 30 from an initial shear rate of 22.5 s⁻¹ to a maximum shear rate of 225 s⁻¹ (curve 1). The shear rate is then decreased every 30 progressively back to the original shear rate (curve 2). The difference between the blood viscosities at each shear rates on curve 1 and curve 2 were calculated for each subject, and then plotted versus

shear rate. The area under the curve for this graph was calculated for each subject and corresponded to the thixotropic index (19,20).

AGEs and Cytokines

ELISA kits were used to measure plasma concentrations of AGEs (Cell Biolabs, Inc., San Diego, CA) and interleukin (IL)-1β (Genetex, Irvine, CA). The ELISA assays were carried out according to the manufacturer instructions. The Bio-Plex Pro Human Cytokine 8-Plex Immunoassay Kit (Bio-Rad) was used to measure concentrations of IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, interferon-γ, and tumor necrosis factor-α (TNF-α) in subjects' plasma. The assays were performed following the instructions with the kit, and cytokine concentrations were measured using the MAGPIX xPONENT 4.2 System (Luminex Corporation, Austin, TX).

Human Aortic Endothelial Cells Culture and Treatment

A subset of 25 plasma samples (control *n* = 7; T2D *n* = 7; SCT *n* = 5; T2D-SCT *n* = 6) was randomly selected from the whole cohort for use in cell culture experiments. Human aortic endothelial cells (HAECs) from PromoCell were grown in Endothelial Cell Medium MV2 (PromoCell) and treated with TNF-α (0.3 ng/mL), either alone or together with plasma (5%), for 4 h.

Flow Cytometry: E-Selectin and Vascular Cell Adhesion Molecule-1

HAECs were detached using accutase (Thermo Fisher Scientific), labeled with FITC-conjugated antibodies against CD106 (vascular cell adhesion molecule-1 [VCAM-1], clone STA; Thermo Fisher Scientific) and phycoerythrin-conjugated antibody against CD62E (E-selectin, clone P2H3; eBioscience), and then analyzed using an Accuri C6 Flow Cytometer (Becton Dickinson).

Flow Cytometry: The Effect of an Antioxidant (Tiron) or AGE Inhibition on HAEC E-Selectin Expression

The effects of reactive oxygen species and AGEs on HAEC E-selectin expression were tested in control subjects (*n* = 5) and individuals with T2D-SCT (*n* = 6) using tiron (ab146234; Abcam), a cell-permeable superoxide scavenger and antioxidant, or aminoguanidine hydrochloride (ab120123; Abcam), an AGE inhibitor. HAECs from PromoCell were grown in

Endothelial Cell Medium MV2 (PromoCell) and treated for 4 h with TNF-α (0.3 ng/mL) together with plasma (5%) from control subjects or subjects with T2D-SCT, with plasma (5%) from control subjects or subjects with T2D-SCT and tiron (5 mmol/L), or with plasma (5%) from control subjects or subjects T2D-SCT and aminoguanidine hydrochloride (5 mmol/L).

HAECs were detached using accutase (Thermo Fisher Scientific), labeled with a phycoerythrin-conjugated antibody against CD62E (E-selectin, clone P2H3; eBioscience), and then analyzed using an Accuri C6 Flow Cytometer (Becton Dickinson).

Real-Time Quantitative PCR: Zonula Occludens-1 and Nuclear Factor-κB

Analysis of HAECs was performed using established protocols. Total RNA was isolated from cells using Tri-Reagent (Sigma-Aldrich, St. Louis, MO). Real-time quantitative PCR was performed using diluted cDNAs and LightCycler 480 SYBR Green I Master Mix (Roche). Triplicate assays were performed for both genes (nuclear factor-κB [NF-κB] and zonula occludens-1 [ZO-1]). Gene expression was normalized with TATA-binding protein.

Western Blotting

HAEC lysate preparation and Western blotting were performed as previously described (21). Semiquantitative assessment of band density was performed using ImageJ software. Band density for the protein of interest was normalized to β-actin. Antibodies against phospho- and total p38 mitogen-activated protein kinase (MAPK) were purchased through Cell Signaling Technology (Danvers, MA). β-Actin antibody was purchased from Sigma-Aldrich.

Statistical Analysis

Results are expressed as the mean ± SD. One-way ANOVA tests were performed with Tukey post hoc tests (to answer the primary objective) and with the Holm-Bonferroni correction (to answer the secondary objective, which is more exploratory) to compare differences among groups for mean age; SBP; DBP; MAP; PWV; anthropometric, biochemical, and biological parameters; microalbumin; serum creatinine; and eGFR. Differences between groups for PWV were also determined by ANCOVA, using MAP as a covariate. χ² tests were used to compare

the sex distribution, and the frequency of hypertension, retinopathy, peripheral neuropathy, peripheral artery disease, and impaired renal function between groups. A multivariate linear regression model was used to predict the independent associations between PWV and several parameters of interest. Statistical significance was defined as $P < 0.05$. Analyses were conducted using SPSS Statistics software (version 24; IBM, Chicago, IL).

RESULTS

Anthropometrics, Blood Pressures, and Biochemical Parameters

There were no significant differences in sex distribution or age among the four groups (Table 1). The duration of

diabetes did not differ between the T2D (6.7 ± 5.7 years) and T2D-SCT (5.9 ± 4.6 years) groups. Furthermore, there was no significant difference between the proportion of subjects receiving metformin (T2D group 63.4%; T2D-SCT group 50.0%), sulfonylurea (T2D group 21.2%; T2D-SCT group 16.7%), or insulin (T2D group 5.7%; T2D-SCT group 10.0%) treatment between the T2D and T2D-SCT groups.

BMI, SBP, MAP, HbA_{1c}, and fasting glucose levels were higher in subjects with T2D compared with those without T2D (Table 1). DBP was significantly higher in the T2D group compared with the SCT group, and was significantly higher in the T2D-SCT group compared with the control and SCT groups. LDL

cholesterol was elevated in the two groups with T2D compared with the control group. Total cholesterol level was higher in the T2D group compared with the control and SCT groups, and was elevated in the T2D-SCT group compared with the control group (Table 1). Triglyceride levels were higher in the T2D-SCT group compared with the three other groups (Table 1). HDL cholesterol was lower in the two groups with T2D compared with the control group (Table 1).

T2D-Associated Complications

Retinopathy, hypertension, and reduced renal function were significantly more frequent in the T2D-SCT group compared with the three other groups (Table 2). The prevalence of neuropathy was elevated

Table 1—Participant characteristics and biochemical parameters

	Control	T2D	SCT	T2D-SCT
N (male/female)	18/38	15/37	13/40	12/48
Age (years)	50.0 ± 6.5	53.1 ± 8.4	49.3 ± 5.7	50.8 ± 8.8
Years since diagnosis		6.7 ± 5.7		5.9 ± 4.6
Weight (kg)	70.6 ± 11.4	74.5 ± 11.7	67.9 ± 10.5	76.3 ± 13.2†
BMI (kg/m ²)	23.7 ± 3.6	26.1 ± 4.6*†	23.3 ± 3.2	27.1 ± 4.5*†
DBP (mmHg)	73 ± 10	79 ± 12†	72 ± 10	82 ± 11*†
SBP (mmHg)	118 ± 11	135 ± 23*†	120 ± 13	131 ± 21*†
Mean arterial BP (mmHg)	87.9 ± 8.6	88.2 ± 9.1*†	97.4 ± 14.3	98.6 ± 12.3*†
HDL cholesterol (mmol/L)	0.8 ± 0.2	0.6 ± 0.2*	0.7 ± 0.3	0.6 ± 0.3*
LDL cholesterol (mmol/L)	0.1 ± 0.5	1.5 ± 0.6*	1.2 ± 0.6	1.5 ± 0.6*
T-chol (mmol/L)	1.6 ± 0.5	2.2 ± 0.6*†	1.8 ± 0.6	2.1 ± 0.6*
TG (mmol/L)	0.9 ± 0.4	1.0 ± 0.6	0.9 ± 0.4	1.3 ± 0.9*†‡
FPG (mg/dL)	93.4 ± 23.3	162.4 ± 75.2*†	87.0 ± 14.8	186.7 ± 80.0*†
HbA _{1c} (%)	5.2 ± 0.6	7.0 ± 1.9*†	5.1 ± 0.8	7.6 ± 2.4*†
HbA _{1c} (mmol/mol)	33.8 ± 6.9	53.4 ± 20.8*†	32.5 ± 9.0	59.6 ± 26.3*†
UAC (mg/L)	13.3 ± 12.3	80.2 ± 96.2*	59.1 ± 51.2*	79.1 ± 100.3*
Serum creatinine (mg/dL)	0.76 ± 0.2	1.1 ± 0.4	0.98 ± 0.3	1.5 ± 1.0*†‡
eGFR (mL/min/1.73 m ²)	115.0 ± 115.0	83.7 ± 26.6*	87.6 ± 25.1*	67.7 ± 34.6*†‡
Hematocrit (%)	40.5 ± 2.5	40.6 ± 2.0	40.3 ± 2.4	41.1 ± 2.3
Plasma viscosity (cP)	1.5 ± 0.34	1.6 ± 0.42	1.4 ± 0.43	1.6 ± 0.36
Blood viscosity (cP) at 5.62 s ⁻¹	9.9 ± 1.3	11.2 ± 1.2*	10.4 ± 1.2	11.6 ± 1.6*†
Blood viscosity (cP) at 11.25 s ⁻¹	8.9 ± 1.2	9.9 ± 1.1*†	9.2 ± 1.1	9.9 ± 1.2*†
Blood viscosity (cP) at 22.5 s ⁻¹	7.8 ± 1.1	8.8 ± 0.98*†	8.0 ± 0.8	8.5 ± 1.1*
Blood viscosity (cP) at 45 s ⁻¹	6.7 ± 1.0	7.3 ± 0.9*	6.9 ± 0.8	7.5 ± 1.0*†
Blood viscosity (cP) at 90 s ⁻¹	5.8 ± 0.7	6.2 ± 0.7*	6.0 ± 0.6	6.7 ± 1.0*†‡
Blood viscosity (cP) at 225 s ⁻¹	4.7 ± 0.6	5.5 ± 0.6*	5.3 ± 0.7*	6.0 ± 1.0*†‡
Blood viscosity (cP) at 375 s ⁻¹	4.1 ± 0.5	4.8 ± 0.5*	4.6 ± 0.7*	5.1 ± 0.9*†‡
Thixotropic index	1,748 ± 416	1,744 ± 433	1,680 ± 495	2,038 ± 381*†‡
AGEs (μg/mL)	1.4 ± 0.4	1.6 ± 0.5	1.5 ± 0.4	1.9 ± 0.7*†
IL-1β (pg/mL)	9.0 ± 1.7	9.5 ± 2.5	9.0 ± 1.6	9.2 ± 1.3
IL-6 (pg/mL)	0.00028 ± 0.0002	0.00031 ± 0.0002	0.00032 ± 0.0002	0.00023 ± 0.0002
IL-8 (pg/mL)	0.0030 ± 0.002	0.0036 ± 0.002	0.0032 ± 0.001	0.0034 ± 0.001
TNF-α	0.0048 ± 0.002	0.0055 ± 0.002	0.0049 ± 0.002	0.0049 ± 0.002

Data are the mean ± SD. cP, centipoise; FPG, fasting plasma glucose; T-chol, total cholesterol; TG, triglyceride. *Different from control. †Different from T2D. ‡Different from SCT, according to the *P* value established by the Holm-Bonferroni correction.

Table 2—Prevalence of hypertension, retinopathy, neuropathy, peripheral artery disease, and reduced renal function in the control, T2D, SCT, and T2D-SCT groups

Target organ complication	Control	T2D	SCT	T2D-SCT	P value
N	56	52	53	60	
Hypertension	14 (25%)	27 (51.9%)	18 (34.0%)	41 (68.3%)	<0.001
Retinopathy	0 (0.0%)	14 (28%)	3 (5.7%)	31 (51.7%)	<0.001
Neuropathy	0 (0%)	7 (17.5%)	0 (0%)	8 (17.4%)	0.001
PAD	0 (0%)	1 (2.5%)	0 (0%)	2 (4.3%)	0.399
Reduced renal function	0 (0%)	10 (19.2%)	3 (5.7%)	23 (38.3%)	<0.001

PAD, peripheral artery disease. Reduced renal function is classified as eGFR <60 mL/min/1.73 m².

in the two T2D groups compared with the control and SCT groups (Table 2). Finally, the prevalence of peripheral artery disease did not vary significantly among the four groups (Table 2). The average values for UAC, serum creatinine concentration, and eGFR for each group are displayed in Table 1. UAC was higher in the T2D and T2D-SCT groups compared with the control group. Serum creatinine concentration was significantly higher in the T2D-SCT group compared with the other three groups. The eGFR was higher in the control group compared with the other three groups, and was lower in the T2D-SCT group compared with the other three groups. Three subjects in the SCT group presented with retinal hemorrhages, which occur in the early stages of diabetic and nondiabetic retinopathy.

PWV

PWV was higher in the T2D group compared with the control group only. Additionally, PWV was significantly higher in the T2D-SCT group compared with the other three groups, even when MAP was fixed as a covariate (Fig. 1).

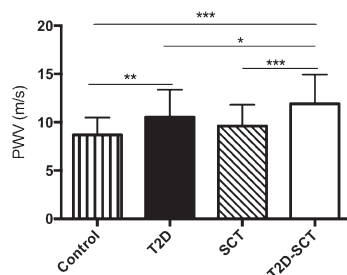


Figure 1—PWV values of patients with T2D with SCT (T2D-SCT) or without SCT (T2D) and of individuals without T2D with SCT or without SCT (Control). The two extremities of the horizontal bars indicate the difference between two given groups (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Results are presented as the mean \pm SD.

Multivariate Analysis

Five variables were included in the model to predict PWV: plasma AGE concentration, blood viscosity at 225 s, age, triglyceride level, and HbA_{1c}. The other variables, which were significantly different between groups by univariate analyses were not included into the model because of the high risk of colinearity effect (variance inflation factor >3) with one of the five variables included. The overall model was statistically significant ($R^2 = 0.09$; $df = 5$; $P < 0.05$). AGE concentration was significantly associated with PWV ($\beta = 0.16$; $P = 0.04$), and blood viscosity was marginally associated with PWV ($\beta = 0.15$; $P = 0.08$).

Hemorheological Parameters

No significant differences in hematocrit or plasma viscosity were observed between groups (Table 1). Whole-blood viscosity was higher in the two T2D groups compared with the control and SCT groups at low shear rates ranging from 5.62 to 45 s with the following exceptions: at 22.5 s⁻¹ blood viscosity was higher in the T2D-SCT group compared with the control group only (Table 1). At higher shear rates (90–375 s⁻¹), blood viscosity in the T2D group was elevated only in comparison with the control group, whereas blood viscosity was higher in the T2D-SCT group compared with the other three groups (Table 1). Furthermore, blood viscosity was higher in the SCT group compared with the control group at the two highest shear rates (225–375 s⁻¹) (Table 1). The thixotropic index was significantly greater in the T2D-SCT group compared with the other three groups (Table 1).

Plasma AGEs and Cytokines

Plasma concentrations of AGEs were significantly increased in the T2D-SCT

group compared with the control and SCT groups, and there was a trend for elevated AGE levels in the T2D-SCT group compared with the T2D group after application of the Holm-Bonferroni correction ($P = 0.03$) (Table 1). Plasma concentrations of IL-1 β did not vary among groups (Table 1). No statistical differences in plasma concentrations of IL-6, IL-8, and TNF- α were found among groups (Table 1). The plasma concentrations of IL-2, IL-4, IL-10, granulocyte-macrophage colony-stimulating factor, and interferon- γ were very low, and below the limits of detection of the kits used.

FACS: E-Selectin and VCAM-1

After incubation with plasma, HAEC expression of E-selectin was significantly higher in the T2D-SCT group compared with the other three groups. No differences in the expression of VCAM-1 were observed among the groups (Fig. 2A).

Incubation of HAECs with plasma and aminoguanidine hydrochloride significantly reduced the HAEC expression of E-selectin in the T2D-SCT group only, whereas incubation of HAECs with plasma and tiron resulted in reduced HAEC expression of E-selectin in both the control and T2D-SCT groups (Fig. 2B).

mRNA Expression

Real-time quantitative PCR revealed no significant difference in the expression of ZO-1 mRNA. NF- κ B mRNA expression was significantly higher in the T2D group compared with the control group (Fig. 2C).

Western Blot

There were no significant differences in expression of phospho- and total p38 MAPK in HAECs among groups (Fig. 2D).

CONCLUSIONS

The primary finding of the current study was that retinopathy, hypertension, and reduced renal function were more prevalent in subjects with SCT and T2D than in those with T2D alone. Our study also found increased arterial stiffness, blood viscosity, thixotropic index, and plasma AGE concentrations in the T2D-SCT group compared with the other three groups. Our results corroborate the findings of a previous study by Diaw et al. (10), which observed amplified vascular dysfunction in individuals with T2D and SCT compared with subjects with T2D, subjects with SCT, and control subjects. However,

few previous studies have evaluated whether SCT could increase the risk of the development of T2D-related complications, and the results of these studies have been contradictory. Indeed, several studies (11,13,14,22) have reported that SCT does not aggravate microvascular or macrovascular complications, whereas several other studies (10,12,23) indicate that SCT could potentially increase the risk of vascular complications in male patients or in all patients with T2D. These conflicting results could be attributed to the fact that the ethnic and racial backgrounds of the study populations varied widely between studies. Furthermore, most of the previous studies had small overall sample sizes, and/or a small number of subjects with T2D-SCT. In the current study, however, all groups were matched for age, ethnicity, and sex.

Interestingly, our results showed no significant differences in urine albumin concentration among the T2D, SCT, and T2D-SCT groups, although eGFR was significantly lower in the T2D-SCT group compared with the other three groups. However, several studies have shown that renal disease in individuals with T2D is heterogeneous, and that renal insufficiency can occur in the absence of albuminuria in individuals with T2D (24).

A large body of evidence suggests that arterial stiffness plays an important role in the pathogenesis of T2D and its complications (25). Indeed, previous studies (25,26) have shown that increased arterial stiffness is associated with the development and progression of hypertension, nephropathy, and retinopathy in individuals with diabetes. Our findings are in accordance with these studies as arterial stiffness was highest in the T2D-SCT group, which also had the highest rates of these complications. PWV, which is considered one of the most reliable measures of arterial stiffness, is strongly associated with blood pressure (27). However, in our study, PWV remained elevated in the T2D-SCT group even when MAP was fixed as a covariate, indicating that differences in arterial pressure are probably not completely responsible for differences in PWV among the groups. In T2D, accelerated arterial stiffening is partially attributed to the formation of AGEs in the arterial wall, which leads to cross-linking

of collagen molecules, resulting in decreased collagen elasticity (26). In our study, plasma AGE concentrations were elevated in the T2D-SCT group. Additionally, the results of our multivariate analysis suggest that the plasma concentrations of AGEs could be the most important factor contributing to the elevated arterial stiffness in the T2D-SCT group. These results suggest that SCT could potentially exaggerate the nonenzymatic glycation cross-linking of collagen, thereby contributing to increased arterial rigidity in these individuals.

Hyperglycemia is one of the primary factors contributing to AGE formation (28). However, in the current study we found no significant differences in HbA_{1c} or fasting glucose levels between the T2D-SCT and the T2D groups, although concentrations of AGEs were significantly higher in the T2D-SCT group. In addition to glucose concentration, AGE concentrations can also be enhanced by oxidative stress (29). Therefore, the elevated AGE concentrations found in the T2D-SCT group could be attributed to the elevated levels of oxidative stress that have been previously observed in individuals with T2D-SCT (10).

Evidence suggests that AGE accumulation promotes the progression of diabetic complications, including nephropathy, retinopathy, and macroangiopathy, both through the formation of cross-links in the basement membrane of the extracellular matrix, and through interaction with the receptor for AGEs (RAGE) (30). AGE-RAGE interaction activates nuclear transcription factors, such as NF- κ B, which can upregulate the transcription of inflammatory proteins, such as VCAM-1 and E-selectin (30). E-selectin is expressed by endothelial cells during inflammation and plays an essential role in mediating leukocyte adhesion to the endothelium (31). Furthermore, increased E-selectin expression may contribute to the pathogenesis of vascular disease in T2D (32). We found that E-selectin expression was upregulated in HAECs treated with plasma from individuals with T2D-SCT compared with HAECs treated with plasma from the other three groups. Furthermore, when the HAECs were treated with plasma from the individuals with T2D-SCT and an inhibitor of AGE formation, the expression of E-selectin significantly decreased; however, there was no significant effect

of AGE inhibition on HAEC E-selectin expression in the control group. These results confirm that AGEs likely contribute to the upregulation of E-selectin expression in the T2D-SCT group.

Our results showed that NF- κ B mRNA expression was not elevated in the T2D-SCT group, and that there were no differences in VCAM-1 expression among groups. These findings suggest that a pathway other than AGE-RAGE activation of NF- κ B could cause the elevated expression of E-selectin found in the T2D-SCT group. The p38 MAPK signaling pathway is another mechanism that modulates the expression of E-selectin (33,34). However, our findings showed no differences in p38 MAPK expression among the groups, suggesting that a different mechanism is most likely responsible for the increased E-selectin protein expression observed in the T2D-SCT group.

Elevated concentrations of proinflammatory cytokines can contribute to the development of vascular dysfunction and microvascular and macrovascular complications in T2D (35). However, in this study, no differences in plasma concentrations of IL-1 β , IL-6, IL-8, or TNF- α were found among groups, indicating that differences in these proinflammatory cytokines likely do not explain the increased arterial stiffness or the increased rates of vascular complications observed in the T2D-SCT group.

Elevated whole-blood viscosity has been shown to play an important role in the pathogenesis of hypertension (36), diabetic retinopathy (37), and chronic kidney disease (38). In our study, whole-blood viscosity was significantly increased at high shear rates (90–375 s) in the T2D-SCT group compared with the other three groups. Whole-blood viscosity is dependent on plasma viscosity, hematocrit, and red blood cell rheological properties, including red blood cell aggregation and deformability (39). However, hematocrit and plasma viscosity did not vary among groups. Therefore, the increased blood viscosity observed in the T2D-SCT group is likely due to alterations in red blood cell rheological properties (39). The impact of red blood cell rheological properties on blood viscosity is highly dependent on shear rate (39). At low-to-moderate shear rates, red blood cell aggregation is the main determinant of blood viscosity, whereas red blood cell deformability plays a more important

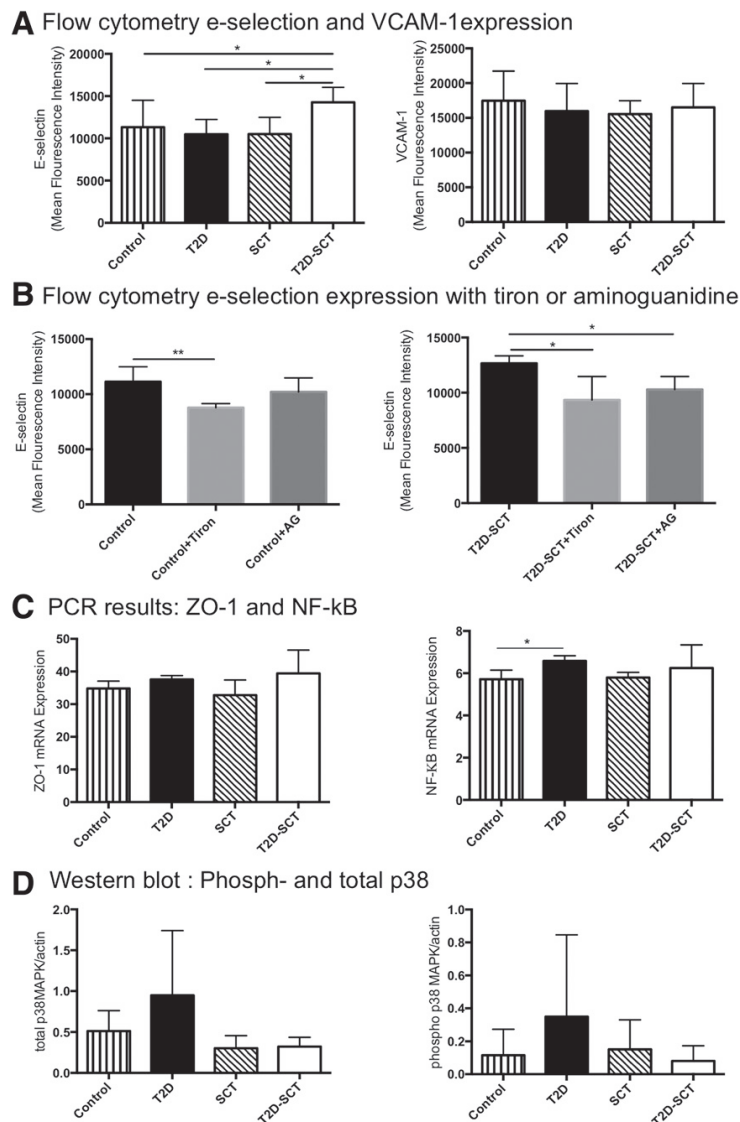


Figure 2—Flow cytometry, real-time quantitative PCR, and Western blot results for subjects with T2D and SCT (T2D-SCT) or without SCT (T2D), and subjects without T2D with SCT (SCT), or without SCT (Control). *A*: Flow cytometry results for E-selectin and VCAM-1 expression in HAECs incubated with TNF- α and plasma from subjects; * $P < 0.05$. *B*: Flow cytometry results for E-selectin expression in HAECs incubated with TNF- α and either control or T2D-SCT plasma (Control or T2D-SCT), control or T2D-SCT plasma and the antioxidant tiron (Control+Tiron or T2D-SCT+Tiron), and control or T2D-SCT plasma with the AGE inhibitor aminoguanidine hydrochloride (Control+AG or T2D-SCT+AG); * $P < 0.05$, ** $P < 0.01$. *C*: Real-time quantitative PCR for results showing relative mRNA expression of NF- κ B and ZO-1 in HAECs incubated with TNF- α and plasma from subjects, * $P < 0.05$. *D*: Western blot results showing the relative expression of phospho- and total p38 MAPKinase in HAECs incubated with TNF- α and plasma from subjects. Results are presented as the mean \pm SD.

role at high shear rates. Decreased red blood cell deformability contributes to increased blood viscosity in both T2D and SCT (8). Indeed, SCT red blood cells contain HbS and have been shown to have increased lactate transporter

activity, which could increase lactate and hydrogen ion accumulation in the red blood cells, thereby decreasing red blood cell deformability (8). Furthermore, in T2D, hyperglycemia leads to increased nonenzymatic glycation of

hemoglobin and membrane proteins in red blood cells, and these alterations are associated with decreased membrane fluidity (39). Evidently, the combination of these factors could potentially amplify decreased red blood cell deformability, and thus increase whole-blood viscosity, in individuals with T2D-SCT. Additionally, the elevated plasma AGE concentrations observed in the T2D-SCT group suggest that nonenzymatic glycation of red blood cell membrane proteins could be increased in these individuals, thereby contributing to the increased red blood cell rigidity (40).

We also observed a higher thixotropic index in the T2D-SCT group compared with the other three groups. Blood is considered a thixotropic fluid because its viscosity depends not only on shear rate, but also on the previous history of motion of the fluid (19). Indeed, at low shear rates, red blood cells form large, reversible aggregates, thereby increasing blood viscosity (19,38). However, as the shear rate rises, viscosity gradually decreases as the aggregates separate into smaller aggregates, and eventually into individual red blood cells (19). If the shear rate is then reduced, red blood cell aggregates will gradually reform, and blood viscosity will increase again (19). Therefore, the increased thixotropic index observed in the T2D-SCT group may reflect the amplified red blood cell alterations present in the T2D-SCT group. Furthermore, the high thixotropic index in the T2D-SCT group could potentially increase vascular disease risk in these individuals because an increased thixotropic index has been linked to the occurrence of microvascular and macrovascular disorders in sickle-cell anemia and coronary artery disease (20, 41).

In conclusion, our study found that hypertension, retinopathy, and decreased renal function were more prevalent in individuals with both SCT and T2D than in those with T2D only. Furthermore, our results suggest that SCT could exacerbate multiple factors that increase the risk of development of T2D-related vascular complications, including arterial stiffness, blood hyperviscosity, AGE accumulation, and increased E-selectin expression. Overall, our results indicate that individuals with T2D-SCT should be monitored more frequently for diabetic retinopathy, diabetic nephropathy, and hypertension. Additionally, SCT is not

only prevalent in Sub-Saharan Africa, but also in the Middle East, India, and populations of African ancestry in Europe and the Americas; the prevalence of T2D is projected to increase in all of these populations (3,4). Therefore, additional studies are needed to confirm whether or not SCT increases the risk of development of T2D-related vascular complications across all populations (4).

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STUDY 3: Altered blood rheology and impaired pressure-induced cutaneous vasodilation in a mouse model of combined type 2 diabetes and sickle cell trait

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The findings of Diaw et al and study 2 demonstrate that T2D-related vascular dysfunction is amplified by SCT, and identify possible underlying mechanisms that could potentially contribute to this vascular dysfunction (Diaw et al. 2015); (Study 2). However, direct evidence to explain the mechanisms of this exaggerated vascular dysfunction is lacking. Therefore, the objectives of study 3 were to create a mouse model of combined T2D and SCT, and then to study vascular function and blood rheology in this model of combined T2D and SCT. This mouse model could ultimately be used to directly study the mechanisms of the alterations in vascular function present in combined T2D and SCT.

Several mouse models of SCD exist, including SAD, BERK, NY1DD, and Townes mice. The SAD mouse was the first murine model of sickle cell disease to be generated, and has been used to study the pathology and potential treatments of SCD (Beuzard 2008). However, SAD mice have no anemia in adulthood and express a very mild disease phenotype (Beuzard 2008). For this reason, several more complex mouse models with more severe phenotypes have been developed, including several “Hb-S only” knock-in and knock-out models (Beuzard 2008). The most relevant “Hb-S only” model is the Townes knock-in model, which was developed by Dr. Tim Townes at the University of Alabama (Wu et al. 2006). Townes mice express only human α and β globin chains, and thus closely mirror the human phenotype of SCD (Wu et al. 2006). Furthermore, during gestation and as newborns Townes mice express human fetal globin, which delays the onset of the symptoms of SCD and increases the chance of survival of the newborn mice with SCD (McConnell et al. 2011). Three genotypes of Townes mice exist:

➤ Townes HbSS mice: In these mice the murine adult α -globin genes were replaced with the human α -globin gene (genotype: *Hba* ha/ha), and the murine adult

β -globin genes were replaced with human sickle β^S - and fetal γ -globin gene fragments linked together (genotype: *Hbb* $h^A\gamma\beta^S/h^A\gamma\beta^S$). These mice present with a severe disease phenotype, accompanied by anemia and a shortened RBC half-life of 2.5 days (Nguyen et al. 2014).

➤ Townes HbAA mice: These “control” Townes mice were created by replacing the murine globin genes with the human α -globin gene (genotype: *Hba* ha/ha) and the murine adult β -globin genes with human sickle β^A - and fetal γ -globin gene fragments linked together (genotype: *Hbb* $h^A\gamma\beta^A/h^A\gamma\beta^A$) (Nguyen et al. 2014).

➤ Townes HbAS mice: These heterozygous “SCT mice” were developed by breeding Townes HbAA and HbSS mice (Nguyen et al. 2014).

For this study we chose to use Townes HbAA as controls and Townes HbAS mice as a model of SCT. The mice came from our own colony, in which two heterozygous β^A/β^S (SCT) Townes mice were crossed to generate AA, SS, and SCT mice. PCR was then used to confirm the genotypes of the mice, and only AA and SCT mice were included in the study.

At 8-12 weeks of age, the AA and SCT were assigned to one of two 12-week diets: standard or high fat high sugar (HFHS) to induce T2D. The AA and SCT mice fed the standard diet made up the control and SCT groups, respectively, while AA and SCT mice fed the HFHS diet became the T2D and combined T2D-SCT groups, respectively. Mice were weighed every week during the diet to calculate changes in body weight. Following the 12-week diet period glucose tolerance tests were used to confirm normoglycemia in the control and SCT groups, or hyperglycemia in the T2D and T2D-SCT groups. Next, laser Doppler flowmetry was used to assess microvascular function *in-vivo* in all mice. Pressure induced vasodilation (PIV) measures the cutaneous microvascular response to local pressure application, which

is highly dependent on the production of endogenous endothelium-derived NO. Therefore, PIV was used to measure endothelium-dependent vasodilation. Endothelium-independent vasodilation was measured using the iontophoretic delivery of sodium nitroprusside (SNP). Following the assessments of microvascular function mice were euthanized, and blood was sampled and used to measure whole blood viscosity, hematocrit, and red blood cell deformability.

The results of this study showed that, after 12-weeks on the assigned diets, the T2D and T2D-SCT mice gained significantly more weight, and had significantly higher glycemia than the mice in the control and SCT groups. Furthermore, no differences in whole blood viscosity, hematocrit, or erythrocyte deformability were observed between the control and T2D mice, or the control and SCT mice. However, whole blood viscosity was significantly higher and red blood cell deformability was significantly lower in the T2D-SCT group compared to the control group. Similarly, although no differences in PIV were observed between the control and T2D groups, or the control and SCT groups, PIV was significantly lower in the T2D-SCT group compared to the control group. Additionally, no differences in endothelium-independent vasodilation were observed between the groups. Overall, these findings indicate that the T2D-SCT mice have significantly altered blood rheology and significantly impaired endothelium-dependent vascular function.



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Altered blood rheology and impaired pressure-induced cutaneous vasodilation in a mouse model of combined type 2 diabetes and sickle cell trait



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ABSTRACT

Objective: Type 2 diabetes (T2D)-related vascular dysfunction and hemorheological abnormalities could possibly be amplified by sickle cell trait (SCT). These alterations could potentially increase the risk of vascular complications in individuals with combined T2D and SCT. Therefore, this study used a mouse model to determine whether vascular function and blood rheology were more severely altered in combined T2D and SCT than in T2D or SCT alone.

Methods: Townes transgenic mice with or without SCT received a 12-week high fat high sucrose or standard diet to create models of combined T2D-SCT, T2D, SCT, and controls. Pressure-induced vasodilation (PIV) and sodium nitroprusside (SNP)-mediated vasodilation *in-vivo*, and hemorheological parameters were measured.

Results: No significant differences in blood viscosity, hematocrit, erythrocyte deformability, or PIV were observed between the control and T2D mice, or the control and SCT mice. However, blood viscosity, erythrocyte deformability, and PIV were significantly altered in the T2D-SCT mice compared to the control mice. There were no differences in SNP response between the groups.

Conclusions: Although neither T2D nor SCT alone had significant effects on blood rheology parameters or vascular function, combined T2D-SCT mice had significantly altered blood rheology and significantly impaired vascular function.

1. Introduction

Sickle cell trait (SCT) is the heterozygous form of sickle cell anemia; an autosomal recessive blood disorder caused by a mutation in the β -globin gene, which results in the production of abnormal hemoglobin, referred to as hemoglobin S (HbS) (Rees et al., 2010). In deoxygenated conditions HbS polymerizes, resulting in red blood cell sickling, which leads to hemolytic anemia and painful vaso-occlusive crises (Rees et al., 2010). Unlike sickle cell anemia, SCT is not considered to be a disease

state, and people with SCT are generally asymptomatic (Key et al., 2015). However, accumulating evidence suggests that SCT could possibly increase the risk of chronic kidney disease, end stage renal disease, venous thromboembolism, and stroke (Naik and Haywood, 2015).

People with SCT are generally protected against the severe consequences of *P. falciparum* malaria infection (Key et al., 2015). Consequently, the HbS allele is prevalent in areas of the world where malaria is common, such as in Sub-Saharan Africa, India, and the Middle East, as well as in populations in the Americas and Europe whose ancestors

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come from these areas (Piel et al., 2013). Current epidemiological data shows that rates of type 2 diabetes (T2D) are already high, or on the rise, in all of these populations (Cho et al., 2018). Therefore, there is likely a large, and growing, population of individuals with both T2D and SCT across the globe.

T2D is characterized by alterations in the metabolic milieu that contribute to impaired vascular function, and augment the risk of developing vascular disease (Sena et al., 2013). Furthermore, blood viscosity is elevated in T2D, and is thought to play a crucial role in the progression of diabetic retinopathy, nephropathy, and neuropathy (Simpson et al., 1987; Simpson, 1988; Williamson et al., 1996). A study conducted by Diaw et al. in 2015 showed that individuals with combined T2D and SCT had significantly higher pulse wave velocity and impaired flow mediated dilation compared to subjects with either SCT or T2D alone (Diaw et al., 2015). Furthermore, the study's findings also showed that blood viscosity was higher in individuals with combined T2D and SCT compared to those with T2D or SCT alone (Diaw et al., 2015). These cross-sectional data suggest that SCT could potentially amplify the blood rheological alterations and impaired vascular function present in T2D. However, there is a lack of direct evidence illustrating that combined SCT and T2D causes more severe alterations in blood rheological parameters and vascular function than T2D alone. For this reason, we conducted a study to evaluate whole blood viscosity, hematocrit, and red blood cell deformability in a mouse model of combined T2D and SCT. Additionally, we evaluated vascular function *in-vivo* using pressure-induced vasodilation, a vasodilatory mechanism that is dependent on endogenous endothelium-derived nitric oxide (NO) production, and with sodium nitroprusside, an exogenous NO donor. We hypothesized that the T2D-SCT mice would exhibit greater alterations in blood rheology and vascular function than control mice or mice with SCT or T2D alone.

2. Methods

2.1. Animals and diet protocol

Townes mice are a transgenic murine model of sickle cell disease, in which the murine adult α - and β -globin genes were replaced with the human α - and γ -globin genes, and either human sickle β^S -globin (SS genotype), or normal β^A -globin (AA genotype) (Nguyen et al., 2014). In the present study, heterozygous β^A/β^S (SCT) Townes mice from our own colony, originally purchased from Jackson Laboratory (Bar Harbor, ME, USA), were used to generate healthy AA mice (control), SS sickle cell disease mice, and SCT mice (Sparkenbaugh and Pawlinski, 2013; Wang et al., 2016). Mouse genotypes were confirmed by PCR, and only male mice with AA or SCT genotypes were used. Mice were maintained in a temperature-controlled facility on a 12-hour light-dark cycle, with free access to food and water. The mice were fed standard mouse chow until the age of 8–12 weeks, at which point both the AA and SCT mice were randomly divided into one of two dietary groups: standard or high fat and high sugar (HFHS). The AA (control) and SCT groups received standard mouse chow (Standard Mouse Diet 5015 Labdiet: 26% fat, 19% protein, 54% Carbohydrates), while the AA type 2 diabetes (T2D) and combined type 2 diabetes and sickle cell trait (T2D-SCT) mice were fed pelleted food containing 45% fat, 16% carbohydrate, and 23% protein (Diet 824,127 Special Diet services, Essex, UK) supplemented with *ad libitum* access to sweetened condensed milk (55% simple carbohydrate, 8% fat, 8% protein, Nestlé®). All mice were given *ad libitum* access to water and their assigned diet for 12 weeks. This diet has been previously shown to result in impaired glucose tolerance and significant weight gain compared to a standard diet in mice (Nguyen-Tu et al., 2013). All experimental procedures were in accordance with the National Institute of Health guide for the care and use of Laboratory animals, and were approved by the regional animal care committee (CEEA-55, Rhône-Alpes, France).

2.2. Glucose tolerance test

All glucose tolerance tests (GTTs) were conducted 11 weeks after the start of the assigned diets. Following a five-hour fast, mice were intraperitoneally injected with a glucose load of 1 g per kg body weight. Blood (0.4 μ L) was sampled from the caudal vein at 0, 15, 30, 60, 90, and 120 min after the injection, and blood glucose concentrations were measured using a glucometer (OneTouch Verio®, LifeScan, France) (Nguyen-Tu et al., 2013). The area under the curve (AUC) for each GTT was calculated using the trapezoidal rule.

2.3. Assessment of cutaneous microcirculation

Assessments were carried out at the end of the 12-week diet. In order to prepare the mice for laser Doppler flow measurements, local pressure application, and iontophoretic deliveries, hair was removed from the top of the skull and back of the mice using a depilatory cream. Hair removal was performed two days before the experiments in order to minimize skin irritation, which could confound results (Demiot et al., 2006).

Mice were anesthetized using an intraperitoneal injection of Pentobarbitol Sodium (60 mg/g) (Gargiulo et al., 2013). The level of anesthesia was confirmed by testing the mice's eye reflexes and tail pinch responses. Following anesthesia, mice were positioned in a warmed incubator to maintain a stable cutaneous temperature of 35 ± 0.5 °C (MMS, Chelles, France). The mice were placed in a prone position with their heads carefully set on a supportive frame. A 20-minute resting period was then carried out to enable temperature and blood pressure stabilization. Mean arterial blood pressure (MABP) was measured throughout all of the assessments using a non-invasive system (XBP 1000 (Kent Scientific, Torrington, CT, USA)).

The cutaneous microvascular response to local pressure application, referred to as pressure-induced vasodilation (PIV), is highly dependent on the production of endogenous endothelium-derived NO, and was therefore used to assess endothelium dependent vasodilation. The PIV was measured using laser Doppler flowmetry (LDF). This method, previously described by Fromy et al. (2000), uses a weighbridge adapted to hold a laser Doppler probe (PF415; Periflux; Perimed, Järfälla, Sweden) at one end, which is connected to a laser Doppler flowmeter (PF5000 Master; Periflux; Perimed, Järfälla, Sweden). The weighbridge was positioned with the laser Doppler probe carefully equilibrated on the hairless skull of the mouse. The external pressure applied by the Doppler probe was then gradually increased at a rate of 132 Pa min^{-1} ($1 \text{ mm Hg} \cdot \text{min}^{-1}$) using a syringe pump. The LDF signal was digitized with a 20-Hz sampling frequency using a computerized acquisition system (Biopac, Santa Barbara, CA, USA). Data collection began with a 1-minute control period before the pressure was gradually increased. The PIV response is calculated as the percent change between basal and peak LDF values (Fromy et al., 2000).

Endothelium-independent response was measured using cathodal sodium nitroprusside (SNP) iontophoresis (67 mmol/L; Nitriate; SERB, Paris, France) with a current of 100 μ A applied for 20 s. Skin blood flow was recorded using a laser Doppler multifiber probe (481-1, Perimed, Järfälla, Sweden), during transcutaneous iontophoresis, applied to a 1.2-cm² area on the hairless back of the mice (Demiot et al., 2006). The LDF signals for PIV and response to SNP were averaged every 10 s to reduce the instantaneous variability of the signals as a result of vaso-motion.

2.4. Blood sampling and hemorheological measures

Blood was sampled *via* a retro-orbital venipuncture and collected into EDTA tubes for hemorheological analysis.

Whole blood viscosity was determined using blood at native hematocrit after complete oxygenation using a cone plate viscometer at a shear rate of 22.5 s^{-1} (Brookfield DVII+ with CPE40 spindle,

Brookfield Engineering Labs, Natick, MA). Hematocrit was determined by microcentrifugation.

Red blood cell deformability was measured at 37 °C at 3 Pa by laser diffraction analysis (ektacytometry), using a Laser-assisted Optical Rotational Analyzer (LORCA MaxSis, RR Mechatronics, Hoorn, The Netherlands). The system has been previously described in detail by Baskurt et al. (2009). Briefly, 7 μ L of oxygenated blood was mixed with 1 mL of polyvinylpyrrolidone (PVP; viscosity = 30 cP, RR Mechatronics, Hoorn, The Netherlands), and sheared into a glass Couette system. A computer was used to analyze the diffraction pattern and calculate an elongation index. All hemorheological measurements were carried out in accordance with recommendations of Baskurt et al. (2009).

2.5. Data analysis

Two-way ANOVA tests with Fisher's LSD post-hoc analyses were used to identify effects of genotype and diet, and between group differences in baseline body weight, average weight gained, GTT AUC, PIV, endothelium independent vasodilation, and hemorheological parameters. A P-value of < 0.05 was considered statistically significant. Data are expressed as means \pm SD. All statistical analyses were conducted using GraphPad Prism version 6 (GraphPad, Sand Diego, CA).

3. Results

3.1. Diet protocol and glucose tolerance test

After 12-weeks on the assigned diets, the HFHS fed T2D and T2D-SCT groups gained significantly more weight than standard chow fed control and SCT groups (Fig. 1a). The GTT AUC was significantly higher in the T2D and T2D-SCT groups compared to the control and SCT groups (Fig. 1b and Table 1). Main effects of diet on weight gain and GTT AUC were observed ($P < 0.001$).

3.2. PIV assessment and endothelium independent response

The SCT and T2D groups tended to have lower PIV responses compared to the control group, although the differences did not reach statistical significance ($P < 0.1$). However, the T2D-SCT group had a significantly lower PIV response than the control group (Fig. 2).

There were no significant differences in endothelium-independent response to SNP between the groups. Basal MABP and LDF values did not differ between groups, and no main effects on these parameters

were observed (Table 1).

3.3. Hemorheology

There were no significant differences in blood viscosity between the control and SCT groups. Blood viscosity tended to be higher in the T2D group compared to the control group ($P < 0.1$). Blood viscosity was significantly higher in the T2D-SCT group compared to the control group and the SCT groups (Fig. 3a).

There were no significant differences in hematocrit between the control and SCT groups. Hematocrit tended to be higher in the T2D group (43.00 ± 2.71) compared to the control group (39.63 ± 2.70) ($P = 0.08$). Hematocrit was significantly higher in the T2D-SCT (42.80 ± 0.84) compared to the SCT group (37.43 ± 3.05) (Fig. 3b), and tended to be higher in the TD-SCT compared to the control group ($P = 0.08$).

There were no significant differences in red blood cell deformability between the control and the SCT groups, or between the control and the T2D groups. However red blood cell deformability was significantly lower in the T2D-SCT group compared to the control group, and compared to the T2D group (Fig. 3c).

4. Discussion

In this study, alterations in vascular function and rheological parameters were more severe in mice with combined T2D-SCT than in mice with SCT or T2D, when compared to control mice. Our assessments of vascular function showed that the PIV response tended to be lower in the T2D and the SCT groups compared to the control group, although these differences were not significant. The PIV response in the T2D-SCT group, on the other hand, was significantly reduced compared to the control group. Therefore, our results suggest that T2D and SCT could potentially have a synergist effect on vascular dysfunction.

The PIV is a protective vasodilatory mechanism that delays the reduction in cutaneous blood flow that follows the application of pressure on the skin.¹⁷ Activation of the PIV response results in endothelium-dependent vasodilation that has been shown to be highly dependent on the availability of endogenously produced NO (Fromy et al., 2000). Unlike PIV, SNP induces vasodilation *via* direct stimulation of smooth muscle cells (Turner et al., 2008). In our study, the vascular response following iontophoretic delivery of SNP did not vary significantly between groups, suggesting a lack of difference in smooth muscle vasodilatory capacity between groups. Therefore, our results indicate that the diminished PIV response in the T2D-SCT group was likely due to

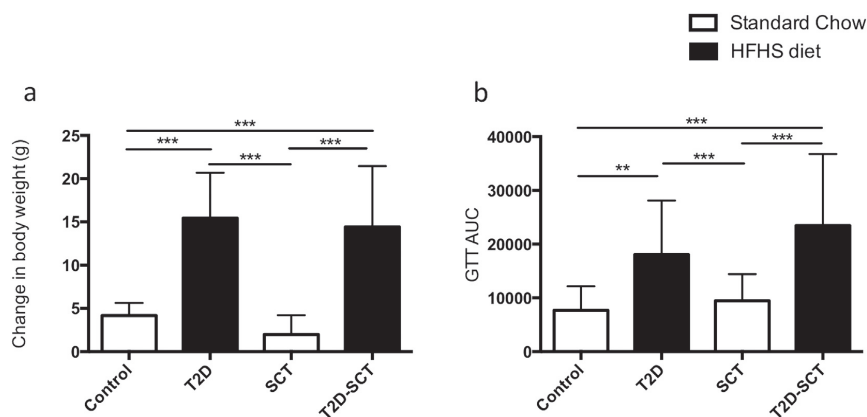


Fig. 1. (a) Change in body weight in grams and (b) glucose tolerance test area under the curve (GTT AUC) in Townes AA and AS after 12-weeks on a standard or high fat high sucrose (HFHS) diet in control ($n = 18$), T2D ($n = 17$), SCT ($n = 15$), and T2D-SCT ($n = 20$) mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 1

Baseline body weight, GTT AUC, MABP, Basal LDF PIV, Basal LDF SNP, and SNP % vasodilation in Townes AA and SCT mice after 12-weeks on a standard or high fat high sucrose diet.

	Control	T2D	SCT	T2D-SCT
Baseline weight (g)	29.32 ± 3.23 (n = 18)	28.70 ± 3.70 (n = 17)	28.88 ± 4.64 (n = 15)	27.03 ± 3.48 (n = 19)
GTT AUC	7687 ± 4475 (n = 18)	18,046 ± 10077 ^{***} (n = 17)	9443 ± 4982 (n = 15)	23,453 ± 13323 ^{***} (n = 19)
MABP (mm Hg)	77.59 ± 15.46 (n = 9)	69.89 ± 12.3 (n = 10)	64.33 ± 12.36 (n = 13)	74.83 ± 15.65 (n = 12)
Basal LDF PIV	102.34 ± 36.67 (n = 9)	109.8 ± 59.14 (n = 10)	79.62 ± 51.38 (n = 13)	105.29 ± 53.29 (n = 12)
Basal LDF SNP	42.48 ± 16.76 (n = 12)	42.17 ± 14.84 (n = 9)	45.14 ± 13.61 (n = 13)	48.39 ± 14.98 (n = 12)
SNP % vasodilation	33.87 ± 10.0 (n = 12)	34.96 ± 15.26 (n = 9)	42.23 ± 18.92 (n = 13)	45.80 ± 24.09 (n = 12)

Control = AA mice fed standard diet; T2D = AA mice fed HFHS diet; SCT = SCT mice fed standard diet; T2D-SCT = SCT mice fed HFHS diet; GTT AUC, glucose tolerance test area under the curve; MABP, mean arterial blood pressure; LDF, laser Doppler flowmetry; PIV, pressure induced vasodilation; SNP, sodium nitroprusside; SNP % vasodilation, percent change in LDF following iontophoretic delivery of sodium nitroprusside.

* P < 0.05, ** P < 0.01, *** P < 0.001 compared to control. # P < 0.05, ## P < 0.01, ### P < 0.001 compared to SCT.

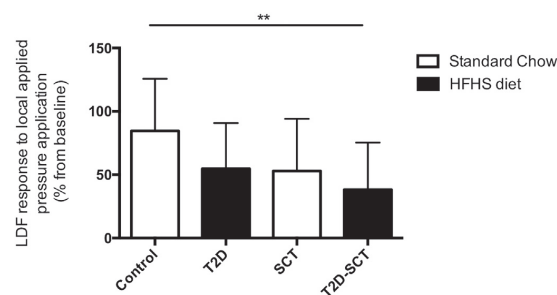


Fig. 2. Percentage of vasodilation during local pressure application (PIV response) in AA and AS mice fed standard (control: n = 9, SCT: n = 13) and high fat high sucrose diets (T2D: n = 10, T2D-SCT n = 12). ** P < 0.01 compared to control.

dysfunction of the endothelium, and not the smooth muscle cells.

Elevated oxidative stress decreases NO availability, and thus contributes to endothelium dysfunction in type 2 diabetes (Sena et al., 2013). Prior studies have found elevated oxidative stress in mice that were fed a HFHS diet for 12 weeks in order to induce T2D (Liu et al., 2015; Lee et al., 2013). Additionally, elevated reactive oxygen species and altered plasma nitric oxide byproducts have been observed in

Townes SCT mice (Charrin et al., 2016). Moreover, a study by Diaw et al. illustrated that individuals with combined T2D and SCT had elevated levels of reactive oxygen species (Diaw et al., 2015). In light of this evidence, it is possible that increased production of reactive oxygen species could decrease NO availability, and consequently lower the PIV response in the T2D-SCT mice.

A large body of evidence indicates that whole blood viscosity is elevated in T2D (Cho et al., 2008; McMillan, 1981; Skovborg et al., 1966). In the current study, whole blood viscosity tended to be higher in the T2D mice compared to the control mice. However, we observed significantly more severe blood hyperviscosity in the T2D-SCT mice than in the T2D mice. These results suggest that blood hyperviscosity in T2D could be amplified by SCT. These findings coincide with the results of Diaw et al., which showed elevated blood viscosity in individuals with combined T2D and SCT compared to those with T2D or SCT only (Diaw et al., 2015).

Whole blood viscosity is highly dependent on hematocrit (Baskurt and Meiselman, 2003). Hematocrit is generally elevated in type 2 diabetes because as blood glucose rises, blood osmolarity increases, resulting in augmented capillary permeability, and a subsequent increase in hematocrit (Meiselman et al., 1967). In this study hematocrit was significantly elevated in the T2D-SCT mice compared to the SCT mice. However, hematocrit only tended to be elevated in the T2D-SCT groups compared to the control group. Therefore, although elevated

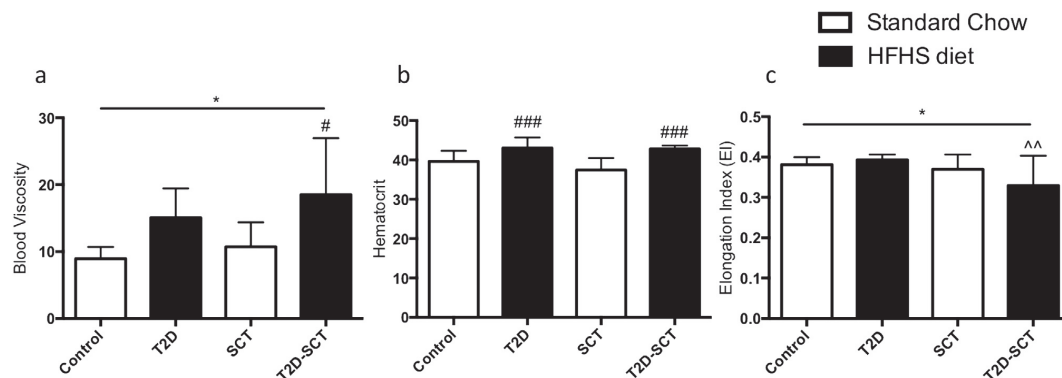


Fig. 3. Hemorheological data (a) Blood viscosity at 22.5 s⁻¹ (control: n = 4, T2D n = 5, SCT n = 6, T2D-SCT n = 5), (b) hematocrit (control: n = 4, T2D n = 4, SCT n = 7, T2D-SCT n = 5), (c) red blood cell deformability at 3 Pa (control: n = 6, T2D n = 7, SCT n = 7, T2D-SCT n = 6). * P < 0.05 compared to control. ** P < 0.01 compared to T2D. # P < 0.05. ### P < 0.001 compared to SCT.

hematocrit likely plays a role in the amplified blood hyperviscosity observed in the T2D-SCT group, it is probable that there are other factors that also contribute to the elevated blood viscosity in combined T2D-SCT.

The capacity of red blood cells to deform under flowing conditions is another crucial element that modulates whole blood viscosity (Baskurt and Meiselman, 2003). Red blood cell deformability is dependent on the internal viscosity, the surface to volume ratio, and the deformability of the membrane of the erythrocyte (Baskurt and Meiselman, 2003; Chien, 1987). Several prior studies have been performed to test the effects of T2D or SCT (but not the effects of the two conditions) on erythrocyte deformability in humans. Hyperglycemia can cause increased hemoglobin glycation as well as glycation of membrane proteins in the erythrocytes, and these changes reduce erythrocyte deformability (Lee et al., 2017; Watala, 1993). Agrawal et al. (2016) also evaluated erythrocytes using dual optical tweezers, and observed a loss of membrane deformability in diabetic erythrocytes. On the other hand, Lee et al. (2017) reported no significant alterations in morphological or biochemical parameters in T2D erythrocytes compared to healthy erythrocytes. However, these results directly contrast with the findings of Neam et al., which showed a high prevalence of morphological changes in diabetic compared to non-diabetic erythrocytes (NeamTu et al., 2015). The reduced erythrocyte deformability observed in T2D patients could contribute to the increased blood viscosity observed in these individuals (Babu and Singh, 2004).

Furthermore, although a previous study published by Charrin et al. found no differences in erythrocyte deformability between Townes AA and SCT mice, several studies have observed reduced red blood cell deformability associated with elevated blood viscosity in individuals with SCT, compared to controls (Charrin et al., 2016; Brandao et al., 2003; Connes et al., 2005). Indeed, in SCT carriers, the amount of intracellular HbS fraction seems to predict the loss of RBC deformability (Hiruma et al., 1995). Additionally, previous evidence suggests that elevated lactate transporter activity could contribute to reduced erythrocyte deformability in individuals with SCT (Connes et al., 2005). However, the findings of the present study contrast with these prior studies, as the results showed that neither the SCT, nor the T2D mice had significantly reduced red blood cell deformability compared to the control mice. It remains unknown why no significant effects of T2D or SCT on erythrocyte deformability were observed in our study, and further studies using our animal models will be required to answer this question. For example, the use of osmotic gradient ektacytometry (Mohandas et al., 1980), in addition to the use of isotonic ektacytometry, as performed in the present study, will be helpful to further explore erythrocyte deformability in these mice (Mohandas et al., 1980). Despite the unexpected findings in our mouse models of isolated T2D and SCT, it is possible that increased hemoglobin and membrane protein glycation (caused by hyperglycemia) combined with the presence of HbS in the T2D-SCT erythrocytes could have resulted in the significantly reduced erythrocyte deformability observed in the T2D-SCT group. Overall, the rheological results suggest that the reduced red blood cell deformability found in the T2D-SCT mice, paired with the increased hematocrit linked to hyperglycemia, could play an important role in the amplified blood hyperviscosity observed in the T2D-SCT mice.

Under normal physiological conditions, elevations in blood viscosity increase shear stress, which results in increased NO production, and subsequent vasodilation (Tsai et al., 2005). However, when NO bioavailability is decreased, vascular function is impaired, and blood vessels cannot effectively vasodilate to protect against the deleterious effects of increased blood viscosity (Connes, 2015). Therefore, in combined T2D-SCT abnormal blood rheology paired with impaired vascular function could possibly result in decreased blood flow and stasis, particularly in the microcirculation, which could potentially increase the risk of developing diabetic microvascular complications (Simpson et al., 1987; Simpson, 1988; Williamson et al., 1996).

In conclusion, neither T2D nor SCT alone had significant effects on blood rheological parameters or vascular function. However, when T2D and SCT were combined, both blood rheology and endothelium-dependent vasodilation were significantly altered compared to the control mice. Both vascular function and blood rheological alterations contribute to the pathogenesis of vascular disease in T2D (Sena et al., 2013; Simpson et al., 1987; Simpson, 1988; Williamson et al., 1996). Therefore, future studies should be conducted to determine whether SCT could increase the risk of developing T2D-related vascular complications.

Declarations of interest

None.

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STUDY 4: Altered acetylcholine-mediated endothelium-dependent vasodilation *in-vivo* in a mouse model of combined type 2 diabetes and sickle cell trait

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[#]Equivalent position

*Equivalent position

Study in progress

The results of Diaw et al indicated that T2D-related macrovascular dysfunction is exaggerated in individuals with both T2D and SCT (Diaw et al. 2015). Furthermore, the findings of Study 2 showed that retinopathy and impaired renal function were more prevalent among subjects with combined T2D and SCT than among subjects with T2D only, which suggests that microvascular dysfunction could also be amplified in individuals with combined T2D and SCT (Study 2, *Diabetes Care*). This hypothesis was reinforced by the results of study 3, which indicated that endothelium-dependent vasodilation is impaired in the microvasculature of mice with combined T2D and SCT (Study 3). However, no studies have been conducted to directly assess the mechanisms of altered microvascular function in combined T2D and SCT. Therefore, the primary objectives of study 4 were to evaluate microvascular function in a mouse model of combined T2D and SCT, and to evaluate possible mechanisms that could be involved in the observed alterations in microvascular function.

As in Study 3, Townes transgenic AA and SCT mice were fed either a standard or HFHS diet for 12 weeks to create four groups of mice: controls (AA mice fed a standard diet), SCT (SCT mice fed a standard diet), T2D (AA mice fed a HFHS diet), and T2D-SCT mice (SCT mice fed a HFHS diet). Mice were weighed every week in order to evaluate changes in body weight. Glucose tolerance tests were conducted in all mice at the end of the 12-week diet period. Next, laser Doppler flowmetry was used to measure microvascular vascular function *in-vivo*. Iontophoretic delivery of SNP and acetylcholine was used to measure endothelium-independent and endothelium-dependent vasodilation, respectively. In order to evaluate the role of COX-2 in modulating acetylcholine-induced vasodilation, SC-58125, a specific inhibitor of inducible COX-2, was injected intraperitoneally into

mice. Acetylcholine-induced vasodilation *in-vivo* was then measured 15 minutes after the injection (Fujino et al. 2004). Following the assessments of microvascular function, samples of sub-cutaneous and visceral adipose tissues were collected to assess macrophage infiltration and COX-2 protein and mRNA expression using western blot and RT-PCR.

After 12-weeks on the assigned diets the T2D and T2D-SCT mice gained significantly more weight and had significantly higher glycemia compared to the control and SCT mice. Endothelium-independent vasodilation did not differ between the groups. However, acetylcholine-mediated endothelium-dependent vasodilation was significantly higher in the T2D-SCT mice compared to the control mice. Inhibition of COX-2 via injection of SC-58125 significantly decreased acetylcholine-mediated vasodilation in the T2D-SCT group to levels comparable to the T2D group. Overall, these results indicate that acetylcholine-mediated vasodilation is enhanced via COX-2 dependent mechanisms in a mouse model of combined T2D and SCT.

Our results are in accordance with the findings of a 2018 study conducted by Nguyen-Tu and colleagues. Their study also observed an enhanced, compensatory vasodilatory response to iontophoresis of acetylcholine that was dependent on COX-2 (Nguyen-Tu et al. 2018). COX-2 enzymes generate prostaglandins, such as PGI₂ and PGE₂, which are potent vasodilators (Szerafin et al. 2006), and COX-2 expression is increased by inflammation (Dubois et al. 1998; Feletou, Huang, and Vanhoutte 2011). Moreover, a previous study has shown that coronary endothelial function is rescued in obese-insulin resistant mice due to the overexpression of COX-2 (Sanchez et al. 2010). Therefore, it is possible that increased inflammation in the T2D-SCT mice could lead to increased COX-2 expression, resulting in an adaptive hyper-vasodilatory response to acetylcholine.

Furthermore, in their study, Nguyen-Tu et al suggested that obesity-related inflammation and the accumulation of pro-inflammatory macrophages could contribute to an adaptive microvascular response mediated by acetylcholine (Nguyen-Tu et al. 2018). Indeed, macrophages have been shown to release COX-2 dependent PGE2 in the presence of acetylcholine, through the stimulation of the alpha-7 nicotinic receptor (Pelletier et al. 2012). Considering this information, we hypothesize that increased inflammation in the T2D-SCT mice could lead to increased macrophage recruitment and increased COX-2 expression. Increased COX-2 expression in the vasculature and/or macrophages in the adipose tissue surrounding the cutaneous blood vessels could result in increased acetylcholine-stimulated vasodilation via the production of PGE2 (Figure 26). In order to test this hypothesis, the next step of this study will be to compare mRNA and protein expression of COX-2, and evaluate macrophage infiltration by measuring the expression of MCP-1, a potent chemokine that affects macrophage accumulation, and F4/80, a highly specific macrophage cell surface marker demonstrating macrophage infiltration density, in the adipose tissue of all of the mice.

Our findings do contradict many reports showing impaired acetylcholine-mediated vasodilation in mice with diabetes (Bourgoin et al. 2008; De Vriese et al. 2000; Sallam et al. 2011). However, previous studies demonstrating impaired acetylcholine-mediated vasodilation in obese T2D mouse models have used *ex-vivo* approaches that generally measure responses in isolated large vessels. These *ex-vivo* methods remove the vessel from its physiological environment, whereas our study measured microvascular function *in-vivo* in cutaneous vessels that are in direct contact with adipose tissue. Furthermore, the few studies that have observed impaired acetylcholine-mediated vasodilation in the microvasculature of diabetic

rodents *in-vivo*, have used non-obese, streptozotocin-induced mouse models of diabetes (Demiot et al. 2006; Sigauco-Roussel et al. 2004).

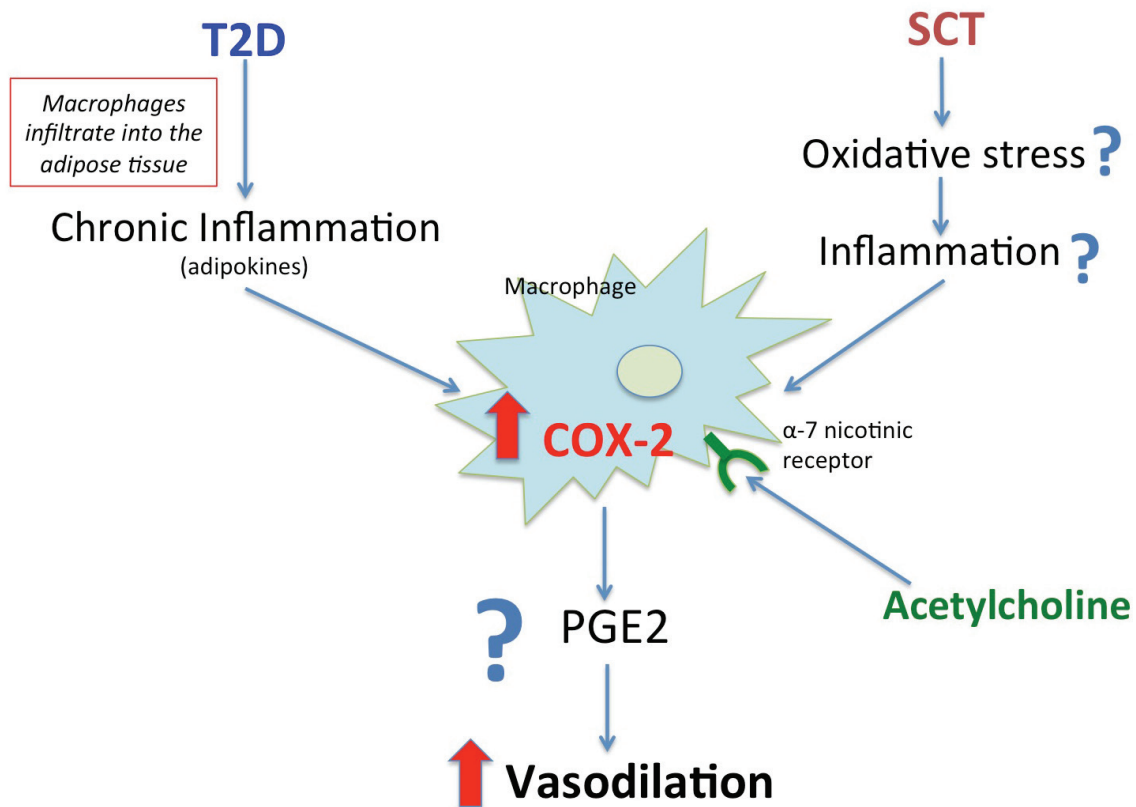


Figure 26: Study 4 hypothesis. Elevated levels of inflammation associated with T2D and SCT could lead to increased macrophage recruitment and increased COX-2 expression, which would result in increased ACH-stimulated vasodilation via the production of prostaglandin E2 (PGE2) in combined T2D and SCT.

Altered acetylcholine-mediated endothelium-dependent vasodilation *in-vivo* in a mouse model of combined type 2 diabetes and sickle cell trait

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1. Introduction

Type 2 diabetes (T2D) is a metabolic disease that causes vascular dysfunction, leading to the pathogenesis of vascular complications (Paneni et al. 2013). T2D is on the rise around the globe, including in populations where sickle cell trait (SCT) is prevalent, such as in Sub-Saharan Africa, India, and the Middle East, as well as among individuals whose ancestors come from these areas (Cho et al. 2018; Piel et al. 2013). SCT is the heterozygous version of sickle cell anemia (Rees, Williams, and Gladwin 2010). Individuals with SCT inherit one regular β^A -globin gene and one mutated β^S -globin gene, and therefore produce both normal hemoglobin A and mutated hemoglobin S. For this reason individuals with SCT do not suffer from the severe complications that are associated with sickle cell anemia, and have a normal life expectancy (Gima and Bemis 1975; Kramer, Rooks, and Pearson 1978). However, accumulating evidence suggests that SCT could increase the risk of venous thromboembolism, chronic kidney disease and end stage renal disease, and thus may not be completely benign, as was originally thought (Naik and Haywood 2015). Furthermore, a study by Diaw et al illustrated that flow mediated dilation is more severely impaired in individuals with both T2D and SCT than in those with T2D alone, suggesting that vascular

dysfunction is exaggerated in the macrovasculature of individuals with combined T2D and SCT (Diaw et al. 2015). A recent study also showed that rates of retinopathy and impaired renal function are higher among individuals with combined T2D and SCT compared to those with T2D only, indicating that microvascular dysfunction could also be exaggerated in people with combined T2D and SCT (Skinner et al, in press *Diabetes Care*). Additionally, we recently showed that endothelium dependent vasodilation is impaired in a mice model of combined T2D and SCT (Skinner et al, Study 3). However, the mechanisms of this dysfunction remain unclear. Therefore, the primary objective of this study is to assess endothelium-independent and endothelium-dependent microvascular function in a mouse model of combined T2D and SCT, and to evaluate potential mechanisms that could be involved in the observed alterations in microvascular function.

2. Methods

2.1 Mice and diet protocol

All experiments were carried out using Townes mice, a transgenic murine model of sickle cell disease. In Townes mice, the murine adult α - and β -globin genes are replaced with the human α - and γ - globin genes, and either human sickle β^S – globin (SS genotype), or normal β^A globin (AA genotype) (Nguyen et al. 2014). For this study, heterozygous β^A/β^S (SCT) Townes mice, originally purchased from Jackson Laboratory (Bar Harbor, ME, USA), were used to generate healthy AA mice (control), SS sickle cell disease mice, and SCT mice (Sparkenbaugh and Pawlinski 2013; Wang et al. 2016). PCR was used to confirm mouse genotypes, and only AA or SCT genotypes were used.

Mice were kept in a temperature-controlled facility, on a 12-h light/dark cycle with ad libitum access to standard mouse chow and water. At 8-12 weeks of age AA and SCT mice were randomly assigned to either a standard diet, or a high fat and high sugar (HFHS) diet to induce type 2 diabetes. The AA (control) and SCT groups were fed a standard diet (Standard

Mouse Diet 5015 Labdiet: 26% fat, 19% protein, 54% Carbohydrates), whereas the AA type 2 diabetes (T2D) and combined type 2 diabetes and sickle cell trait (T2D-SCT) mice were fed pelleted food containing 45% fat, 16% carbohydrate, and 23% protein (Diet 824127 Special Diet services, Essex, UK), supplemented with *ad libitum* access to sweetened condensed milk (55% simple carbohydrate, 8% fat, 8% protein, Nestlé®). The HFHS diet has been shown to induce impaired glucose tolerance and significant weight gain compared to a standard diet in mice (Nguyen-Tu et al. 2013). Mice in all groups were given *ad libitum* access to water and their assigned diet for 12 weeks. The French Ministry of Agriculture guidelines for experimental procedures were followed, and the regional animal care committee approved the protocol (CEEA-55, Rhône-Alpes, France).

2.2 Glucose Tolerance Tests

Glucose tolerance tests (GTT) were conducted following 11 weeks on the assigned diets. After a five-hour fast, mice were injected intraperitoneally with a glucose load of 1g/ kg body weight. Blood (0.4 µL) was sampled from the caudal vein at 0, 15, 30, 60, 90, and 120 minutes after the injection, and blood glucose concentrations were measured using a glucometer (OneTouch Verio®, LifeScan, France) (Nguyen-Tu et al. 2013). The area under the curve (AUC) for each GTT was calculated using the trapezoidal rule.

2.3 Assessment of skin microvascular reactivity

All measures of vascular reactivity were conducted at the end of the 12-week diet. Two days before the assessments were performed, depilatory cream was used to remove the hair from the backs of the mice in order to prepare the skin for the iontophoretic deliveries.

On the day of the assessments mice were anesthetized by intraperitoneal injection of Pentobarbital Sodium (60mg/g) (Gargiulo et al. 2013). Mice were then placed in a prone position inside of a warmed incubator in order to maintain a stable cutaneous temperature of $35\pm 0.5^{\circ}\text{C}$ (MMS, Chelles, France). Mean arterial blood pressure (MABP) was monitored

during all assessments using a non-invasive system (XBP 1000 (Kent Scientific, Torrington, CT, USA).

2.3.1 Endothelium dependent and independent vasodilation

Skin blood flow was recorded using a laser Doppler multifiber probe (481-1, Perimed, Järfälla, Sweden), during transcutaneous iontophoresis applied to a 1.2-cm² area on the hairless back of the mice (Demiot 2006). Endothelium-dependent response was measured using the iontophoresis of anodal acetylcholine (ACH) (5.5 mmol/l; Sigma, Saint Quentin Fallavier, France) with a current of 100 µA, applied for 10 seconds. Endothelium-independent vasodilation was measured using cathodal sodium nitroprusside (SNP) iontophoresis (67 mmol/l; Nitriate; SERB, Paris, France) with a current of 100 µA applied for 20 seconds. The LDF signals were averaged every 10 seconds to reduce the instantaneous variability of the signals as a result of vasomotion.

2.3.2 Pharmacological inhibition of cyclooxygenase 2

The role of cyclooxygenase-2 (COX-2) in modulating ACH-induced vasodilation was studied using SC-58125 (Cayman Chemicals, MI, USA), a specific inhibitor of inducible COX-2 (Fujino et al. 2004). Following anesthesia, the mice were intraperitoneally injected with SC-58125 (10mg/kg). Fifteen minutes later, assessment of endothelial-dependent vasodilation using ACH was conducted, as described above.

2.4 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism version 6 (GraphPad, Sand Diego, CA). Data are expressed as means ± SD. A $P < 0.05$ was considered statistically significant. One-way ANOVA tests with Fisher's LSD post-hoc analyses were used to detect differences between the groups for baseline body weight, weight gained, GTT AUC, endothelium-independent vasodilation, and endothelium-dependent vasodilation.

3. Results

3.1 Diet protocol and GTT

Following the 12-week diet period the T2D and T2D-SCT mice fed the HFHS diet gained significantly more weight than the control and SCT mice fed standard mouse chow (Table 1). The GTT AUC was significantly greater in the T2D and T2D-SCT groups compared to the control and SCT groups (Table 1).

3.2 Endothelium independent and endothelium dependent assessments

No differences in baseline LDF values were observed between the groups (Table 1). The vasodilation mediated by SNP did not vary significantly between groups (Figure 1). There were no significant differences in ACH-mediated vasodilation between the control and T2D groups, or between the SCT and T2D-SCT groups. However, compared to the T2D-SCT group, ACH-mediated vasodilation was 26.8% lower in the control group and 23.9% lower in the T2D group. Inhibition of COX-2 via injection of SC-58125 significantly decreased ACH-mediated vasodilation in the T2D-SCT group by 23.2% (Figure 1).

4. Discussion

This study evaluated endothelium-independent and endothelium-dependent function *in-vivo* in the microvasculature of a mouse model of combined T2D and SCT. The primary finding of this study is that ACH-mediated vasodilation *in-vivo* was amplified in the T2D-SCT mice, whereas SNP-mediated vasodilation *in-vivo* did not vary between the groups. These results indicate that endothelium-dependent, but not endothelium-independent, vasodilation *in-vivo* is altered in the microvasculature of the T2D-SCT mice. This finding is in agreement with previous studies that have also shown impaired endothelium dependent function in humans and a mouse model with combined T2D and SCT (Diaw et al. 2015; Skinner et al. Study 3). Furthermore, our findings demonstrated that acute COX-2 inhibition significantly decreased ACH-mediated vasodilation in the T2D-SCT mice. Therefore, it appears that the COX-2 enzyme could mediate the elevated ACH-induced vasodilation observed in the T2D-SCT

mice. COX-2 is upregulated in inflammatory states, and is a major source of the powerful vasodilating prostaglandins PGE2 and PGI2 (Feletou, Huang, and Vanhoutte 2011). These prostaglandins can compensate for NO as mediators of endothelium-derived relaxation in conditions in which NO is diminished, such as in the vasculature of humans with cardiovascular diseases (Bulut et al. 2003; Szerafin et al. 2006). Additionally, obesity related up-regulation of COX-2 in the coronary arteries has been shown to play a compensatory role in preserving endothelium-dependent vasodilation in obese insulin resistant zucker rats (Sanchez et al. 2010). Our findings are also in agreement with the results of a study published by Nguyen-Tu and colleagues, which observed increased ACH-mediated COX-2 dependent vasodilation *in-vivo* in the cutaneous microvasculature of obese diabetic mice (Nguyen-Tu et al. 2018).

Additional experiments are currently being carried out in order to explore the mechanisms by which COX-2 could mediate the enhanced ACH-mediated vasodilation observed in the T2D-SCT mice.

Table 1: Baseline body weight, change in body weight, GTT AUC, MABP, Basal LDF PIV, Basal LDF SNP, and SNP % vasodilation in Townes AA and AS mice after 12-weeks on a standard or high fat high sucrose (HFHS) diet.				
	Control	SCT	T2D	T2D-SCT
Baseline body weight (g)	29.32 ± 3.23 (n=18)	28.70 ± 3.70 (n=17)	28.88 ± 4.64 (n=15)	27.03 ± 3.48 (n=19)
Change in body weight (g)	4.19 ± 1.46 (n=18)	15.46 ± 5.25***^^^ (n=17)	1.99 ± 2.22 (n=15)	14.43 ± 7.05***^^^ (n=20)
GTT AUC	7687 ± 4475 (n=15)	18046 ± 10077***^^^ (n=17)	9443 ± 4982 (n=18)	23453 ± 13323***^^^ (n=20)
Basal LDF	40.39 ± 4.39	41.28 ± 5.71	36.84 ± 7.20	42.49 ± 8.69

GTT AUC, glucose tolerance test area under the curve; MABP, mean arterial blood pressure; LDF, laser Doppler flowmetry; SNP % vasodilation, percent change in LDF following iontophoretic delivery of sodium nitroprusside.
 * P < 0.05, ** P < 0.01, *** P < 0.001 compared to control. ^ P < 0.05, ^^ P < 0.01, ^^ P < 0.001 compared to SCT

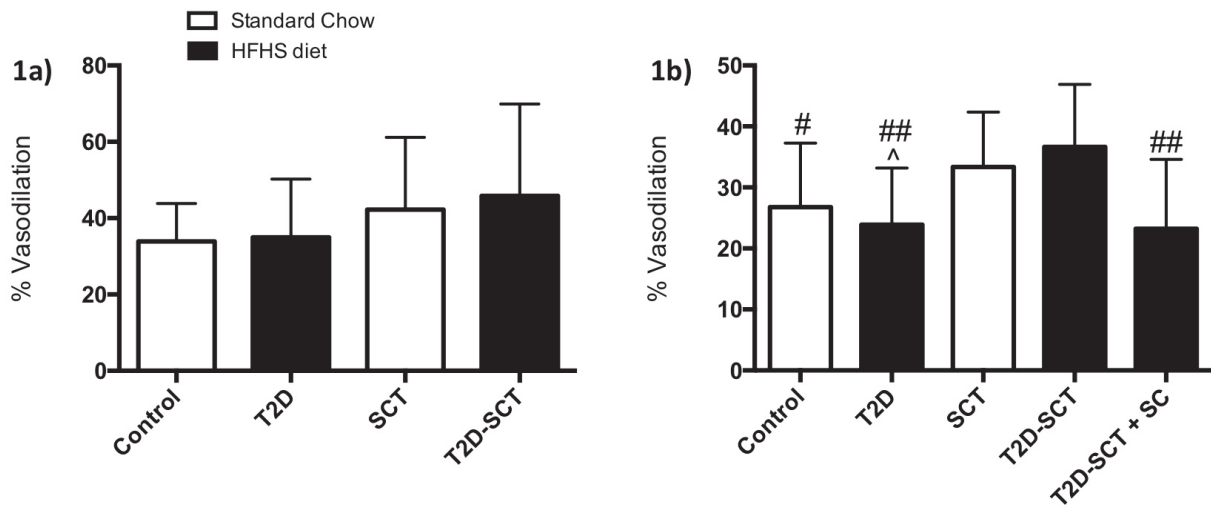


Figure 1a: Percentage of vasodilation in response to iontophoretic delivery of sodium nitroprusside in control, type 2 diabetes (T2D), sickle cell trait (SCT), and combined T2D and SCT (T2D-SCT) mice.

Figure 1b: Percentage of vasodilation in response to iontophoretic delivery of acetylcholine in control, T2D, SCT, and T2D-SCT mice, as well as in mice injected with SC-58125, a specific COX-2 inhibitor (T2D-SCT + SC). ^ P < 0.05 compared to SCT; # P < 0.05, ## P < 0.01 compared to T2D-SCT

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PART 4 – Conclusions and perspectives

General Conclusions

The number of individuals with both T2D and SCT is rapidly rising around the globe. Prior research suggests that the growing population of people with combined T2D and SCT could present a challenge for health care systems and medical professionals for several reasons. First, evidence suggests that HbA1c may perform sub-optimally in individuals with SCT, which could lead to missed diagnoses, and thus an increased risk of developing T2D-related complications in people with SCT (Lacy et al. 2017; Utumatwishima et al. 2018). Furthermore, Diaw et al demonstrated that SCT could exaggerate T2D-related vascular dysfunction, which could potentially increase the risk of developing vascular complications (Diaw et al. 2015). In view of this prior research, the primary objectives of this thesis were to develop a better understanding of how SCT could complicate T2D diagnosis and alter the risk of developing T2D-related vascular complications.

The first part of this thesis consisted of assessing the existing information pertaining to the potential challenges related to diagnosing and monitoring T2D in people with SCT. The findings of this research were summarized in the form of a comment. The comment drew attention to the fact that some HbA1c methods that are classified as not being affected by HbS could still result in a delayed diagnosis of diabetes in individuals with SCT (Lacy et al. 2017). Thus, in order to ensure accurate diagnosis of diabetes in individuals with SCT, HbA1c could be used in combination with classic glucose criteria, such as fasting glucose, and alternative measures of glucose control, like fructosamine (Skinner et al., 2018 *The Lancet Diabetes and Endocrinology*). These conclusions led to the conception of a methodological study designed to compare the relationships and agreement between HbA1c, fructosamine, and fasting glucose in Senegalese adults with and

without SCT. The results of this study showed that although HbA1c, fasting glucose, and fructosamine were positively associated in Senegalese people with and without SCT, there were substantial disparities among all of these biomarkers, and these differences were particularly exaggerated in the SCT group (Skinner et al, *PLOS One In revision*, Study 1). These results indicate that these different measures should not be used interchangeably to assess glycemic control, and that using only one of these measures could lead to imprecise evaluations of glucose control, especially in individuals with SCT. Inaccurate assessments of glucose control could result in delayed diagnoses of T2D, which can increase the risk of developing T2D-related complications. Furthermore, inaccurate assessments of glucose control could provide researchers with erroneous information, resulting in underestimations of the T2D burden in areas where SCT is common. Overall, our findings underscore the need to determine whether combining alternative and classic measures of glycemic control could improve the accuracy of T2D diagnosis, and promote better management of T2D in individuals with SCT.

The second part of this thesis was based on the findings of Diaw et al, which showed that T2D-related vascular dysfunction is exaggerated in individuals with SCT (Diaw et al. 2015). This finding led to the conception of a human cohort study designed to determine whether or not T2D-related vascular complications are more prevalent in individuals with combined T2D and SCT than in people with T2D or SCT alone, or people with neither T2D nor SCT. The secondary objective of this cohort study was to explore the possible mechanisms that could play a role in the pathogenesis of vascular complications in individuals with combined T2D and SCT. The results of this second study illustrated that hypertension, retinopathy, and impaired renal function were more frequent in people with T2D and SCT than in

those with T2D or SCT alone, or neither T2D nor SCT (Skinner et al., *Diabetes Care* 2018 Study 2). Furthermore, the study showed that arterial stiffness was elevated in individuals with combined T2D and SCT compared to people with T2D alone. This finding is in agreement with previous works that have shown that increased arterial stiffness is associated with the development of hypertension, retinopathy and nephropathy in diabetes (Prenner and Chirinos 2015; Stehouwer, Henry, and Ferreira 2008).

Study 2 also measured plasma concentrations of AGEs, which have been previously shown to accelerate arterial stiffening in individuals with T2D (Stehouwer, Henry, and Ferreira 2008). The results showed that plasma concentrations of AGEs were not only higher in the combined T2D and SCT group compared to the other groups, but AGEs were also the most important factor contributing to the elevated arterial stiffness in the T2D-SCT group. Therefore, it is possible that SCT could potentially amplify AGE formation in the arterial wall, thereby resulting in increased arterial stiffness. Furthermore, the results of study 2 also indicated that the interaction of AGEs with RAGEs could result in increased E-selectin expression in combined T2D and SCT. E-selectin is known to play a role in the pathogenesis of vascular disease in T2D, and may therefore play a role in the increased prevalence of hypertension, retinopathy, and impaired renal function observed in the T2D-SCT individuals.

Additionally, the findings of study 2 illustrated that whole blood viscosity was significantly higher in the T2D-SCT group compared to the other groups at high shear rates, indicating that reduced red blood cell deformability could play an important role in the altered whole blood viscosity. These findings confirmed the results of Diaw et al, which also observed elevated blood viscosity at high shear

rates in individuals with both T2D and SCT. Elevated whole blood viscosity has been shown to contribute to the development of hypertension (Merimee 1990), diabetic retinopathy (Gordge et al. 1990), and chronic kidney disease (Baskurt and Meiselman 2003). Therefore, amplified blood hyperviscosity could contribute to the pathogenesis of retinopathy, impaired renal function, and hypertension in individuals with combined T2D and SCT. In addition, in study 2, thixotropic index was increased in the T2D-SCT groups compared to the other groups, indicating that amplified alterations in red blood cell deformability and aggregation likely contribute to the exaggerated blood hyperviscosity in the individuals with combined T2D and SCT.

The findings from Diaw et al and study 2 show that combined T2D and SCT is associated with amplified macrovascular dysfunction and elevated arterial stiffness (Diaw et al. 2015). Additionally, study 2 showed that rates of retinopathy and reduced renal function were higher in people with both T2D and SCT than in those with T2D alone, indicating that microvascular dysfunction is also probably amplified in combined T2D and SCT. However, there was a lack of direct evidence showing that SCT causes more severe microvascular dysfunction in individuals with T2D. For this reason, studies 3 and 4 aimed to study microvascular function in a transgenic mouse model of combined T2D and SCT.

Study 3 (Skinner et al, submitted to *Microvascular Research*) demonstrated that although neither blood rheological parameters nor microvascular function *in-vivo* were significantly altered in mouse models of T2D or SCT, whole blood viscosity and red blood cell rigidity were elevated and vascular function *in-vivo* was impaired in the combined T2D-SCT mice compared to the control mice. Thus, the findings of study 3 confirmed that alterations in blood rheology are exaggerated in a mouse model of combined T2D-SCT. Furthermore, study 3 showed that while there were no

differences in endothelium-independent vasodilation between the different groups of mice, endothelium-dependent vasodilation *in-vivo* was significantly impaired in the microvasculature of mice with combined T2D and SCT compared to the control mice. In this study, endothelium-dependent vasodilation *in-vivo* was measured using PIV, a cutaneous vasodilatory mechanism that is highly dependent on endothelium-derived NO availability. Therefore, these results indicate that reduced NO bioavailability could contribute to impaired endothelial function in the microvasculature of the T2D-SCT mice.

The findings of study 4 (Skinner et al, in progress) confirmed that endothelium-independent vasodilation *in-vivo* is unaltered in mice with combined T2D and SCT. Additionally, the study showed that although acetylcholine-mediated vasodilation *in-vivo* was not significantly altered in the T2D or SCT mice, it was significantly increased in the combined T2D-SCT mice. However, specific inhibition of COX-2 decreased acetylcholine-mediated vasodilation in the T2D-SCT mice. Therefore, the increased acetylcholine-mediated vasodilation in the T2D-SCT mice appears to be COX-2 dependent. COX-2, which is upregulated in inflammation, is a major generator of the vasodilatory prostaglandins PGE2 and PGI2 (Feletou, Huang, and Vanhoutte 2011). These prostaglandins have been shown to play a protective role as compensatory vasodilators when NO bioavailability is diminished in animals and humans (Feletou, Huang, and Vanhoutte 2011; Szerafin et al. 2006). Indeed, the up-regulation of COX-2 has been shown to improve endothelial function in the coronary arteries of obese-insulin resistant mice, and in the mesenteric vascular bed of mice with streptozotocin-induced diabetes (Sanchez et al. 2010; Nacci et al. 2009; Bagi et al. 2005). Considering that the results of study 3 indicated that decreased NO-mediated endothelium-dependent vasodilation was impaired in T2D-SCT mice, it

seems possible that the COX-2 dependent hyper-dilation observed in the T2D-SCT mice could be an adaptive response to counteract diminished NO bioavailability.

In general, the findings of the second part of this thesis support the initial hypothesis that SCT could amplify vascular dysfunction in T2D, and this could lead to an increased risk of developing T2D-related vascular complications. Additionally, the results illuminate possible mechanisms by which SCT could amplify vascular dysfunction, although future research will be required to further clarify these mechanisms. First, study 2 showed that elevated arterial stiffness, blood viscosity, and, most notably, AGEs could possibly contribute to an increased risk of developing two microvascular complications, retinopathy and nephropathy, in individuals with combined T2D and SCT. These findings are in accordance with the results of Diaw et al, which showed amplified macrovascular dysfunction associated with elevated arterial stiffness, blood viscosity, and concentrations of AGEs in subjects with combined T2D and SCT (Diaw et al. 2015). However, no study has been conducted to confirm the presence of microvascular dysfunction in individuals with combined T2D and SCT.

Although microvascular function has yet to be evaluated in individuals with combined T2D and SCT, the results of studies 3 and 4 showed that endothelium-dependent, but not endothelium-independent, vasodilation is impaired in the cutaneous microvasculature of a mouse model of combined T2D and SCT. The results of study 3 suggested that PIV could possibly be impaired in T2D-SCT mice due to reduced NO-bioavailability. The findings of Diaw et al suggest that oxidative stress could possibly be elevated in individuals with combined T2D and SCT, which could consequently decrease NO-bioavailability (Diaw et al. 2015). However, this finding was not replicated in the studies conducted for this thesis.

Additionally, the results of study 4 showed that acetylcholine-mediated vasodilation was exaggerated in T2D-SCT mice via COX-2-dependent mechanisms. A prior study by Shanmugam and colleagues showed that AGEs can amplify inflammatory responses by up-regulating COX-2 via RAGE and several signaling pathways (including NF- κ B) (Shanmugam 2003). AGE concentrations were not measured in the T2D-SCT mice. However, both study 2 and Diaw et al observed elevated levels of AGEs in the individuals with combined T2D and SCT (Diaw et al. 2015). Therefore, it seems plausible that increased AGEs could contribute to altered vascular function in combined T2D and SCT by promoting COX-2 expression.

Furthermore, COX-2 is upregulated by inflammation (Feletou, Huang, and Vanhoutte 2011). However, study 2 showed that the expression of several inflammatory cytokines was not elevated in the T2D-SCT group. On the other hand, the findings of Diaw et al showed that IL-1 β was not only significantly elevated in the T2D-SCT group, but also associated with FMD and PWV (Diaw et al. 2015). Interestingly, a study by Bianchi and colleagues showed that S100B, a ligand of RAGE, upregulates COX-2 expression in circulating monocytes via both IL-1 β -dependent and IL-1 β -independent mechanisms mediated by RAGE (Bianchi 2010). Therefore, it is possible that elevated AGE, and potentially increased IL-1 β , concentrations in combined T2D and SCT could contribute to increased monocyte expression of COX-2.

Acetylcholine can stimulate COX-2 production of vasodilatory prostaglandins in macrophages via the α -7 nicotinic receptor (Pelletier et al. 2012). Therefore, the amplified acetylcholine-mediated vasodilation observed in the cutaneous microvasculature of the T2D-SCT mice in study 4 could be attributed to increased macrophage infiltration into the sub-cutaneous adipose tissue, as suggested in a

previous study by Nguyen-Tu and colleagues (Nguyen-Tu et al. 2018). However the effects of COX-2 on vascular function can be complex. Indeed, COX-2 produces prostaglandins that can result in smooth muscle contraction and relaxation, and certain prostaglandins (like PGE₂) can cause both vasodilation and vasoconstriction depending on the receptor with which they interact (Foudi et al. 2011). Therefore, it is possible that COX-2-mediated production of vasoconstrictor prostaglandins could also play a role in the impaired vascular function observed in the macrovasculature of T2D-SCT individuals in Diaw et al and in the microvasculature of the T2D-SCT mice in study 3; however future studies will need to be conducted to test these hypotheses (Diaw et al. 2015).

Overall the results of this thesis deepen our knowledge of the challenges related to diagnosing and managing T2D in individuals with SCT. They also begin to help us decipher the mechanisms behind the altered vascular function associated with combined T2D and SCT. Ultimately, these findings could lead to further research that will result in improved treatment and care of individuals with combined T2D and SCT.

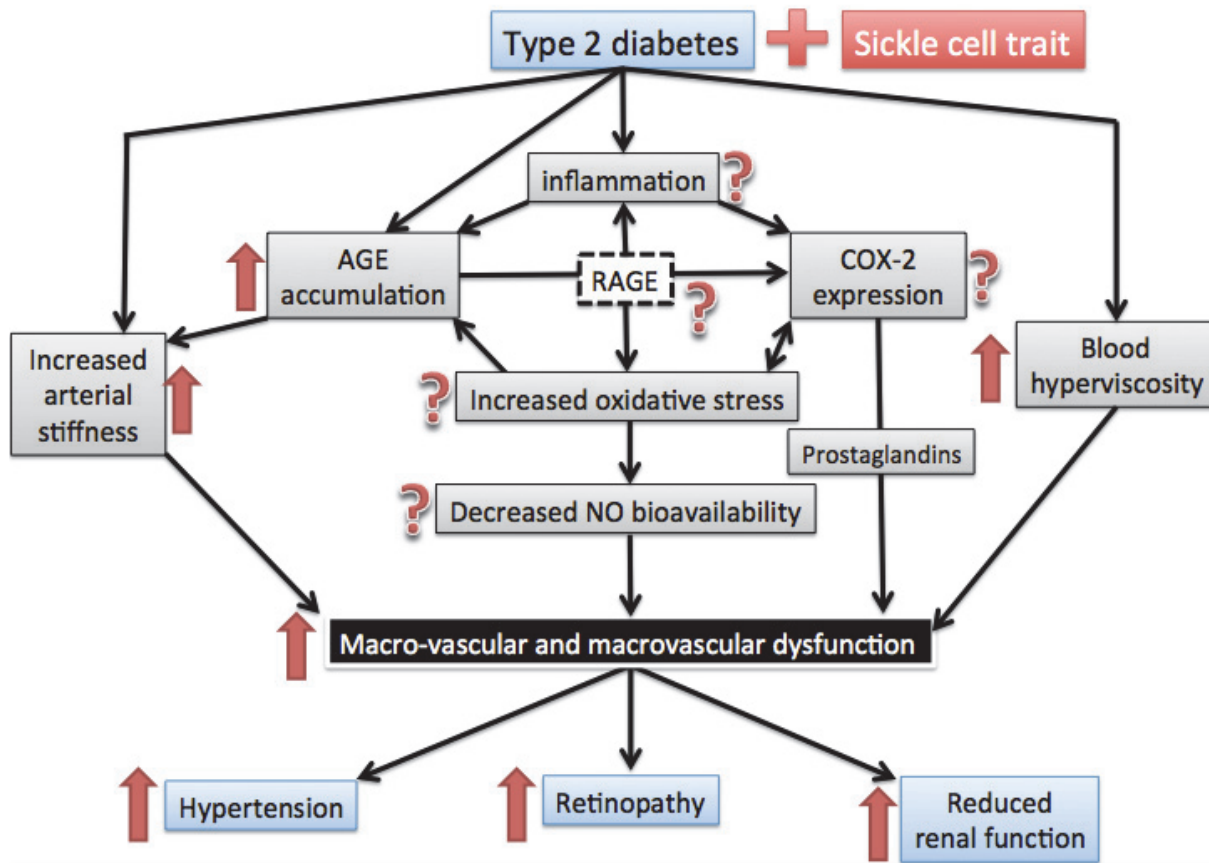


Figure 27: Possible mechanisms contributing to vascular dysfunction and elevated rates of hypertension, retinopathy, and reduced renal function in combined T2D and SCT. Black arrows indicate pathological changes related to T2D. Red arrows indicate an amplifying effect of SCT on these changes. Question marks indicate unknown effects of SCT on a parameter.

Perspectives

The results of study 1 demonstrate an exaggerated lack of agreement between HbA1c, fasting glucose, and fructosamine in Senegalese adults with SCT. However, it remains unknown whether the disparities between these markers could translate to differences in performance as diagnostic tests for T2D. Future studies should be conducted to compare the prognostic sensitivity of each of these markers of glycemic control against the gold standard of diabetes diagnosis, the oral glucose tolerance test, in people with SCT. Furthermore, additional studies should be

conducted to determine whether using multiple glycemic markers could help improve the accuracy of assessments of glycemic control in people with SCT. The ultimate goal of future research should be to determine which measures, or which combination of measures, should ideally be used to prevent delayed diagnosis of T2D and the development of diabetes complications in individuals with SCT. These findings could then be used to develop guidelines for medical professionals.

Evidence from study 2 indicated that plasma concentrations of AGEs were elevated in people with combined T2D and SCT, and could contribute to increased arterial stiffness and the development of vascular complications. Hyperglycemia is one of the main causes of AGE formation (Brownlee 1995). However, the elevated AGE concentrations in the T2D-SCT group cannot be attributed to exaggerated hyperglycemia as no differences in fasting glucose or HbA1c were found between the T2D and T2D-SCT groups. Another factor that can contribute to AGE formation is oxidative stress (Nowotny et al. 2015). Results from Diaw et al have shown that several markers of oxidative stress were elevated in individuals with combined T2D and SCT (Diaw et al. 2015). Therefore, the elevated levels of AGEs in the T2D-SCT group could be attributed to increased levels of oxidative stress. Future studies should be conducted to confirm the link between elevated oxidative stress and increased AGEs in individuals with combined T2D and SCT. If this link is confirmed, both AGE and oxidative stress inhibitors could be considered as possible therapeutic strategies to help prevent the development of vascular complications in individuals with combined T2D and SCT.

Furthermore, physical activity has long been considered an important component of treatment to prevent and manage T2D as it can lower blood glucose, positively affect blood lipids, improve blood pressure, and lower the risk of

cardiovascular events and mortality (Colberg et al. 2010). Indeed, exercise ameliorates vascular health in people with T2D by improving endothelial dysfunction independently of glycemic control, improving insulin sensitivity, increasing anti-inflammatory substances, and decreasing inflammation (De Filippis et al. 2006; Okada et al. 2010). Similarly, in individuals with SCT exercise training has been shown to blunt increased oxidative stress, up-regulate antioxidant enzyme activity and normalize blood viscosity (Chirico et al. 2012). Therefore, future research could be conducted to determine whether regular exercise (and what kind of exercise) could improve vascular function and biological parameters, or even decrease the risk of developing vascular complications in individuals with combined T2D and SCT.

Studies 3 and 4 showed that although endothelium-independent vasodilation was unaltered in the microvasculature of a mouse model of combined T2D and SCT, PIV and acetylcholine-mediated vasodilation were significantly altered. Studies conducted to measure microvascular function in humans with T2D have revealed impaired endothelium-dependent and endothelium-independent vasodilation, measured using acetylcholine and SNP, respectively (Brooks et al. 2008; Morris, Shore, and Tooke 1995; Nitenberg et al. 1993). Furthermore, PIV has been shown to be impaired in humans with diabetes (Fromy et al. 2002). Therefore, it could be pertinent to study whether SCT could amplify the impaired endothelium-dependent, endothelium-independent, and PIV responses in the microvasculature of individuals with combined T2D and SCT.

Study 3 showed that PIV was significantly impaired in mice with combined T2D and SCT compared to control mice. PIV is highly dependent on endothelium-derived NO availability, and is thought to be impaired in diabetes due to increased oxidative stress (Demiot et al. 2006). Furthermore, Demiot and colleagues have shown that

treatment with alpha-lipoic acid (LPA), a potent anti-oxidant, for one week resulted in reduced oxidative stress and a preserved PIV response in streptozotocin-treated diabetic mice (Demiot et al. 2006). However, injection with N^ω-nitro-L-arginine, an acute inhibitor of NOS, abolished the PIV response in the LPA-treated diabetic mice, confirming that reduced oxidative stress could help preserve PIV response by increasing NO bioavailability (Demiot et al. 2006). Overall, these results indicated that increased oxidative stress in the diabetic mice decreased NO bioavailability, which subsequently diminished the PIV response. In light of this evidence, we hypothesized that the diminished PIV response observed in the T2D-SCT mice could be due to reduced NO bioavailability, which could be caused by increased oxidative stress. In order to confirm this hypothesis, a future study could use a protocol similar to the one used by Demiot et al, in which T2D-SCT mice would be treated with an antioxidant, such as LPA, or a placebo (Demiot et al. 2006). Following one week of treatment, levels of oxidative stress would be assayed in the plasma of the mice. Additionally, PIV could be measured in the mice to determine whether decreased oxidative stress would result in an improved PIV response. Finally PIV could be measured following an injection with N^ω-nitro-L-arginine to confirm the role of NO in the PIV response in the T2D-SCT mice.

Study 4 illustrated that acetylcholine-mediated vasodilation was increased *in-vivo* in the microcirculation of mice with combined T2D and SCT via a COX-2 dependent mechanism. A recent study by Nguyen-Tu and colleagues also showed that acetylcholine-mediated vasodilation was increased in obese-insulin resistant mice, and that this hyper-dilation was dependent on COX-2 (Nguyen-Tu et al. 2018). The article suggested that increased adipose tissue in these mice resulted in amplified inflammation and macrophage recruitment (Nguyen-Tu et al. 2018). This

could result in elevated acetylcholine-mediated vasodilation as macrophages release COX-2 derived PGE₂ when stimulated with acetylcholine, via the activation of alpha-7 nicotinic receptors (Pelletier et al. 2012). After considering this evidence, we hypothesized that in T2D-SCT mice, increased macrophage recruitment in adipose tissue surrounding the cutaneous blood vessels could contribute to increased COX-2 dependent acetylcholine-mediated vasodilation. In order to test this hypothesis, we plan to assess COX-2 mRNA and protein expression as well as the mRNA and protein expression of MCP-1, a chemokine that regulates macrophage accumulation, and F4/80, a specific marker of macrophage infiltration, in the adipose tissue of mice with combined T2D and SCT. Furthermore, mouse skin samples could be histologically analyzed to determine whether the expression of MCP-1, F4/80, or inflammatory cytokines, like IL-1, is upregulated in mice with combined T2D and SCT.

Interestingly, in study 4, we did not observe an increase in acetylcholine-mediated vasodilation in the T2D mice (Townes AA mice fed a HFHS diet). This contrasts with the results of Nguyen-Tu and colleagues that demonstrated increased acetylcholine-mediated vasodilation in T2D C57Bl/6J mice fed a HFHS diet for 12 weeks (Nguyen-Tu et al. 2018). However, in the same study, C57Bl/6J mice fed the HFHS diet for only two or four weeks, who were obese but not diabetic, demonstrated a reduced vasodilatory response following iontophoresis of acetylcholine. Thus, the authors suggested that the adaptive microvascular response likely requires a sequential switch from NO-dependent mechanism to COX-2 and prostaglandin-dependent mechanisms (Nguyen-Tu et al. 2018). As inflammation induces COX-2 expression, this switch could be linked to increased inflammation. It is possible that the difference between the T2D and T2D-SCT could be due to

differences in inflammation. Comparing the levels of COX-2, MCP-1, and F4/80 expression in the Townes T2D mice could help give insight into this question.

Finally, AGEs have been shown to amplify inflammation by up-regulating COX-2 expression via RAGE signaling pathways that are dependent on NF- κ B (Shanmugam et al. 2003). IL-1 β has also been shown to mediate COX-2 expression (Bianchi, Giambanco, and Donato 2010). Results from study 2 and Diaw et al indicated that AGE concentrations are elevated and likely play a role in amplified vascular dysfunction in combined T2D and SCT (Diaw et al. 2015). Additionally, study 4 showed that altered acetylcholine-mediated vasodilation in T2D-SCT mice was dependent on COX-2. As prostaglandins produced by COX-2 can mediate both vasoconstriction and vasodilation, it seems pertinent to measure COX-2 expression in individuals with combined T2D and SCT. Additionally, *in-vitro* experiments, could be used to determine whether AGEs and/or IL-1 β could up-regulate COX-2 expression in monocytes of individuals with combined T2D and SCT.

PART 5 – References

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PART 6 – Publications and communications

PUBLICATIONS

2019 **Skinner S**, Diaw M, Mbaye N, Joly P, Renoux C, Masson C, Cuerq C, Lopez P, Ndour M, Diedhiou D, Sow D, Saliou Diop, Samb A, Pialoux V, Connes P. *Evaluation of agreement between hemoglobin A1c, fasting glucose, and fructosamine in Senegalese individuals with and without sickle-cell trait*. PLoS One. In Press. (IF = 2.80)

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- 2018 **Skinner S**, Diaw M, Ndour Mbay M, Ranque B, Philomène Lopez, Malick Ndou, Gueye F, Demba Diedhiou D, Sow D, Diop S, Samb A, Pialoux V, Connes P. *Prevalence of vascular complications in combined sickle-cell trait and type 2 diabetes*. (Communication orale). *Joint Conference of Three Societies: ESCHM-ISB-ISCH 2018. Cracovie, Pologne*. 2-6 juillet 2018

- 2017 **Skinner S**, Pialoux V, Lo M, Liu K, Fromy B, Nader E, Faes C, Martin C, Connes P, Sigaudou-Roussel D. *Mechanisms of altered vascular function in a mouse model of combined type 2 diabetes and sickle cell trait*. (Poster). *Journée scientifique de la SFR Santé Lyon Est. Lyon, France*. 5 décembre 2017

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- 2015 **Skinner S**, Choi S, Chipkin SK, Braun B, Malin SK. *Variations in Dietary Fat Associate with Insulin Sensitivity and Appetite Following Exercise in IGT*

Adults. (Communication orale)

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Résumé: Le taux du diabète de type 2 (DT2) est en augmentation partout dans le monde, y compris dans les régions du monde où le trait drépanocytaire (TD) est très prévalent. Le TD, la forme hétérozygote de la drépanocytose, est généralement considéré comme bénin. Cependant, une dysfonction vasculaire plus importante a été récemment observée chez les patients DT2 porteurs du TD (DT2-TD) par rapport à des patients DT2 non-porteurs du TD. En outre, certaines études démontrent que le TD pourrait rendre le dépistage du DT2 plus complexe. De ce fait, sur la base des travaux précédents, les deux objectifs principaux de ma thèse étaient d'étudier les difficultés liées au dépistage et le suivi du DT2 chez les porteurs du TD, et d'évaluer les mécanismes et conséquences de la dysfonction vasculaire amplifiée chez les porteurs du TD. La 1^{ère} étude a comparé deux mesures typiques (l'HbA1c et la glycémie à jeun) et une mesure atypique (fructosamine) de la glycémie chez des adultes sénégalais avec et sans le TD. Cette étude a démontré une disparité entre ces mesures qui étaient plus marquées chez des porteurs du TD. Dans la 2^{ème} étude, nous avons observé que les prévalences d'hypertension, de rétinopathie, et de néphropathie étaient plus élevées chez les sujets DT2-TD que chez les sujets diabétiques. La dysfonction vasculaire à l'origine de ces complications plus fréquentes semble principalement impliquer les produits de glycation avancés (AGE). Les études 3 et 4 étaient réalisées dans un modèle murin du DT2-TD. L'étude 3 a montré une vasodilatation endothélium-dépendante significativement réduite chez des souris DT2-TD. Enfin, l'étude 4 a montré que la vasodilatation *in-vivo* induite par acétylcholine était augmentée chez la souris DT2-TD via un mécanisme dépendant de la cyclooxygénase-2. Ce travail de thèse a permis de mieux comprendre les difficultés liées au dépistage du DT2 chez les porteurs du TD, de montrer que le TD est un facteur de risque de complications vasculaire dans le DT2 et d'explorer les mécanismes à l'origine de cette dysfonction vasculaire plus marquée. **Mots clés :** Trait drépanocytaire, diabète de type 2, fonction vasculaire, complications vasculaires, HbA1c

Mechanisms and consequences of alterations in vascular function in combined type 2 diabetes and sickle cell trait

Abstract: Rates of type 2 diabetes (T2D) are rapidly increasing worldwide, including in regions, and among populations, of the globe where sickle cell trait (SCT) is prevalent. SCT, the heterozygote form of sickle cell disease, is generally considered a benign condition. However, evidence shows that vascular function is more severely impaired in people with combined T2D and SCT (T2D-SCT) than in those with T2D only. Furthermore, evidence suggests that SCT could complicate screening for T2D, thereby increasing the risk of delayed diagnosis of T2D. In light of this information, the main objectives of this thesis were to study the challenges related to diagnosing and monitoring T2D in individuals with SCT, and to evaluate the mechanisms and consequences of the amplified vascular dysfunction observed in T2D-SCT. Study 1 compared the agreement between two standard measures of glycemia, HbA1c and fasting glucose, and one alternative measure of glycemia, fructosamine, in Senegalese adults with and without SCT. The findings revealed substantial disparities between the markers of glycemia, and these differences were exaggerated in individuals with SCT. Study 2 illustrated that SCT could potentially augment the risk of developing retinopathy, nephropathy, and hypertension in T2D, and demonstrated that AGEs are likely implicated in the vascular dysfunction observed in T2D-SCT. Studies 3 and 4 studied microvascular function in a mouse model of T2D-SCT. Study 3 showed that T2D-SCT mice had significantly impaired endothelium-dependent vasodilation *in-vivo*. Study 4 revealed that ACH-mediated vasodilation *in-vivo* was significantly elevated in the microvasculature of mice with combined T2D and SCT due to cyclooxygenase-2 dependent mechanisms. Overall these findings deepen our understanding about the complexities related to diagnosing and managing T2D in individuals with SCT. Future research should be done to develop reliable guidelines for accurate diabetes diagnosis and prevention of vascular complications in people with T2D-SCT. **Key words:** Sickle cell trait, type 2 diabetes, vascular function, vascular complications, HbA1c