

The impairment of vascular reactivity across the spectrum of cardiometabolic health

by

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Bachelor of Exercise Science (Honours)

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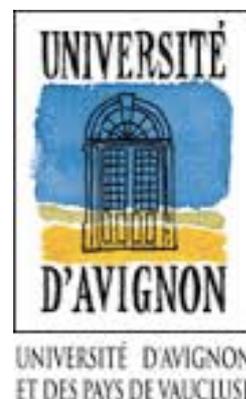
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Statement of authorship and sources

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No parts of this thesis have been submitted towards the awards of any other degree or diploma in any other tertiary institution, except for those pertaining to the experimental procedures and data of Chapter 4 (Study 2) and Chapter 8.2 (Study 2: Extended methodology); a single study that is comprised of both clinical research that I completed as leading author and experimental research, which was completed in a collaboration with another PhD candidate at an international institution and submitted towards her Doctorate.

No other person's work has been used without due acknowledgment in the main text of the thesis.

All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees (where required).

Jordan Luke Loader

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List of publications related to this thesis

1. **Loader J**, Montero D, Lorenzen C, Watts R, Meziat C, Reboul C, Stewart S, Walther G. Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: systematic review and meta-analysis. *Arter Thromb Vasc Biol.* 2015;35(9):2060-72. Doi: 10.1161/ATVBAHA.115.305530
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1. Meziat C, **Loader J**, Reboul C, Walther G. Acute hyperglycemia impairs flow-mediated dilation through an increase in vascular oxidative stress: winter is coming for excess sugar consumption. *J Thorac Dis.* 2016;8(9):E1103-E1105. Doi: 10.21037/jtd.2016.07.99.
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1. **Loader J**, Roustit M, Baltzis D, Veves A. Vascular dysfunction, inflammation and exercise in diabetes. In: Reusch J, Regensteiner J, Stewart K, Veves A. Diabetes and Exercise. Contemporary Diabetes. Human Press, Cham. 2018:137-150.
2. Roustit M, **Loader J**, Baltzis D, Zhao W, Veves A. Microvascular changes in the diabetic foot. In: Contemporary Diabetes, The Diabetic Foot: Medical and Surgical Management, Fourth Edition. Springer; accepted for publication.

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Abstract

Cardiovascular disease (CVD) remains the single leading cause of mortality and is strongly associated with obesity and cardiometabolic disease (inclusive of metabolic syndrome [MetS], impaired glucose tolerance and type 2 diabetes [T2D]). Impairments in vascular reactivity, stemming from such endothelial dysfunction, as well as possible disruptions to endothelium-independent activity and maladaptation to the vascular smooth muscle, increase susceptibility to endothelial injury and, thus, promote atherosclerotic change. Several large cross-sectional studies provide evidence that chronic impairments in vascular reactivity may present early in the decline of cardiometabolic health, such as in those considered overweight. However, whether there is a pathophysiological continuum in the impairment of vascular reactivity between early stages and diabetic complications has never been addressed. Excess sugar consumption is one of the main dietary factors driving the increased prevalence of obesity, insulin resistance and CVD, but the overall effect of acute hyperglycemia on vascular reactivity is currently unclear due to discrepant findings. Considering this, the primary aim of this research program was to examine the extent and nature of vascular impairment across the spectrum of cardiometabolic health; with a focus on the mediating effects of excess sugar consumption and the validity and accuracy of current methods used to quantify vascular reactivity in affected individuals.

Study one evaluated the effect of acute hyperglycemia on vascular reactivity through a systematic review and meta-analysis; presenting evidence that excess sugar consumption may transiently impair endothelium-dependent vascular reactivity in the macrocirculation of those considered healthy or obese and of those with cardiometabolic disease (30 studies; $n = 884$; standardized mean difference [SMD], -1.40 ; 95% CI, -1.68 to -1.12 ; $P < 0.01$); while microvascular endothelium-dependent (9 studies; $n = 181$; SMD, -0.63 ; 95% CI, -1.36 to 0.11 ; $P = 0.09$) and endothelium-independent vascular reactivity (6 studies; $n = 144$; SMD, -0.07 ; 95% CI, -0.30 to 0.16 ; $P = 0.55$) remain unaffected. However, these findings were ultimately inconclusive due to inadequate reporting of macrovascular data in nearly all studies included in the analyses and considerable heterogeneity in microvascular endothelial data; and due to a limited availability of endothelium-independent data.

Considering this, study two aimed to clarify the effect of acute hyperglycemia on vascular function, by assessing the impact of sugar-sweetened beverage (SSB) consumption, one of the most prominent sources of added sugar in the human diet. Compared to water consumption, a single 600 mL SSB impaired microvascular and macrovascular endothelial function in a healthy population, as indicated by a decrease in the vascular response to acetylcholine iontophoresis (208.3 ± 24.3 vs. 144.2 ± 15.7 %, $P < 0.01$) and by reduced flow-mediated dilation (0.019 ± 0.002 vs. 0.014 ± 0.002 %/ s^{-1} , $P < 0.01$), respectively. Similar to findings in study one, endothelium-independent vascular reactivity remained unaffected. Experimental models suggested that this SSB-mediated endothelial dysfunction is partly due to an acute hyperglycemic-mediated increase in oxidative stress that reduces the bioavailability of nitric oxide, a key vasodilating agent.

Combined, study one and two demonstrated that excess sugar consumption transiently impairs endothelial function in those considered healthy or obese and in those with cardiometabolic diseases; and, subsequently, that it may have a significant role in the initial and ongoing development of chronic vascular dysfunction, obesity, MetS, T2D and CVD. Despite this, whether there is a pathophysiological continuum in vascular dysfunction between early stages and diabetic complications was still unclear. Therefore, study three assessed, in a systematic review and network meta-analysis, the extent and nature of impairment in vascular reactivity across the spectrum of cardiometabolic health (i.e. overweight, obesity, impaired glucose tolerance, MetS, T2D and T2D with complications). One hundred and ninety three articles (7226 healthy subjects and 19,344 overweight or obese patients, or those with cardiometabolic diseases) were analysed, revealing a pathophysiological continuum where there is progressive impairment in endothelium-dependent and endothelium-independent vascular reactivity throughout the pathogenesis of T2D and its complications. Interestingly, meta-regressions revealed that for every 1 mmol/l increase in fasting blood glucose concentration, flow-mediated dilation decreased by 0.52 %.

One of the most commonly used techniques to assess microvascular reactivity is iontophoresis of a vasoactive agent; a methodology that, within itself, presents a range of varying protocols for researchers to choose from, with no standardization of the

protocols or data expression. In addition to a lack of standardization amongst the variety of techniques used to assess microvascular reactivity, the considerable heterogeneity, observed in the microvascular data from studies one and three, may have also been influenced by non-specific vasodilatory effects that can be induced by the iontophoresis technique. Indeed, there is no consensus as to which protocols of iontophoresis are free of confounding, non-specific vasodilatory effects. Considering this, study four evaluated commonly used protocols of iontophoresis finding that seven out of the ten published methods assessed in this present study induce non-specific vasodilatory effects that may confound vascular data. Providing updated methodological recommendations, this study found that iontophoresis of acetylcholine or sodium nitroprusside in sodium chloride (0.02 mA for 200 and 400 s, respectively) and acetylcholine in deionized water (0.1 mA for 30 s) mediate the least non-specific vasodilatory effects when iontophoresis is coupled with laser speckle contrast imaging; while the cutaneous microvascular responses to each tested insulin protocol were mediated mainly by non-specific effects.

In conclusion, this program of research demonstrates that there is a pathophysiological continuum in vascular dysfunction; where endothelium-dependent and endothelium-independent vascular reactivity are progressively impaired throughout the pathogenesis of T2D and its complications. Additionally, this research confirmed that excess sugar consumption induces transient impairments in endothelial function, which may contribute to the development of chronic vascular dysfunction, obesity, MetS, T2D and CVD. Further research is needed to assess if transient impairments in vascular function, mediated by several dietary and lifestyles factors, develop into chronic impairments, when the individual is still considered clinically healthy, or after the clinical onset of an overweight state. However, several issues within the current methodology for assessing vascular reactivity should be addressed first. Regardless, this research continues to inform public health policy that transient and chronic impairments of vascular function, which are representative of critical events in the pathogenesis of CVD and are associated with CVD mortality, occur early, long before the clinical onset of obesity, MetS and T2D. Furthermore, from a clinical perspective, the findings of this research program importantly provide further understanding of the development of vascular dysfunction, which may enhance the timing and, subsequent, effectiveness of treatment strategies that aim to improve vascular health and CVD outcomes.

Abstract (Français)

Les maladies cardiovasculaires est la première cause de mortalité dans le monde et fortement associées à l'obésité et aux pathologies métaboliques (incluant l'intolérance au glucose, le syndrome métaboliques [SMET] et le diabète de type 2 [DT2]). La dysfonction vasculaire, au-delà de son caractère essentiel dans l'homéostasie vasculaire, est également largement reconnue comme un précurseur et un marqueur précoce de l'athérosclérose et des pathologies cardiovasculaires. Cette dysfonction vasculaire est fortement influencée par différents facteurs de risque tels que l'hyperglycémie, l'obésité, l'insulino-résistance ou encore l'inflammation, mais peut aussi initier le développement de l'obésité et surtout de l'insulino-résistance. Cependant, il n'est pas clairement démontré comment la dysfonction vasculaire évolue le long du continuum de la maladie cardiometabolique depuis un excès de poids, initiateur de l'obésité, jusqu'à la pathologie DT2 présentant des complications. Le développement de la dysfonction vasculaire chronique pourrait débuter notamment par des altérations transitoires de la réactivité endothéliale. La consommation importante de sucre, le facteur nutritionnel le plus largement associé avec l'obésité, les pathologies cardiometaboliques ou cardiovasculaires, pourrait être un médiateur majeur de la dysfonction vasculaire transitoire. Néanmoins, les effets de l'hyperglycémie aiguë sur le continuum de la dysfonction vasculaire restent à ce jour peu décrits. Dans ce contexte, un premier objectif de ce travail a été de déterminer le degré et la nature de la dysfonction vasculaire au cours du continuum de la pathologie cardiometabolique entre une situation physiologique chez des sujets sains, et des situations pathologiques au cours du développement de l'obésité, de l'insulino-résistance, du syndrome métabolique jusqu'au DT2 sans et avec des complications associées.

La première étude basée sur une revue systématique et une méta-analyse a donc évalué les effets d'une hyperglycémie aiguë sur la fonction vasculaire. Ce travail a confirmé qu'un excès de consommation de sucre pouvait de manière transitoire altérer la fonction endothéliale de la macrocirculation (30 études; $n=884$; standardized mean difference [SMD], -1.40; 95% IC, -1.68 à -1.12; $P<0.01$), sans modifications de la fonction endothéliale au niveau microcirculatoire (9 études; $n = 181$; SMD, -0.63; 95% IC, -1.36 to 0.11; $P=0.09$) de la réactivité du muscle lisse vasculaire (6 études; $n = 144$; SMD, -0.07; 95% IC, -0.30 to 0.16; $P=0.55$). Néanmoins, ces résultats étaient à prendre avec

précaution puisque les données macro- et micro-vasculaires présentaient une hétérogénéité importante et un nombre d'études limité, notamment chez le sujet sain.

Ainsi, notre second travail s'est intéressé à l'aide d'une approche translationnelle chez l'homme et l'animal à mieux comprendre les effets d'une hyperglycémie aiguë, induite par une consommation de boisson sucrée, sur la fonction vasculaire macro- et microcirculatoire. En comparaison avec une consommation d'eau, une boisson sucrée de 600 mL chez des sujets jeunes et sains a provoqué une altération de la fonction vasculaire au niveau des vaisseaux de conduits (baisse de la réactivité flux-dépendante, 0.019 ± 0.002 vs. 0.014 ± 0.002 %/s⁻¹, $P < 0.01$) et de la microcirculation (baisse de la réactivité de la microcirculation cutanée à une iontophorèse d'acétylcholine, $(208.3 \pm 24.3$ vs. 144.2 ± 15.7 %, $P < 0.01$). La réactivité du muscle lisse, quels que soient les territoires vasculaires considérés, n'a pas été modifiée, confirmant ainsi l'origine endothéliale des altérations observées. L'étude sur un modèle de rongeur a par ailleurs démontré que cette baisse de fonction vasculaire en réponse à cette consommation aiguë de boisson sucrée était liée à une augmentation du stress oxydant vasculaire et une diminution de la biodisponibilité du monoxyde d'azote, un agent vasodilatateur majeur du système circulatoire. Ainsi, les deux premières études ont démontré que les phénomènes hyperglycémiques aigus, induits par une consommation excessive de sucre, pouvaient de manière transitoire altérer la fonction endothéliale chez les patients atteints de pathologies cardiométaboliques, mais également chez les sujets sains. Ces premiers résultats ont également renforcé l'hypothèse que ces mécanismes pouvaient être à l'origine du développement d'une dysfonction vasculaire chronique chez les patients obèses, syndrome métabolique et DT2.

Ainsi, la troisième étude a cherché à évaluer par une revue systématique associée à une méta-analyse, comment la fonction vasculaire évolue le long de ce continuum de pathologies cardiométaboliques. 193 études ont été analysées pour inclure 7226 sujets sains et 19344 patients présentant une pathologie cardiométabolique. Une méta-analyse par réseau a montré, pour la première fois dans la littérature, une dysfonction vasculaire endothéliale progressive au cours du développement physiopathologique menant au DT2 avec complications. Les analyses post-hoc ont démontré également une évolution identique de la réactivité du muscle lisse, et ces résultats étaient retrouvés dans tous les territoires vasculaires. Néanmoins, il est apparu qu'une des techniques couramment

utilisée dans la littérature pour explorer la fonction vasculaire microcirculatoire présentait des méthodologies d'application très variables (protocoles non standardisés, ni d'expression homogène des données. De plus, cette technique qui associe une mesure de la perfusion microcirculatoire cutanée par Laser Doppler ou Laser speckle contrast imaging avec une iontophorèse de drogues vasoactives peut être influencée par des effets de vasodilatation non-spécifiques et liés au courant ou au protocole utilisé.

Ainsi, notre quatrième étude a consisté à comparer les différents protocoles existant dans la littérature scientifique afin de mieux discriminer les effets propres des drogues vasoactives sur la microcirculation des effets non spécifiques, majoritairement courant-dépendants. Ce travail a permis de proposer une méthodologie reproductible et sans effets « courant » sur la microcirculation cutanée évaluée par le Laser speckle contrast imaging pour les agents vasoactifs endothélium dépendant (acétylcholine dans de l'eau désionisée, 0,02 mA pendant 200 secondes) et indépendant (nitroprussiate de sodium dans de l'eau désionisée, 0,02 mA pendant 400 secondes). L'insuline ayant des effets spécifiques sur la microcirculation, il nous a semblé également important de valider une méthodologie valide et reproductible. Parmi les deux protocoles existant dans la littérature, aucun n'a pu démontré d'effets spécifiques à l'insuline, ne permettant ainsi aucune recommandation quant à l'utilisation de cette approche pour mesurer les effets vasculaires de l'insuline.

En conclusion, l'ensemble de ces études ont démontré une atteinte progressive de la fonction vasculaire endothéliale et musculaire lisse au niveau des lits macro- et microcirculatoires le long du continuum de la maladie cardiométabolique menant les patients d'un stade de surpoids corporelle à un diabète de type 2 avec complications macro- et micro-angiopathiques. Ces travaux montrent également, que même une seule boisson sucrée provoquant une hyperglycémie aiguë, était capable chez des sujets jeunes et en bonne santé d'altérer de manière transitoire la fonction vasculaire endothéliale, confirmant ainsi le caractère très précoce de la dysfonction vasculaire de la pathologie métabolique. Néanmoins d'autres études sont nécessaires pour mieux comprendre comment un stress aigu, telle que l'hyperglycémie aiguë, peut provoquer de manière répétée l'installation d'une dysfonction vasculaire chronique. Il est également nécessaire de mieux appréhender les mécanismes sous-jacents explicatifs de ces altérations en alliant des approches cliniques et expérimentales afin de proposer des

stratégies de prévention adéquates pour améliorer la santé vasculaire dès le début de l'installation de la pathologie cardiométabolique. Par conséquent, ces travaux permettent de contribuer à un message de santé publique pour mieux appréhender le risque cardiovasculaire et ainsi prévenir la morbi-mortalité cardiovasculaire.

List of abbreviations and nomenclature

AGEs	Advanced glycation end products
ANOVA	Analysis of variance
CAMs	Cellular adhesion molecules
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
COX	Cyclooxygenase
CRP	C-reactive protein
CVC	Cutaneous vascular conductance
CVD	Cardiovascular disease
CYP	Cytochrome P450
eNOS	Endothelial nitric oxide synthase
eNOS ^{ser1177}	eNOS and its activation by phosphorylation at serine 1177
ET-1	Endothelin-1
EETs	Epoxyeicosatrienoic acids
EDHF	Endothelium-derived hyperpolarizing factors
DNA	Deoxyribonucleic acid
FMD	Flow-mediated dilation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GRADE	Grading of Recommendations Assessment, Development & Evaluation
HDL	High-density lipoprotein
LDF	Laser Doppler flowmetry
LDI	Laser Doppler imaging
LDL	Low-density lipoprotein
LSCI	Laser speckle contrast imaging
ICAM-1	Intracellular adhesion molecule-1
MetS	Metabolic syndrome
NAD ⁺	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
NMD	Nitrate-mediated dilation

NO	Nitric oxide
PARP	Poly (adenosine diphosphate ribose) polymerases
PGI ₂	Prostacyclin
PKC	Protein kinase C
PORH	Post-occlusive reactive hyperemia
PRISMA	Preferred Reporting Items for Systematic Review & Meta-Analysis
PU	Perfusion units
ROS	Reactive oxygen species
SAQOR	Systematic Appraisal for Observational Research
SMD	Standardized mean difference
SSB	Sugar-sweetened beverages
TcPO ₂	Transcutaneous oxygen tension
TNF- α	Tumor necrosis factor- α
T2D	Type 2 diabetes
VCAM	Vascular cellular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle

Chapter 1 – Introduction and overview

The increasing prevalence of obesity and cardiometabolic disease (inclusive of metabolic syndrome [MetS], impaired glucose tolerance and type 2 diabetes [T2D]) represents one of the greatest public health issues of the modern era. While high incidences of obesity and cardiometabolic disease have traditionally affected those in developed regions such as North America and Western Europe, these epidemics have now become truly global with cases increasing at an alarming rate across developing countries.¹⁻³ Recent statistics from the World Health Organization clearly demonstrate this trend, indicating that while approximately 39 % of the global adult population (aged 18 years or more) are currently overweight, obesity rates have more than doubled in the past three decades and now affect about 13 % of the world's adult population.⁴ Moreover, while the prevalence of obesity is, on occasion, exceeding 50 % amongst adults in developing nations, overweight and obesity rates in children from developing countries have also increased from 8.1 % to 12.9 % in boys and 8.4 % to 13.4 % in girls.⁵ At the same time, the global incidence of diabetes has nearly quadrupled in recent decades, increasing from 108 million people worldwide in 1980 to 422 million in 2014,³ with approximately 90-95 % of that population being accounted for by those with T2D.⁶ Indeed, while metabolic issues such as insulin resistance may also effect those with clinically normal weight, obesity is one of the primary factors driving this prevalence of T2D.⁷ Once defined as 'adult onset diabetes', a growing number of T2D cases are now also being observed in children and adolescents,⁸ further potentiating a situation where a majority of the world's population will face complications and/or premature death stemming from poor cardiometabolic health.

The urgency of this public health crisis is well recognised and authorities have set global targets to halt the rise in obesity and T2D at their 2010 levels by the year 2025.^{2,3} However, if post-2000 trends continue, the likelihood of achieving these targets is virtually zero for obesity and approximately 1 % for T2D.^{2,3} Subsequently, the burden of obesity and T2D is only projected to worsen.⁹ Although this trend is driven by a complex interaction of numerous lifestyle and socioeconomic factors, it is now well accepted that habitual overconsumption of sugar-sweetened beverages (SSB) is the dietary factor that is most consistently found to be associated with an increased

incidence of obesity, MetS and T2D.¹ From the mid-1960's, the prevalence of obesity and cardiometabolic disease has closely paralleled the steady increase in per capita calories from SSB consumption in both adults and children of developed nations; a trend that has been markedly more rapid in developing nations over the past few decades as traditional diets become more westernized.¹⁰⁻¹² Overconsumption of SSB has also been directly linked with an increased risk of developing cardiovascular disease (CVD),¹⁰ which remains the single leading cause of mortality, accounting for 70 % of all deaths amongst those with T2D and 31 % of the overall global mortality rate each year.¹³⁻¹⁵ It is now well accepted that endothelial dysfunction may be considered one of the earliest discernible, pathophysiological, precursors to the pathogenesis of CVD.¹⁶ Indeed, impaired vascular reactivity may also contribute to the development of obesity and insulin resistance; in what may, indeed, be considered a vicious cycle where each compounds the other.¹⁷

In normal vascular function, the endothelium and perivascular adipose tissue continuously interact with the vascular smooth muscle (VSM) cells, regulating the diameter of the blood vessel under the influence of several vasodilating (nitric oxide [NO], prostacyclin [PGI₂] and endothelium-derived hyperpolarizing factors [EDHF]) and vasoconstricting (endothelin-1 [ET-1], angiotensin II, prostanoids and isoprostanes) substances;^{18,19} ultimately, to maintain optimal vascular tone and organ perfusion.^{20,21} However, during vascular dysfunction, the reactivity of the blood vessel is impaired, reducing its capacity to vasodilate when challenged (e.g. when blood flow shear stress, exerted on the arterial wall, increases).¹⁴ Subsequent increases in overall vascular tone, in conjunction with additional hematologic events (e.g. abnormal increases in viscosity of the blood),²² may heighten the risk of injury to the arterial walls.²³ Vascular dysfunction may also be accompanied by increases in the expression of plasma and urinary biomarkers that are representative of general endothelial dysfunction and inflammation.²⁰ These inflammatory responses, which compound the primary vascular dysfunction, are key indicators of atherosclerotic changes and are characterized by lesion formation, plaque rupture, and thrombosis; all of which represent significant events in the pathogenesis of CVD.^{24,25} Importantly, it must be recognised that the adverse impact of vascular dysfunction is not only limited to the coronary circulation. Indeed, similarly to that in those with type 1 diabetes, vascular dysfunction also plays a major role in the development of other complications that are

associated with T2D, such as diabetic nephropathy, retinopathy, peripheral neuropathy and diabetic foot ulceration.²⁶⁻²⁹

Many studies have compared vascular reactivity between health groups providing strong evidence that chronic impairments in vascular reactivity may present early, even in those considered overweight and metabolically healthy;^{30,31} a health condition that is often considered less serious than obesity, MetS or T2D. However, even if much of the research has shown differences, cross-sectional studies may often lack the power to accurately estimate the effect size of vascular impairment between health groups. Moreover, whether there is a pathophysiological continuum in the impairment of vascular reactivity between early stages of obesity and diabetic complications has never been addressed. Furthermore, it has also been suggested that acute hyperglycemia induced by the excess consumption of added sugar, such as that in the form of SSB, may transiently impair vascular reactivity in humans; contributing to the initial and ongoing development of chronic vascular dysfunction. Hyperglycemia-mediated vascular dysfunction, whether induced by diet or by dysglycemia in those with poor cardiometabolic health, may be explained by increases in oxidative stress that reduce the bioavailability of the vasodilator, NO.^{24,32} Ultimately, though, the effect of acute hyperglycemia on vascular reactivity and the mechanisms that may underlie any dysfunction remain unclear due to discrepant results in previous research and a lack of data, respectively.

It is important from a clinical perspective that vascular impairments are fully understood to enhance the timing and, subsequent, effectiveness of treatment strategies that aim to improve vascular health; and to ascertain which clinical measures of vascular reactivity may best detect primary markers of vascular impairment, long before the development of chronic dysfunctions and significant detrimental cardiovascular events. Considering this, the primary aim of this research program is to examine the extent and nature of vascular impairment across the spectrum of cardiometabolic health (i.e. health classifications including overweight, obese, MetS, impaired glucose tolerance, T2D and T2D with complications); with a focus on the mediating effects of SSB consumption and the validity and accuracy of current methods used to quantify vascular reactivity in affected individuals.

Chapter 2 – Literature review

Publication statement:

The content of this chapter is comprised of two book chapters that were completed during the course of my PhD program in collaboration with the laboratory of Professor Aristidis Veves (Microcirculation Laboratory and Rongxiang Xu, MD, Center for Regenerative Therapeutics, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA). The content has been modified to ensure that it meets the flow and formatting of this thesis.

Loader J, Roustit M, Baltzis D, Veves A. Vascular dysfunction, inflammation and exercise in diabetes. In: Reusch J, Regensteiner J, Stewart K, Veves A. Diabetes and Exercise. Contemporary Diabetes. Human Press, Cham. 2018:137-150.

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2.1 Normal structure and physiology of the vascular network

Early research into the role of vascular dysfunction in CVD often assessed vascular reactivity directly in the region of interest (e.g. the coronary circulation). Common techniques included intracoronary infusion of a vasoactive substance (e.g. acetylcholine, nitroprusside etc.), performed in conjunction with a coronary angiography or intravascular ultrasound to measure coronary macrovascular reactivity; and with Doppler wires or cineangiographic frame counts to assess coronary microvascular reactivity.³³ Such tools measure changes in vessel diameter or coronary blood flow in response to the aforementioned pharmacological stimuli or even exercise, which induces shear stress; with decreased responses indicating impaired vascular reactivity.³³ However, these early methods are considered highly invasive and technically challenging; and are not an option in patients who do not require an angiography. More recently, vascular research has consistently used non-invasive surrogate markers of coronary vascular function, assessing peripheral vascular reactivity in conduit arteries and in the microcirculation. Indeed, measures of peripheral vascular reactivity may also provide important information about the decline in vascular health, directly in the region of interest, in those with T2D and complications such as diabetic neuropathy or foot ulceration. Considering this as a whole, the following review will focus primarily on aspects of the peripheral circulation.

2.1.1 Anatomy of the vascular network

The vascular network is typically categorized by size and defined as the macrocirculation, which includes both conduit arteries (>1000 μm) and small arteries (300 – 1000 μm), or the microcirculation, consisting of smaller arteries, arterioles and venules (10 – 300 μm), and capillaries (\approx 6 μm).³⁴ In the cutaneous microcirculation, these small arteries, arterioles and venules collectively form two horizontal plexuses in the dermis.³⁵ The upper plexus in the papillary dermis, from which nutritive capillary loops arise, is connected by ascending arterioles and descending venules to a lower network located at the dermal-hypodermal interface.³⁵ Arterio-venous anastomoses provide direct connections between the arterial and venous networks.³⁵ Each blood vessel has three distinct layers defined as the outer tunica adventitia, the central tunica media and the inner tunica intima.³⁶ The adventitia's proportion of the vascular wall is

variable, dependent on the vascular bed and is comprised of elastin, collagen, fibroblasts, mast cells, and macrophages.³⁶ Compared to other tissues, the adventitia of blood vessels within the cutaneous microcirculation also present a high density of sensory, sympathetic and parasympathetic nerve axons that do not penetrate the media.³⁷ However, these nerve fibres do pass close to the media, which is comprised predominantly of VSM cells, demonstrating the major influence of autonomous neural control in cutaneous microvascular function.³⁸

As blood vessels decrease in diameter, the proportion of the vessel wall occupied by VSM cells remains similar due to a decrease in the number of VSM cell layers to as little as one layer in the arterioles.³⁶ However, the actual volume fraction of VSM cells within the media typically increases to 70-85% of the total media volume demonstrating an increased ability of microcirculatory blood vessels to control vessel diameter.³⁶ Deep to the media and an internal elastic lamina is the intima, which consists of a layer of endothelial cells that forms a continuous internal cover over the vascular wall. The endothelium, which sometimes forms ridges that project into the lumen, also frequently projects through fenestrations of the internal elastic lamina regularly making contact with VSM cells of the media.³⁶ This important anatomical feature allows for an interaction between the endothelium and VSM cells that is crucial to maintaining normal vascular tone. Finally, blood vessels are surrounded by perivascular adipose tissue, which may also have vasoactive and vasoprotective roles.³⁹ The composition of the perivascular adipose tissue varies by region with the amount surrounding the blood vessel increasing with greater general adiposity.⁴⁰ While the microcirculation contains mainly white adipose tissue, the macrocirculation is characterized by white, brown or beige adipose tissue.^{39,40}

2.1.2 Normal vascular physiology

In addition to neural control, vascular tone is modulated by shear stress, metabolic mechanisms and the arteriolar myogenic response.²⁰ Shear stress, the force exerted on the endothelial wall by vascular blood flow, is considered the predominant regulator of vasomotion.¹⁸ Together, with other agonists such as insulin, acetylcholine, adenosine triphosphate, adenosine, bradykinin and histamine (Figure 2-1), shear stress acts on the endothelium to stimulate the synthesis of several vasodilating (NO, PGI₂, and EDHF) and vasoconstricting (ET-1, angiotensin II, prostanoids, and isoprostanes) substances

that are released to the VSM, mediating widening and narrowing of the blood vessel, respectively.^{18,19} Additionally, vasoactive substances such as NO, adiponectin, hydrogen sulphide and others yet to be identified, may also be released by the perivascular adipose tissue surrounding the blood vessel.³⁹ Although both vasodilators and vasoconstrictors are synthesized simultaneously, the net result of these two antagonistic effects in normal healthy conditions is usually vasodilation.⁴¹ Indeed, the balance of this mechanism is not only critical to maintaining normal vascular tone, but also essential in promoting optimal vascular health through the regulation of pro-inflammatory cytokines, leukocyte recruitment, platelet aggregation and adhesion, angiogenesis and VSM cell proliferation.¹⁸

Given its status in those with insulin resistance, the vasoactive role of insulin is important to consider when exploring underlying mechanisms of microvascular dysfunction. A key component of insulin's metabolic action is its ability to dilate resistance vessels and pre-capillary arterioles to increase total blood flow and the microvascular exchange surface perfused within the skeletal muscle, respectively.⁴² Thus, allowing for optimal postprandial-nutrient delivery to the most peripheral vascular beds such as those of the skeletal system and cutaneous microcirculation. Unique to other agonists, insulin achieves its vasodilatory role by synthesizing NO exclusively via a calcium-independent pathway.⁴³ In brief, circulating insulin signals the insulin receptor of the endothelial cell, activating G protein-phospholipase interactions that stimulate the phosphatidylinositol 3-kinase pathway.^{43,44} This cascade of signalling activates protein kinase B to phosphorylate and activate endothelial NO synthase (eNOS), which ultimately synthesizes NO from the amino acid, L-arginine.^{44,45} Further to its vasodilatory action, insulin also simultaneously induces vasoconstrictive mechanisms via mitogen-activated protein kinase, which stimulates the secretion of ET-1.⁴²

Shear stress and agonists of vasomotion, such as acetylcholine, adenosine triphosphate, adenosine, bradykinin, and histamine, also stimulate NO synthesis via the phosphatidylinositol 3-kinase, calcium-independent pathway.¹⁸ However, unlike insulin, these agonists also stimulate the synthesis of both NO and PGI₂ via a calcium-dependent, cyclooxygenase (COX) pathway. In brief, agonist-mediated G protein-phospholipase interactions deplete the endothelial cell calcium concentration, inducing

a calcium influx via store-operated channels and potassium channel activity.⁴⁶ Free intracellular calcium then binds to calmodulin and liberates arachidonic acid, activating eNOS to synthesize NO and initiating the COX pathway to synthesize PGI₂, respectively.^{46,47} Once synthesized in the endothelial cell, NO and PGI₂ diffuse to the VSM cell where they increase the formation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), respectively. The actions of cGMP and cAMP are identical, mediating a reduction in intracellular calcium concentration that induces VSM relaxation. Noting that cGMP also promotes cAMP activity to increase the overall sensitivity of the mechanism demonstrates the synergistic influence of NO and PGI₂ to modulate cutaneous microvascular tone.¹⁸

In healthy conditions, NO is the dominant agonist in macrovascular vasodilation,⁴⁸ while both NO and PGI₂ are the dominant mediators of microvascular dilation. However, EDHF, which include epoxyeicosatrienoic acids (EETs), also contribute to inducing vasodilation.³⁸ In brief, arachidonic acids that are liberated by an increase in endothelial intracellular calcium concentrations also stimulate several cytochromes to synthesize EETs in the endothelium.⁴⁹ Diffusion of EETs to the VSM cell induces hyperpolarization and subsequent VSM relaxation via opening of potassium channels and closure of calcium channels, which causes a decrease in VSM intracellular calcium concentrations. Indeed, normal NO bioavailability suppresses cytochrome activity and the subsequent synthesis of EETs. However, when NO activity is disrupted, EETs production and its vasodilatory influence may increase in order to maintain normal vascular reactivity.⁴⁹ Although these dynamic systems with multiple regulators allow for vasodilation and vasoconstriction to still occur normally in the event of weakening of a vasoactive pathway, a significant dysfunction within a central mechanism may still substantially impair overall vascular function.¹⁸

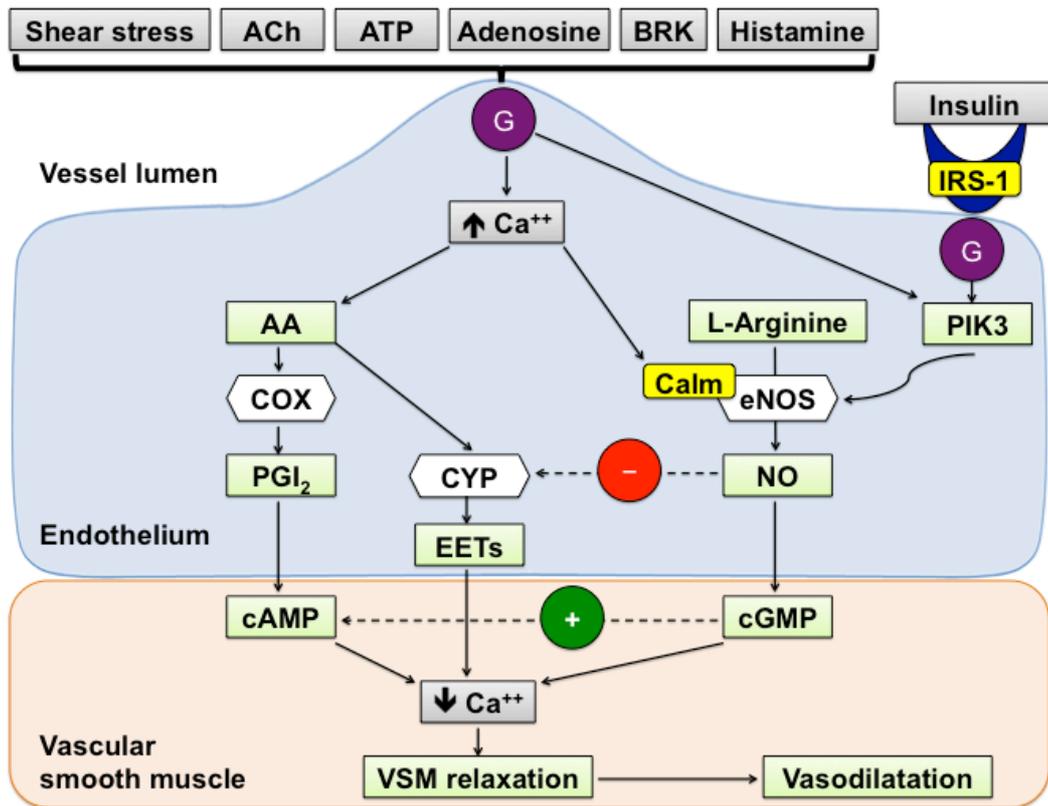


Figure 2-1. Schematic diagram illustrating the interaction between the three main vasodilatory pathways in normal healthy vascular function Adapted from Hellsten *et al.*¹⁸ ACh, acetylcholine; ATP, adenosine triphosphate; BRK, bradykinin; IRS-1, insulin receptor substrate-1; G, G-protein phospholipase; Ca⁺⁺, free intracellular calcium; AA, arichidonic acid; PIK3, phosphatidylinositol 3-kinase; COX, cyclooxygenase; Calm, calmodulin; eNOS, endothelial nitric oxide synthase; PGI₂, prostacyclin; CYP, cytochrome metabolites; NO, nitric oxide; EETs, epoxyeicosatrienoic acids; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; VSM, vascular smooth muscle; ↑, increase; ↓, decrease; -, down-regulates; +, up-regulates.

2.2 Methods of assessing peripheral vascular reactivity

The peripheral vascular network has become widely accepted as a model of systemic vascular function due, mainly, to its superior accessibility allowing for simple non-invasive investigations. Indeed, the peripheral circulation may also provide an index, specifically, of coronary vascular reactivity. Previous vascular research has typically utilized techniques that assess peripheral macrovascular reactivity. However, emerging evidence suggests that coronary microvascular disease may explain the occurrence of myocardial ischemia, heart failure and CVD mortality following myocardial infarction without apparent coronary macrovascular disease; highlighting the microcirculation, which represents most of the arterial vascular network and exerts dominant control over local blood flow to the nutritive network, as an equally important concentration in the study of vascular function. When interpreting data acquired from tests of peripheral microvascular and macrovascular reactivity, it must be acknowledged that the physiology may vary between vascular regions.^{45,50} The following section of this review will detail common tests of microvascular and macrovascular function in the peripheral circulation.

2.2.1 Assessing peripheral macrovascular reactivity

In recent decades, flow-mediated dilation (FMD) in conjunction with ultrasound measurements of the brachial artery has become the standard non-invasive test to assess macrovascular endothelial reactivity.^{51,52} Briefly, this test consists of comparing the brachial artery diameter at rest (baseline) to that following occlusion of an artery with the patient remaining in a supine position for the duration of the test (Figure 2-2). Occlusion is usually of the brachial artery and is achieved by inflating a sphygmomanometric cuff below, or sometimes above, the antecubital fossa. The cuff is typically inflated to 50 mmHg above systolic blood pressure, or to 200-300 mmHg, for 5 minutes before being rapidly deflated to induce shear stress and subsequent vasodilation.⁵³ Ultrasound images are taken pre-occlusion and post-occlusion using a high-resolution ultrasound machine with the ultrasound probe positioned in the longitudinal plane of the brachial artery above the antecubital fossa.

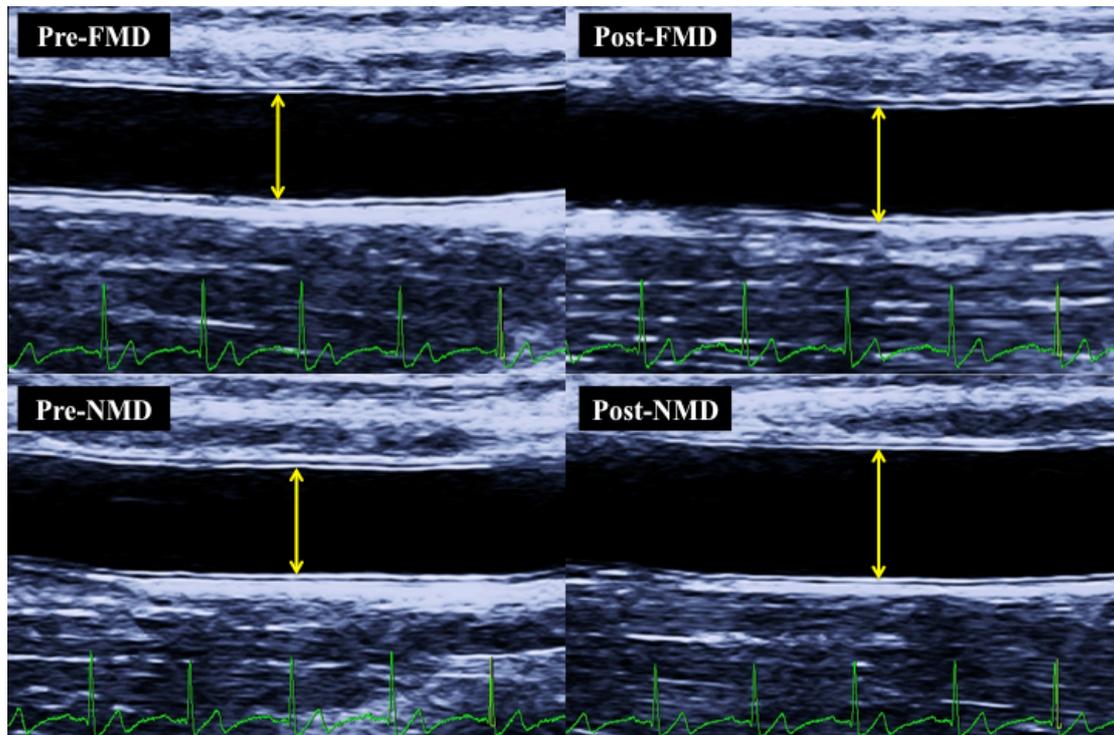


Figure 2-2. Ultrasound images of the brachial artery before and after the flow-mediated dilation (FMD) and nitrate-mediated dilation (NMD) procedures. Simultaneous electrocardiogram recordings are performed (illustrated by the green line) during each phase (pre- and post-occlusion/nitroglycerin administration) of FMD and NMD.

Whereas FMD assesses endothelial function, nitrate-mediated dilation (NMD) is often used to examine VSM reactivity in the macrocirculation.⁵⁴ Nitrate-mediated dilation is performed using similar protocols to that in FMD, but the occlusion is substituted with a pharmacological stimulus, namely a NO donor (e.g. sublingual nitroglycerine) that bypasses the endothelium and directly stimulates the VSM cells.⁵⁴ To ensure ultrasound image analyses, which determine brachial artery diameter, are performed at a consistent phase of the cardiac cycle, ultrasound recordings should be acquired in conjunction with electrocardiogram during both FMD and NMD measurements. The percentage change between baseline and post-challenge artery diameter is the key variable of interest in both tests and is considered as the maximal vasodilator response; irrespective of endothelial function, in NMD only.⁵²

Evidence suggests that mechanisms underlying FMD predominantly involve the NO pathway as infusion of the NO synthase inhibitor N-monomethyl-L-arginine blunts vasodilation following occlusion. In contrast, aspirin infusion had no effect, suggesting a decreased role of PGI₂ in macrovascular function.^{48,55} More recently, cytochrome P450 (CYP) metabolites and, putatively, other hyperpolarizing factors (e.g. EETs), have been suggested to closely interact with the NO pathway in the response to artery occlusion.⁵⁶ It must also be recognized that FMD does not only measure mechanisms of conduit artery vascular function *per se*, but also, indirectly, the limb microcirculation, due to the stimulus (i.e. shear stress) being highly dependent on maximal forearm resistance.³³ To facilitate between-study comparisons and optimize the evaluation of macrovascular function, it has been suggested that the change in brachial artery diameter should be normalized to the shear stress stimulus.^{57,58} Despite these efforts to optimize and standardize the test,^{34,52,59} the FMD technique still suffers from a lack of homogeneity and high inter-operator variability.

2.2.2 Assessing peripheral microvascular reactivity

Recent technological advances have allowed researchers to perform non-invasive assessment of cutaneous microcirculatory health in specific regions with improved accuracy; a development that is of considerable importance given that global measurement of microvascular perfusion may not reflect regional deficits observed in those with diabetes complications (e.g. diabetic foot ulceration).⁶⁰ Despite these advances, the heterogeneity of the cutaneous vascular anatomy itself represents a key challenge for achieving reproducible measurements.

2.2.2.1 Strain gauge plethysmography

Strain gauge plethysmography, also referred to as venous occlusion plethysmography, is an old, minimally invasive method that permits extrapolation of blood flow (mL/min), usually from the forearm muscle microcirculation. This test consists of inflating a cuff around the upper arm above venous blood pressure but below diastolic blood pressure (typically 10 s at 40 mmHg). Venous return from the forearm is then briefly interrupted while arterial blood inflow is preserved, resulting in a linear increase in forearm blood volume. A second cuff placed around the wrist excludes the hand circulation. Changes in forearm blood volume are measured by a strain gauge located between the cuffs and connected to a plethysmograph.⁶¹ Strain gauge plethysmography

is particularly interesting to assess the response to vasodilators, which requires intra-arterial infusion of drugs and makes the procedure more difficult. Finally, heterogeneity in the vascular response due to differences in initial arterial pressures, forearm blood flow, or size of the forearm, limits the value of the technique.³³

2.2.2.2 Digital plethysmography

More recently, a digital form of plethysmography has emerged as an additional technique for assessing peripheral microvascular reactivity. Briefly, arterial pulse wave amplitude is measured before and after brachial artery occlusion, and the signal is corrected with simultaneous measurement on the contralateral hand. A marketed proprietary device (Endo-PAT, Itamar Medical) has been used to show that digital vascular dysfunction correlates with metabolic risk factors, including obesity, diabetes mellitus and ratio of total to high-density lipoprotein (HDL) cholesterol.⁶² Digital plethysmography also has the advantage of being operator-independent; however, it is only partly dependent on the NO pathway.⁶³

2.2.2.3 Laser Doppler flowmetry

One of the most common methods adopted by researchers over recent decades to quantify changes in cutaneous microvascular reactivity has been laser Doppler flowmetry (LDF). The laser Doppler principle is based upon the phenomenon that when a laser beam emitted by the device hits moving red blood cells in the cutaneous vessels, the light undergoes a change in wavelength (Doppler shift) and the backscatter is detected.⁶⁴ The laser Doppler signal, quantified as the product of mean red blood cell velocity and concentration, provides an index of cutaneous perfusion referred to as *flux*, rather than a direct measure of cutaneous blood flow as measured by strain gauge plethysmography.⁶⁵ Using a single-point laser probe and a high sampling frequency of approximately 32 Hz, LDF is capable of accurately quantifying rapid variations in cutaneous blood flow within a volume of 1 mm³ or smaller. However, considering the anatomical heterogeneity of the cutaneous microcirculation and the relatively small vascular region that can be assessed, LDF is subject to increased spatial variability and, thus, presents relatively poor reproducibility between measurements.⁶⁵

2.2.2.4 Laser Doppler imaging

Laser Doppler imaging (LDI) is an alternative laser-based technology that scans a tissue bed of interest (e.g. the volar surface of the forearm) to produce a 2D image and map cutaneous blood flux within that region, with each pixel representing a separate perfusion value.⁶⁵ In contrast to LDF, where the laser unit is in direct contact with the skin, LDI emits a laser beam at a set distance above the skin surface. Therefore, given that LDI is capable of assessing a large area of the cutaneous microvasculature in a single scan, the spatial variability associated with LDF is reduced. However, the image rate of LDI is much slower than that of LDF and therefore it is not possible to detect rapid changes in cutaneous perfusion. Furthermore, research commonly performs a single scan to acquire baseline and post-intervention perfusion values, resulting in images that correspond to a single time-point during the assessment of microvascular function. Consequently, critical events (e.g. peak responses to tests of vascular reactivity) may be completely missed, introducing temporal variability and severely limiting the reproducibility and interpretation of LDI data.

2.2.2.5 Laser speckle contrast imaging

The most recently developed laser-based imaging technology, laser speckle contrast imaging (LSCI), allows for near real time analysis of cutaneous blood perfusion.³⁸ Based on the same fundamental operating principles as LDF and LDI, the LSCI head unit emits a laser beam above the skin surface from which speckle pattern images are acquired to provide a perfusion index proportional to the mean velocity of red blood cells.⁶⁶ Given that LSCI continuously measures cutaneous microvascular blood perfusion over a large area (>100 cm²) using a high sampling frequency, it theoretically combines the primary advantages of LDF and LDI, reducing the spatial variability and temporal variability associated with each technology, respectively.^{38,66} Indeed, such design features may contribute to the excellent reproducibility of LSCI, when compared to that of LDF and LDI.^{38,65-68} Furthermore, recent research has also established the relevance of LSCI for monitoring the health of the superficial microvasculature in those with T2D-related vascular complications such as foot ulceration, via human and animal models (Figure 2-3).^{69,70}

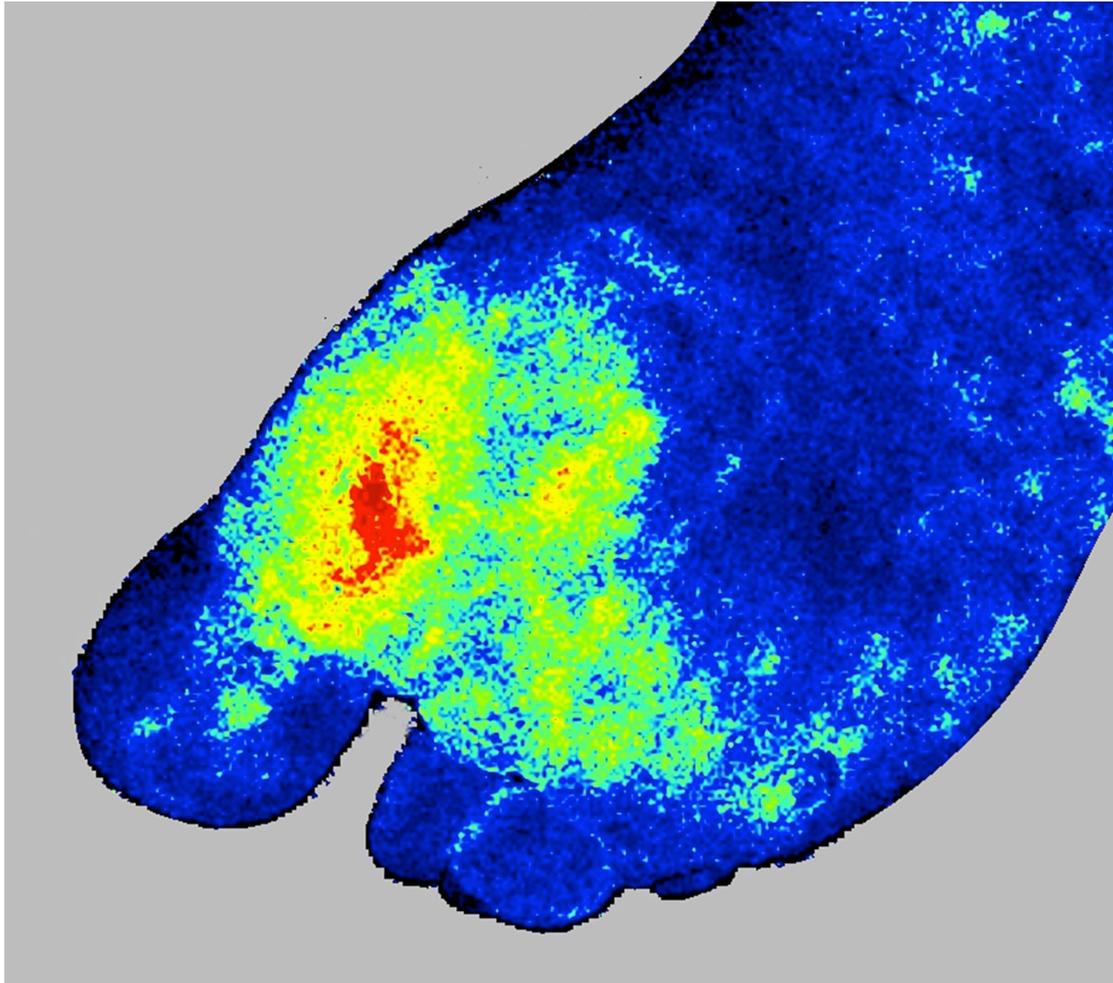


Figure 2-3. Measurement of skin perfusion with laser speckle contrast imaging on the plantar region of the foot in a diabetic patient with an active ulcer that is healing properly. Colours range from dark blue (no perfusion) to red (high perfusion).

2.2.2.6 Capillaroscopy

Whereas laser Doppler and laser speckle provide an index of general cutaneous perfusion, capillaroscopy allows for researchers to noninvasively perform direct *in vivo* assessment of the density, recruitment and blood flow velocity of the capillaries;⁶⁵ the normal function of which are critical to maintaining adequate gas and nutrient exchange between the microcirculation and the tissues; and promote optimal tissue health. Using a microscope with epi-illumination and imaging systems, capillaroscopy is often performed at the periungueal region where nailfold capillary loops are oriented parallel to the skin, imaging the width of a few millimetres.⁷¹ As capillaroscopy visualizes erythrocytes, rather than providing an image of the capillary wall, only capillary loops with circulating erythrocytes at the time of assessment will be captured.⁷² For a nailfold

capillary pattern to be considered normal, capillary loops ranging from 6 – 15 μm in diameter should be homogeneously distributed.⁷² Although nailfold capillaroscopy has been shown to have diagnostic applications in diseases that affect the digital cutaneous microcirculation, capillaroscopy outside this periungueal region has not been found to have clinical applications. Indeed, capillary loops in these other cutaneous regions are oriented perpendicular to the skin and, thus, visualization of capillary perfusion is limited to the top of the loop, only providing an index for the density of functioning capillaries per region of interest.

2.2.2.7 Transcutaneous oxygen tension

Given that oxygen is vital to maintaining optimal tissue health and, in those with diabetic foot ulceration, to promoting the wound healing processes, assessing the oxygenation in the cutaneous microcirculation may be considered as an important index of skin blood perfusion. Transcutaneous oxygen tension (TcPO_2) is an established technique that allows for non-invasive evaluation of the partial pressure of oxygen in cutaneous tissue. Correlating well with other T2D-related complications such as peripheral arterial disease, TcPO_2 may also have value in predicting healing rates in those suffering from diabetic foot ulceration and amputation rates in those with peripheral arterial disease or ischaemic ulcers.⁷³ In brief, using a probe that is applied to the surface of the skin and heated to 45°C in order to induce vasodilation, TcPO_2 measures the transfer of oxygen molecules from the blood vessels to the skin surface with a decreased TcPO_2 reading indicating reduced oxygenation.⁷³ Given that TcPO_2 only assesses the area of tissue directly under the probe, it may be more clinically relevant to perform multiple measurements across varied regions rather than conducting a single assessment. Indeed, a regional perfusion index, calculated by dividing the foot TcPO_2 value by a baseline TcPO_2 value measured at the chest, may provide more reliable data.⁶⁰ It must be noted that TcPO_2 may be less reliable in warm ambient environments and in those who are active smokers, have autonomic neuropathy or vascular calcification, with or without peripheral arterial disease; or in those who have an active infection, oedema, or callus, due to arteriolar shunting that causes TcPO_2 readings to be less representative of the true state of microvascular health.^{60,74} The ‘oxygen challenge’, in which patients are administered 100 % oxygen during the TcPO_2 assessment, has been proposed as a strategy to more accurately detect true values that represent peripheral artery diseases in such conditions.⁶⁰

2.2.2.8 Near-infrared spectroscopy

Cutaneous oxygen concentration can also be non-invasively assessed using near-infrared spectroscopy, a technique that has traditionally been the most popular method of estimating or measuring tissue oxygenation.⁷⁵ Near-infrared spectroscopy, which may also provide an indirect method of evaluating mitochondrial function,⁷⁶ uses near-infrared light emitted from a probe placed on the skin and is based on the principles that specific wavelengths of red and near-infrared light have the ability to penetrate through biological tissue; that absorption of these specific red and near-infrared wavelengths are dominated by haemoglobin; and that absorption varies between oxygenated and deoxygenated haemoglobin.⁷⁷ Light emitted by the probes typically penetrates the tissue to a depth of 2 cm and is detected by photodetectors, which can provide estimations of total haemoglobin, oxyhaemoglobin, deoxyhaemoglobin and tissue oxygen saturation.⁷⁷

Near-infrared spectroscopy may have particularly interesting applications in those with T2D-related complications. Traditionally, evaluation of diabetic foot ulceration and monitoring of healing rates has been based on surface assessments that involve a clinician manually measuring the length and width of the wound; a method that may be limited by the fact that irregular wound shapes may lead to inaccurate estimations of size and unfavourable recommendations for wound treatment.⁷⁸ The diffuse photon density wave methodology of near-infrared spectroscopy allows for measurement of oxyhaemoglobin and deoxyhaemoglobin at depths of up to ~3 cm; and, thus, may provide more clinically valuable information about the wound on a subcutaneous level (e.g. revascularization) and a more advanced method of evaluating the evolution of diabetic foot ulceration.⁷⁹ The efficacy of diffuse near-infrared spectroscopy was evaluated in a recent study that monitored the progression of human diabetic foot ulceration longitudinally over 24 weeks, finding that the technique had an 82 % predictive value for diabetic foot ulceration outcomes within 4 weeks of wound monitoring.⁷⁸

2.2.2.9 Hyperspectral imaging

Hyperspectral imaging allows for measurement of cutaneous oxygen saturation over a wide area and may also have interesting applications in those with T2D-related

complications (Figure 2-4). In the lower limb of patients with diabetic foot ulceration, there was a negative association between tissue oxygenation assessed by hyperspectral imaging at baseline and healing at 12 weeks.⁸⁰ An index derived from hyperspectral imaging measurements indicated very good sensitivity and specificity for predicting healing of ulcerations in a small group of patients with type 1 diabetes,⁸¹ which needs to be validated on a larger scale.

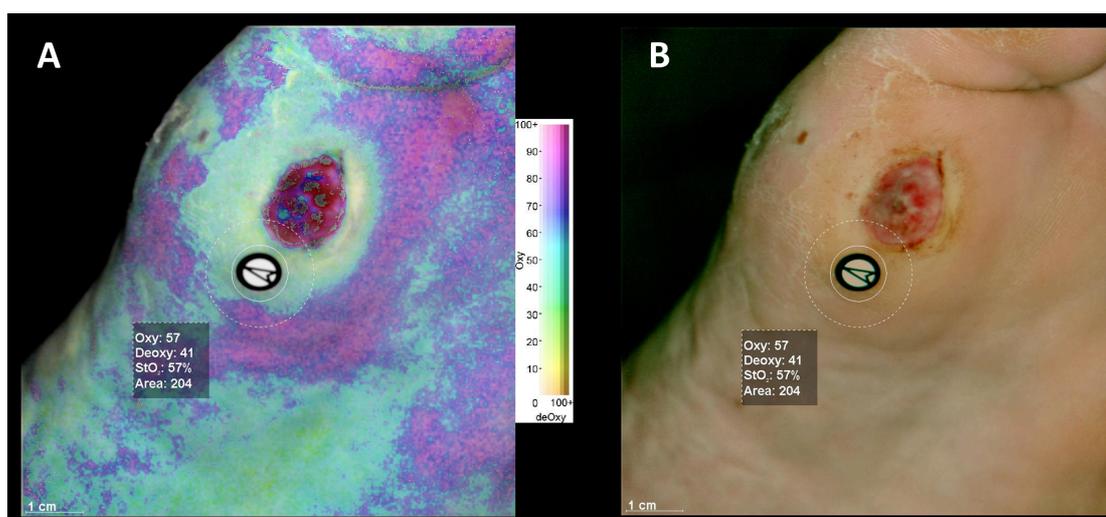


Figure 2-4. Measurement of cutaneous oxygenation in a peri-wound area using hyperspectral imaging (A) and a corresponding photo of the ulcer (B). The image shows poor skin oxygenation around the wound.

2.2.3 Challenging microvascular reactivity

Given that resting skin blood flux is highly variable, most interest in technologies that assess the microcirculation has been shown when challenging the functionality of the microvessels with various tests of vascular reactivity. Indeed, the combination of a variety of mechanical, thermal, electrical, or pharmacological stimuli offers a wide range of methods that explore different physiological pathways of the microcirculation, with varying reproducibility.³⁸ By analogy, arterial occlusion in FMD has been proposed as a test of endothelium-dependent microvascular reactivity. This simple test, commonly referred to as post-occlusive reactive hyperemia (PORH), involves sensory nerves and CYP metabolites, putatively EETs. Local heating of the skin to a temperature of 42-44°C is another interesting, reproducible test characterized by a biphasic rise in cutaneous blood flow. The initial peak depends on sensory nerves and

involves transient receptor potential vanilloid type 1 channels. This peak is followed by a transient nadir, and after 10 to 20 minutes the flux stabilizes. The delayed plateau is a good indicator of endothelial function, with an involvement of both NO (accounting for approximately two thirds of the response) and EDHF (the other third, half of which is dependent on EETs).³⁸

To date, most studies that have assessed microvascular reactivity in humans have used iontophoresis coupled with laser Doppler. This non-invasive technique is based on the transfer of charged molecules, driven by a low-intensity electrical current, across the uppermost layers of the skin. Acetylcholine and sodium nitroprusside are the most widely utilized vasoactive drugs and are used to assess endothelium-dependent and endothelium-independent vascular reactivity, respectively (Figure 2-5). There are several methodological issues that should be considered when using iontophoresis. Firstly, the vascular response to acetylcholine iontophoresis has long been primarily attributed to a NO-dependent endothelial response, when indeed, the involvement of a COX-dependent pathway is likely.³⁸ Recently, acetylcholine-mediated vasodilation in human skin has also been shown to involve EDHF.⁸² Interestingly, the relative contribution of NO and PGI₂ to acetylcholine-mediated vasodilation varied according to the concentration and duration of acetylcholine infusion.⁸² This suggests that using acetylcholine iontophoresis, as a test of NO-dependent vasodilation, is somewhat oversimplified. Moreover, additional methodological issues are related to non-specific, current-induced vasodilation. This is particularly true in patients with diabetes, in whom neuropathy may decrease the vasodilatory response to C-fibre activation (axon reflex). Indeed, findings from studies using iontophoresis to assess microvascular reactivity are inconsistent; highlighting that there are still no standardized protocols for iontophoresis and, consequently, there is an array of methods for iontophoresis being utilized in the literature with no consensus as to which are free of non-specific vasodilatory effects. Other methods, such as microinjections or microdialysis, allow for direct delivery of the vasoactive drug into the dermis. Although they are free from the risk of current-induced vasodilation, permitting administration of any kind of vasoactive substance, they can be more invasive and difficult to implement.

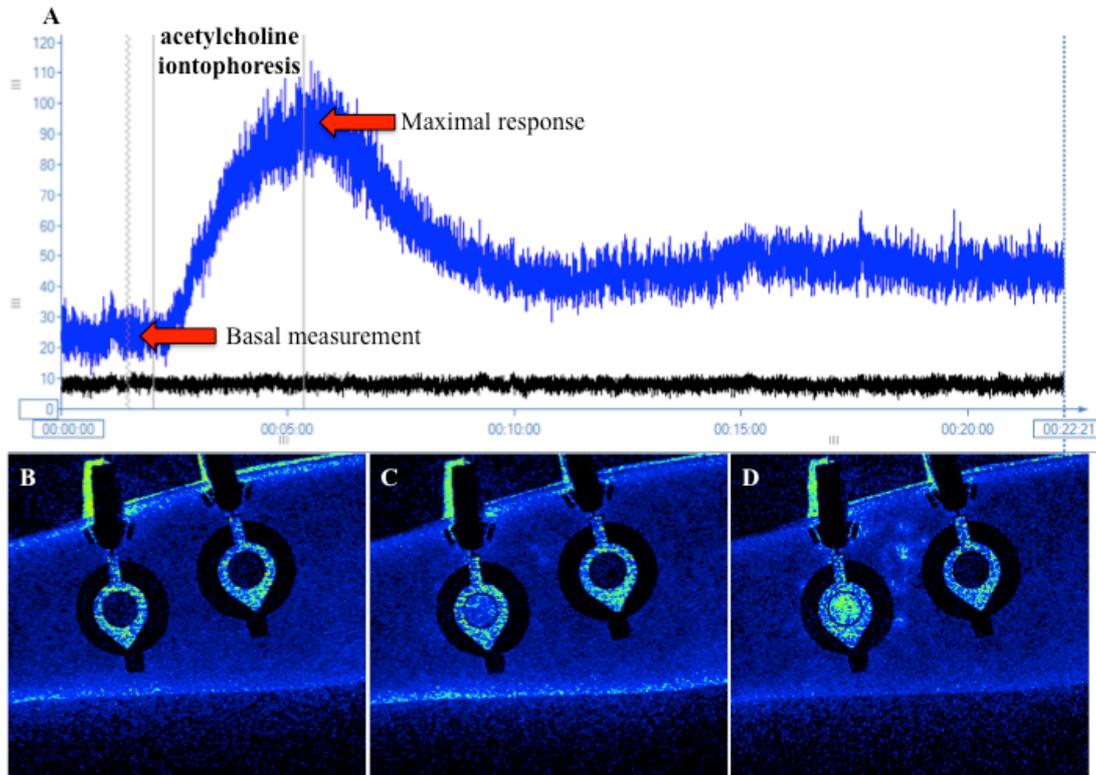


Figure 2-5. (A) The graphed response, in perfusion units, to iontophoresis of acetylcholine, which typically induces rapid vasodilation. Laser speckle contrast imaging illustrates the increase in cutaneous blood flow from the (B) basal state, to the (C) intermediate, and (D) maximal responses to acetylcholine iontophoresis. Note that the speckle pattern colour varies by cutaneous region with the brighter green speckles within the electrode during and immediately following acetylcholine iontophoresis indicating increased cutaneous blood flow as compared to the darker (black and blue) colours within the electrodes during basal measurements.

2.3 Changes in vascular function across the spectrum of cardiometabolic health

Currently, evidence indicates that chronic impairments in vascular reactivity may present early, even in those considered overweight or obese, long before the clinical onset of T2D or its associated complications (e.g. diabetic neuropathy, nephropathy, retinopathy and foot ulceration).³¹ However, whether there is a pathophysiological continuum in vascular impairment between early stages and diabetic complications is less clear due, mostly, to a lack of comparisons between all key health classifications across the spectrum of cardiometabolic health. The pathogenesis of a chronic vascular dysfunction may begin with transient impairments in endothelial function, which can be induced by numerous dietary and behavioural factors (e.g. excess sugar, salt and lipid consumption, smoking, sedentary behaviour etc.). However, the interaction between vascular function and these lifestyle factors are also unclear due to discrepant results and, ultimately, a lack of research in this field.

Vascular dysfunction, transient or chronic, reduces the capacity of the blood vessel to react to specific stimuli (e.g. when shear stress from blood flow, exerted on the arterial wall, increases),¹⁴ potentiating a situation in which risk of injury to the arterial walls is heightened,²³ an event that is significant in the process of atherosclerotic change. In addition to contributing to the pathogenesis of CVD, impaired vascular reactivity also plays a major role in the development of other complications that are associated with T2D, such as diabetic nephropathy, retinopathy, peripheral neuropathy and diabetic foot ulceration.²⁶⁻²⁹ Furthermore, impaired vascular reactivity may also contribute to the initial and ongoing development of obesity and/or insulin resistance.¹⁷ The mechanisms underlying vascular dysfunction, whether endothelium-dependent, endothelium-independent, perivascular adipose tissue-dependent or neurovascular, are complex and multifactorial; and are directly affected by hyperglycemia, obesity, insulin resistance and low-grade inflammation.

2.3.1 Effects of hyperglycemia on vascular physiology

Although there are numerous dietary factors that may affect vascular reactivity, the effect of hyperglycemia on vascular function is of primary importance considering that

habitual overconsumption of excess sugar, commonly in the form of SSB, is the factor that is most consistently found to be associated with an increased incidence of obesity, MetS, T2D and CVD.¹ From the mid-1960's, the prevalence of these cardiometabolic diseases have closely paralleled the steady increase in per capita calories from SSB consumption in both adults and children of developed nations; a trend that has been markedly more rapid in developing nations over the past few decades as traditional diets become more westernized.¹⁰⁻¹² Moreover, concerning data from the United States demonstrates that these dietary habits are being instilled at a young age, with children and adolescents aged 2-19 years consuming an average of 80 g of added sugar daily.⁸³ It is important to note that cardiometabolic disease is primarily characterized by dysglycemia, which causes frequent hyperglycemic excursions due to poor glycemic control. Furthermore, fasting blood glucose concentrations may also be elevated in those with cardiometabolic disease.

Considering that little research has examined the acute effects of excess sugar consumption on vascular function, this section of the review will detail mechanisms of hyperglycemia-mediated vascular dysfunction that may explain vascular dysfunction resulting from both acute hyperglycemia-mediated and dysglycemia-mediated hyperglycemic excursions. Traditionally, there have been four pathways to explain the mechanisms through which hyperglycemia damages the blood vessels and causes impairment to vascular function; namely, an abnormal elevation in oxidative phosphorylation in the mitochondria, activation of the polyol pathway and aldose reductase, protein kinase C (PKC) activation and the formation of advanced glycation end products (AGEs).⁸⁴ All of these pathways contribute to the production of reactive oxygen species (ROS), such as superoxide, in the vascular wall and are also involved in nerve damage in diabetes, which itself impairs microvascular reactivity; the latter depending on intact sensory nerves.⁴²

When blood glucose concentrations increase, oxidative metabolism initiates oxidative phosphorylation of adenosine triphosphate at the electron transport chain of the mitochondria, which via the phenomenon of electron leakage causes a superoxide generation of ROS.⁸⁵ These ROS are normally readily detoxified by antioxidant systems that suppress oxidative stress;⁸⁵ however, elevated activity within this mechanism, such as that during hyperglycemia, may result in an abnormal increase in

the production of ROS to a rate beyond the suppressive capacities of the antioxidant systems.⁸⁶ Furthermore, hyperglycemia may also induce an inflammatory response, which itself can contribute to a pro-oxidative stress environment.²⁴ Ultimately, increases in oxidative stress may disrupt normal vascular reactivity by decreasing the bioavailability of the vasodilator NO through several mechanisms including the oxidation of eNOS cofactors (e.g. tetrahydrobiopterin), competitive inhibition of eNOS by asymmetric N^G,N^G-dimethylarginine, and reducing arginine availability in an arginase-dependent manner.²⁴ Indeed, these mechanisms contribute to the uncoupling of eNOS, which may also promote the production of ROS, compounding the existing deleterious effects.²⁴

Endothelial dysfunction may also be induced by increased activity in the polyol pathway. Glucose metabolism through the polyol pathway is very low in those considered healthy. However, in the presence of cardiometabolic disease-related hyperglycemia, glucose conversion to the polyol sorbitol by aldose-reductase is increased using NADPH as a cofactor. This may deplete cytosolic NADPH, which is necessary for regenerating reduced glutathione, a potent cellular antioxidant.⁸⁶ Moreover, NADPH is a cofactor for the synthesis of the vasodilator, NO. Thus, cytosolic depletion in the endothelium of this essential cofactor of NO synthesis in conjunction with a decreased antioxidant capacity favours eNOS uncoupling, shifting NO synthesis activity towards decreased NO production and increased superoxide generation.

Protein kinase C is a collection of ubiquitously expressed regulatory enzymes involved in cellular signal transduction. It plays a central role in several vascular functions, such as the regulation of vascular cell permeability, extracellular matrix synthesis, angiogenesis, and the regulation of VSM contractility.⁸⁷ While increased lipid diacylglycerol causes PKC activation to be sustained in diabetes, hyperglycemia also increases PKC activation through upregulated transcription.⁸⁸ Such sustained activation of endothelial PKC results in endothelium-dependent vascular dysfunction through inhibition of the NO and EDHF pathways. Moreover, it also activates the ET-1 pathway and enhances the production of ROS, resulting in increased vascular tone.⁸⁷

Hyperglycemia also induces the formation of intracellular and extracellular AGEs through both enzymatic and non-enzymatic reactions. In endothelial cells, AGEs alter the structure and function of intracellular and extracellular matrix proteins causing abnormal interactions with other matrix proteins and with integrins.⁸⁶ Moreover, activation of receptors for AGEs by extracellular AGEs leads to a signalling cascade that stimulates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This increases the production of ROS, which contributes to eNOS uncoupling, as described earlier in this section of the review. The role of NADPH oxidase-derived oxidative stress in diabetic kidney disease and in diabetic peripheral neuropathy has been established.⁸⁹ Another target of AGEs receptor signalling is nuclear factor- κ B (NF- κ B) translocation to the nucleus, which increases the transcription of proteins including ET-1 and intracellular adhesion molecule-1 (ICAM-1); and activates inflammatory pathways.⁹⁰

Generation of ROS appears to be a unifying pathway between hyperglycemia and endothelial dysfunction, as well as a key player in endothelial cell damage (Figure 2-6). Indeed, in addition to reducing the bioavailability of NO, superoxide rapidly reacts with NO to form peroxynitrite, which exerts a variety of deleterious effects in endothelial cells. Peroxynitrite-mediated alterations include depletion of the eNOS cofactor, tetrahydrobiopterin, which further promotes eNOS uncoupling, deoxyribonucleic acid (DNA) injury and activation of poly (adenosine diphosphate ribose) polymerases (PARP).³² In addition to their role in DNA repair, PARP are involved in pro-inflammatory reactions in endothelial cells (e.g. ICAM-1 production in response to tumor necrosis factor- α [TNF- α]). Over-activation of PARP by peroxynitrite depletes the cell of nicotinamide adenine dinucleotide (NAD⁺), therefore impairing mitochondrial electron transport leading to cell death by necrosis.⁸⁴ Interestingly, neutralization of peroxynitrite or inhibition of PARP have demonstrated interesting effects in the treatment of microvascular complications in various experimental models.⁸⁴

To counter the adverse effects of increased oxidative stress, protective mechanisms that naturally occur in endothelial cells may also be initiated in an effort to maintain equilibrium in vasomotion. In the skin of the lower limb, mitochondrial superoxide dismutase, which converts superoxide into hydrogen peroxide (accounting for EDHF-

dependent vasodilation), is overexpressed in patients with recently diagnosed T2D, while the subepidermal endothelial cell area is preserved.⁹¹ This suggests that increased mitochondrial superoxide dismutase is an early mechanism protecting against the formation of mitochondrial ROS. In contrast, reduced levels of serum mitochondrial superoxide dismutase have been associated with a higher incidence of microvascular complications.⁹²⁻⁹⁴ Activity within the EDHF pathway may also increase, playing a compensatory role and maintaining vascular function in those with cardiometabolic disease, when NO-dependent vascular function is impaired. Supporting this, recent experimental data in an overweight hyperglycemic mouse model of NO deficient conditions suggest that inhibiting the degradation of EETs, which also accounts for EDHF activity, prevents the onset of complications associated with chronic vascular dysfunction, such as microalbuminuria and renal inflammation.⁹⁵ Indeed, with chronic stress, these protective mechanisms may eventually be overwhelmed, potentiating the pathogenesis of worse states of cardiometabolic disease, atherosclerotic change and the development of complications associated with T2D.

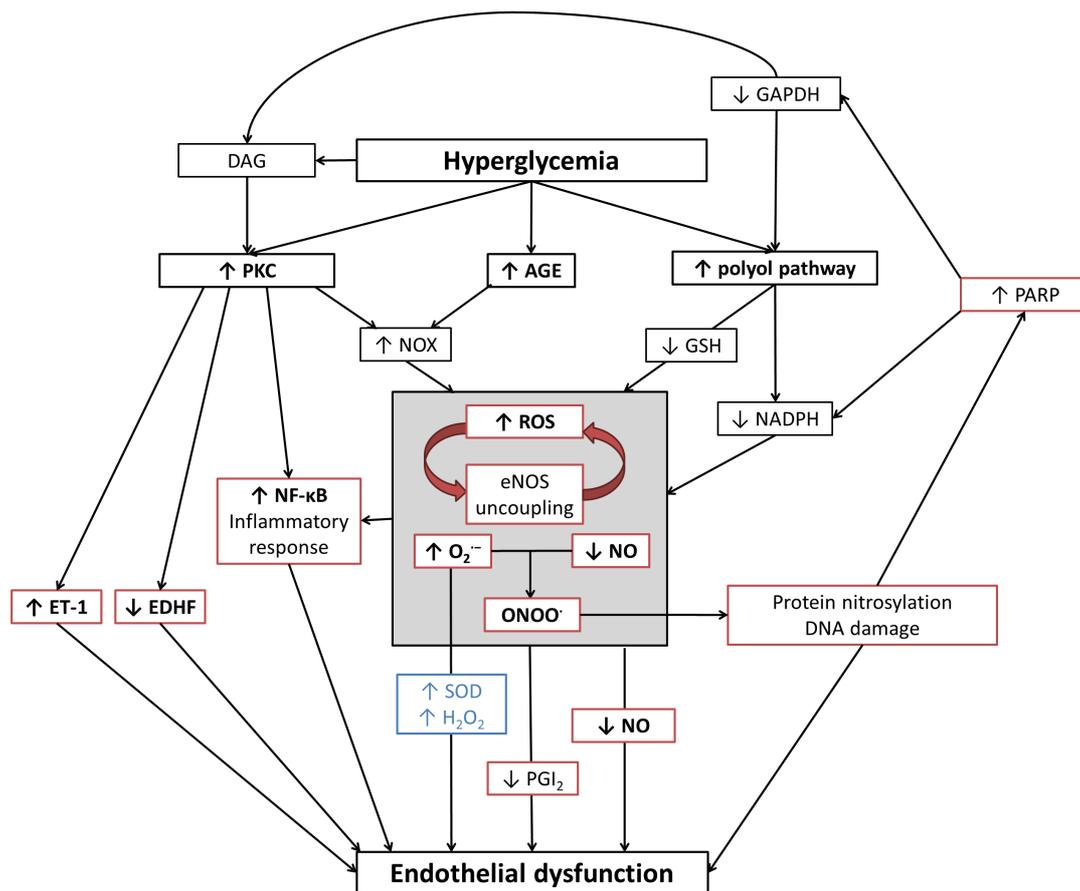


Figure 2-6. Mechanisms linking hyperglycemia to endothelial dysfunction involve reactive oxygen species (ROS) as a key agonist. Deleterious pathways appear in red, while protective mechanisms are in blue. AGE: advanced glycation end-product; DAG: diacylglycerol; EDHF: endothelium-derived hyperpolarizing factor; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GSH: glutathione; NADPH: nicotinamide adenine dinucleotide phosphate; NF- κ B: nuclear factor-kappa B; NO: nitric oxide; NOX: NADPH oxidase; O_2^- : superoxide; ONOO $^-$: peroxynitrite; PARP: Poly (ADP-ribose) polymerase; PKC: protein kinase C; SOD: superoxide dismutase.

2.3.2 The role of insulin resistance and obesity in chronic vascular dysfunction

It must be acknowledged that while obesity is one of the primary driving factors behind the increasing prevalence of T2D,⁷ insulin resistance may also be observed without obesity. This section of the review focuses purely on the interaction of obesity and insulin resistance. As described earlier in this review, insulin has a pivotal role in regulating cell metabolism and in mediating a wide range of hemodynamic effects including increased NO-dependent vasodilation and capillary recruitment.⁹⁶ In endothelial cells, insulin up-regulates gene expression of eNOS, vascular endothelial growth factor (VEGF) and ET-1, and down-regulates vascular cellular adhesion

molecule-1 (VCAM-1); noting that up-regulation of ET-1 does not exert beneficial effects on endothelial function. Interestingly, targeted knockout of the insulin receptor in the vascular endothelium of mice led to accelerated atherosclerosis without changes in insulin levels or sensitivity, suggesting an overall beneficial effect of insulin on endothelial function, independently of its metabolic effects.⁹⁷

The ability of insulin to enhance endothelium-dependent vasodilation in the lower-limb is impaired, even in those considered obese, with a negative correlation between leg blood flow during metacholine injection and percentage of body fat.⁹⁸ Interestingly, endothelium-dependent vasodilation is similarly reduced by 40-50% in obese patients with or without T2D, while endothelium-independent responses remain preserved.⁹⁸ In contrast, insulin resistance in patients with T2D has been associated with impaired endothelium-dependent and endothelium-independent vascular reactivity, independently from obesity, in parallel with low-grade inflammation.⁹⁹ This suggests that obesity, which plays a key role in the development of insulin resistance, potentiates the deleterious effect of insulin resistance on vascular function. Indeed, elevated free fatty acids impaired capillary recruitment and acetylcholine-mediated vasodilation in the skin of lean subjects, while they were improved after free fatty acids were lowered in obese subjects.¹⁰⁰

The main mechanism underlying this effect involves the binding of elevated free fatty acids to toll-like receptors, which initiate a pro-inflammatory environment through NF- κ B activation. They also activate PKC, which inhibits signalling of the phosphatidylinositol 3-kinase pathway and, subsequently, downregulates eNOS, while the mitogen-activated protein kinase pathway is preserved. Hence, decreased NO and increased ET-1 disrupts the endothelium's ability to properly vasodilate in response to stimuli. Finally, intracellular oxidation of free fatty acids generates ROS, which compounds the aforementioned deleterious mechanisms.⁷

2.3.3 Increases in inflammatory cytokines, growth factors, and biomarkers of vascular dysfunction in obesity and cardiometabolic diseases

This review has introduced the interaction between hyperglycemia, insulin resistance and the inflammatory response. However, given that low-grade inflammation signals the onset of vascular injury and the atherosclerotic changes that contribute to the pathogenesis of CVD,¹⁰¹ this section will provide special focus on inflammatory cytokines, growth factors and biomarkers of vascular dysfunction that develop with prolonged cardiometabolic stress; and that accompany and promote chronic impairment of vascular reactivity in obesity and cardiometabolic disease (Table 2-1). Previous research demonstrated that serum C-reactive protein (CRP), an inflammatory biomarker, correlates negatively with the forearm blood flow response to acetylcholine, suggesting a relationship between systemic inflammation and endothelial function.¹⁰² Moreover, hyperglycemia and insulin resistance are both known to contribute to microvascular dysfunction through several mechanisms, including the induction of a chronic state of vascular inflammation.¹⁷ Thus, highlighting inflammatory biomarkers as an acceptable surrogate for tests of vascular reactivity to track cardiovascular health in diabetes.

Increased expression of intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 is observed in diabetic populations and has been shown to independently predict myocardial infarction.^{103–105} Activated by cytokines and developing atherosclerotic lesions, the endothelium produces cellular adhesion molecules (CAMs) that mediate the attachment and transmigration of leukocytes across the endothelial surface, an important event in atherogenesis.¹⁰⁵ Found firmly bound to the membrane of the endothelial cell in greatest concentration around the borders of atherosclerotic lesions and lesion prone areas, CAMs may also be released by activated endothelial cells from cell surface adhesions in a soluble form that circulates throughout the plasma.^{106,107} Therefore, the presence of soluble CAMs is thought to reflect their increased expression on the endothelial cell surface and provide a valuable marker of atherosclerotic changes.¹⁰⁶

Further to being acted upon, the endothelium contributes to the inflammatory process and, consequently, to the pathophysiology of vascular complications in diabetes.

Indeed, proinflammatory molecules such as TNF- α and CRP may promote endothelial cells to express an atherogenic phenotype.¹⁰⁸ When deciding which inflammatory biomarkers to track, it is important to note that TNF- α , CRP, interleukin-1, interleukin-6, monocyte chemoattractant protein-1, and macrophage migration inhibition factor are the main markers that have been proven to provide prognostic information about the progression and outcome of CVD in diabetic populations.¹⁰⁹ Considering the role of perivascular adipose tissue in maintaining normal vascular function, it is important to recognise that as an active endocrine and paracrine organ, adipose tissue has a major role in releasing these cytokines and biomarkers that mediate a pro-oxidant, pro-inflammatory phenotype.^{110,111}

Noting that diabetes is also associated with pro-thrombotic and anti-fibrinolytic states, several molecules such as plasminogen activator inhibitor-1, tissue factor, fibrinogen, P-selectin and von Willebrand factor may provide an indication of endothelial function.¹¹² Further to this, assessment of micro-ribonucleic acids and circulating microparticles may reveal the status of endothelial apoptosis, mechanical injury, and cellular activation by cytokines. Endothelial dysfunction is also associated with elevated levels of homocysteine, which may share a relationship with increased concentrations of asymmetrical dimethylarginine. Elevated asymmetrical dimethylarginine disrupts NO synthesis, decreasing its bioavailability and thus impairing endothelial function. Decreased concentrations of tetrahydrobiopterin, an essential co-factor in the regulation of eNOS, also provides an indication of reduced NO bioavailability and endothelial function.¹¹³ More recently, endothelial progenitor cells, which are of lower concentration in those with diabetes, were also highlighted as viable biomarkers of endothelial dysfunction.^{108,114,115}

Table 2-1. Biomarkers of endothelial dysfunction and inflammation in diabetes that indicate key events in the pathogenesis of cardiovascular disease

Biomarker	Status in diabetes	Diagnostic marker of:
ICAM-1	↑	Adhesion of leukocytes
VCAM-1	↑	Adhesion of leukocytes
IL-1	↑	Inflammation
IL-6	↑	Inflammation
TNF-a	↑	Inflammation
CRP	↑	Inflammation
MCP-1	↑	Inflammation
MIF	↑	Inflammation
VEGF	↑	Inflammation
Homocysteine	↑↑	Inflammation/hyper-coagulation
Adiponectin	↓	Inflammation/endothelial dysfunction
E-selectin	↑	Inflammation/adhesion of leukocytes
P-selectin	↑	Procoagulant state/adhesion of leukocytes
ET-1	↑	Elevated free fatty acids
PAI-1	↑	Altered coagulation process
Fibrinogen	↑	Altered coagulation process
vWF	↑↑	Altered coagulation process
BH4	↓	Decreased NO bioavailability
EPC	↑	Decreased NO bioavailability
ADMA	↑↑	Decreased NO bioavailability
miRs	↓↑	Either enhanced or inhibited eNOS activity
MPs	↑	Endothelial apoptosis, mechanical injury, and cellular activation by cytokines

ICAM-1, intercellular cell adhesion molecule; VCAM-1, vascular cell adhesion molecule; IL-1, interleukin-1; IL-6, interleukin-6; TNF-a, tumor necrosis factor-a; CRP, C-reactive protein; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibition factor; VEGF, vascular endothelial growth factors; ET-1, endothelin-1; PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand factor; BH4, tetrahydrobiopterin; EPC, endothelial progenitor cells; ADMA, asymmetrical dimethylarginine; miRs, micro-ribonucleic acids; MPs, circulating microparticles; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; ↑ increased/up-regulated; ↓ decreased/down-regulated.

2.3.4 The role of diabetic peripheral neuropathy in chronic vascular dysfunction

The ability of the skin to adequately regulate blood flow in response to temperature variations or to a variety of mechanical and chemical stimuli is highly dependent on the existence of intact neurovascular function. Indeed, diabetes is also associated with nerve dysfunction that contributes to impaired reactivity of the cutaneous microvasculature and has long been observed through disturbances in cold and heat pain thresholds.¹¹⁶ Although diabetic neuropathy has been classically defined as a microvascular complication, the relationship between skin microvascular dysfunction

and neuropathy in diabetes is complex and has not yet been fully elucidated. Indeed, diabetic peripheral neuropathy is a complication that typically presents later in the pathophysiological continuum when diabetes is well established.

From a mechanistic perspective, peripheral neuropathy and endothelial dysfunction share similar pathophysiological pathways. Indeed, some of the mechanisms described above for endothelial cells are also encountered in neurons.¹¹⁷ For example, increased intracellular glucose increases the polyol pathway flux. In addition to depleting the cellular NADPH reserve, increased aldose-reductase transformation of glucose leads to sorbitol accumulation, which de-differentiate Schwann cells into immature cells.¹¹⁸ Oxidative stress and AGEs also play an important role in the pathophysiology of diabetic peripheral neuropathy.¹¹⁷ It is therefore tempting to think that endothelial dysfunction and diabetic peripheral neuropathy are two concomitant phenomenon of the same origin.

Pioneering work using electron micrographs of sural nerve capillaries showed that endoneural microangiopathy was related to the severity of neuropathy, thus supporting a causal relationship between impaired microvasculature and diabetic neuropathy.¹¹⁹ Recent experimental data provided further insight into the complex relationship between endothelial function and neuropathy, suggesting that endothelium impairment is sufficient to cause neuropathy through the involvement of the Desert Hedgehog pathway.¹²⁰ This is consistent with a large observational study conducted in participants with prediabetes, diabetes with or without neuropathy, and controls, demonstrating that endothelial function was a strong, independent predictor of diabetic peripheral neuropathy. It further suggested that endothelial dysfunction mediates the deleterious effects of diabetes on cardiovascular risk and diabetic peripheral neuropathy.³⁰

A unifying hypothesis could involve neuronal sensors that are also present at the surface of endothelial cells. In recent years, growing evidence has suggested that transient receptor potential vanilloid subfamily member-1 may play a key role in vascular health and metabolism; with possible involvement in the pathogenesis of T2D.¹²¹ Other sensors may also be involved. Indeed, pressure-induced vasodilation, an early microvascular response that delays the decrease in cutaneous blood flow produced by local low pressure, is abnormal at the foot level in diabetic patients with or without

diabetic peripheral neuropathy.^{122,123} This reflects microvascular fragility in the skin and involves acid-sensing ion channel-3, a voltage-insensitive cation channel that has been shown to be a neuronal sensor for appropriate adjustment to pressure changes in the cutaneous microcirculation.¹²⁴

2.3.5 Anatomical changes to the vascular network in cardiometabolic diseases

The following section will focus on anatomical changes in the vascular network that may present in those with a history of T2D. Structural abnormalities of the arterioles were observed in the mid-twentieth century in the retina and the kidneys; and arteriolar hyalinization, corresponding to the thickening of the walls of arterioles from amputated diabetic limbs, was also described.¹²⁵ Such arteriolar remodelling was confirmed decades later in patients with T2D, who had systemic structural alterations of subcutaneous small resistance arteries, as indicated by an increased media-to-lumen ratio. These abnormalities are characterized by hypertrophic remodelling and are associated with impaired endothelium-dependent vasodilation in vitro. Of note, they affect patients with and without hypertension.¹²⁶ Another structural abnormality, that has been inconsistently reported, is the decrease in skin capillary density that was observed in the lower limb muscle of patients with diabetes when compared to controls.¹²⁷ Additionally, endo-neural capillary density was also found to be reduced in diabetic patients with neuropathy.¹²⁸ Aside from density, abnormal morphology of cutaneous capillaries in the dorsum of the foot such as capillary enlargement, a sign of hypoxia, has also been reported.¹²⁹

One of the most notable structural changes of the microvasculature in diabetes involves thickening of the capillary basement membrane. These abnormalities are more pronounced in the leg, likely because of the higher hydrostatic pressure and the inability of the skin microvasculature of diabetic patients to respond adequately to postural changes.¹³⁰ Capillary basement membrane thickness in the nerve and the skin of the lower limb correlates with the extent of neuropathy in diabetes.¹²⁸ Such thickening of the basement membrane may affect oxygen and nutrient exchanges. Moreover, it may also limit the compensatory arteriolar dilation in response to reduced perfusion pressure.¹²⁹ Despite these deleterious adaptations, structural microcirculatory changes are not likely to have a primary role in the pathophysiology of more severe complications associated with T2D, such as diabetic foot ulceration; rather, they

potentiate the functional impairment that affects different parts of the cutaneous microcirculation, most prominently, the arterioles and arterio-venous anastomoses.

2.3.6 Regional differences in the decline of vascular health

Popular hypotheses suggest that microvascular dysfunction may precede macrovascular dysfunction. However, whether there is a pathophysiological continuum in microvascular and macrovascular dysfunction between early stages and diabetic complications has never been addressed; and, thus, it remains unclear whether microvascular dysfunction precedes or parallels large vessel impairment. In contrast, regional difference between anatomical regions (e.g. upper body versus lower body) may be clearer. Differences in the cutaneous microcirculation between the upper and the lower limb may explain why diabetic ulcers develop on the foot. In healthy subjects, absolute perfusion and TcPO₂ of non-glabrous skin is similar between the upper and the lower limb.¹³¹ However, the reactivity of the microvasculature expressed as the percentage change from baseline measurement in response to reactivity tests is lower in the foot than in the forearm; and this difference is consistent in diabetic patients with and without diabetic peripheral neuropathy.¹³¹ In patients with T2D, the vasodilatory response to acetylcholine iontophoresis was more altered in the foot than in the forearm when neuropathy was present; while diabetic peripheral neuropathy was associated with reduced microvascular reactivity both on the foot and the forearm.¹³² Similarly, local anaesthesia had no effect on microvascular reactivity on the foot of patients with diabetic peripheral neuropathy when assessing sensory nerve-dependent vasodilation, showing further impairment in these patients.¹³³ Ultimately, differences between upper and lower extremities may be explained by higher hydrostatic pressure in the lower limb that leads to microvascular remodelling and a subsequent decrease in the ability to respond to stimuli. Furthermore, differences in the density of arterio-venous anastomoses between the foot and the forearm may also be an explanation.¹³⁴

2.4 Summary

In normal vascular function, the endothelium and perivascular adipose tissue interact with the VSM cells, releasing vasodilating and vasoconstricting substances that maintain optimal blood vessel tone and organ perfusion. In recent decades, several techniques have been developed that allow researchers to assess changes in vascular reactivity, non-invasively in the peripheral vasculature, providing a model of coronary vascular health and, in certain cases (e.g. neuropathy and foot ulceration), a measure of vascular health directly in the region of interest. While the guidelines for assessing macrovascular reactivity appear clear, this review has demonstrated that there is far less consensus in the methodology for assessing microvascular reactivity and, subsequently, there are a range of techniques presented in the literature.

In vascular dysfunction, the reactivity of the blood vessel is decreased, heightening the risk of injury to the vessel wall and, subsequent, atherosclerotic change. Many studies have explored vascular reactivity across different populations from healthy subjects to patients with T2D, providing evidence that chronic impairments in vascular reactivity develop early, even in those considered overweight and metabolically healthy. While dysglycemia may have a primary role in the ongoing development of chronic vascular dysfunction, it has been suggested that acute hyperglycemia, induced by excess sugar consumption, may also cause transient impairments to vascular reactivity. Indeed, any hyperglycemic excursion may cause an elevation in oxidative stress that disrupt pathways crucial to maintaining normal vascular function (e.g. the NO pathway). Ultimately, though, the effect of acute hyperglycemia on vascular function is unclear due to discrepant results and a lack of data. Furthermore, whether there is a pathophysiological continuum in microvascular and macrovascular dysfunction between early stages and diabetic complications has never been addressed. Considering that vascular dysfunction is one of the earliest discernible pathophysiological precursors to the pathogenesis of CVD and a potentiating factor in the ongoing development of obesity and insulin resistance, highlights the importance of this research program's objective to further understand the changes in vascular reactivity across the spectrum of cardiometabolic health.

Chapter 3 – Study one: Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: systematic review and meta-analysis

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Loader J, Montero D, Lorenzen C, Watts R, Meziat C, Reboul C, Stewart S, Walther G. Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: systematic review and meta-analysis. *Arter Thromb Vasc Biol*. 2015;35(9):2060-72. Doi: 10.1161/ATVBAHA.115.305530.

3.1 Linking paragraph

It has been suggested that the development of a chronic impairment in vascular reactivity, which may potentiate atherosclerotic change and the pathogenesis of obesity and cardiometabolic disease, begins with transient impairments induced by a combination of dietary and lifestyle factors. Excess sugar consumption is the dietary factor that is most consistently associated with the pathogenesis of obesity, cardiometabolic disease and CVD. Indeed, it has been suggested that acute hyperglycemia, mediated by excess sugar consumption, may transiently impair vascular function. However, the overall effect of acute hyperglycemia on vascular function is not entirely clear due to discrepant results in previous research. Considering this, the first study of this research program consolidates existing data in a systematic review and meta-analysis, aiming to determine the effect of acute hyperglycemia on vascular function in those considered healthy, obese or in those with cardiometabolic diseases.

3.2 Abstract

Objectives

Controversy exists over the effect of acute hyperglycemia on vascular function. In this systematic review, we compared the effect of acute hyperglycemia on endothelium-dependent and endothelium-independent vascular reactivity across healthy and cardiometabolic diseased subjects.

Approach and Results

A systematic search of MEDLINE, EMBASE and Web of Science from inception until July 2014 identified articles evaluating endothelium-dependent and endothelium-independent vascular reactivity during acute hyperglycemia and normoglycemia. Meta-analyses compared the standardized mean difference (SMD) in endothelium-dependent and endothelium-independent vascular reactivity between acute hyperglycemia and normoglycemia. Subgroup analyses and meta-regression identified sources of heterogeneity. Thirty nine articles (525 healthy and 540 cardiometabolic subjects) were analysed. Endothelium-dependent vascular reactivity was decreased (39 studies; $n = 1065$; SMD, -1.25 ; 95% confidence interval [CI], -1.52 to -0.98 ; $P < 0.01$), whereas endothelium-independent vascular reactivity was preserved (6 studies; $n = 144$; SMD, -0.07 ; 95% CI, -0.30 to 0.16 ; $P = 0.55$) during acute hyperglycemia compared with normoglycemia. Significant heterogeneity was detected among endothelium-dependent data ($P < 0.01$). A subgroup analysis revealed that endothelium-dependent vascular reactivity was decreased in the macrocirculation (30 studies; $n = 884$; SMD, -1.40 ; 95% CI, -1.68 to -1.12 ; $P < 0.01$), but not in the microcirculation (9 studies; $n = 181$; SMD, -0.63 ; 95% CI, -1.36 to 0.11 ; $P = 0.09$). Similar results were observed according to health status. Macrovascular endothelium-dependent vascular reactivity was inversely associated with age, blood pressure, and low-density lipoprotein (LDL) cholesterol and was positively associated with the post-occlusion interval of vascular assessment.

Conclusions

To our knowledge, this is the first systematic review and meta-analysis of its kind. In healthy and diseased subjects, we found evidence for impairment of macrovascular, but not microvascular, endothelium-dependent reactivity during acute hyperglycemia.

3.3 Introduction

The prevalence of T2D represents a major public health issue, directly affecting an estimated 312 million people worldwide.¹³⁵ This burden is projected to worsen due, in part, to increasingly sedentary lifestyles and unhealthy dietary habits predominantly characterized by an excess consumption of added sugars.^{9,11,136} Habitual consumption of added sugars, most commonly in the form of SSB, is strongly associated with an increased risk in developing T2D, as well as MetS and obesity.^{137–140} In addition, consumption of added sugars has been linked to an increased risk of developing CVD, which is the leading cause of mortality amongst those with cardiometabolic disease.^{135,141,142} Consumption of excess added sugars leads to acute hyperglycemia, which is considered a better predictor of future CVD events than fasting glycemia in healthy and diabetic populations.^{24,143} Indeed, such acute hyperglycemic stress has also been proposed to contribute to vascular dysfunction,¹⁴⁴ which represents one of the main precursors to CVD.¹⁴⁵

In normal vascular function, the endothelium and VSM cells continuously interact to regulate vasodilation and vasoconstriction, maintaining optimal organ perfusion and vascular tone.^{20,21} During acute hyperglycemia, increased oxidative stress has been proposed as a key trigger of vascular dysfunction by reducing NO production or NO bioavailability.^{21,146,147} Furthermore, animal and *in vitro* studies suggest that acute hyperglycemia may also impair endothelium-independent reactivity by disrupting its pathways and VSM cell apoptosis, causing subsequent VSM cell proliferation and desensitization to NO.^{25,148,149} However, whether endothelium-dependent and endothelium-independent vascular reactivity are transiently impaired during acute hyperglycemia in humans is unclear because of discrepant results. Given this, we conducted a systematic review and meta-analysis of available studies comparing endothelium-dependent vascular reactivity alone or in combination with tests of endothelium-independent vascular reactivity during acute hyperglycemia in healthy and cardiometabolic diseased individuals. To our knowledge, this represents the first systematic review and meta-analysis to assess the effect of acute hyperglycemia on vascular function.

3.4 Methods

This review was conducted according to the Preferred Reporting Items for Systematic Review & Meta-Analysis (PRISMA) statement.¹⁵⁰

3.4.1 Data sources and searches

The systematic search included MEDLINE, EMBASE, and Web of Science databases, from their inception until July 2014. The search consisted of combinations of subject headings including: ‘postprandial hyperglycemia’, ‘acute hyperglycemia’, ‘oral glucose tolerance test’, ‘high glucose’, ‘glucose load’, ‘vasodilation’, ‘vascular reactivity’, ‘FMD’, ‘acetylcholine’, ‘endothelium-dependent’, ‘endothelium-independent’, ‘nitroglycerin’, and ‘sodium nitroprusside’. Searches were limited to ‘human’ studies only; but were not limited by study design. A manual search of reference citations in identified reviews and original articles selected for full text retrieval was also performed.⁵⁴

3.4.2 Study selection

To be included in this review, studies had to assess endothelium-dependent and endothelium-independent vascular reactivity in states of acute hyperglycemia and normoglycemia in humans. Blunted FMD and sublingual NMD of the brachial artery indicated impaired macrovascular endothelium-dependent and endothelium-independent reactivity, respectively. A blunted increase in blood perfusion in response to PORH or the administration of acetylcholine, metacholine or serotonin intravenously or by iontophoresis suggested impairment of microvascular endothelium-dependent reactivity. Impairment of endothelium-independent vascular reactivity was demonstrated if there was a blunted increase in blood perfusion in response to iontophoresis or intravenous administration of sodium nitroprusside. Studies following the above criteria, but including other interventions deemed likely to influence vascular function, such as exercise training programs combined with hyperglycemic tests, were excluded. In the event of multiple publications pertaining to the same research, the first published or more comprehensive study was included. If multiple trials inducing acute hyperglycemia were conducted for the same sample in a single study, data for the initial acute hyperglycemia and normoglycemia trials were included in the meta-analysis.

Where vascular reactivity was tested multiple times during a single period of acute hyperglycemia, data for vascular reactivity measured during peak hyperglycemia was included. Inclusion was limited to studies published in the English language. Study selection was performed independently by two investigators (J. L.) and (D. M.). Discrepancies in inclusion/exclusion were solved by consensus or through consultation with a third reviewer (G. W.).

3.4.3 Data extraction and quality assessment

The following variables were summarized into a pre-formatted spreadsheet by two investigators (J.L.) and (D.M.): authors, year of publication, characteristics of participants (n, % female, age, body mass index, blood pressure, fasting insulin, fasting glucose, HbA_{1c} %, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, smoking, medication and health status) and vascular variables (region assessed, method used, length of time for post-occlusion interval of vascular assessment, endothelium-dependent and endothelium-independent vascular reactivity data). Additionally, the type of carbohydrate and dosage used to induce acute hyperglycemia were recorded. If data were unclear or were not available in the published reports, the corresponding and/or first author was contacted by email to request this information. A Systematic Appraisal for Observational Research (SAQOR),¹⁵¹ previously applied in meta-analyses of observational studies evaluating vascular function,^{152,153} was performed to provide assessment of study quality. The SAQOR was scored out of 12, quality deemed better with a greater score. The Grading of Recommendations Assessment, Development & Evaluation (GRADE) was performed to provide assessment of the quality of evidence for outcomes investigated by this meta-analysis.¹⁵⁴ The GRADE for each outcome was classified as high quality, moderate quality, low quality, or very low quality. Results from studies designed as randomized controlled trials began as high-quality evidence, whereas observational studies began as low-quality evidence. Quality of evidence decreased due to: (1) study limitations; (2) inconsistency of results; (3) indirectness of evidence; (4) imprecision; or (5) reporting bias. Quality of evidence was upgraded if there was: (1) a large magnitude of effect; (2) a dose-response gradient; or if (3) plausible biases would decrease the magnitude of an apparent treatment effect. Data extraction (J.L. and D.M.) and assessment of study quality (J.L. and C.L.) were performed independently by two investigators. Discrepancies were solved through consultation with a third reviewer (G.W.).

3.4.4 Data synthesis and analysis

The meta-analyses and meta-regression analyses were performed using Review Manager (RevMan 5.3; Cochrane Collaboration, Oxford, UK) and Comprehensive Meta-Analysis software (Comprehensive Meta-Analysis 2.2.064; Biostat, Englewood NJ, USA), respectively. The primary outcomes were the SMD in endothelium-dependent and endothelium-independent vascular reactivity between acute hyperglycemic and normoglycemic states. The SMD summary statistic allowed for standardization of values obtained using different methodologies into uniform scales to complete the meta-analyses.¹⁵⁵ Each SMD was weighted according to the inverse variance method and they were pooled with a random-effects model.¹⁵¹ A negative SMD corresponded to decreased vascular reactivity in the acute hyperglycemic state compared to the normoglycemic state.

Heterogeneity between studies was assessed using the chi-squared test for heterogeneity and the I^2 statistic. In case of considerable heterogeneity ($I^2 > 75\%$) after subgroup analyses, the adequacy of pooling study estimates was determined in light of the consistency of the direction and size of the intervention effect.¹⁵⁵ In addition, pre-defined potential moderating factors (*n*, age, gender, body mass index, systolic blood pressure, diastolic blood pressure, fasting insulin, fasting glucose, HbA_{1c} %, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, length of time for post-occlusion interval of vascular assessment, methodological quality score, year of publication) influencing the SMD in endothelium-dependent vascular reactivity were evaluated ad-hoc by subgroup and meta-regression analyses. In all meta-regression models, studies were weighted by the inverse variance of the dependent variable. Potential moderating factors were individually entered as independent variables in regression models with the SMD in endothelium-dependent vascular reactivity as the dependent variable. Publication or other biases were evaluated by funnel plots, the Begg and Mazumdar's rank correlation test, and Egger's regression test.¹⁵⁶ A *P*-value of less than 0.05 was considered statistically significant.

3.5 Results

3.5.1 Study selection and characteristics

A flow-chart of study selection is shown in Figure 3-1. The systematic search resulted in the inclusion of 39 from 394 potential articles.^{157–195} Fourteen of these articles reported vascular data for multiple subgroups of a given or diverse health status; thus, they were assessed as individual studies.^{157,160–163,166,172,173,180,187,191–194} The main characteristics and clinical data for these studies are shown in Table 3-1 and Table 3-2, respectively. Three potentially relevant studies were not available for full text reading and, thus, could not be included.^{196–198} All studies assessed endothelium-dependent and endothelium-independent vascular reactivity during acute hyperglycemia and normoglycemia in a total of 1065 individuals classified as healthy (n = 525), obese (n = 72), or having impaired glucose tolerance (n = 104), T2D (n = 229), hypertension (n = 94), MetS (n = 30), or type 1 diabetes (n = 11).

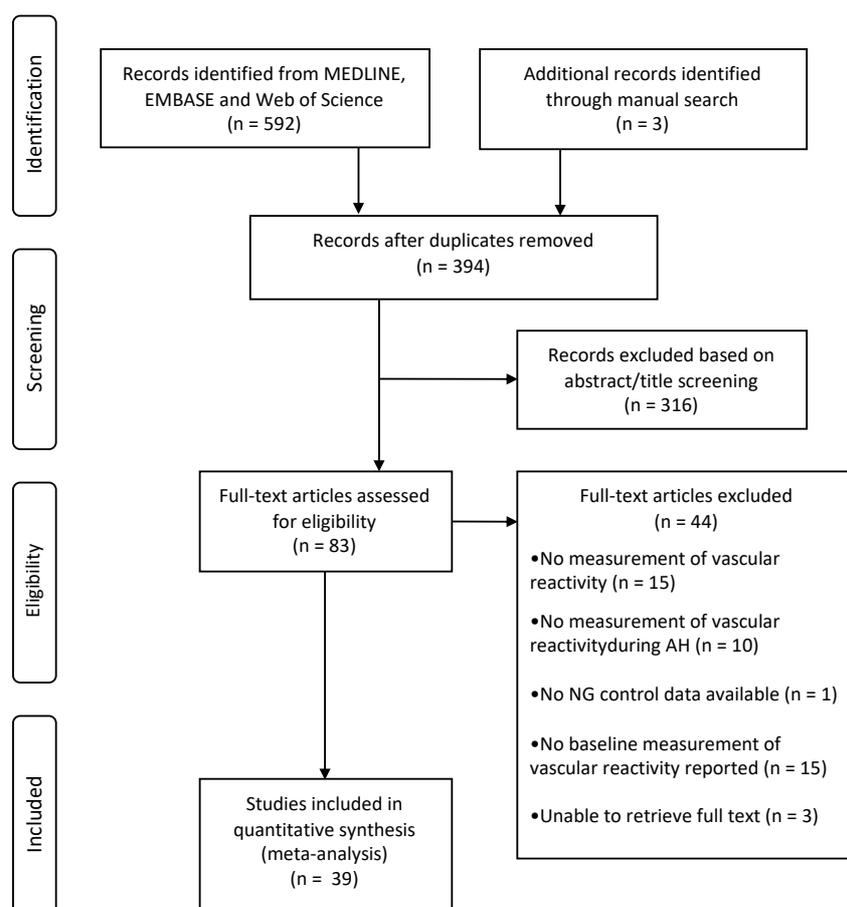


Figure 3-1. Flow Diagram of the study selection process. AH, acute hyperglycemia; NG, normoglycemia.

Table 3-1. The main characteristics of the studies included in the meta-analysis

Study, year of publication	Study design	Health status	Medication	Quality	Vascular region	Inducing AH		EDVR during AH	EIVR during AH
				Score (0 – 12)		Method	Dose		
Grasser et al, ¹⁶⁹ 2014	RCT	Healthy	None	9	Micro	Energy drink	Sucrose/glucose 39.1g	↑ ACh	NA
Nakayama et al, ¹⁷⁸ 2013	OBS	Healthy	None	6	Macro	Sugar drink	Maltose 75g	↓ FMD	NA
Mah et al, ¹⁷⁷ 2013	RCT	Healthy	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Wang et al A, ¹⁸⁷ 2013	RCT	Healthy	None	9	Macro	OGTT	Glucose 75g	↓ FMD	NA
Zhang et al A, ¹⁹⁴ 2013	RCT	Healthy	None	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
De Marchi et al, ¹⁶⁵ 2012	RCT	Healthy	None	8	Micro	OGTT	Glucose 75g	↓ ACh	↔ SNP
Grassi et al, ¹⁷⁰ 2012	RCT	Healthy	None	9	Macro	OGTT	Glucose 75g	↓ FMD	NA
Suzuki et al, ¹⁸⁴ 2012	OBS	Healthy	None	8	Macro	OGTT	Glucose 75g	↓ FMD	NA
Ceriello et al A, ¹⁶³ 2011	RCT	Healthy	None	6	Macro	OGTT	Glucose 75g	↓ FMD	NA
Mah et al, ¹⁷⁶ 2011	RCT	Healthy	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Watanabe et al, ¹⁸⁸ 2011	OBS	Healthy	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Baynard et al A, ¹⁶⁰ 2009	OBS	Healthy	3 statin, 1 hydrochlorothiazide, 1 angiotensin II receptor blocker, 1 angiotensin converting enzyme inhibitor	9	Macro	Test meal	Carbohydrate 80g	↓ FMD	NA
Ceriello et al (1) A, ¹⁶² 2008	OBS	Healthy	None	8	Macro	IV infusion	Glucose 15mmol/L	↓ FMD	NA
Ceriello et al (2) A, ¹⁶¹ 2008	RCT	Healthy	None	8	Macro	IV infusion	Glucose 15mmol/L	↓ FMD	NA
Natali et al A, ¹⁸⁰ 2008	OBS	Healthy	Unknown	11	Micro	OGTT	Glucose 75g	↔ ACh	↓ SNP
Weiss et al, ¹⁸⁹ 2008	RCT	Healthy	None	10	Macro	Candy/sugar drink	Carbohydrate 101g	↓ FMD	NA
Xiang et al (1) A, ¹⁹² 2008	RCT	Healthy	None	11	Macro	OGTT	Glucose 75g	↔ FMD	NA
Xiang et al (2) A, ¹⁹¹ 2008	OBS	Healthy	None	11	Macro	OGTT	Glucose 75g	↓ FMD	↔ NMD
Xiang et al (2) B, ¹⁹¹ 2008	OBS	Healthy	None	11	Macro	OGTT	Glucose 75g	↔ FMD	↔ NMD
Dengel et al A, ¹⁶⁶ 2007	OBS	Healthy	None	11	Macro	OGTT	Glucose 75g	↑ FMD	NA

Zhu et al, ¹⁹⁵ 2007	RCT	Healthy	None	7	Macro	OGTT	Glucose 75g	↓ FMD	NA
Arora et al A, ¹⁵⁷ 2006	OBS	Healthy	None	10	Micro	OGTT	Glucose 75g	↓ PORH	NA
Fujimoto et al, ¹⁶⁸ 2006	OBS	Healthy	None	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
Tushuizen et al, ¹⁸⁶ 2006	RCT	Healthy	None	11	Macro	Test meal	Carbohydrate 55g	↓ FMD	NA
Napoli et al, ¹⁷⁹ 2004	RCT	Healthy	None	6	Micro	Test meal	Carbohydrate 60g	↔ ACh	↔ SNP
Siafarikas et al, ¹⁸² 2004	RCT	Healthy	None	9	Macro	OGTT	Glucose 75g	↔ FMD	NA
Ihlemann et al, ¹⁷¹ 2003	RCT	Healthy	None	7	Micro	OGTT	Glucose 75g	↓ Serotonin	↓ SNP
Bagg et al, ¹⁵⁸ 2000	RCT	Healthy	None	9	Macro	IV infusion	Dextrose 10% 238mL	↔ FMD	NA
Title et al, ¹⁸⁵ 2000	RCT	Healthy	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Kawano et al A, ¹⁷³ 1999	OBS	Healthy	None	10	Macro	OGTT	Glucose 75g	↔ FMD	NA
Williams et al, ¹⁹⁰ 1998	OBS	Healthy	None	10	Micro	IV infusion	Glucose 16.7mmol/L	↔	NA
Metacholine									
Lavi et al, ¹⁷⁵ 2009	RCT	Obese	None	8	Macro	Sugar drink	Glucose 50g	↓ FMD	NA
Dengel et al B, ¹⁶⁶ 2007	OBS	Obese	None	11	Macro	OGTT	Glucose 75g	↑ FMD	NA
Wang et al B, ¹⁸⁷ 2013	RCT	IGT	None	9	Macro	OGTT	Glucose 75g	↓ FMD	NA
Natali et al B, ¹⁸⁰ 2008	OBS	IGT	Unknown	11	Micro	OGTT	Glucose 75g	↔ ACh	↓ SNP
Xiang et al (1) B, ¹⁹² 2008	RCT	IGT	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Arora et al B, ¹⁵⁷ 2006	OBS	IGT	None	10	Micro	OGTT	Glucose 75g	↓ PORH	NA
Kawano et al B, ¹⁷³ 1999	OBS	IGT	None	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
Wang et al C, ¹⁸⁷ 2013	RCT	T2DM	None	9	Macro	OGTT	Glucose 75g	↓ FMD	NA
Ceriello et al B, ¹⁶³ 2011	RCT	T2DM	6 metformin discontinued 4 weeks prior 5 excluded angiotensin converting enzyme inhibitor	6	Macro	OGTT	Glucose 75g	↓ FMD	NA
Chittari et al, ¹⁶⁴ 2011	OBS	T2DM	17 oral agents	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
Kato et al A, ¹⁷² 2010	RCT	T2DM	None	9	Macro	Cookie	Carbohydrate 75g	↓ FMD	NA
Kato et al B, ¹⁷² 2010	RCT	T2DM	None	9	Macro	Cookie	Carbohydrate 75g	↓ FMD	NA
Kato et al C, ¹⁷² 2010	RCT	T2DM	None	9	Macro	Cookie	Carbohydrate 75g	↓ FMD	NA
Ceriello et al (1) B, ¹⁶² 2008	OBS	T2DM	None	8	Macro	IV infusion	Glucose 15mmol/L	↓ FMD	NA
Ceriello et al (2) B, ¹⁶¹ 2008	RCT	T2DM	None	8	Macro	IV infusion	Glucose 15mmol/L	↓ FMD	NA
Ceriello et al (2) C, ¹⁶¹ 2008	RCT	T2DM	None	8	Macro	IV infusion	Glucose 10mmol/L	↓ FMD	NA

Ceriello et al (2) D, ¹⁶¹ 2008	RCT	T2DM	None	8	Macro	IV infusion	Glucose 10mmol/L	↓ FMD	NA
Natali et al C, ¹⁸⁰ 2008	OBS	T2DM	Unknown	11	Micro	OGTT	Glucose 75g	↔ ACh	↓ SNP
Stirban et al, ¹⁸³ 2006	RCT	T2DM	13 insulin, 11 aspirin, 9 ACE inhibitors, 1 angiotensin receptor blockers, 6 hydroxymethylglutaryl-CoA inhibitors, 5 Beta-blockers, 5 diuretics, 3 calcium channel blockers	11	Macro	Test meal	Carbohydrate 48g	↓ FMD	NA
Kim et al, ¹⁷⁴ 2003	OBS	T2DM	None	9	Micro	IV infusion	Glucose 12/mg/kg/min	↓ PORH	NA
Kawano et al C, ¹⁷³ 1999	OBS	T2DM	None	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
Shige et al, ¹⁸¹ 1999	OBS	T2DM	None	8	Macro	Test meal	Sucrose 75g	↓ FMD	↔ NMD
Zhang et al B, ¹⁹⁴ 2013	RCT	Hypertensive	None	10	Macro	OGTT	Glucose 75g	↓ FMD	NA
Zhang et al A, ¹⁹³ 2012	RCT	Hypertensive	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Zhang et al B, ¹⁹³ 2012	RCT	Hypertensive	None	11	Macro	OGTT	Glucose 75g	↓ FMD	NA
Ballard et al, ¹⁵⁹ 2013	RCT	MetS	None	11	Macro	Rice milk	Mixed sugars 23g	↓ FMD	NA
Baynard et al B, ¹⁶⁰ 2009	OBS	MetS	3 metformin, 2 sulfonylurea, 4 Statin	11	Macro	Test meal	Carbohydrate 80g	↓ FMD	NA
Dye et al, ¹⁶⁷ 2012	OBS	T1DM	Insulin monotherapy	9	Micro	IV infusion	Dextrose 200mg/dL	↓ PORH	NA

Some studies presented multiple health groups comparing normoglycemia and acute hyperglycemia vascular function and were therefore evaluated as individual studies (distinguished by A, B, C, or D). Authors who published multiple studies in a single year were distinguished by (1) or (2). ↓, indicates significant decrease of vascular function during acute hyperglycemia compared to normoglycemia; ↔, no significant difference in vascular function between acute hyperglycemia and normoglycemia; ↑, significant increase of vascular function during acute hyperglycemia compared to normoglycemia; ACE, angiotensin-converting enzyme; ACh, acetylcholine; AH, acute hyperglycemia; EDVR, endothelium-dependent vascular reactivity; EIVR, endothelium-independent vascular reactivity; FMD, flow-mediated dilation; IGT, impaired glucose tolerance; IV, intravenous; MetS, metabolic syndrome; NA, vascular data not available; NMD, nitroglycerin-mediated dilation; OBS, observational study; OGTT, oral glucose tolerance test; PORH, post-occlusive reactive hyperemia; RCT, randomized controlled trial; SNP, sodium nitroprusside; T1DM, type 1 diabetes mellitus; and T2DM, type 2 diabetes mellitus.

Table 3-2. Clinical data for each study included in this meta-analysis

Study, year of publication	Health status	n (% female)	Age	BMI	Blood pressure (mmHg)			Fasting plasma	Fasting plasma	HbA _{1c}	Cholesterol (mmol/l)			Triglycerides
			(years)	(kg/m ²)	SBP	DBP	MAP	insulin (pmol/l)	glucose (mmol/l)	(%)	Total	HDL	LDL	(mmol/l)
Grasser et al, ¹⁶⁹ 2014	Healthy	25 (48)	22.5±3	23.3±10	114±10	87±5	96±7	NA	NA	NA	NA	NA	NA	NA
Nakayama et al, ¹⁷⁸ 2013	Healthy	23 (0)	44±10	23±2.1	111±8	71±6	84±7	NA	5.1±0.4	NA	NA	NA	NA	NA
Mah et al, ¹⁷⁷ 2013	Healthy	16 (0)	21.8±3.2	24.8±NA	117±4	79±4	92±4	142.5±94.4	5.3±0.4	NA	3.7±0.8	NA	NA	3.3±5
Wang et al A, ¹⁸⁷ 2013	Healthy	33 (64)	51.36±7.15	24.76±3.6	NA	NA	NA	NA	5.4±0.5	6±0.3	5.5±0.6	1.4±0.4	3.7±0.6	1.5±0.5
Zhang et al A, ¹⁹⁴ 2013	Healthy	31 (48)	47.87±10.95	23.9±2.1	124±8	79±5	94±6	NA	4.8±1.1	NA	4.7±0.4	1.4±0.3	2.9±0.4	1.3±0.3
De Marchi et al, ¹⁶⁵ 2012	Healthy	34 (50)	32.4±3.5	19±3	119±4	79±9	92±7	34.8±6.6	5.1±0.6	NA	4.3±0.7	1.5±0.2	NA	1.5±0.1
Grassi et al, ¹⁷⁰ 2012	Healthy	12 (58)	28±2.7	23.2±4.2	NA	NA	NA	NA	4.2±0.4	NA	NA	1.5±0.4	2.5±0.4	0.7±0.3
Suzuki et al, ¹⁸⁴ 2012	Healthy	14 (57)	33.4±11.9	20.7±2.3	106±9	64±6	78±7	NA	4.8±0.6	5.4±0.3	5±0.6	1.7±0.4	2.7±0.5	0.8±0.4
Ceriello et al A, ¹⁶³ 2011	Healthy	12 (50)	50.5±8.66	28.5±10.7	117±19	78±8	91±11	73.4±15.2	4.5±1.4	4.8±0.7	4.5±2.1	1.4±0.7	2.5±1	0.9±0.7
Mah et al, ¹⁷⁶ 2011	Healthy	16 (0)	21.6±3.2	28.7±NA	117±4	79±4	92±4	147±96	5.3±0.4	NA	3.6±0.7	NA	NA	NA
Watanabe et al, ¹⁸⁸ 2011	Healthy	14 (43)	33.4±11.9	20.7±2.3	106±9	64±6	78±7	29.7±11.9	4.7±0.4	NA	5±0.6	1.7±0.4	2.7±0.5	0.8±0.4
Baynard et al A, ¹⁶⁹ 2009	Healthy	10 (NA)	53±3.32	32.7±3.5	117±13	72±3	87±6	NA	4.6±0.3	5.2±0.4	5.2±1	1.3±0.3	3.2±0.95	1±0.3
Ceriello et al (1) A, ¹⁶² 2008	Healthy	22 (45)	50.5±11.73	28.5±14.5	117±26	78±10	91±15	NA	4.5±1.4	4.8±0.9	4.5±2.8	1.4±0.9	2.5±1.4	0.9±0.9
Ceriello et al (2) A, ¹⁶¹ 2008	Healthy	10 (40)	50.3±8.22	27.5±9.8	115±14	76±11	89±12	NA	4.5±1	4.8±0.6	4.8±1.9	1.4±1.6	2.4±1.3	0.9±1.6
Natali et al A, ¹⁸⁰ 2008	Healthy	20 (70)	49±8.94	27.9±4	129±13	78±9	95±10	NA	5.3±NA	5.6±0.5	4.9±0.9	1.2±0.2	3.3±0.8	1.1±0.5
Weiss et al, ¹⁸⁹ 2008	Healthy	13 (62)	48±17	24±2.2	114±14	66±7	82±10	NA	5.5±1.4	NA	NA	NA	NA	NA
Xiang et al (1) A, ¹⁹² 2008	Healthy	26 (46)	50±6	24.2±2.3	114±7	72±6	86±7	NA	4.6±0.5	NA	4.4±0.9	1.2±0.1	1.9±0.7	1.4±1.2
Xiang et al (2) A, ¹⁹¹ 2008	Healthy	17 (41)	39±12.37	24.1±6.6	115±20	68±12	84±15	NA	5.1±1.7	5.1±0.4	4.7±2.7	1.2±0.7	1.9±2.4	1.4±3.8
Xiang et al (2) B, ¹⁹¹ 2008	Healthy	15 (47)	40±11.62	23.7±7.4	111±20	24±7	53±12	NA	4.6±1.9	4.8±0.8	4.5±1.9	1.2±0.7	1.9±1.8	1.4±3
Dengel et al A, ¹⁶⁶ 2007	Healthy	15 (53)	11.3±1.55	17.5±1.9	110±12	59±8	76±9	41.2±15.1	4.8±0.3	NA	3.8±0.8	1.2±0.3	2.3±0.6	0.8±0.4
Zhu et al, ¹⁹⁵ 2007	Healthy	11 (0)	22.6±2.3	22.5±1.5	113±7	79±6	90±6	NA	5.2±0.2	NA	4±0.8	1.4±0.2	2.1±0.6	0.9±0.3
Arora et al A, ¹⁵⁷ 2006	Healthy	10 (0)	27±N/A	22.4±NA	122±NA	68±NA	86±NA	NA	4.8±NA	NA	4.1±NA	1.8±NA	2.2±NA	1.1±NA
Fujimoto et al, ¹⁶⁸ 2006	Healthy	10 (0)	30±2	NA	111±12	65±8	80±9	NA	5.1±0.6	NA	4.3±0.7	1.2±0.3	NA	1.2±0.7
Tushuizen et al, ¹⁸⁶ 2006	Healthy	17 (0)	25.4±3	23.6±1.8	116±8	75±7	89±7	33±10	4.8±0.3	5.1±0.2	4±0.6	1.4±0.2	2.2±0.6	0.8±0.3
Napoli et al, ¹⁷⁹ 2004	Healthy	10 (40)	23±3.16	23.6±1.9	124±6	60±3	81±4	NA	5±NA	NA	NA	NA	NA	NA
Siafarikas et al, ¹⁸² 2004	Healthy	32 (66)	19.1±1.7	22.9±4.2	NA	NA	NA	NA	NA	5±0.3	4±0.8	1.3±0.4	2.2±0.7	1.2±0.7
Ihlemann et al, ¹⁷¹ 2003	Healthy	10 (40)	53±6.96	22.7±1.9	144±17	75±11	98±13	NA	NA	5.2±0.3	5.2±0.6	1.7±0.3	3.2±0.6	0.8±0.3
Bagg et al, ¹⁵⁸ 2000	Healthy	10 (20)	26±6	22±2	111±10	65±8	80±9	34.2±11.4	5.2±0.3	NA	4.7±1	NA	NA	NA
Title et al, ¹⁸⁵ 2000	Healthy	10 (40)	25.5±3.1	24±3	118±8	72±7	87±7	NA	5.3±0.7	NA	5.1±1.1	1.3±0.1	3.3±0.9	1.2±0.5
Kawano et al A, ¹⁷³ 1999	Healthy	17 (35)	52.6±7.42	NA	NA	NA	NA	51.6±9.9	5±0.3	NA	4.9±0.4	1.2±0.1	3±0.4	1.5±0.2
Williams et al, ¹⁹⁰ 1998	Healthy	10 (30)	33±6.32	NA	NA	NA	NA	NA	3.9±1.2	3.6±0.6	4.2±0.7	1.1±0.2	2.6±0.7	1±0.4
Lavi et al, ¹⁷⁵ 2009	Obese	56 (0)	47.9±5.8	32.1±4.3	134±13	82±6	99±8	NA	5.4±0.2	NA	5.1±0.7	1.1±0.2	3.2±0.7	1.7±0.9
Dengel et al B, ¹⁶⁶ 2007	Obese	16 (56)	10.1±1.6	19.3±6.4	120±12	65±8	83±9	61.6±33.2	4.8±0.2	NA	4.2±0.6	1.1±0.2	2.6±0.5	1±0.6
Wang et al B, ¹⁸⁷ 2013	IGT	33 (64)	52.88±9.2	27.8±3.1	NA	NA	NA	NA	6.1±0.5	6.5±0	5.5±1	1.2±0.3	3.1±0.8	1.8±0.9
Natali et al B, ¹⁸⁰ 2008	IGT	16 (63)	52±20	29.5±4.8	122±12	79±8	93±9	NA	5.7±NA	5.9±1.2	5.6±1.4	1.3±0.5	3.7±1.3	1.3±0.6
Xiang et al (1) B, ¹⁹² 2008	IGT	21 (48)	51±6	24.8±3.1	110±8	72±6	85±7	NA	5.9±0.9	NA	5.2±1.1	1.2±0.2	2.3±0.7	2±1.1
Arora et al B, ¹⁵⁷ 2006	IGT	10 (0)	65±N/A	23.2±NA	134±NA	72±NA	93±NA	NA	5.3±NA	NA	4.3±NA	1.2±NA	2.1±NA	2.6±NA
Kawano et al B, ¹⁷³ 1999	IGT	24 (38)	58.5±7.84	NA	NA	NA	NA	66±11.8	5.8±0.8	NA	5.3±1	1.1±0.1	3.4±0.5	1.7±0.2
Wang et al C, ¹⁸⁷ 2013	T2DM	43 (42)	53.4±8.99	25.7±2.9	NA	NA	NA	NA	7.5±1.2	7.4±0.1	5.8±1.7	1.2±0.3	3.2±0.7	2.2±1.2
Ceriello et al B, ¹⁶³ 2011	T2DM	16 (44)	51.3±10.4	29.5±13.2	123±26	80±14	95±18	107.3±15.2	7.8±8.8	8.4±1.2	5.1±3.2	1.2±0.3	2.6±0.4	1.2±1.6
Chittari et al, ¹⁶⁴ 2011	T2DM	21 (43)	46.4±9.62	30.1±5	NA	76±8	NA	NA	7.8±1.8	7.8±1.4	4.5±0.9	1.2±0.5	2.5±0.9	1.6±0.5
Kato et al A, ¹⁷² 2010	T2DM	10 (50)	68±7.7	26.8±3.2	144±29	89±19	107±22	45±17.4	6.1±0.8	5.8±0.6	5.5±0.4	1.4±0.3	3.5±1.3	1.5±0.1
Kato et al B, ¹⁷² 2010	T2DM	10 (30)	67.6±6.2	25.8±2.5	141±26	88±23	106±24	57.6±33.6	6.1±1	6±0.3	5.2±0.7	1.5±0.3	3.7±1.8	1.3±0.2

Kato et al C, ¹⁷² 2010	T2DM	10 (30)	67.8±8.6	25.8±3.3	141±29	88±12	106±17	39.6±24.6	6.6±1	6.1±0.6	5.3±0.7	1.4±0.3	3.9±2	1.4±0.2
Ceriello et al (1) B, ¹⁶² 2008	T2DM	27 (48)	51.3±13.51	29.5±17.2	123±33	80±19	95±24	NA	7.8±11.4	7.7±1.6	5.1±4.2	1.2±0.3	2.6±0.4	1.2±1.6
Ceriello et al (2) B, ¹⁶¹ 2008	T2DM	10 (50)	50.3±6.96	27.5±10.1	118±17	77±12	91±14	NA	6.8±7	7.3±1	5±2.5	1.3±0.2	2±0.5	1±1.9
Ceriello et al (2) C, ¹⁶¹ 2008	T2DM	10 (50)	50.2±14.23	28.4±13	121±7	79±9	93±8	NA	7.7±1.3	7.9±1.6	5±2.2	1.2±0.3	2.7±0.4	1.3±0.95
Ceriello et al (2) D, ¹⁶¹ 2008	T2DM	10 (60)	51±17.71	28.6±11.7	122±11	80±12	94±12	NA	6±1	7.7±1.9	5.1±2.9	1.2±0.3	2.6±0.8	1.2±2.2
Natali et al C, ¹⁸⁰ 2008	T2DM	17 (71)	58±8.25	29.3±4.1	139±17	81±8	100±11	NA	8.3±NA	7.2±2.1	4.9±1.2	1±0.3	3.29±1.1	1.5±0.4
Stirban et al, ¹⁸³ 2006	T2DM	13 (NA)	56.9±10.1	30.3±3.2	136±22	79±13	98±16	NA	NA	8.5±1.8	NA	NA	NA	NA
Kim et al, ¹⁷⁴ 2003	T2DM	8 (50)	55.9±3.7	25.4±3.2	134±7	82±6	99±7	NA	7.2±2.6	6.6±0.8	5.5±0.3	1.3±0.2	NA	2.1±0.6
Kawano et al C, ¹⁷³ 1999	T2DM	17 (29)	62.2±4.95	NA	NA	NA	NA	84±22.3	7.1±0.3	NA	5.6±0.4	1.1±0.1	3.7±0.4	1.9±0.2
Shige et al, ¹⁸¹ 1999	T2DM	7 (29)	49.3±8	26±5	128±9	72±9	91±9	47.4±16.8	7.1±1.3	NA	4.9±13	1±0.2	NA	1.6±0.6
Zhang et al B, ¹⁹⁴ 2013	Hypertensive	34 (50)	47.44±10.96	24.7±3.5	156±9	96±8	116±8	NA	4.7±0.6	NA	4.7±0.5	1.3±0.2	3±0.4	1.3±0.3
Zhang et al A, ¹⁹³ 2012	Hypertensive	26 (50)	47.92±8.05	24.6±2.6	160±8	97±6	118±7	NA	5.3±0.5	NA	4.7±0.6	1.4±0.3	2.6±0.4	1.4±0.2
Zhang et al B, ¹⁹³ 2012	Hypertensive	34 (47)	49.29±8	24.8±2.5	159±9	99±6	119±7	NA	5.2±0.6	NA	4.9±0.5	1.3±0.3	2.7±0.5	1.3±0.3
Ballard et al, ¹⁵⁹ 2013	MetS	19 (26)	28.5±9.59	35±3.9	125±11	85±8	98±8	55±25.7	6±0.9	NA	4.7±0.9	1±0.1	2.8±0.2	1.9±0.9
Baynard et al B, ¹⁶⁰ 2009	MetS	11 (NA)	52±3.16	34.4±5	123±7	78±3	93±4	NA	6.1±1.3	5.9±1.1	5.1±1	1±NA	3.1±0.7	2.4±1.3
Dye et al, ¹⁶⁷ 2012	T1DM	11 (36)	14.5±3.32	21.5±9.6	105±7	61±10	76±9	NA	NA	8.3±1.2	NA	NA	NA	NA

Data are expressed as mean ± standard deviation or *n*. Some studies presented multiple health groups comparing normoglycemia and acute hyperglycemia vascular function and were therefore evaluated as individual studies (distinguished by A, B, C, or D). Authors who published multiple studies in a single year were distinguished by (1) or (2). BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; LDL, low-density lipoprotein; MAP, mean arterial pressure; MetS, metabolic syndrome; NA, data not available; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; and SBP, systolic blood pressure.

3.5.2 Quality assessment and potential bias

The quality of the studies was moderate-to-high. The mean score was 9.4 ± 1.5 of a possible 12 points (Table 3-1). The quality of evidence for outcomes demonstrating the effect of acute hyperglycemia on vascular reactivity was low-to-moderate (Table 3-3). As for the evaluation of potential bias, the funnel plot (Figure 3-2), Begg and Mazumdar's rank correlation test, and the Egger's regression test suggested the presence of publication bias or other biases for the SMD in endothelium-dependent vascular reactivity in the studies included in the meta-analysis ($P < 0.01$ and $P < 0.01$, respectively). There was no evidence of publication or other biases when assessing the SMD in endothelium-independent vascular reactivity in the studies included in the meta-analysis.

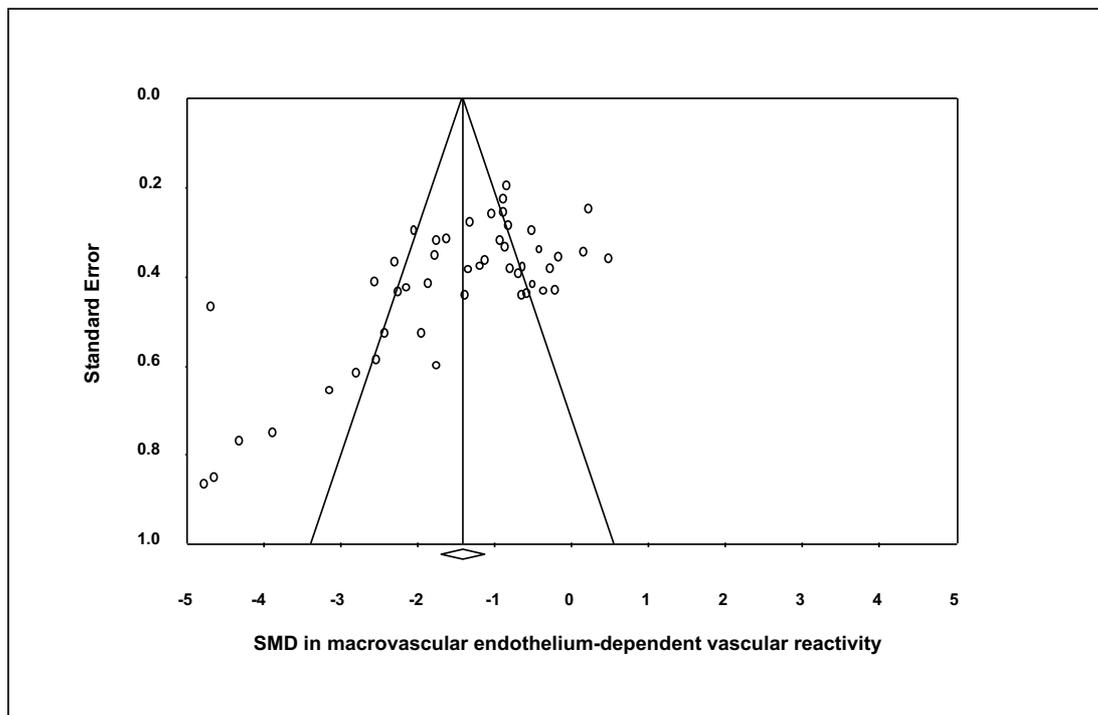


Figure 3-2. Funnel plot of the standardized mean difference (SMD) in macrovascular endothelial function in studies included in the meta-analysis. Funnel plot asymmetry: $P = 0.0002$ and $P = 0.00005$ according to Begg and Mazumdar's rank correlation test and Egger's test, respectively.

Table 3-3. Effect of acute hyperglycemia on vascular reactivity in healthy and cardiometabolic populations: Quality of Evidence

Outcome Among Participants	Design (No. of Studies)	Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations ^a	Quality of Evidence (GRADE)
Decreased EDVR	RCT (23)	Serious†	Not serious	Not serious	Not serious	Publication bias likely¶	Low
Decreased EDVR	OBS (16)	Not serious	Not serious	Not serious	Not serious	None	Low
Decreased macro- EDVR	RCT (19)	Serious†	Not serious	Not serious	Not serious	Publication bias likely¶	Low
Decreased macro- EDVR	OBS (11)	Not serious	Not serious	Not serious	Not serious	None	Low
Preserved micro- EDVR	RCT (4)	Very serious†	Serious§	Not serious	Very serious✚	Publication bias likely¶	Very low
Preserved micro- EDVR	OBS (5)	Serious≠	Not serious	Not serious	Serious✚	None	Very low
Preserved EIVR	RCT (3)	Serious†	Not serious	Not serious	Not serious	None	Moderate
Preserved EIVR	OBS (3)	Not serious	Not serious	Not serious	Not serious	None	Low

EDVR indicates endothelium-dependent vascular reactivity; endothelium-independent vascular reactivity; GRADE, Grading of Recommendations Assessment, Development and Evaluation; OBS, observational study; and RCT, randomized controlled trial.

*Large magnitude of effect, dose-response, plausible biases decreasing the magnitude of effect, publication bias.

†Method for allocation of concealment and participant/assessor blinding unclear or not performed. Incomplete outcome data and selective reporting when assessing endothelium-dependent vascular reactivity, macrovascular endothelium-dependent vascular reactivity and microvascular endothelium-dependent vascular reactivity or endothelium-independent vascular reactivity during acute hyperglycemia.

≠No control population included, failure to adequately control for confounding, and incomplete follow up when assessing microvascular endothelial function.

§Large I^2 and point estimates vary widely across studies assessing microvascular endothelial function suggesting benefit, harm and no effect of acute hyperglycemia.

✚The 95 % confidence interval of the pooled risk ratio includes both positive and negative effects of acute hyperglycemia.

¶Publication bias is strongly suspected due to the presence of asymmetry in funnel plots for randomized control trials assessing endothelial, macrovascular endothelial, and microvascular endothelial function during hyperglycemia.

3.5.3 Endothelial function

After data pooling, endothelium-dependent vascular reactivity was significantly decreased during acute hyperglycemia when compared with normoglycemia (39 studies; n = 1065; SMD, -1.25; 95% CI, -1.52 to -0.98; $P < 0.01$; Figure 3-3). There was no difference between health groups in the SMD in endothelium-dependent vascular reactivity ($P = 0.13$), but significant heterogeneity was detected ($I^2 = 87\%$, $P < 0.01$). Subgroup analysis of the SMD in endothelium-dependent vascular reactivity revealed that macrovascular endothelium-dependent reactivity was significantly decreased during acute hyperglycemia when compared with normoglycemia (30 studies; n = 884; SMD, -1.40; 95% CI, -1.68 to -1.12; $P < 0.01$; Figure 3-4), whereas no significant decrease was found in the studies that assessed microvascular endothelium-dependent reactivity (9 studies; n = 181; SMD, -0.63; 95% CI, -1.36 to 0.11; $P = 0.09$; Figure 3-5). Heterogeneity was detected in the SMD in endothelium-dependent reactivity for both macrovascular ($I^2 = 84\%$, $P < 0.01$) and microvascular reactivity studies ($I^2 = 90\%$, $P < 0.01$). Of note, the heterogeneity in microvascular endothelium-dependent reactivity was primarily explained (~40 %) by a single study;¹⁶⁵ and the exclusion of such study did not significantly alter the pooled effect size (8 studies; n = 147; SMD, -0.18; 95% CI, -0.53 to 0.17; $P = 0.30$).

3.5.4 Endothelium-independent vascular reactivity

After data pooling, endothelium-independent vascular reactivity was preserved during acute hyperglycemia versus normoglycemia (6 studies; n = 144; SMD, -0.07; 95% CI, -0.30 to 0.16; $P = 0.55$; Figure 3-6). There was no significant difference between health groups in the SMD in endothelium-independent vascular reactivity ($P = 0.49$) and no heterogeneity was observed ($I^2 = 0\%$, $P = 0.85$). Due to a limited availability of data, it was not possible to analyse endothelium-independent reactivity in the macrocirculation and microcirculation separately.

3.5.5 Meta-regression analyses

The SMD in macrovascular endothelium-dependent reactivity was inversely associated with age ($\beta = -0.03$; $P < 0.01$), systolic blood pressure ($\beta = -0.04$; $P < 0.01$), diastolic blood pressure ($\beta = -0.05$; $P < 0.01$), mean arterial pressure ($\beta = -0.05$; $P < 0.01$) and LDL cholesterol ($\beta = -0.93$; $P < 0.01$; Figure 3-7). In turn, it was positively associated with the post-occlusion interval of vascular assessment ($\beta = 0.36$; $P = 0.01$).

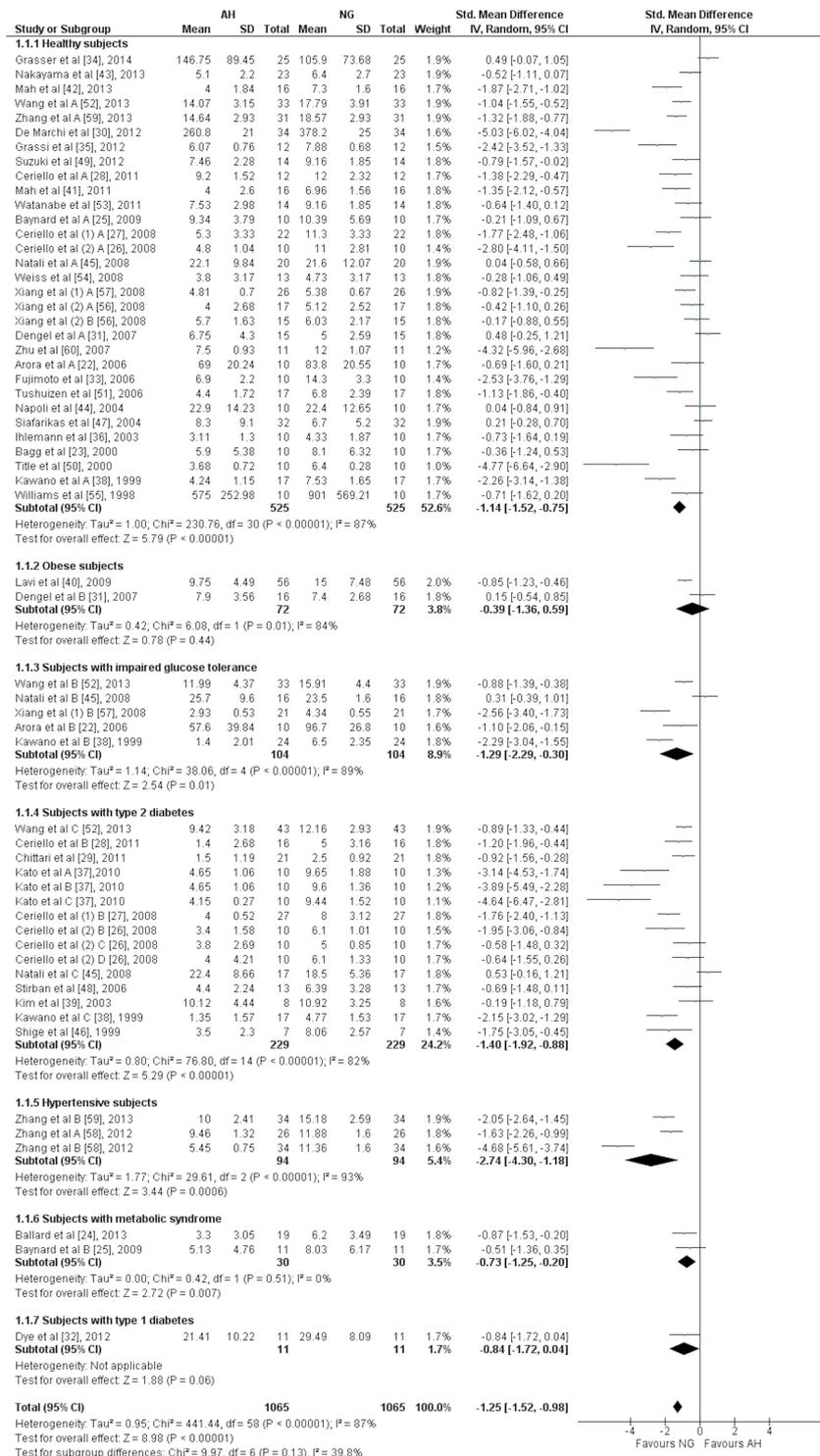


Figure 3-3. Forest plot of the standardized mean difference (SMD) in endothelium-dependent vascular reactivity between acute hyperglycemic and normoglycemic states for all included health groups. Squares represent the SMD in endothelium-dependent vascular reactivity of each study. The diamond represents the pooled SMD in endothelium-dependent vascular reactivity by health group and overall. Some studies presented multiple subgroups according to health status; thus, they were evaluated as individual studies (distinguished by A, B, C or D). Authors who published multiple studies in a single year had studies distinguished by numerical values (1 and 2). AH indicates acute hyperglycemia; CI, confidence interval; IV, inverse variance; and NG, normoglycemia.

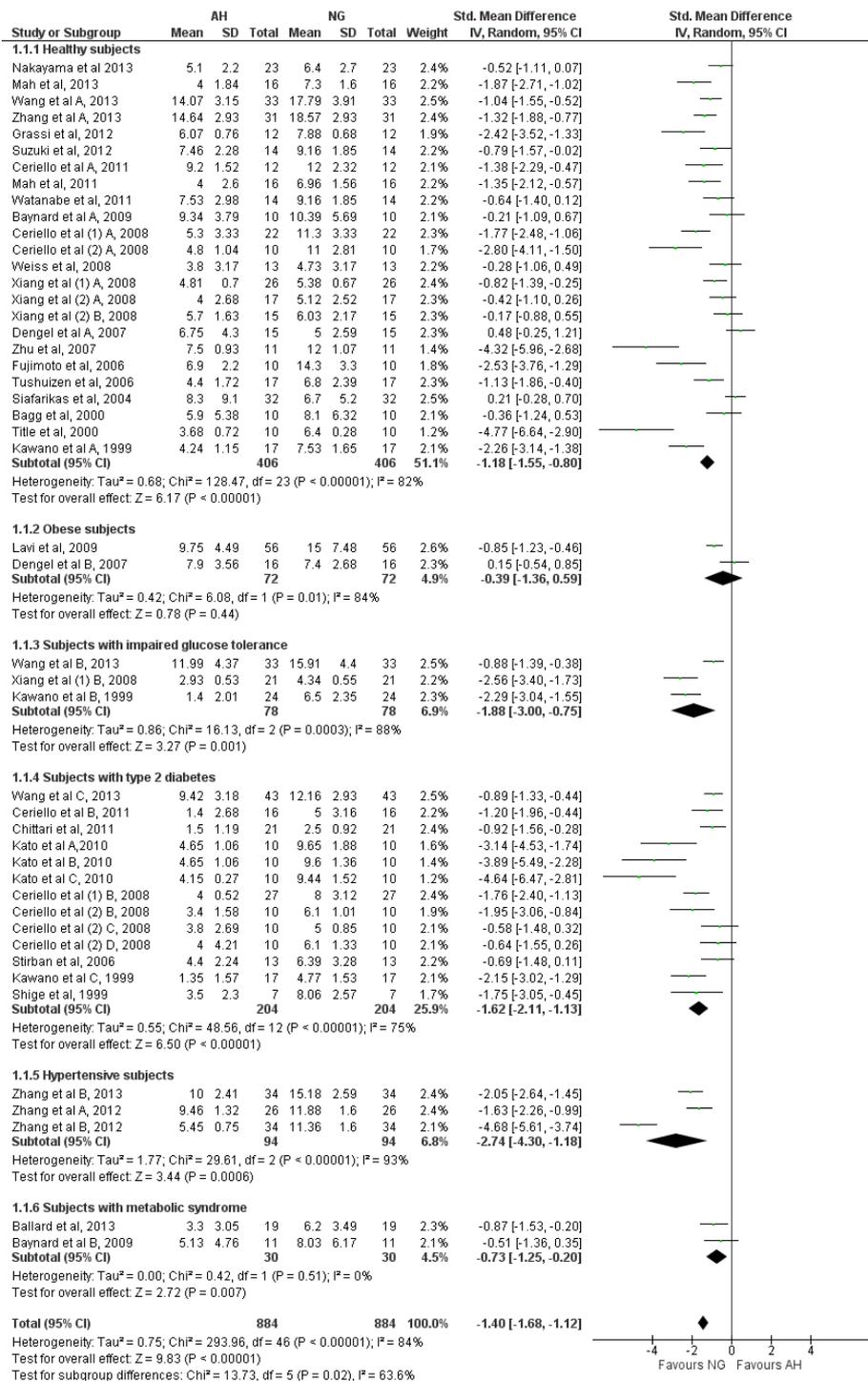


Figure 3-4. Forest plot of the standardized mean difference (SMD) in macrovascular endothelium-dependent reactivity between acute hyperglycemic and normoglycemic states for all included health groups. Squares represent the SMD in endothelium-dependent vascular reactivity of each study. The diamond represents the pooled SMD in endothelium-dependent vascular reactivity by health group and overall. Some studies presented multiple subgroups according to health status; thus, they were evaluated as individual studies (distinguished by A, B, or C). Authors who published multiple studies in a single year had studies distinguished by numerical values (1 and 2). AH, acute hyperglycemia; CI, confidence interval; IV, inverse variance; and NG, normoglycemia.

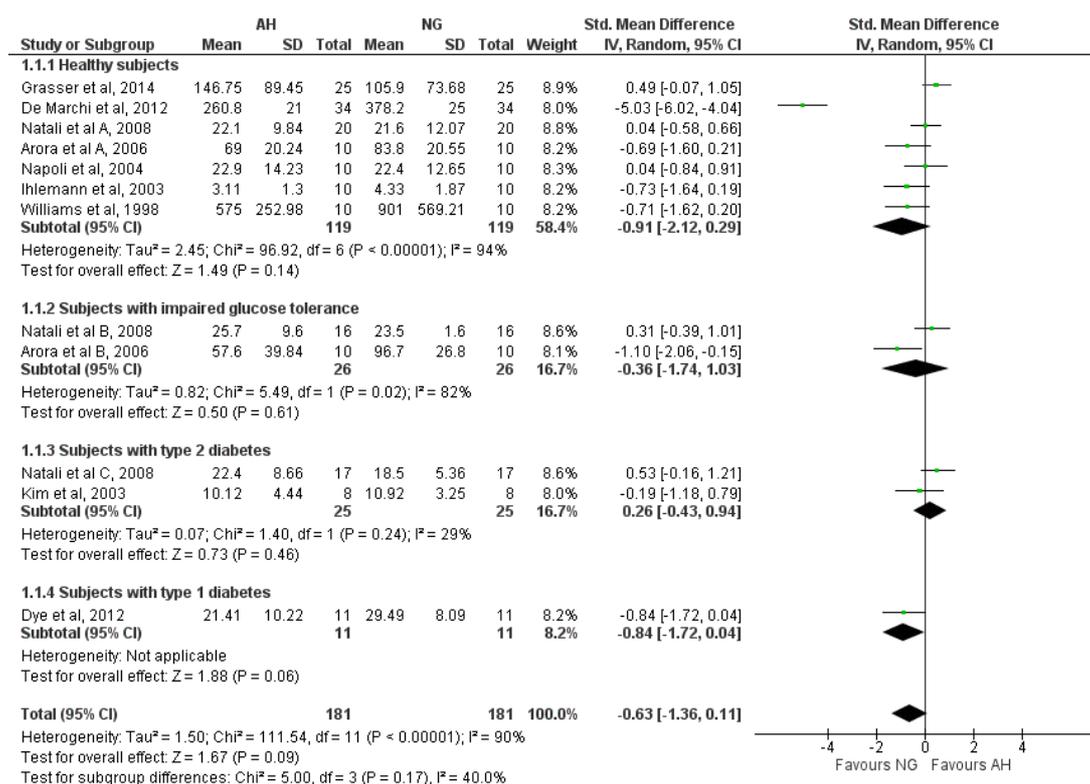


Figure 3-5. Forest plot of the standardized mean difference (SMD) in microvascular endothelium-dependent reactivity between acute hyperglycemic and normoglycemic states for all included health groups. Squares represent the SMD in endothelium-dependent vascular reactivity of each study. The diamond represents the pooled SMD in microvascular endothelium-dependent vascular reactivity by health group and overall. Some studies presented multiple subgroups according to health status; thus, they were evaluated as individual studies (distinguished by A, B, or C). Authors who published multiple studies in a single year had studies distinguished by numerical values (1 and 2). AH, acute hyperglycemia; CI, confidence interval; IV, inverse variance; and NG, normoglycemia.

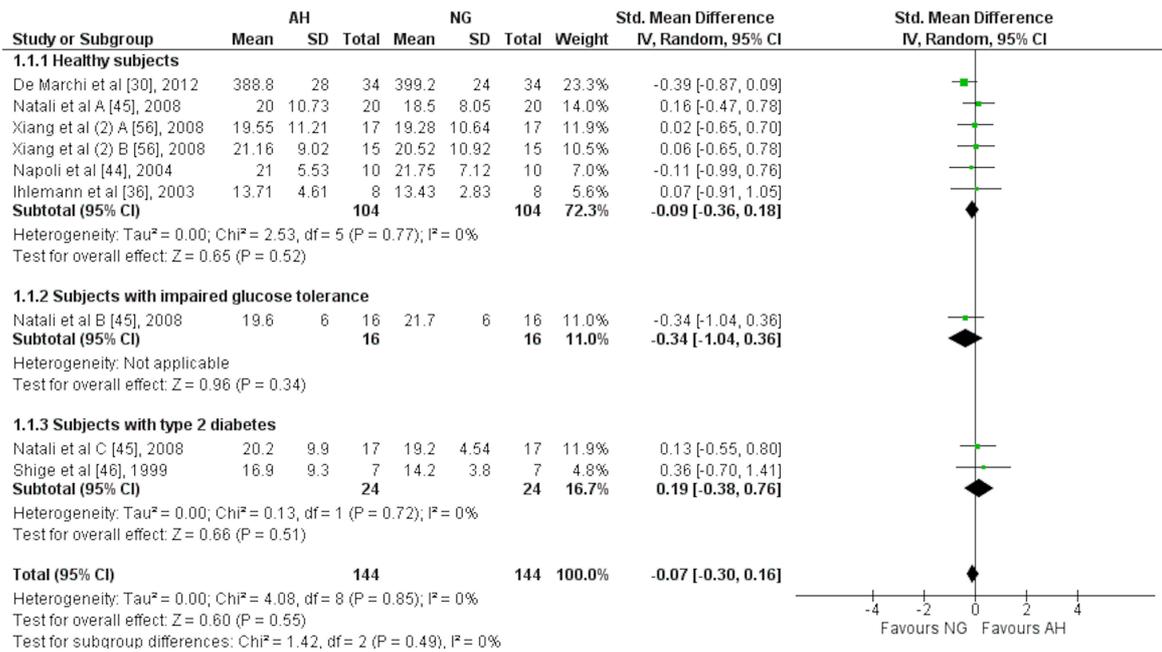


Figure 3-6. Forest plot of the standardized mean difference (SMD) in endothelium-independent vascular reactivity between acute hyperglycemic and normoglycemic states for all included health groups. Squares represent the SMD in endothelium-independent vascular reactivity of each study. The diamond represents the pooled SMD in endothelium-independent vascular reactivity by health group and overall. Some studies presented multiple subgroups according to health status; thus, they were evaluated as individual studies (distinguished by A, B, or C). Authors who published multiple studies in a single year had studies distinguished by numerical values (1 and 2). AH, acute hyperglycemia; CI, confidence interval; IV, inverse variance; and NG, normoglycemia.

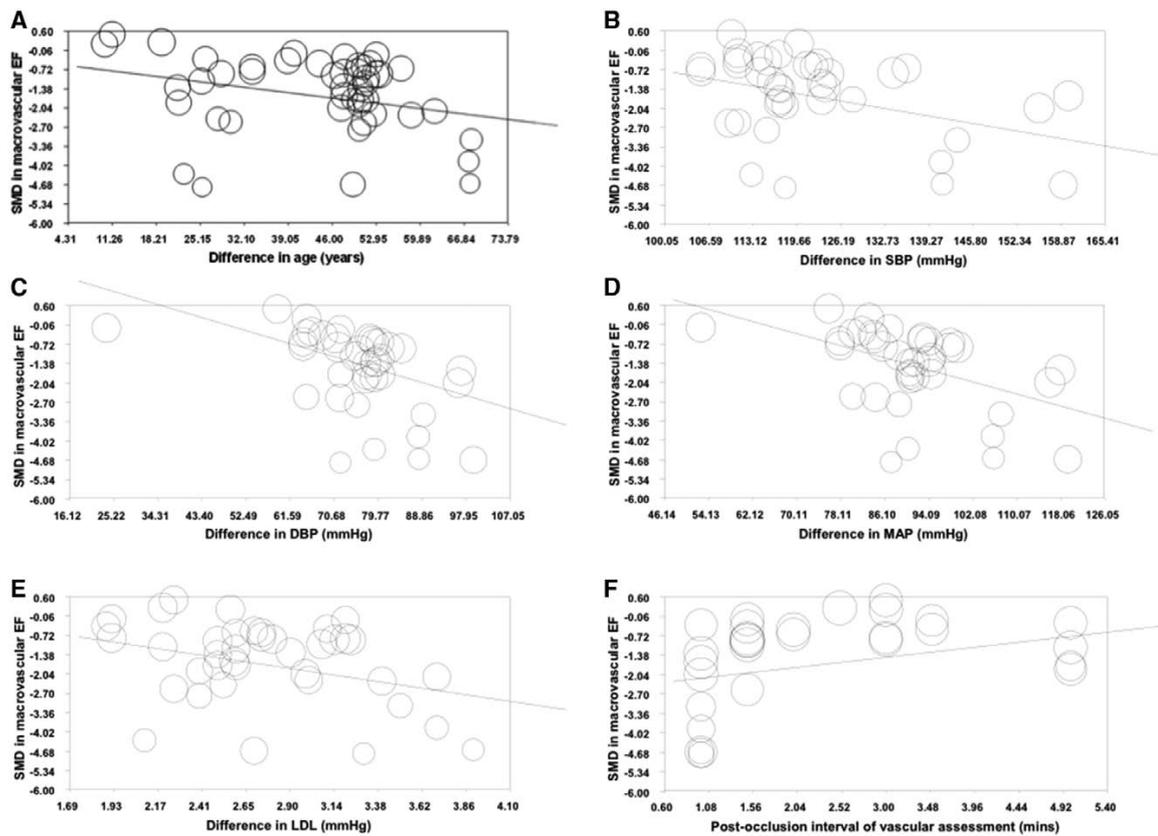


Figure 3-7. Meta-regression plots of the standardized mean difference (SMD) in macrovascular endothelium-dependent reactivity according to the difference in **(A)** age ($\beta=-0.03$, $P=0.005$), **(B)** systolic blood pressure (SBP; $\beta=-0.04$, $P=0.0004$), **(C)** diastolic blood pressure (DBP; $\beta=-0.05$, $P<0.00001$), **(D)** mean arterial pressure (MAP; $\beta=-0.05$, $P=0.00001$), **(E)** low-density lipoprotein cholesterol (LDL; $\beta=-0.93$, $P=0.005$) and **(F)** post-occlusion interval of vascular assessment ($\beta=0.36$, $P=0.01$). The size of each circle is proportional to the study's weight.

3.6 Discussion

To our knowledge, this is the first systematic review and meta-analysis to assess the effect of acute hyperglycemia on vascular function. Data from 39 studies assessing endothelium-dependent vascular reactivity alone or in combination with tests of endothelium-independent vascular reactivity during acute hyperglycemia in healthy and cardiometabolic diseased individuals were pooled and analysed. The meta-analysis provided evidence that the average effect of acute hyperglycemia on endothelial function is impaired function, whereas endothelium-independent vascular reactivity was preserved in healthy and diseased individuals. Due to evidence of heterogeneity, the interpretation of the pooled effects of hyperglycemia on endothelium-dependent vascular reactivity should be made cautiously. Considerable heterogeneity was identified for most subgroups suggesting the variability across studies was due to not only sampling variability, but also differences in treatment effect within each study.¹⁹⁹ Nevertheless, the large effects sizes and 95 % CIs consistently favoured normoglycemia, providing evidence of treatment effect.¹⁹⁹ Exploration of heterogeneity with meta-regression indicated that the variability across studies could be explained by differences in age, blood pressure, LDL cholesterol and the post-occlusion interval of vascular assessment.

Currently, there is no consensus on the effect of acute hyperglycemia on endothelium-dependent vascular reactivity and endothelium-independent vascular reactivity as studies assessing vascular reactivity during acute hyperglycemia have presented confounding results. This meta-analysis demonstrated evidence of macrovascular endothelial dysfunction during acute hyperglycemia in healthy people, as well as in patients with cardiometabolic disease, suggesting that the pathogenesis of CVD may begin, among others, with acute hyperglycemia-mediated transient decreases in endothelial function long before the onset of morbidities such as obesity, hypertension, or T2D. Interestingly, the inverse relationship between macrovascular endothelial function and several traditional cardiovascular risk factors demonstrates the degree to which acute hyperglycemia mediates macrovascular endothelial dysfunction correlates with increases in age, blood pressure, or LDL cholesterol levels. This is consistent with previous research revealing that elderly, hypertensive and subjects with dyslipidemia

all exhibited significantly impaired endothelium-dependent vasodilation at rest compared with healthy populations,^{200–202} indicating that any existing macrovascular endothelial dysfunction may therefore be compounded by an acute hyperglycemic stress. The fact that microvascular endothelial dysfunction during acute hyperglycemia was not detected contradicts previously published data that associates decreased microvascular function with incident T2D and suggests a role for microvascular dysfunction in the pathogenesis of T2D.²⁰³ Although macrovascular endothelial function was affected in healthy populations and in those with cardiometabolic disease, in this meta-analysis, endothelium-independent vascular reactivity remained preserved during acute hyperglycemia when compared to normoglycemia. In contradiction to previous findings in animal and *in vitro* studies, which found VSM impairment mediated by VSM cell proliferation may occur in as little as 6 hours.^{148,187}

Macrovascular endothelial dysfunction observed during acute hyperglycemia by methods assessing endothelium-dependent vascular reactivity primarily implicate decreased NO bioavailability as a central mechanism of endothelial dysfunction in healthy and cardiometabolic diseased populations.⁴⁸ This may be attributed to acute hyperglycemia increasing oxidative stress and its role in disrupting pathways of NO synthesis.²⁴ The fact that even healthy people exhibited impaired macrovascular endothelial function during acute hyperglycemia demonstrates how acutely NO bioavailability may be affected by excess sugar consumption. The magnitude of macrovascular endothelial dysfunction induced by acute hyperglycemia may be compounded when cardiovascular risk factors such as increased age, blood pressure or LDL cholesterol are present. This may be partly due to the fact that health groups exhibiting these clinical markers demonstrate decreased NO bioavailability and therefore impaired endothelial function, even at rest.^{200–202} Although NO is the predominant vasodilator in macrocirculation, it has been demonstrated to have significantly less influence in the microcirculatory system.²⁰⁴ The increased influence of other chemical mediators of vasodilation such as endothelial derived hyperpolarizing factor and prostaglandin I₂,²⁰⁵ may, however, explain why microvascular endothelium-dependent vascular reactivity remained preserved during acute hyperglycemia. Consideration should also be given to the spatial variability associated with the techniques used in several of the studies using skin microcirculation as a model of assessing microvascular reactivity.²⁰⁶ The large spatial variability in single-point LDF

for example, may have limited findings.⁶⁷ Despite this, final conclusions on the effect of acute hyperglycemia on microvascular reactivity should not be made due to the limited availability of microcirculation data. Furthermore, it must be acknowledged that shear stress was not considered when performing analyses of FMD data in many studies. Shear stress, which is responsible for inducing synthesis of the NO release that causes FMD, is dependent on variability of the hyperemic blood flow response in the microcirculation.²⁰⁷ Therefore, macrovascular endothelial dysfunction observed during acute hyperglycemia may partially be mediated by a decrease in shear stress stimulus reflecting microvascular dysfunction.²⁰⁷ The fact that endothelium-independent vascular reactivity was preserved during acute hyperglycemia indicates that endothelial dysfunction precedes impairment to endothelium-independent vascular reactivity, further supporting its role as a primary mechanism of CVD pathogenesis. Although disruptions in NO bioavailability mediated by acute hyperglycemia and the resulting endothelial dysfunction may initially be transient, if repeated often enough, may lead to cumulative adverse outcomes, including proinflammatory responses and VSM cell proliferation.^{24,54} It has been suggested that acute hyperglycemia may induce VSM cell proliferation by disrupting VSM cell apoptosis, which is a key mechanism to prevent increased neointimal formation and stenosis.²⁵ Moreover, decreased NO delivery by the endothelium to the VSM, may contribute to VSM cell proliferation through increased periods of higher vasoconstrictive tone.⁵⁴ Ultimately, VSM cell proliferation may signal the beginning of a detectable and significant impairment in endothelium-independent vascular reactivity, representing a critical event in vascular remodelling and the development of CVD.²⁰⁸

Given that CVD is the single leading cause of death, accounting for 30 % of the annual global mortality rate,²⁰⁹ and that even healthy populations are subject to vascular dysfunction during acute hyperglycemia, there is a clear need to further investigate the effects of acute hyperglycemia on the underlying mechanisms of vascular function, *in vivo*. Due to a surge in added sugar consumption in recent decades, predominantly in the form of SSB,¹³⁶ humans are more often in a state of acute hyperglycemia and therefore are more frequently inducing endothelial dysfunction. Considering this, future research should quantify what frequency and dosage of sugar consumption mediate atherosclerotic vascular changes in healthy populations and in those with cardiometabolic diseases. Previously, certain ethnicities have demonstrated decreased

vascular reactivity at rest compared with white subjects.²¹⁰ Whether this exacerbates any vascular dysfunction mediated by acute hyperglycemia is still unknown and requires further research. To provide more comprehensive conclusions as to how microvascular and microvascular function are affected by acute hyperglycemia, future research should consider shear stress as a covariate of conduit artery FMD data during statistical analyses.^{34,58} Furthermore, noting that vascular reactivity is not entirely mediated by NO,²⁴ future research may also investigate the effect of consuming SSB on numerous mechanisms of vasodilation (e.g. NO, endothelium derived hyperpolarizing factor, prostaglandin I₂) and vasoconstriction (e.g. ET-1), the influence of which vary from the microcirculation to the macrocirculation.

There are a number of inherent limitations to our analyses that require comment. As previously discussed, significant heterogeneity was observed among studies that assessed endothelium-dependent vascular reactivity. Studies published in languages other than English were not included and the quality of evidence for outcomes assessed in this meta-analysis was low-to-moderate. Sub-analyses of microcirculatory and endothelium-independent data were limited due to the low number of studies assessing microvascular reactivity and endothelium-independent vascular reactivity in normoglycemic and acute hyperglycemic states. Furthermore, some studies used methods of assessing the microcirculation that can be easily influenced by spatial variability and thus may limit results when assessing microvascular function. The risk of publication or other biases were detected when assessing the SMD in endothelial function. However, the quality of studies was evaluated by specific tools for the quality assessment of observational research,^{151,153} revealing a predominantly low bias risk. It must be acknowledged that the ethnicity of the populations was poorly reported by studies included in this meta-analysis and, thus, conclusions on the effect of ethnicity cannot be drawn from these data. Finally, many studies using FMD as a method of assessing macrovascular endothelial function did not report shear stress. Therefore, it was not possible to comprehensively conclude whether macrovascular endothelial dysfunction found during acute hyperglycemia is due to intrinsic abnormalities of macrovascular endothelial function or if it is partially attributable to microvascular dysfunction and decreased stimulus for conduit artery dilation.⁵⁸

In conclusion, based on studies included in this meta-analysis, current evidence suggests that acute hyperglycemia decreases macrovascular endothelial function with no changes in microvascular endothelial function and systemic endothelium-independent vascular reactivity across healthy and cardiometabolic diseased populations. This further supports endothelial dysfunction mediated by decreased NO availability as a primary mechanism in the pathogenesis of CVD, which may begin long before vascular remodelling is detectable or the onset of cardiometabolic diseases. Noting that microvascular data were limited, the microcirculatory system should therefore not be dismissed as a possible site of vascular dysfunction. Considering this, future studies should investigate the effects of consuming SSB on the underlying mechanisms of human vascular reactivity at microvascular and macrovascular levels. These studies will provide a better understanding of how acute hyperglycemia induces vascular dysfunction and how it contributes to the pathogenesis of CVD from healthy populations to those with cardiometabolic diseases.

Chapter 4 – Study two: Effects of sugar-sweetened beverage consumption on microvascular and macrovascular function in a healthy population

Publication statement:

This chapter is comprised of a manuscript that is published in *Arteriosclerosis, Thrombosis and Vascular Biology*.

Loader J*, Meziat C*, Watts R, Lorenzen C, Sigaud-Roussel D, Stewart S, Reboul C, Meyer G, Walther G. Effects of sugar-sweetened beverage consumption on microvascular and macrovascular function in a healthy population. *Arter Thromb Vasc Biol*. 2017;37(6):1250-60. Doi: 10.1161/ATVBAHA.116.308010.

*Both authors contributed to this work. It must also be acknowledged that the experimental models of this study were primarily executed by Cindy Meziat; and, thus, some of the data included in Chapter 4 has been previously published in her Doctoral Thesis. However, as first author of this study, I had significant involvement in the production of all aspects of this manuscript, including those of an experimental nature.

4.1 Linking paragraph

Study one of this research program provided evidence that acute hyperglycemia, induced by excess sugar consumption, transiently impairs endothelial function in the macrocirculation, but not in the microcirculation; while systemic endothelium-independent vascular reactivity remains unaffected. This effect was consistent in those with cardiometabolic diseases and in those considered healthy, suggesting that acute hyperglycemia induces endothelial dysfunction across the spectrum of cardiometabolic health; and, subsequently, that it has a significant role in the initial and ongoing development of chronic vascular dysfunction, obesity, MetS and T2D. However, study one summarized that this evidence, describing the effect of acute hyperglycemia on endothelial function, was inconclusive due to the fact that previous research had inadequately reported macrovascular data. Indeed, changes in FMD were not adjusted for possible differences in shear stress between assessments and, therefore, it was not possible to confirm whether the acute hyperglycemia-mediated dysfunction was local to the macrocirculation or if was simply due to a lack of stimulus for vasodilation, possibly caused by an impairment in microvascular endothelial function. Additionally, the availability of microvascular data was limited, suggesting that further research was needed. Considering this, study two aimed to clarify the vascular impact of acute hyperglycemia by examining the effect of commercial SSB consumption, the predominant source of sugar in our diets, on endothelium-dependent vascular reactivity and endothelium-independent vascular reactivity in the microcirculation and macrocirculation.

4.2 Abstract

Objective

To assess vascular function during acute hyperglycemia induced by consuming commercial SSB and its effect on underlying mechanisms of the NO pathway.

Approach and results

In a randomized, single-blind, crossover trial, twelve healthy male participants consumed 600 mL (20 oz.) of water or a commercial SSB across two visits. Endothelial function and endothelium-independent vascular reactivity were assessed in the microcirculation using LSCI coupled with iontophoresis and in the macrocirculation using brachial artery ultrasound with FMD and NMD. Compared to water, SSB consumption impaired microvascular and macrovascular endothelium-dependent reactivity as indicated by a decrease in the vascular response to acetylcholine iontophoresis (208.3 ± 24.3 vs. 144.2 ± 15.7 %, $P < 0.01$) and reduced FMD (0.019 ± 0.002 vs. 0.014 ± 0.002 %/s⁻¹, $P < 0.01$), respectively. Systemic endothelium-independent vascular reactivity remained preserved. Similar decreases in endothelial function were observed during acute hyperglycemia in an *in vivo* rat model. However, function was fully restored by treatment with the antioxidants, N-acetylcysteine and apocynin. Additionally, *ex vivo* experiments revealed that while the production of ROS was increased during acute hyperglycemia, the bioavailability of NO in the endothelium was decreased, despite no change in the activation state of eNOS.

Conclusions

To our knowledge, this is the first study to assess the vascular effects of acute hyperglycemia induced by commercial SSB consumption alone. These findings suggest that SSB-mediated endothelial dysfunction is partly due to increased oxidative stress that decreases NO bioavailability.

4.3 Introduction

Commercial SSB are one of the most frequently consumed drinks worldwide.^{136,211} As such, they represent a major source of added sugar in the modern diet. Habitual consumption of SSB induces frequent episodes of acute hyperglycemia and is linked to an increased risk of developing obesity, MetS, and T2D.^{12,211,212} Furthermore, excessive SSB consumption is also associated with a higher incidence of advanced forms of CVD,²¹¹ which remains the single leading cause of death representing 31 % of the global mortality rate.²⁰⁹ Impaired vascular reactivity is considered to be a main precursor to the pathogenesis of CVD and may be present long before atherosclerotic vascular changes occur.¹⁴⁵

Normal vascular function involves a continuous interaction between the endothelium and VSM that is regulated by numerous vasodilators and vasoconstrictors.^{205,213} In a recent systematic review and meta-analysis, our research group provided evidence that acute hyperglycemia induced by an oral sugar load transiently impairs endothelial function in not only patients with cardiometabolic disease, but also in those considered healthy.²¹⁴ It has been suggested that such endothelial dysfunction may be attributed to increased oxidative stress mediated by acute hyperglycemia.^{24,176} Indeed, NO bioavailability in the vascular wall is highly sensitive to redox modulation of the cellular environment.^{215,216} However, due to a limited availability of microcirculatory data and discrepant reporting of macrovascular data, the impact of acute hyperglycemia on vascular reactivity remains inconclusive and the underlying mechanisms not fully understood.

In this context and considering the surge in commercial SSB consumption, as well as its potential link to a sustained epidemic of CVD, this present study aimed to provide a comprehensive assessment of the effect of SSB-mediated acute hyperglycemia on microvascular and macrovascular reactivity in a healthy population. Additionally, considering that experimental exploration of healthy vascular tissue in humans is difficult to perform, due to ethical barriers, this study also aimed to further investigate the underlying mechanisms of the interaction between acute hyperglycemia and endothelial function via an *in vivo* and *ex vivo* rat model.

4.4 Methods

This study was comprised of two separate protocols in order to comprehensively investigate the effect of acute hyperglycemia induced by consuming SSB on vascular function. Protocol one was a clinical study conducted at the Australian Catholic University, Melbourne, which assessed the effect of SSB consumption on microvascular and macrovascular reactivity in healthy humans. Protocol two was conducted at Avignon University, France, providing deeper exploration into the effect of acute hyperglycemia on underlying mechanisms of vascular function through *in vivo* and *ex vivo* experimental rat models.

4.5 Protocol One - Clinical Study

4.5.1 Participants and screening

Healthy non-smoking, sedentary (<2 hours/week of exercise) males (n = 12) aged 18-55 years were recruited. Participants were excluded if they had any history of CVD; a 5 % weight gain or loss in the 6 months prior to beginning the protocol; or if they were currently using or being treated with any vasoactive medications. All participants provided informed written consent. The Human Research Ethics Committee at the Australian Catholic University, Australia, approved this protocol. This study was registered with the Australian New Zealand Clinical Trial Registry: #ACTRN12614000614695.

4.5.2 Study design

Following overnight fasting (≥ 10 hours), participants presented to the cardiovascular laboratory on two occasions to perform this randomized, single-blind, crossover trial. To standardize responses between trials, participants were instructed to maintain their current level of physical activity and to abstain from strenuous exercise for 48 hours, alcohol consumption for 24 hours, and caffeine consumption in the 12 hours prior to each trial. Clinical measurements were performed at the beginning of the first trial to collect anthropometric and hemodynamic data; and to assess fasting blood glucose concentrations. At visit one, participants were randomized using an online sequence generator,²¹⁷ by a researcher not involved in data collection or data analyses (J.L.), to

consume either 600 mL (20 oz.) of water or 600 mL of a commercial SSB from an opaque container. At visit two, the participants returned to consume 600 mL of the beverage type that was not consumed at visit one. Participants were allowed five minutes to consume each beverage. Blood glucose concentrations, blood pressure, heart rate and vascular function were assessed throughout each visit (Figure 4-1). A 600 mL volume of each beverage was administered, as it represents the common volume of SSB commercially available for purchase by the public.

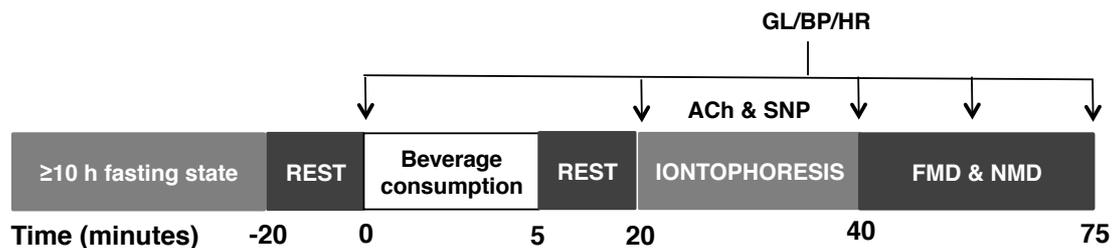


Figure 4-1. Study design: sequence of testing. In a fasted state (≥ 10 h) participants rested for 20 minutes before consuming 600 mL (20 oz.) of water or sugar-sweetened beverage (SSB) within a 5 minute period. Participants then rested for a further 15 minutes before iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) were performed. Immediately following the conclusion of iontophoresis, flow-mediated dilation (FMD) and nitrate-mediated dilation (NMD) were also performed. Blood glucose (GL), blood pressure (BP), and heart rate (HR) were measured after the 20 minutes resting period, 15 minutes after consuming the test beverage, at the end of iontophoresis, at the end of FMD and at the end of NMD. All 12 participants were administered water or the commercial SSB in a randomized order.

4.5.3 Clinical measurements

Height and mass were measured using a stadiometer and a calibrated scale, respectively. Body mass index was calculated as mass (kg) divided by height² (m). Blood glucose concentrations were assessed with a handheld blood glucose monitoring system (Freestyle Optium, Abbott Diabetes Care Ltd, UK). Systolic and diastolic blood pressure, and heart rate were measured using a digital sphygmomanometer (Dinamap, GE Medical Systems, Milwaukee, USA). Blood pressure variables were expressed as mean arterial pressure (mmHg) calculated by $[(2 \times \text{diastolic blood pressure}) + \text{systolic blood pressure}] / 3$.

4.5.4 Vascular reactivity

Microvascular and macrovascular measurements were conducted in a temperature-controlled room maintained at 22-24°C and proceeded 15 minutes following the consumption of water or the commercial SSB, during the measured time period of acute hyperglycemia. Each participant remained in a supine position from the moment they finished consuming the 600 mL test beverage until measurements of vascular reactivity were complete. Microvascular reactivity was assessed on the ventral surface of the right forearm using iontophoresis in conjunction with LSCI. Assessment of macrovascular reactivity commenced next on the upper right arm using brachial artery ultrasound coupled with FMD and NMD. Blood glucose concentrations and hemodynamic changes were monitored throughout.

4.5.4.1 Assessment of cutaneous microvascular reactivity

Iontophoresis delivers a pharmacologically charged solution to the skin allowing for non-invasive assessment of cutaneous microvascular endothelium-dependent reactivity and endothelium-independent reactivity.²¹⁸ The vascular response to iontophoresis was quantified by LSCI using a 70mW system (PeriCam PSI System[®], Perimed, Järfälla, Sweden) with a laser wavelength of 785 nm and laser head working distance set at 15 cm. The LSCI head unit emits and detects light scattered in the tissue that is partially backscattered by moving blood cells, causing a change in frequency from which microvascular blood flux is calculated.²¹⁹ Laser speckle measurements were recorded continuously at a frequency of 18 Hz using an interfaced computer with data acquisition software (PimSoft 1.2.2.0[®], Perimed, Järfälla, Sweden). Two adhesive drug delivery electrodes (LI 611, Perimed, Järfälla, Sweden) were installed on the ventral surface of the right forearm, avoiding any hair, broken skin, areas of increased skin pigmentation and visible veins. A dispersive electrode (PF 384, Perimed, Järfälla, Sweden) was positioned approximately 15 cm from each drug delivery electrode to complete the electrical current circuit. The arm was then immobilized with a vacuum cushion prior to commencing the protocol to ensure the participant was positioned as recommended. Following two minutes of baseline measurement of basal blood flux, iontophoresis of acetylcholine 2 % dissolved in sodium chloride 0.9 % (saline) and sodium nitroprusside 1 % dissolved in saline were performed to assess cutaneous microvascular endothelium-dependent reactivity and endothelium-independent reactivity, respectively. An anodal current of 0.02 mA for acetylcholine and a cathodal current of

0.02 mA for sodium nitroprusside were administered simultaneously for 200 and 400 seconds, respectively, using a Micropharmacology system (PF 751 PeriIont USB Power Supply, Perimed, Järfälla, Sweden), avoiding nonspecific vasodilation observed with higher cathodal electrical charges.²²⁰ Laser speckle contrast imaging was performed throughout. The manufacture's software was used to set regions of interest at 30 mm² that were adjusted retrospectively to find the area of max blood flux during iontophoresis. Data were exported to Microsoft Excel and analysed off-line. Cutaneous microvascular blood flux values were averaged for the 30 seconds immediately prior to the beginning of iontophoresis for baseline and for the five seconds at the maximal blood flux plateau. Data were reported as blood flux at rest, peak blood flux in response to iontophoresis, and the relative percentage increase in cutaneous blood flux from baseline measurements; and expressed as perfusion units (PU) and cutaneous vascular conductance (CVC), which is the flux in PU divided by the mean arterial pressure (mmHg), to account for differences in blood pressure between tests.³⁸

4.5.4.2 Assessment of macrovascular reactivity

All macrovascular measurements were performed as previously described using high-resolution vascular ultrasonography (Vivid I, GE Medical Systems, Milwaukee, USA), with a 10-MHz multi-frequency probe.²²¹ Briefly, B-mode images and Doppler signals were simultaneously recorded with ECG data. Arterial diameter was measured on B-mode images in the region of the artery running perpendicular to the ultrasound beam. The operator searched for the largest diameter, strong wall signals and the longitudinal section of the artery in each image. Time-averaged mean velocity (cm.s⁻¹) was recorded, at the same level, by pulsed wave Doppler with a 45-60° insonation angle. Measurements were corrected for the insonation angle, and the pulsed Doppler sample volume was adjusted to cover the entire width of the vessel. The high-pass Doppler frequency filter was kept at the lower value ensuring rejection of arterial wall motion artefacts, with a cut-off value usually below 100 Hz. The same, well-trained operator (G.W.) performed all measurements. To assess macrovascular endothelium-dependent reactivity, brachial FMD was performed according to the International Brachial Reactivity Task Force Guidelines.⁵² A pneumatic cuff was placed around the right forearm distal to the elbow and the ultrasound probe was positioned approximately midway between the antecubital and axillary regions before baseline measurements of basal brachial artery diameter were performed. The cuff was then inflated to 250 mmHg

for five minutes before sudden cuff deflation induced reactive hyperemia and measurements of brachial artery diameter were performed again. Fifteen minutes later, baseline measurements of basal brachial artery diameter were repeated before NMD was performed to assess macrovascular endothelium-independent vascular reactivity. As described previously, this procedure involves sublingual delivery of 0.4 mg of glyceryl trinitrate (Nitrolingual® Pumpspray, G. Pohl-Boskamp GmbH & Co, KG, Germany).^{222,223} Data were analysed off-line as the mean of five consecutive measurements using dedicated software (EchoPac 6.0, GE Healthcare, Horten, Norway). Volume blood flow ($\text{mL}\cdot\text{min}^{-1}$) was measured as the mean of the five cardiac cycles with the highest systolic velocity following cuff release and reported for the basal state and during peak reactive hyperemia; and expressed as the percentage change from basal measurements. Shear rate (s^{-1}) was calculated as $(4 \times \text{mean systolic velocity})/\text{mean diameter}$ to estimate the shear stress induced by hyperemia; and was expressed as the change from basal measurements (Peak shear rate minus basal shear rate).²²⁴ Flow-mediated dilation and NMD in response to PORH and nitrate administration, respectively, were expressed as the percentage change in brachial artery diameter from baseline measurements, with and without respect to the change in shear rate.

4.6 Protocol Two - Experimental exploration

4.6.1 Animals

Thirty seven male Wistar rats (12 weeks old, 300 ± 50 g; Laboratoire Janvier, France) were housed in controlled conditions at 23-24°C with a normal 12 hour light/dark cycle, free access to water and a commercial standard diet conforming to current French legislation. The local Research Ethics Committee at Avignon University approved this protocol (84004).

4.6.2 Study design

Each rat was randomized into one of three treatment groups using an online sequence generator prior to *in vivo* assessment of cutaneous microvascular function (Figure 4-2).²¹⁷ Rats designated as normoglycemic controls received an intra-peritoneal injection of a 1.5 mL bolus of saline one hour prior to vascular assessment; while rats designated

to perform a glucose tolerance test were treated with either an intra-peritoneal injection of a 1.5 mL bolus of saline one hour prior to vascular assessment or a 1.5 mL bolus of the antioxidant, N-acetylcysteine, at 50 mg.kg⁻¹ body weight, 48 hours and one hour prior to vascular assessment. These treatments prior to the glucose tolerance test allowed for assessment of the vascular effect of acute hyperglycemia alone, as well as the interaction between oxidative stress and microvascular function during acute hyperglycemia. Following overnight fasting (12-16 hours) and the designated treatment preparations, each rat was inspected to ensure that the skin on the back was hairless and the hind legs were intact. Experiments were performed in a temperature-controlled enclosure set at 34°C to maintain the skin temperature of each rat at 36-37°C. The rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital at 60 mg.kg⁻¹ and were maintained in a prone position for the duration of the experiment.

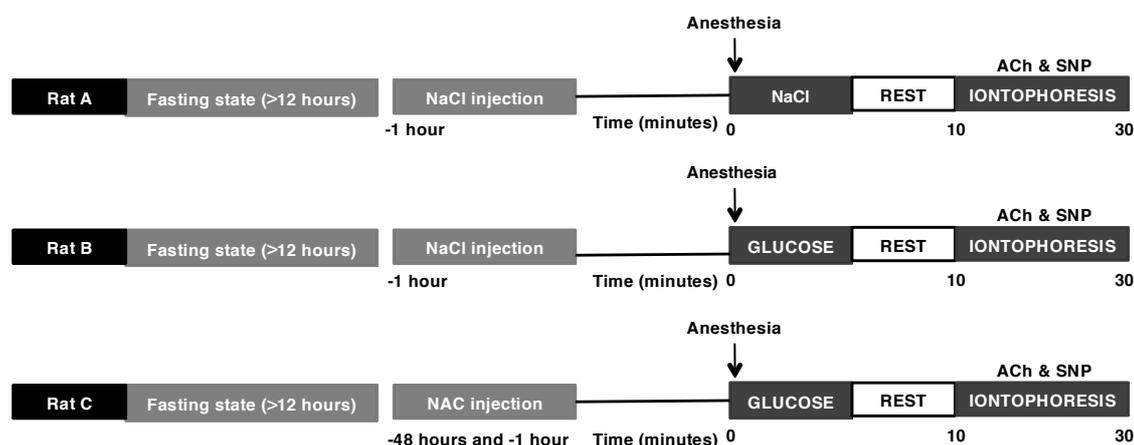


Figure 4-2. Study design of experimental exploration: In a fasted state (≥ 12 hours), following pre-treatments with saline (NaCl) or N-acetylcysteine (NAC), rats were anesthetized using sodium pentobarbital (60mg.kg⁻¹). Rats then received an intra-peritoneal injection of NaCl or glucose (2g/kg) before acclimatizing for 10 minutes in an incubator to maintain skin temperature at 37°C. Iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) were then performed. Rats were randomized into each treatment group.

4.6.3 Glucose tolerance test

Rats designated to receive the glucose tolerance test were administered an intra-peritoneal injection of a 1.5 mL bolus of 40 % glucose solution at 2g.kg⁻¹ body weight. Rats designated as normoglycemic controls received a second intra-peritoneal injection of saline in lieu of the glucose tolerance test. Blood samples were collected from the tail for measurement of blood glucose concentrations at 0, 10, 15, 20, 30, 60, 120

minutes following the induction of acute hyperglycemia or administration of the control (saline) solution.

4.6.4 In vivo vascular exploration

Cutaneous microvascular function was assessed 10 minutes following the induction of acute hyperglycemia or the administration of the control (saline) treatment using a LDF system (Periflux PF5000; Perimed, Järfälla, Sweden) and a thermostatic LDF probe (PF 481; Perimed) with an effective surface area of 0.95 cm²; in conjunction with iontophoresis. Two adhesive drug delivery electrodes containing acetylcholine 2 % dissolved in saline and sodium nitroprusside 2 % dissolved in saline were positioned on the inferior-posterior surface of the right and left leg of each rat. Each adhesive drug delivery electrode was inserted with a LDF probe. Dispersive electrodes were placed on the front paws to complete the electrical current circuit and were connected to a battery powered iontophoresis device (Perilont, Perimed, Järfälla, Sweden). For 60 seconds prior to the commencement of iontophoresis, baseline cutaneous blood flux of the leg was measured using LDF. A single pulse of a 0.1 mA anodal and cathodal current was administered for 20 seconds for acetylcholine and sodium nitroprusside, respectively,²²⁵ followed by a further 20 minutes of LDF measurements. Data was exported to Microsoft Excel and analysed off-line. Cutaneous blood flux values were averaged for the 10 seconds immediately prior to the beginning of iontophoresis for baseline measurements and for the period at the maximal blood flux plateau. Data were reported as PU expressed as the percentage increase in cutaneous blood flux relative to baseline measurements.

4.6.5 Ex vivo vascular exploration

Following *in vivo* assessment, rats were anesthetized using an intra-peritoneal injection of sodium pentobarbital at 120 mg.kg⁻¹ and the intact thoracic aorta was quickly removed and placed in cold Krebs-Henseleit bicarbonate buffer (composition in mM: NaCl 118; NaHCO₃ 25; Glucose 11; KCl 4.8; KH₂PO₄ 1.2; MgSO₄7H₂O 1.1; CaCl₂ 1.25). Following removal of adherent tissue, the thoracic aorta was cut into 2-3 mm rings and suspended between two wire hooks. The suspended rings were then mounted in an organ chamber with 5 mL of Kreb's solution with a pH level of 7.4 at 37°C and continuously gassed with 95 % oxygen and 5 % carbon dioxide, under a resting tension of 2 g. The rings were connected to an isometric force transducer (EMKA technologies,

EMKA Paris, France) and linked to an amplifier (EMKA technologies, EMKA Paris, France), as well as a computer acquisition system that recorded changes in isometric force. The rings were then allowed to equilibrate for 45 minutes. During this period, the tension was verified every 15 minutes and washed with Krebs's solution. Following the equilibration period, the rings were pre-contracted with phenylephrine (10^{-6} M) and relaxed with acetylcholine (10^{-5} M) to functionally confirm the muscular and endothelium integrity. Each ring was then pre-contracted with phenylephrine (10^{-6} M). When a plateau in the effect of pre-contraction was reached, endothelial function was examined by challenging the aortic rings with two cumulative concentration-response curves to administration of acetylcholine (10^{-10} to 10^{-5} M), firstly in the normoglycemic state and then during acute hyperglycemia. Acute hyperglycemia was induced by one hour of incubation in a hyperglycemic Krebs's solution at glucose concentrations of 30 mM, 100 mM and 200 mM. The interaction between microvascular function and oxidative stress was further examined during acute hyperglycemia, induced by incubation in hyperglycemic Krebs's solution (100 mM), in aortic rings that were pre-treated with N-acetylcysteine (20 mM) for 30 minutes, or with apocynin (100 μ M) for 15 minutes. Data acquisition was performed using IOX (EMKA technologies, EMKA Paris, France). The relaxation response was expressed as a percentage of the pre-contraction induced by phenylephrine.

4.6.6 Western blotting analysis

Proteins from aorta homogenates were separated on polyacrylamide-sodium dodecyl sulphate gels and transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight with primary antibodies, anti-eNOS III and anti-eNOS-P^{Ser1177} (1:1000 and 1:500, respectively; BD Transduction Laboratory), at 4°C in 10 % milk with Tris-buffered saline containing 0.05 % Tween 20. The eNOS-P^{Ser1177} protein content was expressed relative to the eNOS content and the eNOS protein content was expressed relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content (anti-GAPDH antibody 1: 5000; incubated in 3 % bovine serum albumin, Santa Cruz Biotechnology). Low-temperature SDS-PAGE (LT-PAGE) was performed for detection of eNOS dimers. Briefly, total proteins, were prepared in 1 \times SB buffer without 2-mercaptoethanol. The samples were then subjected to SDS-PAGE with 6 % gel. Gels and buffers were equilibrated at 4°C before electrophoresis; and the buffer tank was placed in an ice bath during electrophoresis to

maintain the temperature of the gel at $<15^{\circ}\text{C}$. Subsequent to LT-PAGE, the gels were transferred, and the blots were probed as a routine Western blot. Immunodetection was performed using an ECL or ECL Plus System (Supersignal West Pico Chemiluminescence Substrate, Thermo Scientific); and the membranes were exposed to X-ray films for visualization.

4.6.7 Measurement of reactive oxygen species

Production of ROS in both normoglycemic and hyperglycemic aortic ring preparations were evaluated by electron paramagnetic resonance in fresh frozen aortic homogenates as previously described.^{226,227} Aortic ring preparations were then treated with 1 mM CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine solution) (1:1 v/v), put in the electron paramagnetic resonance glass capillary tube (Noxygen Science Transfer and Diagnostics, Germany) and placed inside the e-scan spectrometer (Bruker, Germany) for data acquisition. Production of ROS was normalized to the protein content of each sample and then expressed in $\mu\text{mol}/\text{mn}/\text{mg}$.

4.6.8 Measurement of Nitrites

Quantification of nitrite in aortic lysates was performed using the Measure-iT™ High-Sensitivity Nitrite Assay Kit (Invitrogen) according to the manufacturer's instructions.

4.6.9 Statistical analyses

Sample size was calculated using the results of a similar intervention, which demonstrated that FMD decreased from $6.96 \pm 1.56\%$ to $4.0 \pm 2.6\%$ during acute hyperglycemia when compared to the normoglycemic state.¹⁷⁶ Considering this, it was estimated that a total of six participants would be needed to detect a difference between trials, with a one-tailed α of 0.05 and a $1-\beta$ of 0.80. All clinical microvascular and macrovascular data were exported and coded by a researcher (J.L.) not involved in data collection and analyses in order to blind the investigator. Following checks of distribution, clinical data was then analysed using repeated measures analysis of variance (ANOVA) with a post-hoc Tukey's multiple comparison test to assess differences over time in vascular, hemodynamic and blood glucose responses to water or SSB consumption. A one-way ANOVA with a Fisher post hoc test was performed to determine differences in the cutaneous microvascular blood flow response to the induction of acute hyperglycemia in rats designated with normoglycemic, acute

hyperglycemic only, or acute hyperglycemic-N-acetylcysteine treatments. Differences in *ex vivo* vascular reactivity between the hyperglycemic and normoglycemic conditions were determined using a two-way ANOVA with Bonferroni post hoc tests. All statistical analyses were performed using MedCalc software (bvba, Mariakerke, Belgium) and significance was accepted at $P < 0.05$. All values are reported as mean \pm SEM.

4.7 Results

Twelve healthy male participants were recruited and completed this randomized, single-blind, crossover trial between June 9th and July 4th, 2014 (Figure 4-3). To examine the acute vascular effects of commercial SSB consumption in humans, variables of blood glucose concentration, heart rate, arterial pressure and vascular function were assessed after the ingestion of 600 mL (20 oz.) of a commercial SSB and compared to values measured following water consumption. Nutritional information for each beverage is presented in Table 4-1.

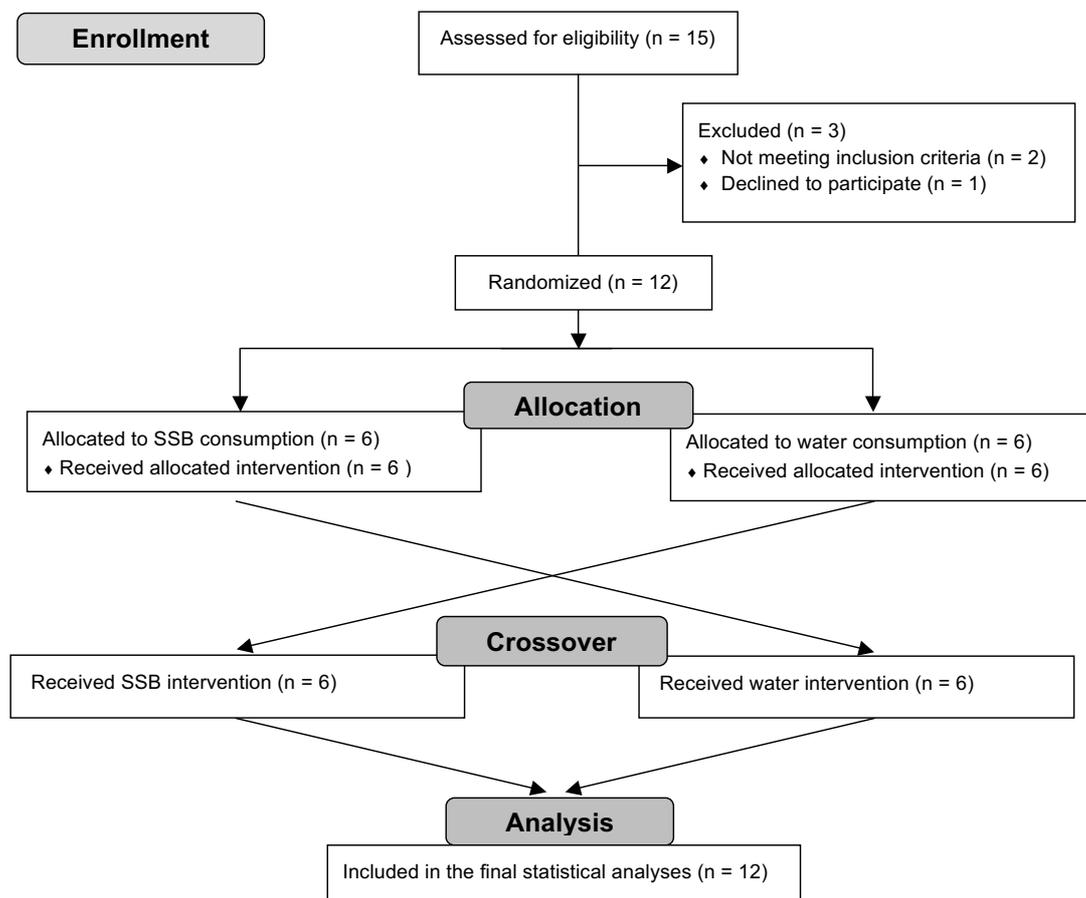


Figure 4-3. Flow diagram illustrating participant recruitment and randomized allocation to sugar-sweetened beverage (SSB) or water consumption interventions; and the participant retention to the conclusion of the crossover trial.

Table 4-1. The nutritional composition of each test beverage

	WATER	SSB
Volume, <i>mL</i>	600	600
Energy, <i>kJ</i>	0	1200
Protein, <i>g</i>	0	0.30
Fat		
- Total, <i>g</i>	0	0
- Saturated, <i>g</i>	0	0
Carbohydrate, <i>g</i>	0	72.4
- Sugars, <i>g</i>	0	72.4
Calcium, <i>mg</i>	18	N/A
Magnesium, <i>mg</i>	6	N/A
Sodium, <i>mg</i>	24	108
Zinc, <i>mg</i>	0.06	N/A
Ingredients	Tap water	Carbonated water, sugar, reconstituted lemon juice (5 %), food acids (330,331, natural flavour, preservatives (211, 223), natural colour (safflower extract)

Nutrient composition of water was obtained from the United States Department of Agriculture Nutrient Database Standard Reference Release 28.²²⁸ Nutrient composition for the commercial sugar-sweetened beverage (SSB) was obtained from the nutrition information label on packaging. Participants consumed 600 mL of water or commercial SSB 15 minutes prior to the beginning of vascular assessment. Participants completed trials on separate days for each test beverage in a randomized order.

4.7.1 Participant characteristics

Participants included in the study were aged 31 ± 1.9 years with a body mass index of 24.68 ± 0.71 kg/m². At each visit, prior to the consumption of the test beverage, basal measurements of fasting blood glucose (4.95 ± 0.15 vs. 4.78 ± 0.16 mmol/L, $P > 0.05$), heart rate (64.08 ± 3.1 vs. 62.25 ± 3.2 beats.min⁻¹, $P > 0.05$), and mean arterial pressure (80.50 ± 1.66 vs. 79.86 ± 1.48 mmHg, $P > 0.05$) were found to be similar (Figure 4-4).

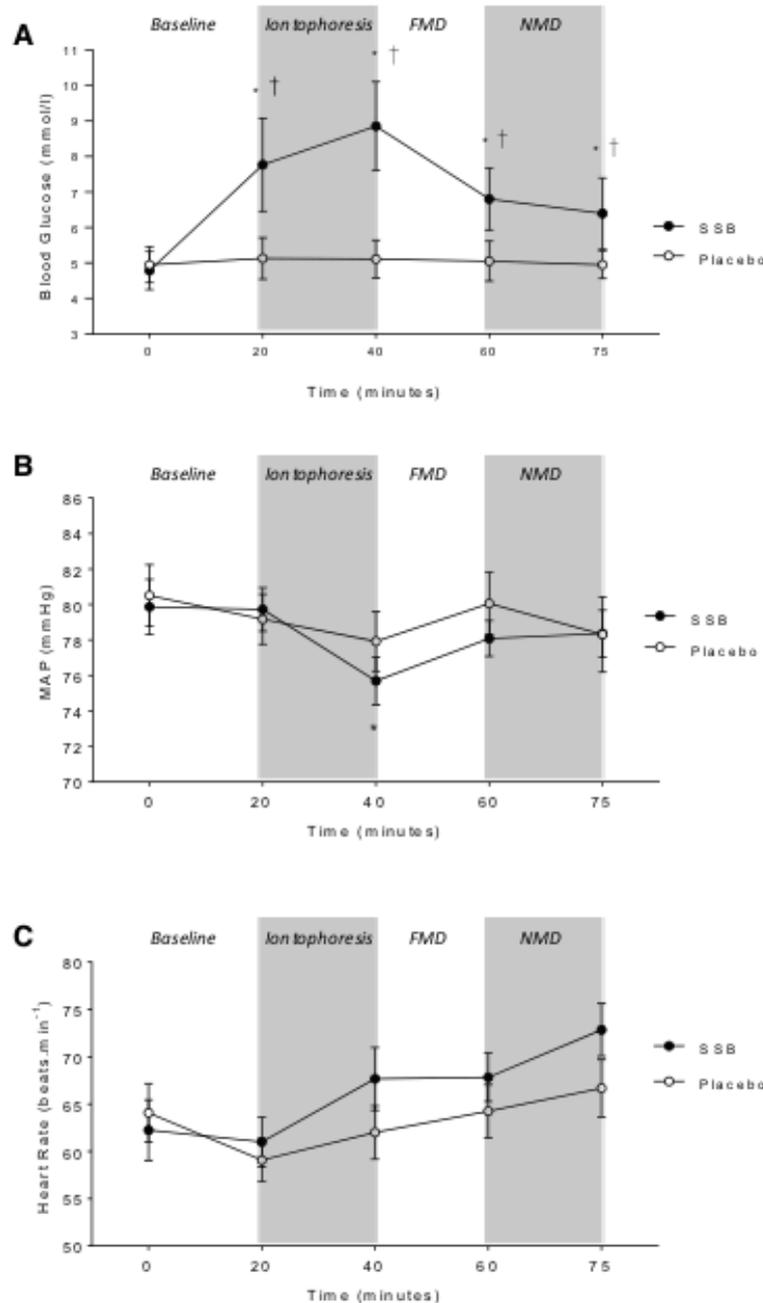


Figure 4-4. Changes in (A) blood glucose concentrations, (B) mean arterial pressure (MAP), and (C) heart rate over time in response to consumption of 600 mL of water or commercial sugar-sweetened beverage (SSB). * $P < 0.05$ vs. baseline; † $P < 0.05$ vs. water.

4.7.2 Sugar-sweetened beverage consumption increased blood glucose concentration with no major effect on heart rate or blood pressure.

Ingestion of the SSB significantly elevated blood glucose concentrations above basal values 20 minutes (7.77 ± 0.38 vs. 4.78 ± 0.16 mmol/L, $P < 0.05$) following the beginning of consumption, with peak hyperglycemia (8.85 ± 0.36 mmol/L, $P < 0.05$) recorded at 40 minutes (Figure 4-4A). A progressive decrease in blood glucose concentrations was observed between 40 and 75 minutes thereafter. However, blood glucose concentrations at 75 minutes were still significantly greater than basal blood glucose values (6.39 ± 0.3 vs. 4.78 ± 0.16 mmol/L, $P < 0.05$) and all blood glucose values measured over time following water consumption. Blood glucose concentrations did not deviate from basal measurements following water consumption. Acute hyperglycemia had no major effect on mean arterial pressure. However, a slight but significant reduction in mean arterial pressure was observed during peak hyperglycemia when compared to that at the baseline measurement ($P < 0.05$) (Figure 4-4B). No variations in heart rate were observed during acute hyperglycemia (Figure 4-4C).

4.7.3 Sugar-sweetened beverage consumption decreased microvascular and macrovascular endothelial function

All assessments of microvascular and macrovascular function were completed between 20 and 75 minutes following the beginning of SSB consumption, during acute hyperglycemia. Vascular function was assessed in the same time period following water consumption to allow for comparison between the two test beverages.

4.7.3.1 Assessment of cutaneous microvascular reactivity

Cutaneous microvascular endothelium-dependent reactivity and endothelium-independent reactivity were assessed using LSCI in conjunction with iontophoresis of acetylcholine and sodium nitroprusside, respectively. Prior to the beginning of iontophoresis, there were no differences in baseline measurements of basal cutaneous blood flux between each visit (23.6 ± 1.8 vs. 26.4 ± 1.6 PU $P = 0.23$) (Table 4-2). An increase in cutaneous blood flux was observed following iontophoresis of acetylcholine and sodium nitroprusside (Figure 4-5A). However, the relative percentage increase in cutaneous blood flux in response to acetylcholine iontophoresis was significantly lower during SSB-mediated acute hyperglycemia when compared to that during

normoglycemia following water consumption (129.76 ± 11.18 vs. 196.78 ± 20.61 %, respectively, $P < 0.01$) (Figure 4-5B). Even after accounting for differences in blood pressure between visits by converting cutaneous blood flux from PU to CVC, the relative increase in cutaneous blood flux mediated by acetylcholine iontophoresis was still lower following ingestion of the SSB than that measured after water consumption (144.2 ± 15.7 vs. 208.3 ± 24.3 %, respectively, $P < 0.01$). In contrast, the vascular responses to iontophoresis of sodium nitroprusside were similar following the consumption of each test beverage.

Table 4-2. Microvascular and macrovascular reactivity in response to water and commercial SSB consumption in healthy subjects

	WATER	SSB	<i>P</i> -value
n	12	12	
Microcirculation			
Basal CBF (PU)	23.6 ± 1.8	26.4 ± 1.6	$p=0.231$
Peak ACh CBF (PU)	70.9 ± 8.0	63.0 ± 6.4	$p=0.346$
Peak SNP CBF (PU)	101.2 ± 6.0	101.9 ± 7.4	$p=0.934$
ACh CVC increase (%)	208.3 ± 24.3	$144.2 \pm 15.7^*$	$p=0.008$
SNP CVC increase (%)	360.3 ± 26.7	355.9 ± 29.3	$p=0.926$
Skin resistance (Ω)	399.4 ± 41.3	395.6 ± 30.2	$p=0.872$
Macrocirculation			
Basal brachial artery diameter (mm)	4.7 ± 0.1	4.7 ± 0.1	$p=0.684$
Basal brachial blood flow (ml.min ⁻¹)	48.0 ± 5.7	53.0 ± 6.5	$p=0.673$
Peak brachial blood flow (ml.min ⁻¹)	408.3 ± 27.6	445.1 ± 34.5	$p=0.194$
Δ shear rate (%/s ⁻¹)	484.3 ± 32.8	501.6 ± 37.1	$p=0.506$
FMD/ Δ shear rate (%/s ⁻¹)	0.019 ± 0.002	$0.014 \pm 0.002^*$	$p < 0.001$
Brachial hyperemia (%)	886.4 ± 132.3	855.5 ± 107.7	$p=0.693$

Values are mean \pm SEM. SSB indicates sugar-sweetened beverage; CBF, cutaneous blood flux; PU, perfusion units; ACh, acetylcholine; SNP, sodium nitroprusside; CVC, cutaneous vascular conductance; FMD, flow-mediated dilation. Δ shear rate means peak brachial shear rate minus resting brachial shear rate. P-value was estimated by repeated measures ANOVA followed by post-hoc Tukey's multiple comparison tests. * $P < 0.05$ vs. water.

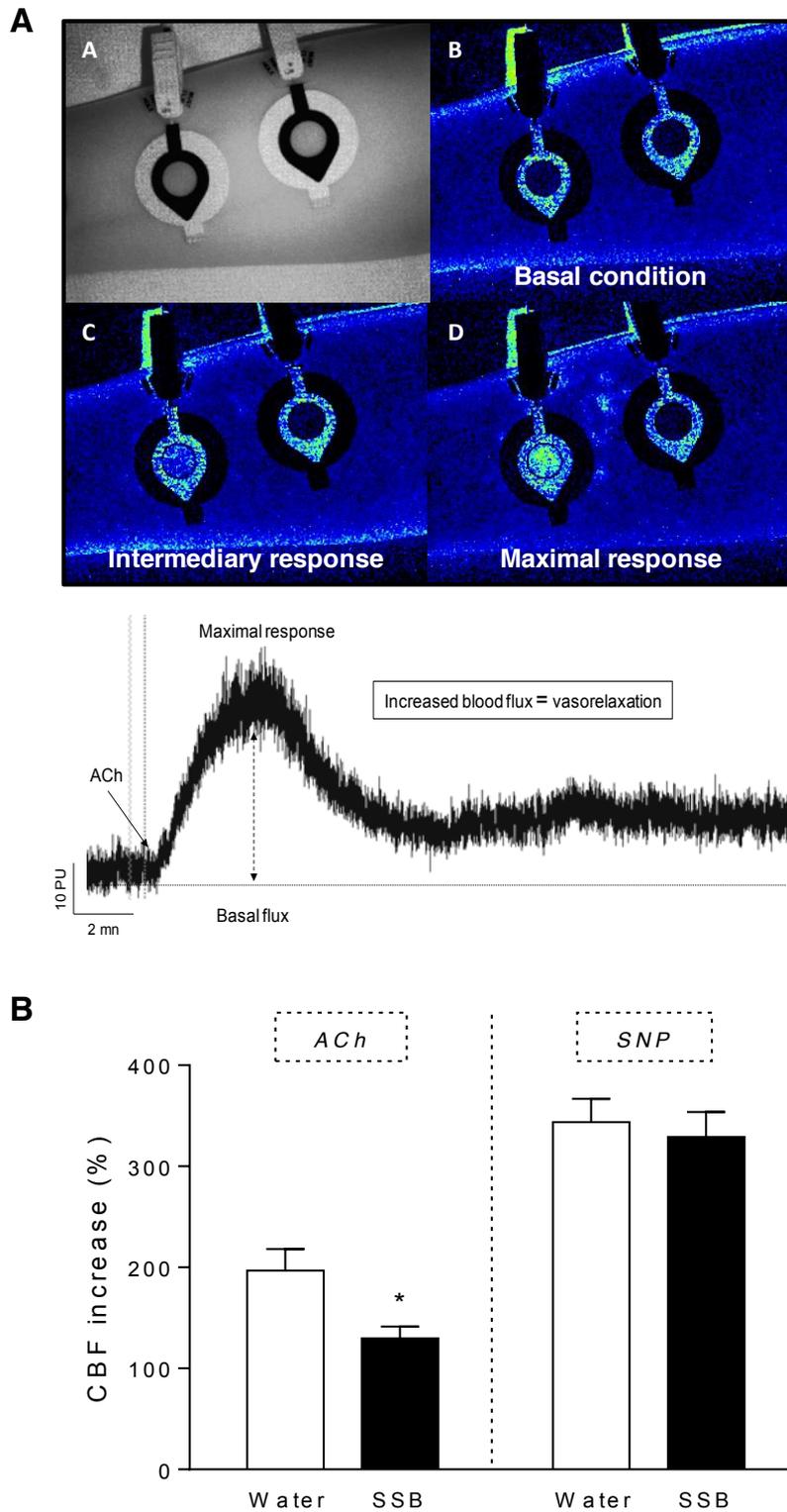


Figure 4-5. Microvascular reactivity in healthy participants following consumption of 600 mL of water or a commercial sugar-sweetened beverage (SSB). **(A)** Top: Laser speckle contrast imaging electrode position on the right forearm. Bottom: Representative curve of cutaneous blood flux (CBF) during acetylcholine (ACh) iontophoresis. **(B)** The percentage increase from baseline in CBF in response to iontophoresis of ACh and sodium nitroprusside (SNP). * $P < 0.01$ vs. water.

4.7.3.2 Macrovascular measurements

Endothelium-dependent and endothelium-independent vascular reactivity were then assessed in the macrocirculation using ultrasound of the brachial artery in conjunction with FMD and NMD, respectively. Following the consumption of each test beverage, there were no differences in basal brachial artery diameter or blood flow between visits (4.7 ± 0.1 vs. 4.7 ± 0.1 mm, $P=0.68$; and 48.0 ± 5.7 vs. 53.0 ± 6.5 ml.min⁻¹, $P=0.67$, respectively) (Table 4-2). Increases in brachial blood flow and the Δ shear rate induced during hyperemia were also similar between each visit (408.3 ± 27.6 vs. 445.1 ± 34.5 ml.min⁻¹, $p=0.19$; and 484.3 ± 32.8 vs. 501.6 ± 37.1 %/s⁻¹, $P=0.51$, respectively). However, FMD was significantly reduced (-23.86 ± 5.33 %) during SSB-mediated acute hyperglycemia when compared to that during normoglycemia following water consumption; when expressed solely as the percentage change in diameter (6.53 ± 0.61 vs. 8.56 ± 0.54 %, respectively, $P<0.01$) (Figure 4-6) and as the percentage change in diameter with respect to the change in shear rate (0.014 ± 0.002 vs. 0.019 ± 0.002 %/s⁻¹, respectively, $P<0.01$). In contrast, there were no differences in the responses to NMD between each visit.

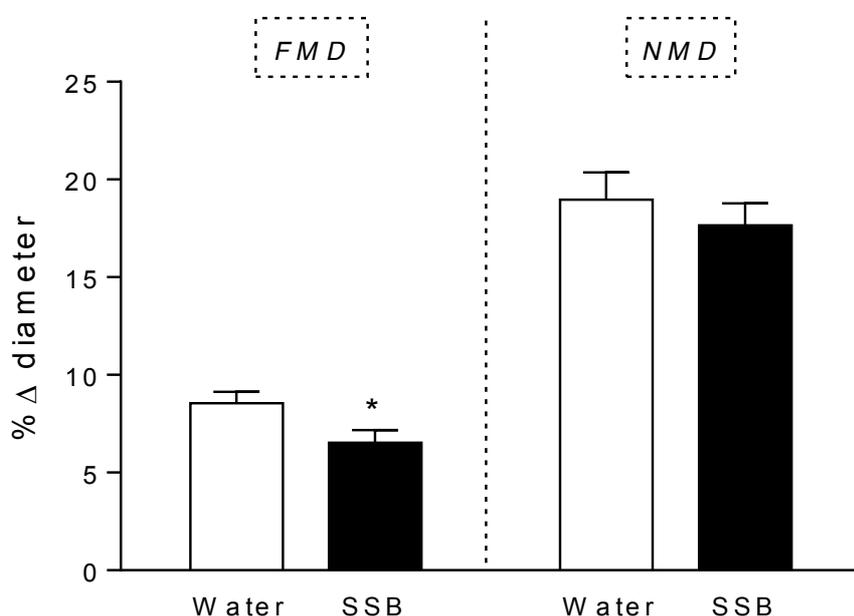


Figure 4-6. Macrovascular reactivity in healthy participants following consumption of 600 mL of water or commercial sugar-sweetened beverage (SSB). The percentage change from baseline brachial artery diameter in response to flow-mediated dilation (FMD) and nitrate-mediated dilation (NMD). * $P<0.01$ vs. water.

4.7.4 Endothelial dysfunction following SSB consumption may be associated with an acute hyperglycemic-mediated increase in oxidative stress that decreases NO bioavailability

Given that both FMD and the vascular response to acetylcholine iontophoresis were reduced during SSB-mediated acute hyperglycemia, whilst the vascular responses to nitrate administration were preserved, clinical findings clearly suggest that acute hyperglycemia impairs microvascular and macrovascular reactivity via an endothelium-dependent pathway. However, considering that further experimental exploration within vascular tissues of healthy humans is, for ethical reasons, very difficult to perform, the underlying mechanisms by which acute hyperglycemia mediates endothelial dysfunction were evaluated further, both *in vivo* and *ex vivo*, using an experimental rat model. Furthermore, as it is not possible to administer rats with an intra-peritoneal injection of commercial SSB, this study used an intra-peritoneal injection of glucose to examine the underlying mechanisms of SSB-mediated endothelial dysfunction. Importantly, previous research has revealed that acute changes in vascular reactivity following SSB consumption are more related to the glucose, than the fructose, in sucrose;²²⁹ and that fructose has a minimal effect on endothelial function in rats.²³⁰ Moreover, the adverse vascular effects of glucose consumption in humans has been further demonstrated in this study with supplementary data indicating that macrovascular endothelial function is decreased during glucose-mediated acute hyperglycemia, compared to that during normoglycemia, when expressed as the percentage change in diameter (6.17 ± 0.66 vs. 9.92 ± 1.26 %, respectively, $P < 0.05$) and as the percentage change in diameter with respect to the change in shear rate (0.011 ± 0.001 vs. 0.018 ± 0.003 %/ s^{-1} , respectively, $P = 0.07$) (Figure 4-7).

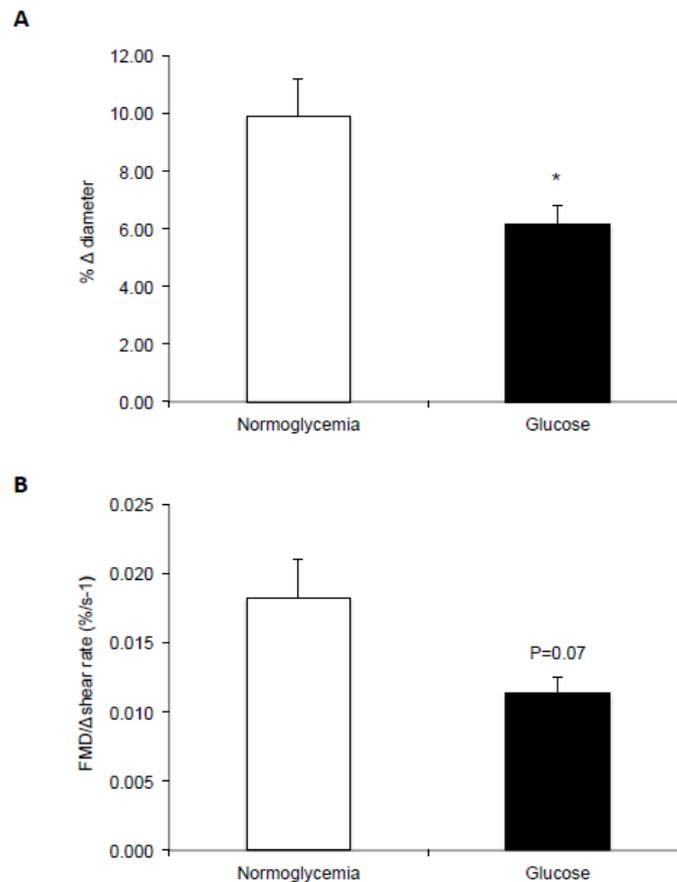


Figure 4-7. The vascular response to flow-mediated dilation (FMD) during normoglycemia and glucose-mediated acute hyperglycemia, induced by consumption of 72.4 g of glucose diluted in 600 mL of water, in six healthy males when expressed as **(A)** the percentage change in diameter and **(B)** the percentage change (Δ) in diameter with respect to the change in shear rate. * $P < 0.05$ vs. normoglycemia.

4.7.4.1 In vivo experimental exploration

Acute hyperglycemia was induced in each rat using an intra-peritoneal injection of glucose that mediated a hyperglycemic plateau between 10 and 20 minutes following administration, with peak hyperglycemia occurring at 15 minutes (Figure 4-8A). To allow for comparisons between the acute hyperglycemic and normoglycemic conditions, a group of control rats received a placebo injection of sodium chloride 0.9 % (saline). During the acute hyperglycemic plateau, iontophoresis of acetylcholine and sodium nitroprusside were performed inducing a significant increase in cutaneous microvascular blood flux (Figure 4-9A). However, similar to the findings obtained in human trials, the relative increase in cutaneous blood flux in response to iontophoresis of acetylcholine was decreased during acute hyperglycemia when compared to normoglycemic controls, whilst the vascular response to sodium nitroprusside

iontophoresis remained preserved (Figure 4-9B). Such endothelial dysfunction may be primarily caused by an acute hyperglycemic-mediated increase in oxidative stress.²³¹ Considering this, a third group of rats were treated with a dose of the antioxidant, N-acetylcysteine, during normoglycemia and prior to the induction of acute hyperglycemia. Treatment with N-acetylcysteine had no effect on vascular function during normoglycemia. However, although the acute hyperglycemic responses to the intra-peritoneal injection of glucose between N-acetylcysteine-treated and untreated rats were similar (Figure 4-8B), the increase in cutaneous blood flux in response to acetylcholine iontophoresis was fully restored in rats treated with the antioxidant (Figure 4-9B); supporting the implication of increased oxidative stress in acute hyperglycemia-mediated endothelial dysfunction.

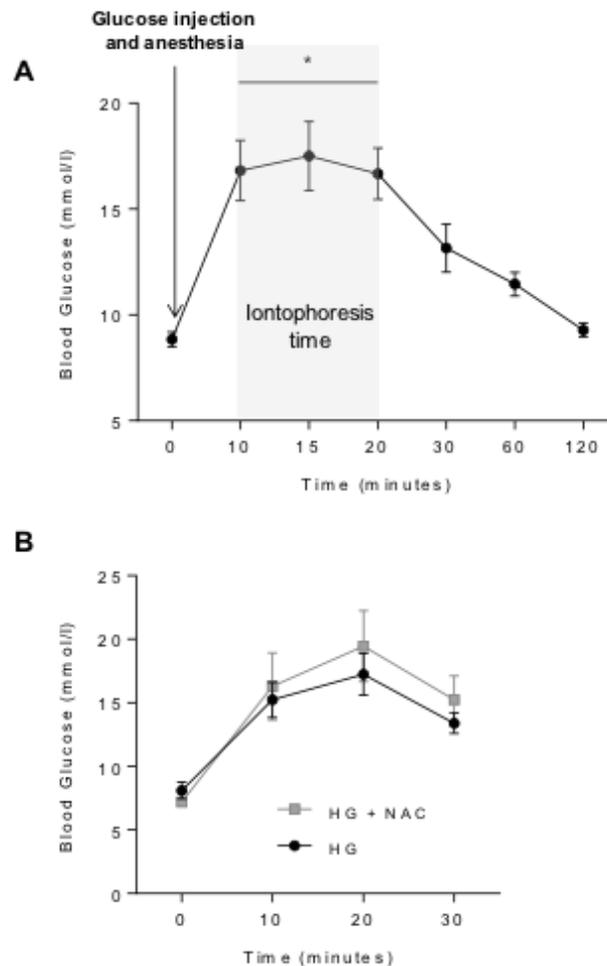


Figure 4-8. (A) Time course of blood glucose concentrations following glucose injection (2 g/kg, 1.5 ml) in rats. (B) Time course of blood glucose concentrations following glucose injection in rats pre-treated with saline only (HG) or N-acetylcysteine (HG-NAC). *P<0.05 vs. baseline.

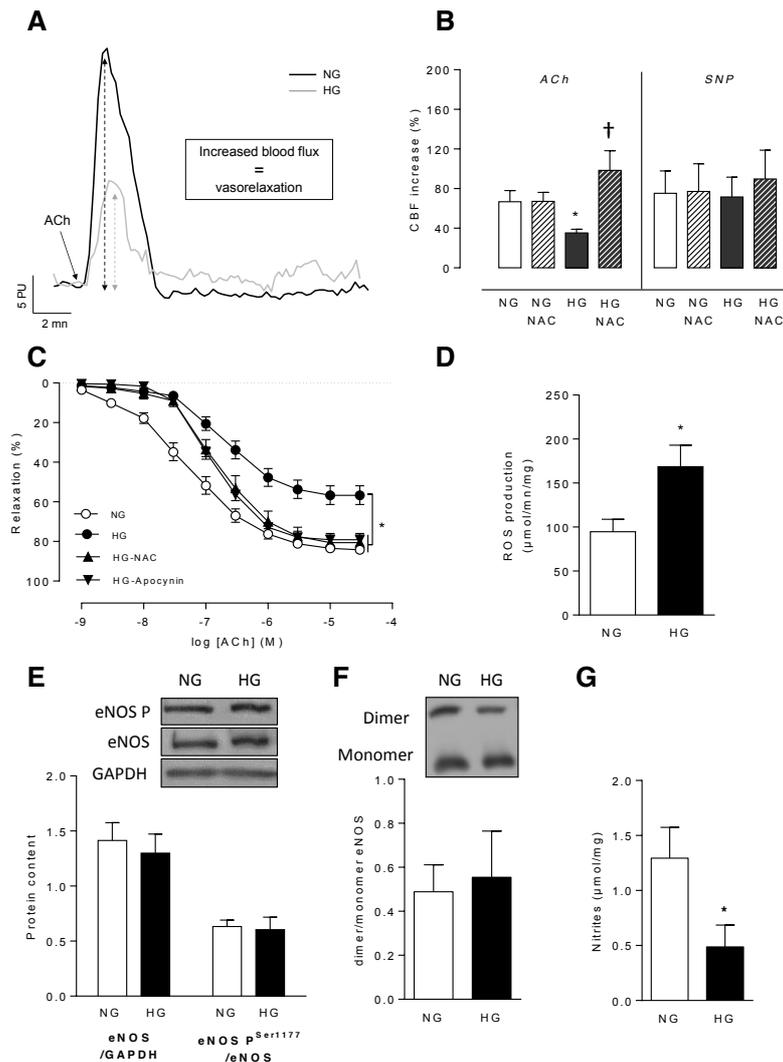


Figure 4-9. Implication of oxidative stress in endothelial dysfunction during acute hyperglycemia. **(A)** Representative curve of cutaneous microvascular blood flux (CBF) during normoglycemia (NG) and acute hyperglycemia (HG) in response to acetylcholine (ACh) iontophoresis in rats **(B)** The percentage increase from baseline in CBF in response to iontophoresis of acetylcholine and sodium nitroprusside following administration of sodium chloride 0.9 % (saline) in NG rats and following the induction of HG in rats pre-treated with saline in order to examine the effect of HG alone, or in rats pre-treated with N-acetylcysteine (NAC) to evaluate implication of oxidative stress in endothelial dysfunction. **(C)** A dose response curve to ACh during NG and HG (100 mM) in pre-contracted aortic rings and during HG (100 mM) in pre-contracted rings pre-incubated in NAC (20 mM) or in apocynin (100 μ M) **(D)** Production of reactive oxygen species (ROS) evaluated by electron paramagnetic resonance in NG aortic preparations; and aortic preparations pre-incubated with HG Krebs's solution (glucose concentration: 30 mM). **(E)** Expression and activation of endothelial nitric oxide synthase (eNOS) by phosphorylation (P) at serine 1177 analysed by western blotting in NG aortic preparations and aortic preparations pre-incubated in HG Krebs's solution (100 mM). **(F)** eNOS dimer/monomer ratio analysed by detecting SDS-resistant eNOS dimers using low-temperature SDS-PAGE in the aortas of NG and HG rats. **(G)** Nitrite concentration in NG aortic preparations and aortic preparations pre-incubated with HG Krebs's solution or HG (100 mM). * $P < 0.05$.

4.7.4.2 Ex vivo experimental exploration

Given that endothelial function is highly dependent on NO bioavailability, which itself is dependent on eNOS and its activation by phosphorylation at serine 1177 (eNOS^{ser1177}), *ex vivo* experiments were then performed on rat aortic tissue to investigate the interaction between oxidative stress and the NO pathway; and whether this interaction contributes to the endothelial dysfunction observed during acute hyperglycemia. Similar to that observed following SSB consumption in human trials and the intraperitoneal injection of glucose in *in vivo* experimental exploration, acute hyperglycemia induced by the hyperglycemic solution decreased the relaxation response to acetylcholine administration in pre-contracted aortic rings in a dose-dependent manner (Figure 4-10). However, pre-treatment with the non-specific antioxidant, N-acetylcysteine restored the relaxation response in aortic rings during acute hyperglycemia. Interestingly, similar results were observed when apocynin, a specific inhibitor of NADPH oxidase,²³² was administered (Figure 4-9C). Although these results were supported by electron paramagnetic resonance assessments, which measured an elevation in the production of ROS during acute hyperglycemia when compared to the normoglycemic condition (Figure 4-9D), western blotting revealed that acute hyperglycemia had no effect on the expression of eNOS or its activation in aortic rings (Figure 4-9E). Moreover, eNOS dimerization, evaluated by the SDS resistant dimer/monomer ratio, was not impacted by acute hyperglycemia (Figure 4-9F). Despite this, there was still a marked reduction in the bioavailability of NO in the aortic tissue, indicated by a lower concentration of nitrites, during acute hyperglycemia (Figure 4-9G).

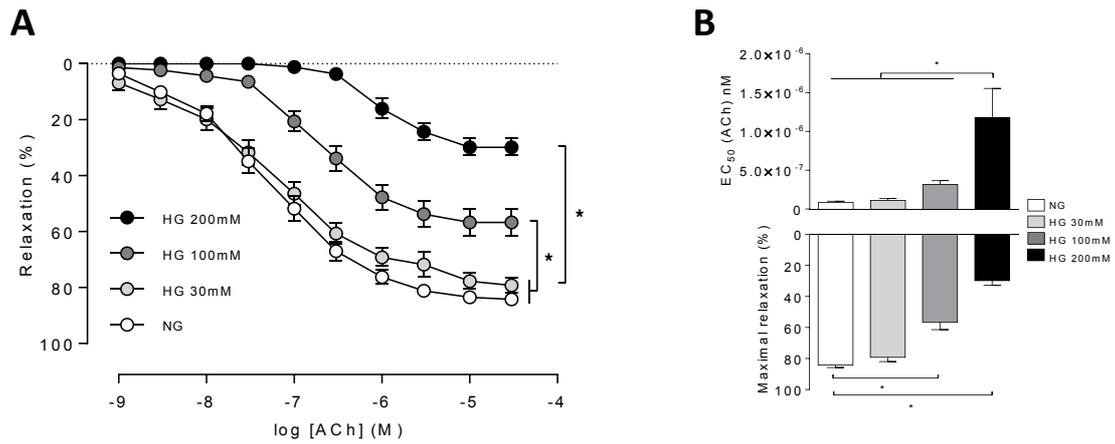


Figure 4-10. (A) Dose response curve to acetylcholine (ACh) in normoglycemic (NG) pre-contracted aortic ring preparations; and pre-contracted aortic preparations incubated with incremental concentrations of a hyperglycemic (HG) Krebs's solution (glucose concentrations: 30, 100 and 200 mM). **(B) Up:** Concentration of ACh administered to induce 50 % of maximal relaxation response to ACh in NG aortic ring preparations; and aortic preparations pre-incubated with incremental concentrations of a HG Krebs's solution (glucose concentrations: 30, 100 and 200 mM). **Down:** Maximal relaxation of aortic ring preparations in response to administration of ACh expressed as the percent relaxation relative to the measured pre-contraction with phenylephrine (10^{-6} mM). * $P < 0.05$.

4.8 Discussion

The present study aimed to assess the effect of acute hyperglycemia induced by the ingestion of a commercial SSB on vascular function in healthy sedentary participants. It was found that SSB consumption decreased both microvascular and macrovascular endothelium-dependent reactivity, whereas endothelium-independent vascular reactivity remained unaffected during acute hyperglycemia. Considering these findings, *in vivo* and *ex vivo* explorations were then performed in an experimental rat model to further investigate the interaction between acute hyperglycemia and vascular function; and examine the underlying mechanisms that may mediate such endothelial dysfunction. Similar to that observed in human trials, acute hyperglycemia impaired endothelial function in both *in vivo* and *ex vivo* experimental rat models. Interestingly, endothelial function was fully restored during acute hyperglycemia in rats that were pre-treated with the antioxidant, N-acetylcysteine; and in aortic rings that were pre-treated with either N-acetylcysteine or a specific inhibitor of NADPH oxidase, apocynin. Further to this, it was found that acute hyperglycemia was associated with increased production of arterial ROS and reduced bioavailability of NO. Collectively, these findings suggest that acute hyperglycemia induces endothelial dysfunction by mediating an increase in oxidative stress that disrupts the NO pathway.

It is currently unknown how acute hyperglycemia induced by commercial SSB consumption affects vascular function due to limited and discrepant data. Previous research has most commonly assessed vascular function during acute hyperglycemia induced by a typical oral glucose load using measures of macrovascular reactivity, such as brachial artery ultrasound with FMD.²¹⁴ However, emerging evidence suggests that coronary microvascular disease may explain the occurrence of myocardial ischemia, heart failure and CVD mortality following myocardial infarction without apparent coronary macrovascular disease;²³³ highlighting the need to assess the microcirculation, which represents most of the arterial vascular network and exerts dominant control over local blood flow,³⁸ in conjunction with the macrocirculation when investigating mechanisms that contribute to the pathogenesis of CVD. The results of this present study are consistent with those in previous research, which demonstrated that commercial SSB consumption mediates a decrease in macrovascular endothelial

function as indicated by reduced FMD during acute hyperglycemia.¹⁸⁹ Importantly, it should be noted that this previous study induced acute hyperglycemia by administering a commercial SSB in conjunction with a high caloric commercial candy bar. In contrast, the microcirculatory findings contradict a separate study, which found that consumption of commercial SSB enhances microvascular endothelial function, as indicated by an increased cutaneous blood flux response to iontophoresis of acetylcholine during acute hyperglycemia.¹⁶⁹ However, in this study, acute hyperglycemia was induced using a commercial SSB that is defined as an energy drink containing caffeine, which is an ingredient that has been found to increase microvascular reactivity in healthy participants.²³⁴ Considering the possible confounding vascular effects of these additional foods or ingredients, this present research is, to our knowledge, the first to examine the effects of acute hyperglycemia induced by commercial SSB consumption alone on microvascular and macrovascular reactivity. The results of this study also contribute to clarifying the effect of acute hyperglycemia on systemic vascular endothelial function, which in a recent systematic review and meta-analysis was found to be inconclusive due to limited microvascular data and discrepant reporting of shear stress data in studies that used FMD to assess changes in vascular function from normoglycemic to hyperglycemic states.²¹⁴ Given that this present study found a decrease in FMD during acute hyperglycemia with no reduction in shear stress, a disruption of the NO pathway may be implicated in SSB-mediated endothelial dysfunction.^{34,58}

Current evidence suggests that acute hyperglycemia induces endothelial dysfunction by mediating an abnormal elevation in oxidative stress that disturbs normal underlying mechanisms of NO synthesis.^{235,236} In the postprandial state, oxidative metabolism initiates oxidative phosphorylation of adenosine triphosphate at the electron transport chain of the mitochondria, which via the phenomenon of electron leakage causes superoxide generation of ROS.⁸⁵ Moreover, it has been clearly reported that increased glycemia is responsible for the activation of NADPH oxidase, which also contributes to production of the superoxide anion.²⁴ Although these ROS are normally readily detoxified, elevated activity within this mechanism such as that following commercial SSB consumption increases production to a rate beyond suppressive capabilities of the antioxidant systems.⁸⁶ The implication of oxidative stress in acute hyperglycemia-mediated endothelial dysfunction is further supported by *ex vivo* findings in this present

study, which demonstrated that acute hyperglycemia increased ROS in rat aortic rings. Moreover, treatment with the antioxidants, N-acetylcysteine and apocynin, were found to attenuate the impaired relaxation response to acetylcholine iontophoresis observed during acute hyperglycemia. Importantly, the applicability of this oxidative stress-dependent mechanism to acute hyperglycemia-mediated endothelial dysfunction in living organisms was also demonstrated in this study, which revealed for the first time in an *in vivo* experimental rat model that antioxidant treatment also fully restores cutaneous microvascular endothelium-dependent reactivity during acute hyperglycemia. This study also found that eNOS^{ser1177} and the eNOS dimer/monomer ratios remained preserved during acute hyperglycemia, while the concentration of nitrites was decreased. Such experimental findings provide evidence that SSB-mediated endothelial dysfunction is due, at least in part, to decreased bioavailability of NO that is not caused by a disruption to the synthesis of NO via the eNOS pathway. Indeed, the reaction between NO and free radical superoxide results in the formation of peroxynitrite, a potent cytotoxic molecule.²³⁷ This nitro-oxidative stress could be a primary mechanism responsible for the decrease in NO bioavailability that was observed in our model of acute hyperglycemic stress.

Despite not being affected in this present study, previous research has demonstrated impaired endothelium-independent vascular reactivity mediated by VSM cell proliferation in as little as 6 hours following the induction of hyperglycemia in animal and *in vitro* studies,¹⁴⁸ suggesting a need to extend the typical assessment period of vascular function following SSB consumption in future research. Moreover, considering the global rate of commercial SSB consumption and its role in transient endothelial dysfunction, even in a healthy population, research must also quantify the relative loading of SSB over time that mediates significant vascular remodelling and contributes to the pathogenesis of CVD in humans.²¹⁴ In addition to examining the underlying mechanisms of NO-mediated microvascular reactivity, future research may also assess the effect of SSB consumption on other main vasoactive mediators such as prostaglandin I₂, endothelium derived hyperpolarizing factor, and ET-1, all of which have varying influence between the microcirculation and macrocirculation.^{176,205,238} Finally, these studies need to be conducted across a variety of ethnicities, some of which have previously demonstrated decreased vascular reactivity even at rest; and, therefore,

may be more severely impacted by the deleterious vascular-related effects of acute hyperglycemia than that observed in Caucasian populations.^{210,239}

Several inherent limitations must be considered when interpreting this data. Whereas the main sugar in SSB is sucrose, which is comprised of glucose and fructose, only glucose was used to induce acute hyperglycemia in the experimental rat model that aimed to explain the effects of SSB consumption on underlying mechanisms of vascular reactivity. However, it has been suggested that acute deleterious cardiovascular effects of sucrose are more related to glucose rather than fructose;^{176,229} with previous findings also demonstrating that fructose has no significant effect on endothelial function in rats.²³⁰ Nevertheless, although endothelium-independent vascular reactivity remained preserved following SSB consumption in this present study, previous research has found that fructose can decrease the endothelium-independent vascular relaxation response to the administration of sodium nitroprusside in rats;²³⁰ and, therefore, it can't be completely discounted that fructose may also contribute, to some extent, to the observed SSB-mediated endothelial dysfunction. It must also be considered that ingredients other than sucrose that comprise commercial SSB were not evaluated individually in this research and, therefore, it is not known how they may contribute to the observed SSB-mediated endothelial dysfunction. Indeed, increases in plasma sodium may also contribute to a decrease in NO bioavailability.²⁴⁰ Additionally, it was not possible to blind participants to the intervention by using a sugar-free placebo such as a commercial diet soda due to previous research suggesting that even artificial sweeteners may interact with taste receptors stimulating insulin secretion, which may induce a vascular response.²⁴¹ Given that changes in blood insulin concentration in response to SSB consumption were not monitored in this study, it was not possible to explore what effect SSB consumption may have on mechanisms of insulin-mediated vasodilation. Finally, vascular reactivity was assessed in a focused sample and, therefore, the effect of SSB consumption may vary across health groups, ethnicities and genders.

In conclusion, this present study is, to our knowledge, the first to assess vascular function during acute hyperglycemia induced by SSB consumption alone. The findings of this study demonstrate that commercial SSB consumption induces microvascular and macrovascular endothelial dysfunction in a healthy population. Furthermore, data from

the experimental rat model suggest that this commercial SSB-mediated endothelial dysfunction is partly due to increased oxidative stress, which reduces NO bioavailability. Ultimately, these results inform international public health policy on the adverse effects of both commercial SSB and general excess sugar consumption; and how they contribute so acutely, even in those considered healthy, to the upregulation of mechanisms that are the primary precursors to the pathogenesis of CVD.²⁴²

Chapter 5 – Study three: The continuums of impairment in vascular reactivity across the spectrum of cardiometabolic health: a systematic review and network meta-analysis

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5.1 Linking paragraph

To this point, this program of research has demonstrated that acute hyperglycemia induces transient endothelial dysfunction across the spectrum of cardiometabolic health; and, subsequently, that it has a significant role in the initial and ongoing development of chronic vascular dysfunction, obesity, MetS and T2D. However, whether there is a continuum in microvascular and macrovascular dysfunction between early stages and diabetic complications is still not clear. Indeed, comprehensive understanding of the development of vascular dysfunction is highly important to improve the timing and, subsequent, effectiveness of prevention and treatment strategies. Considering this, the following study examined vascular reactivity across the spectrum of cardiometabolic health, from those considered healthy, to those with obesity, MetS, impaired glucose tolerance, T2D and T2D with complications; by combining direct and indirect comparisons in a systematic review and network meta-analysis.

5.2 Abstract

Objective

Impaired vascular reactivity has a key role in the development of obesity, insulin resistance and cardiovascular disease. However, as its exploration has mostly been conducted via cross-sectional studies, it isn't entirely clear how vascular reactivity changes from early stages right through to diabetic complications. Considering this, the objective of this study was to assess vascular reactivity across the spectrum of cardiometabolic health.

Approach and results

A systematic search of MEDLINE and EMBASE from inception until March 2017 identified articles that evaluated endothelium-dependent and endothelium-independent vascular reactivity in two or more health groups of interest (healthy, overweight, obese, impaired glucose tolerance, MetS and T2D with or without complications). Pairwise meta-analyses and network meta-analyses compared indexes of vascular reactivity between each health group. One hundred and ninety-three articles (7226 healthy subjects and 19,344 patients considered overweight, obese and/or insulin resistant) were included. The network meta-analyses revealed a progressive impairment in vascular reactivity (FMD data) from the clinical onset of an overweight status (-0.41 %, 95% CI -0.98, 0.15) to the development of vascular complications in those with T2D (-4.26 %, 95% CI -4.97, -3.54). Meta-regressions revealed that for every 1 mmol/l increase in fasting blood glucose concentration, FMD decreased by 0.52 %.

Conclusions

To our knowledge, this study is the first to demonstrate a continuum where the severity of impairment in vascular reactivity progressively increases throughout the pathogenesis of obesity and/or insulin resistance, through to the development of diabetic complications.

5.3 Introduction

It is well established that endothelial dysfunction is an early predictor of cardiovascular events in at-risk patients.^{243–245} Impairments in vascular reactivity, stemming from such endothelial dysfunction, as well as possible disruptions to endothelium-independent activity and maladaptation to the VSM, increase susceptibility to endothelial injury and, thus, promote atherosclerotic change. Furthermore, impaired vascular reactivity may also contribute to the development of obesity and insulin resistance; in what may, indeed, be considered a vicious cycle where each compounds the other.^{246,247} It is therefore important from a clinical perspective that the interaction between cardiometabolic health and vascular reactivity is fully understood to enhance the timing and, subsequent, effectiveness of treatment strategies that aim to improve vascular health and CVD outcomes.

Currently, numerous cross-sectional studies provide evidence that, in comparison to healthy controls, vascular reactivity is significantly impaired early in the development of obesity and/or insulin resistance. Although such data may suggest that impaired vascular reactivity precedes the development of overt disease, many cross-sectional studies often lack the power to accurately estimate the effect size of impairment between groups. Moreover, given that no single study has compared vascular reactivity across the full spectrum of cardiometabolic health, whether there is a continuum in the impairment of macrovascular and microvascular reactivity between early stages and diabetic complications has not yet been properly addressed.

Therefore, considering the large number of vascular studies that have been conducted, the primary objective of this present research is to combine direct and indirect comparisons of vascular reactivity in a network meta-analysis to test this hypothesis and to further understand the development of vascular dysfunction.

5.4 Methods

The protocol for this systematic review and network meta-analysis was registered on PROSPERO (Registration number: CRD42017053411) and was conducted according to the PRISMA statement.²⁴⁸

5.4.1 Population and outcomes

Seven health groups that represent key stages in the pathogenesis of T2D were included in this study: healthy, overweight, obese, impaired glucose tolerance, MetS, T2D and T2D with complications. Complications of interest, in those with T2D, were microvascular (diabetic neuropathy, retinopathy or nephropathy), macrovascular (peripheral artery disease or coronary artery disease), or both (diabetic foot ulceration). Considering that definitions for these conditions have evolved over time and between countries and, thus, vary between research, a homogenous classification for each health group was applied to all studies included in this network meta-analysis by comparing the average value of the key clinical characteristics (e.g. body mass index, fasting blood glucose concentration etc.) from each health group against the World Health Organization guidelines for classification of overweight and obesity;²⁴⁹ the joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention, harmonizing the criteria for defining MetS;²⁵⁰ and the American Diabetes Association criteria for impaired glucose tolerance and diabetes.²⁵¹

The objective of this study was to assess differences in endothelium-dependent and endothelium-independent vascular reactivity between each of the seven health groups of interest. Outcomes of interest included commonly used tests of vascular reactivity in the microcirculation and macrocirculation. Microvascular endothelium-dependent reactivity could be evaluated using post-occlusive reactive hyperemia, pressure-induced vasodilation, local thermal hyperemia, or the administration of acetylcholine, delivered intravenously or by iontophoresis. Microvascular endothelium-independent reactivity may have been assessed with the administration of sodium nitroprusside, also delivered intravenously or by iontophoresis. The microvascular response to each test of reactivity could be measured with strain gauge plethysmography or a laser-based perfusion monitoring technology (e.g. LDF, LDI or LSCI).³⁸ Macrovascular

endothelium-dependent reactivity and endothelium-independent reactivity were assessed using FMD and NMD, respectively;³³ each in conjunction with ultrasound of the brachial artery.

5.4.2 Data sources and searches

The systematic search was performed in MEDLINE and EMBASE databases from their inception until March 13th, 2017, using a combination of subject headings for health status (obesity, prediabetes, MetS and T2D) and methods of assessing vascular reactivity (FMD, NMD, brachial artery ultrasound, post-occlusive reactive hyperemia, iontophoresis, skin microdialysis, intradermal injection, pressure-induced vasodilation, local thermal hyperemia, current-induced vasodilation, nerve-axon reflex, LDF, LDI, LSCI, Doppler wires, strain gauge plethysmography and venous occlusion plethysmography). Searches were limited to ‘human’ studies only; but were not limited by study design. The search strategy is presented in Supplemental Table 5-S1. A manual search of reference citations in identified reviews and original articles selected for full text retrieval was also performed.

5.4.3 Study selection

Two investigators (J. L. and F.T) independently performed study selection using Covidence[®], an online, Cochrane approved, software for conducting systematic reviews.²⁵² Discrepancies in inclusion/exclusion were solved through consultation with a third (G. W.) or fourth reviewer (M.R.). To be included in this review, each study had to assess vascular function in the basal state, in two or more of the health groups of interest. Only data from vascular assessments completed on those aged ≥ 18 years were included. The complete inclusion and exclusion criteria are available as supplemental methods.

5.4.4 Data extraction and quality assessment

Characteristics of the population, outcomes and covariates of interest were summarized from each study into a pre-formatted spreadsheet by two investigators (J.L. and M.R.). If data were unclear or were not available in the published manuscripts, the corresponding or first author was contacted by email to request this information. To minimize heterogeneity, research using methods of assessing microvascular function that were not often used in the literature (i.e. in <5 studies) were excluded from the

network meta-analyses. The full list of variables extracted and details about data extraction are available as supplemental methods.

A SAQOR, previously applied in meta-analyses of observational studies evaluating vascular function,^{54,214} was performed to provide assessment of study quality. The SAQOR was scored out of 17; quality deemed better with a greater score. The GRADE was performed to provide assessment of the quality of evidence for outcomes investigated by this meta-analysis.¹⁵⁴ The GRADE for each outcome was classified as high quality, moderate quality, low quality, or very low quality. More details about the GRADE quality assessment are available as supplemental methods.

5.5.5 Data synthesis and analysis

All statistical analyses were performed using R statistical software (version 3.2.4), using the Metafor, Meta and Netmeta packages. Considering that macrovascular endothelium-dependent and endothelium-independent reactivity were each assessed with a single method, FMD and NMD, respectively, macrovascular data was synthesized using the mean difference. In contrast, microvascular reactivity was assessed with various techniques; and, thus, the SMD summary statistic was used. Direct, pairwise meta-analyses were performed first to assess pooled mean differences or SMD, as well as 95% CI, in macrovascular and microvascular data, respectively, between healthy controls and each other health group. A DerSimonian and Laird random-effects model was used when substantial heterogeneity was detected (I^2 statistic, >50 %; or p-value of the Q statistic, <0.10). A negative mean difference or negative SMD indicated that vascular reactivity was impaired in that health group when compared to another.

A frequentist network meta-analysis was then performed using the graph theoretical method developed by Rucker *et al.* A network evidence plot was produced with the nodes indicating the health groups being assessed and the thickness of lines referring to the number of direct comparisons between each health group (e.g. the thicker the line, the more direct comparisons). The hypotheses of homogeneity and consistency were explored by the Q statistic and netheat plots. Additionally, the node splitting method assessed the consistency between direct and indirect comparisons, with a p-value of <0.05 deemed inconsistent. A Hasse diagram, using partial order sets (posets),

was used to rank the severity of impairment in vascular reactivity for each health group included in the network meta-analysis. Publication bias was evaluated by funnel plot asymmetry and by using Egger's regression test, with a p-value of <0.05 suggesting publication bias when more than 10 studies were available in each health group.

Several post hoc meta-regressions were performed on the following potential effect modifiers of macrovascular reactivity using a Bayesian approach: age, body mass index, brachial artery diameter at rest, blood pressure, fasting glucose, HbA1c %, total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides. The Bayesian network meta-analysis was performed using four chains, 10,000 burn-in and 50,000 iterations using gemtc package (version 0.8-2). Convergence was assessed using the Gelman-Rubin-Brooks plot.

5.5 Results

5.5.1 Study selection and characteristics

The systematic search resulted in the inclusion of 193 from a total of 4641 potential articles (Figure 5-1). From the 193 studies included in the analyses, vascular reactivity was assessed in a total of 26,570 patients that were considered healthy ($n = 7226$), overweight ($n = 7605$) or obese ($n = 1758$), or that were diagnosed with MetS ($n = 2405$), impaired glucose tolerance ($n = 936$), T2D ($n = 5254$) or T2D with vascular complications ($n = 1386$). The main characteristics for each study are presented in Supplemental Table 5-S2. Flow-mediated dilation of the brachial artery was the most frequently used test of vascular reactivity ($n = 120$), while an array of tests was used to assess microvascular reactivity.

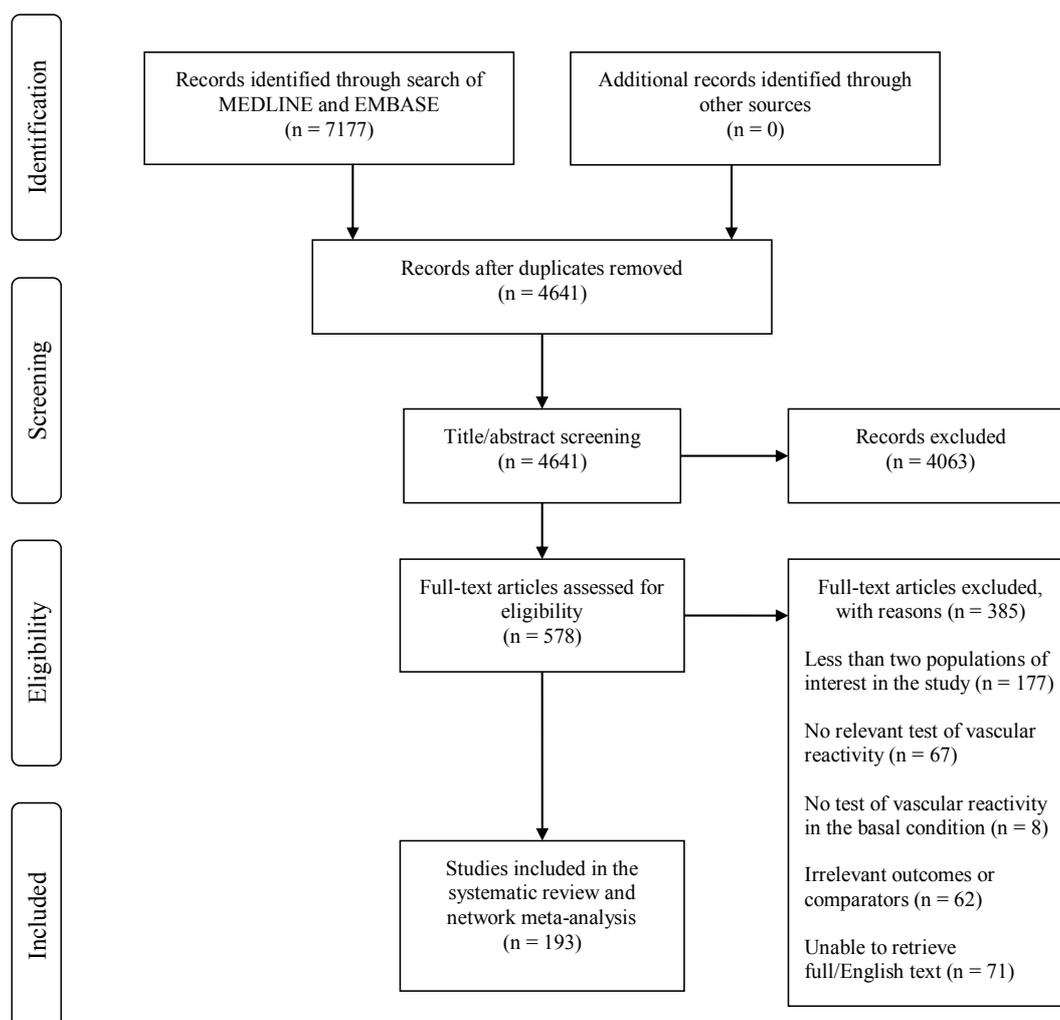


Figure 5-1. Flow diagram of the study selection process.

5.5.2 Quality assessment and potential bias

The quality score and risk of bias for each study are reported in Supplemental Table 5-S2. The mean quality score was 14.3 ± 2.1 out of a possible 17 points. Quality assessments graded two studies with a high risk of bias, 53 with moderate risk of bias, and 138 with low risk of bias. Overall, the quality of evidence for outcomes demonstrating the impairment of vascular reactivity throughout the pathogenesis of T2D and its complications was low-to-very low (Supplemental Table 5-S3 to 5-S6). Evaluation of funnel plot asymmetry and the Egger's regression test suggested a possible publication bias for microvascular and macrovascular endothelium-dependent reactivity in those with T2D, compared with healthy controls (Supplemental Figure 5-S2). No major asymmetry was found in data for other health groups.

5.5.3 Pairwise meta-analyses results

Results of the pairwise meta-analyses, summarized in Table 5-1, demonstrate that macrovascular endothelium-dependent reactivity is impaired in all health groups when compared to healthy controls. In contrast, endothelium-independent vascular reactivity is not significantly affected in overweight and obese subjects. In the microcirculation, abnormal endothelium-dependent and endothelium-independent vascular reactivity were detected in obese patients and in all health groups with cardiometabolic disease, except for patients with impaired glucose tolerance.

5.5.4 Network meta-analyses

The networks of available comparisons for endothelium-dependent and endothelium-independent vascular reactivity, in the microcirculation and macrocirculation, are represented in Figure 5-2. These network meta-analyses indicate a progressive impairment of endothelium-dependent reactivity in both the microcirculation and macrocirculation, throughout the pathogenesis of T2D and its related complications (Table 5-2 and 5-3, Figure 5-3). A similar pattern was observed for endothelium-independent vascular reactivity in large vessels, while fewer differences were seen in the microcirculation. Forest plots for comparisons of endothelium-dependent and endothelium-independent vascular reactivity between all health groups, using each health group as the reference, are represented in Supplemental Figure 5-S2; demonstrating similar patterns in the impairment of endothelium-dependent and endothelium-independent vascular reactivity at each comparison.

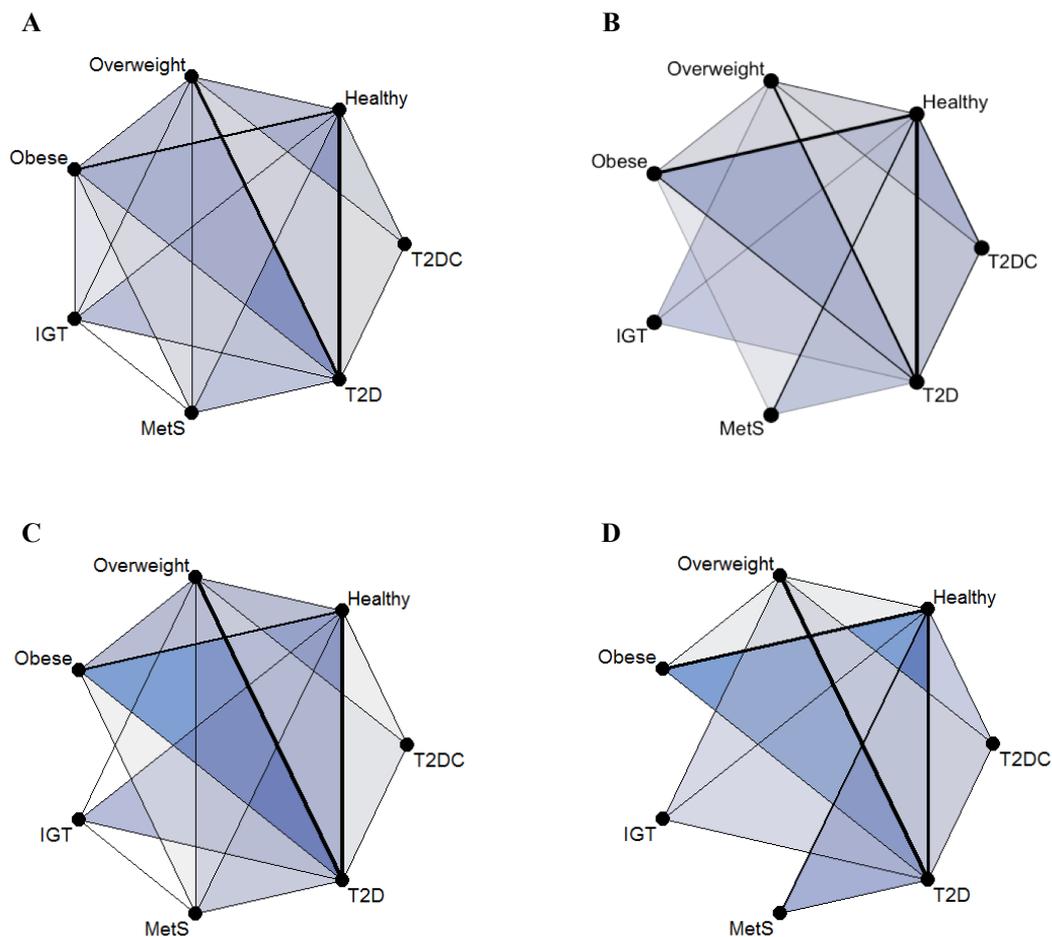


Figure 5-2. The networks of available comparisons between each health group from studies included in the network meta-analysis; for (A) macrovascular and (B) microvascular endothelium-dependent reactivity; as well as (C) macrovascular and (D) microvascular endothelium-independent reactivity. The thickness of lines refers to the number of direct comparisons between each health group; with thicker lines indicating more comparisons. Where there is no line joining two health groups, there was no previous direct comparison of vascular reactivity between those health groups in the literature. IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

The consistency between direct and indirect comparisons was assessed by the node-splitting method (Supplemental Table 5-S7 to 5-S10). Although several comparisons had significant heterogeneity between direct and indirect evidence, the difference was primarily driven by the magnitude of the effect size and not by the direction of effect, suggesting consistency in this study's results. Furthermore, only four comparisons differed in direction: type 2 diabetes vs type 2 diabetes with complications, in 1) macrovascular endothelium-dependent reactivity and 2) macrovascular endothelium-

independent vascular reactivity; 3) healthy vs obese, in macrovascular endothelium-independent vascular reactivity; and 4) obese vs overweight, in microvascular endothelium-independent vascular reactivity. Netheat plots confirmed the overall consistency in the results (Supplemental Figure 5-S3).

Considering the risk of bias related to the outcome assessment, sensitivity analyses, only including studies in which the outcome assessors were blinded to the health group classification, were conducted (Supplemental Figure 5-S4). Indeed, a similar trend in the impairment of endothelium-dependent vascular reactivity was observed, but the effect size was smaller. For example, in patients with T2D, the mean difference for FMD was about 20% lower when outcome assessment was blinded. Similar results were found for endothelium-independent vascular reactivity.

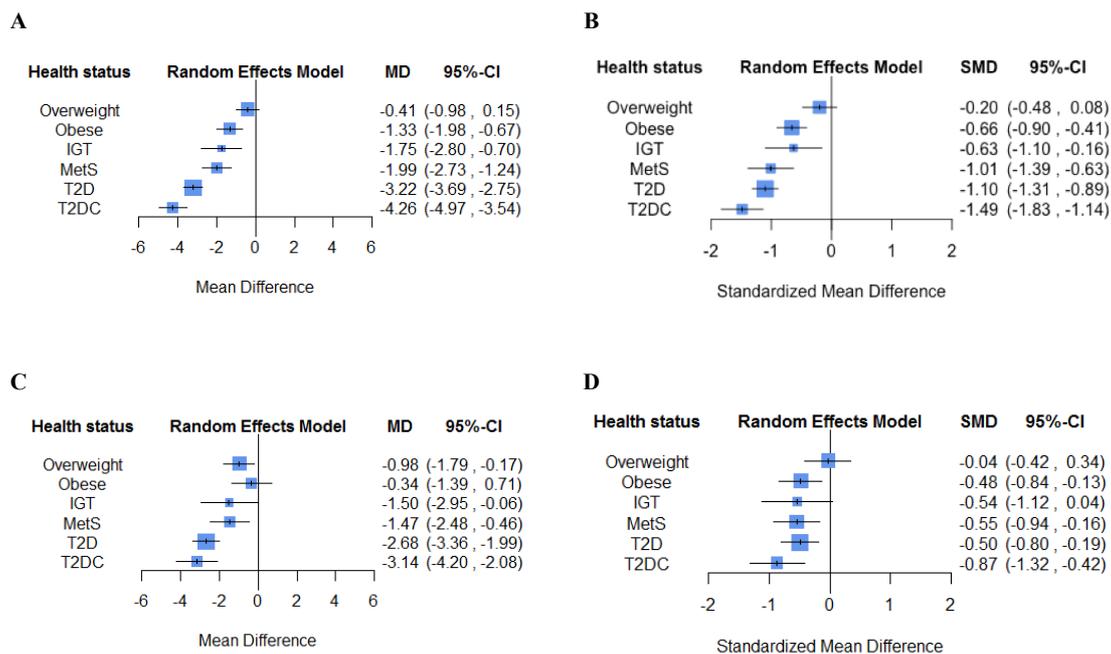


Figure 5-3. Forest plots of the mean difference (MD) in (A) macrovascular endothelium-dependent reactivity and (C) macrovascular endothelium-independent reactivity; and the standardized mean difference (SMD) in (B) microvascular endothelium-dependent reactivity and (D) microvascular endothelium-independent reactivity between each health group considered overweight or obese, or with cardiometabolic disease, as compared to the healthy group in the network meta-analyses. CI, confidence interval; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

5.5.5 Meta-regressions

Given that methods for assessing macrovascular endothelium-independent reactivity and microvascular reactivity remain largely unstandardized and that data, in some cases, were limited, meta-regressions were only performed on FMD data. Among the 11 potential effect modifiers, only fasting blood glucose was significantly correlated to FMD (Supplemental Table 5-S11); suggesting that the severity of impairment in macrovascular reactivity worsened as fasting blood glucose concentrations increased (Supplemental Figure 5-S5). Indeed, for every 1 mmol/l increase of fasting blood glucose concentration, there is a 0.52 % decrease in FMD.

5.5.6 Microvascular *versus* macrovascular dysfunction

To explore whether the pattern of impairment in vascular reactivity is similar between the microcirculation and the macrocirculation, when compared across all cardiometabolic health groups, ranks for macrovascular endothelium-dependent reactivity were plotted against ranks for microvascular endothelium-dependent reactivity (Supplemental Figure 5-S6A). They were subsequently computed as posets. Overall, the ranks were similar between the macrocirculation and the microcirculation: healthy status preceded impaired glucose tolerance and an overweight status, which preceded both MetS and obesity, which preceded T2D, which finally preceded T2D with vascular complications, as demonstrated in the Hasse diagram (Supplemental Figure 5-S6B).

Table 5-1. Meta-analysis results of pairwise comparisons *versus* healthy controls

	Macrovascular						Microvascular					
	Endothelium-dependent			Endothelium-independent			Endothelium-dependent			Endothelium-independent		
	n	MD (95% CI)	I ²	n	MD (95% CI)	I ²	n	SMD (95% CI)	I ²	n	SMD (95% CI)	I ²
Overweight	9	-1.26 (-1.75, -0.77)	0	6	-1.38 (-3.48, 0.72)	51	6	0.04 (-0.30, 0.38)	45	1	-0.17 (-1.88, 1.53)	.
Obese	22	-0.88 (-1.51, -0.26)	65	10	-1.68 (-3.83, 0.47)	75	20	-0.70 (-0.99, -0.41)	78	9	-0.65 (-1.13, -0.18)	68
IGT	2	-1.09 (-1.40, -0.77)	0	2	-1.02 (-2.02, 0.02)	44	3	-0.73 (-1.50, -0.03)	70	1	-0.79 (-1.72, 0.14)	.
MetS	9	-1.78 (-3.17, -0.40)	94	3	-1.21 (-2.00, -0.43)	0	10	-1.05 (-1.49, -0.61)	78	7	-0.58 (-0.96, -0.19)	65
T2D	38	-3.67 (-4.34, -3.00)	93	19	-2.34 (-3.32, -1.37)	68	25	-1.27 (-1.63, -0.91)	81	10	-0.31 (-0.55, -0.06)	0
T2DC	7	-3.29 (-4.68, -1.90)	90	1	-4.40 (-6.37, -2.43)	.	9	-1.07 (-1.35, -0.78)	41	3	-1.27 (-2.01, -0.52)	0

Values presented in bold font are significantly different from healthy subjects. n, number of studies; MD, mean difference; CI, confidence interval; SMD, standardized mean difference; I², results of heterogeneity statistic; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-2. Network meta-analysis results for macrovascular reactivity. Results are mean difference (95% CI)

Macrovascular endothelium-dependent reactivity						
Healthy	-0.41 (-0.98, 0.15)	-1.33 (-1.98, -0.67)	-1.75 (-2.80, -0.70)	-1.99 (-2.73, -1.24)	-3.22 (-3.69, -2.75)	-4.26 (-4.97, -3.54)
-0.98 (-1.79, -0.17)	Overweight	-0.91 (-1.67, -0.16)	-1.34 (-2.33, -0.34)	-1.57 (-2.31, -0.84)	-2.80 (-3.29, -2.32)	-3.84 (-4.57, -3.12)
-0.34 (-1.39, 0.71)	0.64 (-0.52, 1.81)	Obese	-0.42 (-1.59, 0.74)	-0.66 (-1.57, 0.25)	-1.89 (-2.61, -1.17)	-2.93 (-3.84, -2.02)
-1.50 (-2.95, -0.06)	-0.52 (-2.00, 0.96)	-1.17 (-2.89, 0.56)	IGT	-0.24 (-1.40, 0.93)	-1.47 (-2.50, -0.44)	-2.51 (-3.68, -1.33)
-1.47 (-2.48, -0.46)	-0.49 (-1.42, 0.43)	-1.13 (-2.43, 0.16)	0.03 (-1.61, 1.67)	MetS	-1.23 (-1.99, -0.47)	-2.27 (-3.21, -1.33)
-2.68 (-3.36, -1.99)	-1.69 (-2.35, -1.04)	-2.34 (-3.45, -1.23)	-1.17 (-2.63, 0.29)	-1.20 (-2.18, -0.23)	T2D	-1.04 (-1.69, -0.38)
-3.14 (-4.20, -2.08)	-2.16 (-3.12, -1.20)	-2.80 (-4.18, -1.43)	-1.64 (-3.31, 0.03)	-1.67 (-2.92, -0.42)	-0.47 (-1.36, 0.43)	T2DC
Macrovascular endothelium-independent reactivity						

Values presented in bold font are significantly different. CI, confidence interval; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-3. Network meta-analysis results for microvascular reactivity. Results are SMD (95% CI)

Microvascular endothelium-dependent reactivity						
Healthy	-0.20 (-0.48, 0.08)	-0.66 (-0.90, -0.41)	-0.63 (-1.10, -0.16)	-1.01 (-1.39, -0.63)	-1.10 (-1.31, -0.89)	-1.49 (-1.83, -1.14)
-0.04 (-0.42, 0.34)	Overweight	-0.46 (-0.79, -0.13)	-0.43 (-0.90, 0.04)	-0.81 (-1.27, -0.35)	-0.90 (-1.16, -0.64)	-1.29 (-1.67, -0.90)
-0.48 (-0.84, -0.13)	-0.45 (-0.90, 0.01)	Obese	0.03 (-0.48, 0.54)	-0.35 (-0.79, 0.09)	-0.44 (-0.72, -0.17)	-0.83 (-1.23, -0.42)
-0.54 (-1.12, 0.04)	-0.50 (-1.05, 0.04)	-0.06 (-0.71, 0.59)	IGT	-0.38 (-0.98, 0.22)	-0.47 (-0.94, 0.00)	-0.86 (-1.41, -0.30)
-0.55 (-0.94, -0.16)	-0.51 (-1.04, 0.01)	-0.07 (-0.59, 0.45)	-0.01 (-0.70, 0.68)	MetS	-0.09 (-0.51, 0.32)	-0.48 (-0.98, 0.03)
-0.50 (-0.80, -0.19)	-0.46 (-0.74, -0.18)	-0.01 (-0.43, 0.41)	0.04 (-0.50, 0.58)	0.05 (-0.42, 0.52)	T2D	-0.38 (-0.73, -0.04)
-0.87 (-1.32, -0.42)	-0.84 (-1.26, -0.42)	-0.39 (-0.93, 0.15)	-0.33 (-0.97, 0.31)	-0.32 (-0.90, 0.26)	-0.38 (-0.77, 0.01)	T2DC
Microvascular endothelium-independent reactivity						

Values presented in bold font are significantly different. CI, confidence interval; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

5.6 Discussion

The objective of this study was to compare vascular reactivity across the spectrum of cardiometabolic health, from healthy populations, through those with obesity, impaired glucose tolerance, MetS and T2D, to those with T2D with complications. Combining direct and indirect comparisons from 193 studies, the findings of this network meta-analysis indicate a progressive impairment in microvascular and macrovascular endothelium-dependent reactivity throughout the pathogenesis of T2D and its complications. A similar pattern was also observed in endothelium-independent vascular reactivity in the macrocirculation, but not in the microcirculation; which remained relatively unaffected across the health groups.

When interpreting these findings, however, it is important to acknowledge from the outset that this network meta-analysis does not properly address whether impairment in the microcirculation precedes that of the large vessels, a widely accepted hypothesis.²³³ Indeed, while FMD of the brachial artery is a more standardized procedure than most methods of assessing vascular reactivity, it must be noted that a majority of studies (116 of the 120 included in this network meta-analysis) did not account for changes in shear rate during their data analyses. Subsequently, it is not possible to comprehensively conclude that the impairment in macrovascular endothelium-dependent reactivity is due to intrinsic abnormalities of macrovascular function, or if they are partially attributable to downstream abnormalities (e.g. microvascular dysfunction) and/or simply a decrease in the stimulus for conduit artery dilation.⁵⁸ Considering this, future vascular studies and the accurate interpretation of their data would be improved by adopting previously standardized methodology for evaluating vascular reactivity. In a similar vein, it must also be noted that the progressive decline in FMD is associated with a decrease in NMD, suggesting that impaired vascular reactivity could be caused by one or several factors; including abnormalities in endothelial function, endothelium-independent function or structural changes within the blood vessel itself. Regardless, this study is the first to demonstrate a continuum where the severity of impairment in vascular reactivity progressively increases throughout the pathogenesis of obesity and/or insulin resistance, through to the development of diabetic complications. Furthermore, this data reinforces that

chronic impairments in vascular reactivity present early in the decline of cardiometabolic health, long before the clinical onset of overt diseases. The impact of such chronic vascular impairment on the pathogenesis of CVD, even in these early stages, may be significant with data indicating that the risk of coronary heart disease is increased in those who are metabolically healthy, but are considered obese.²⁵⁴

Notably, meta-regression analyses of FMD data and potential effect modifiers found one significant, negative correlation, that between FMD and fasting blood glucose concentration; indicating that as fasting blood glucose concentration increases by 1 mmol/l, FMD decreases by 0.52 %. Indeed, hyperglycemia is one of several other factors, including insulin resistance, obesity and low-grade inflammation, which may influence vascular function throughout the decline of cardiometabolic health. Elevated generation of ROS appears to be a unifying pathway between each of these factors and impaired vascular reactivity.⁷ Oxidative stress may induce endothelial dysfunction by disrupting the synthesis of the potent vasodilator, NO; thus, reducing its bioavailability.⁷ Additionally, hyperglycemia-mediated increases in the concentration of the superoxide anion may deactivate available NO, converting it to the oxidant, peroxynitrite, which induces substrate nitration and, subsequently, further disrupts endothelial NO synthase and enzyme activity.⁷ Acknowledging that other signalling pathways of vasomotion may be affected by oxidative stress, reduced NO bioavailability is considered a strong predictor of CVD outcomes.⁷ Cardiometabolic diseases are characterized by abnormally frequent hyperglycemic excursions. Such exposure to a hyperglycemic environment that is 'chronic' in nature may also induce VSM cell proliferation by disrupting its natural apoptosis.²⁵⁵ Furthermore, hyperglycemia may enhance the production of advanced glycation end products and collagen cross-linking.²⁵⁵ Collectively, these mechanisms stiffen the arterial wall, possibly explaining why vascular reactivity may be reduced in those with impaired glucose tolerance, MetS and T2D. Ultimately, this increase in myogenic tone elevates the risk of injury to the endothelial wall; an event that is significant in the pathogenesis of CVD.

There are several inherent limitations to this research that must be addressed. Many studies included in this network meta-analysis used control groups that had no specific health classification. Considering this, mean clinical data from all included studies

assessing health groups not defined as T2D, with or without complications, were checked against current definitions for the different health groups. Although this approach allowed us to obtain more homogeneous health groups while acknowledging the definitions and criteria that have evolved over time, it does not take into account the heterogeneity between subjects within each study arm. Furthermore, most studies did not include parameters that define MetS and are known to have an influence on vascular reactivity, such as insulin resistance; and, therefore, the effect of such factors could not be accounted for in this analysis. It must also be recognised that while there is a large amount of data focusing on endothelium-dependent vascular reactivity, the number of studies that assessed endothelium-independent vascular reactivity was limited; thus, reducing the power to detect differences between health groups and develop conclusions about the changes in endothelium-independent vascular reactivity. Similarly, few studies assessed vascular reactivity in those with impaired glucose tolerance. Although potential bias was detected in several outcomes in this study, the SAQOR revealed a predominantly low risk of bias. Finally, the GRADE indicates that the quality of evidence for outcomes assessed in this study is low-to-very low; as is the nature of observational data. Although this does not affect the conclusion regarding the trends, the confidence in the effects estimates is more limited. Similarly, sensitivity analyses including only studies in which the outcome assessors were blinded to the health group indicate that the trends remain the same, but with lower effects estimates. This highlights evaluation biases, which is a limitation in techniques such as FMD. Similarly, funnel plot asymmetry and the Egger's regression test suggest possible publication bias in patients with T2D (endothelium-dependent reactivity data). However, there is no major asymmetry for other health groups; and, therefore, the impact of publication bias on these findings is difficult to evaluate.

This present study demonstrates that vascular reactivity may be impaired early, even in those considered overweight; while previous research from our laboratory has demonstrated that acute hyperglycemia, induced by excess sugar consumption, transiently blunts endothelium-dependent vascular reactivity, even in those considered healthy.²⁵⁶ Considering this, further research is needed to assess if transient impairments in vascular reactivity, mediated by several dietary and lifestyle factors, develop into chronic vascular impairment before, when someone is still considered clinically healthy, or after the clinical onset of an overweight state or insulin resistance.

Furthermore, given that there may be differences in vascular function between ethnicities and gender,²¹⁰ future research may also assess vascular reactivity, as well as the mechanisms that underlie any impairment (e.g. disruption of the NO pathway), in varying health populations from a range of ethnic backgrounds.

In conclusion, this network meta-analysis demonstrates a continuum where vascular reactivity is progressively impaired throughout the pathogenesis of obesity and/or insulin resistance; where for every 1mmol/l increase in fasting blood glucose concentration, FMD decreases by 0.52 %. Ultimately, though, these results support the need for early interventions in at-risk populations, to overturn the progressive deterioration of vascular health and, subsequently, improve cardiovascular outcomes.

5.7 Supplemental material

5.7.1 Supplemental methods

5.7.1.1 Study selection

Two investigators (J. L. and F.T) independently performed study selection using Covidence[®], an online, Cochrane approved, software for conducting systematic reviews.²⁵² Discrepancies in inclusion/exclusion were solved through consultation with a third (G. W.) or fourth reviewer (M.R.). To be included in this review, each study had to assess vascular reactivity in the basal state, in two or more of the health groups of interest. Only data from vascular assessments completed on those aged ≥ 18 years were included.

To avoid confounding factors that are known to influence vascular reactivity, health groups of interest were excluded from the review if it included patients with a history of cancer or transplant (e.g. kidney, bone marrow, or blood transfusion), patients with the human immunodeficiency virus, blood disorders (e.g. anaemia, sickle-thalassemia, or hemophilia), rheumatoid arthritis, lupus, polycystic ovary syndrome, non-diabetic chronic kidney disease or end stage renal disease (kidney failure), or sleep apnea. Health groups that included patients with type 1 diabetes, gestational diabetes or who were pregnant, or postpartum, at the time of assessment were also excluded. In any circumstance where a study included a cohort comprised of more than one health group of interest (e.g. MetS with and without T2D or T2D with and without complications), that group was excluded from all analyses; and, indeed, if that study subsequently presented less than two health groups of interest, it was excluded from the review. Finally, inclusion was limited to studies originally published in the English language or where translated copies had been made available.

5.7.1.2 Data extraction and quality assessment

Where available, the following variables were summarized from each study into a pre-formatted spreadsheet by two investigators (J.L. and M.R.): authors, year of publication, characteristics of participants (n, health status, % female, menstruation status, age, ethnicity, aerobic fitness [VO₂ max], weight, height, body mass index, waist circumference, waist-to-hip ratio, body fat %, resting blood pressure, resting heart rate,

fasting blood glucose, HbA_{1c} %, fasting blood insulin, HOMA-IR, total cholesterol, concentration of HDL cholesterol, LDL cholesterol, triglycerides, history of hypertension, dyslipidemia, neuropathy, retinopathy, nephropathy, diabetic foot ulceration, stroke, adverse cardiovascular events, smoking status and details of medication use, as well as blood biomarkers of inflammation, oxidative stress, endothelial dysfunction, liver function, and kidney function) and vascular variables (vascular region assessed, method used [test of vascular reactivity, occlusion period, temperature applied or dose of vasoactive drug, iontophoretic protocol, length of time post-vascular reactivity test to vascular assessment] and the vascular response to each test of reactivity). If data were unclear or were not available in the published manuscripts, the corresponding or first author was contacted by email to request this information.

In the event of multiple publications pertaining to the same research, the first published or more comprehensive study was included. If two cohorts of the same health group were included in a single study along with a third health group of interest (e.g. three health groups in a single study: two groups with T2D and one healthy group), the sample of the third group (e.g. healthy) was evenly divided into two separate (healthy) groups to compare to the two groups with T2D; allowing for each separate health group to be included in the network meta-analysis, whilst maintaining the same power. Indeed, if the study included three cohorts that had the same health classification, three separate studies were subsequently created for the same reason. If a single study examined vascular reactivity, using the same methodology in two different vascular regions (e.g. calf and forearm), in all health groups of interest included in that research, then each set of data (classified by vascular region) were treated as separate studies. Where a single study assessed basal vascular reactivity multiple times in the same sample, only data for the initial assessment was included in the analysis. If blood perfusion was measured following varying concentrations of acetylcholine or sodium nitroprusside intravenous infusion, then vascular data measured at the strongest concentration was included in the analysis. To minimize heterogeneity, research using methods of assessing microvascular reactivity that were not often used in the literature (i.e. in <5 studies: oral administration of berapost sodium [number of studies, n = 2]; iontophoresis of insulin [n = 2]; intravenous infusion of BQ123 [n = 2], methacholine [n = 3], verapamil [n = 2], isoprenaline [n = 2], endothelin-1 [n = 4], norepinephrine [n

= 3], insulin [n = 2], bradykinin [n = 2], serotonin [n = 3] or serotonin with insulin [n = 1]; pressure-induced vasodilation [n = 1]; and near-infrared spectroscopy [n = 1]), were excluded from the network meta-analyses.

A SAQOR, previously applied in meta-analyses of observational studies evaluating vascular function,^{54,214} was performed to provide assessment of study quality. The SAQOR was scored out of 17, quality deemed better with a greater score. The GRADE was performed to provide assessment of the quality of evidence for outcomes investigated by this meta-analysis.¹⁵⁴ The GRADE for each outcome was classified as high quality, moderate quality, low quality, or very low quality. As the design of all studies included in this review are observational, the quality of evidence was rated low before being downgraded or upgraded. Quality of evidence decreased due to: 1) study limitations, 2) inconsistency of results, 3) indirectness of evidence, 4) imprecision, or 5) reporting bias. Quality of evidence was upgraded if there was: 1) a large magnitude of effect, 2) a dose-response gradient, or 3) plausible biases would decrease the magnitude of an apparent treatment effect. Assessments of study quality and quality of evidence were performed independently by two investigators (J.L. and F.T.). Discrepancies were solved by consensus or through consultation with a third reviewer (G. W.).

5.7.2 Supplemental figures

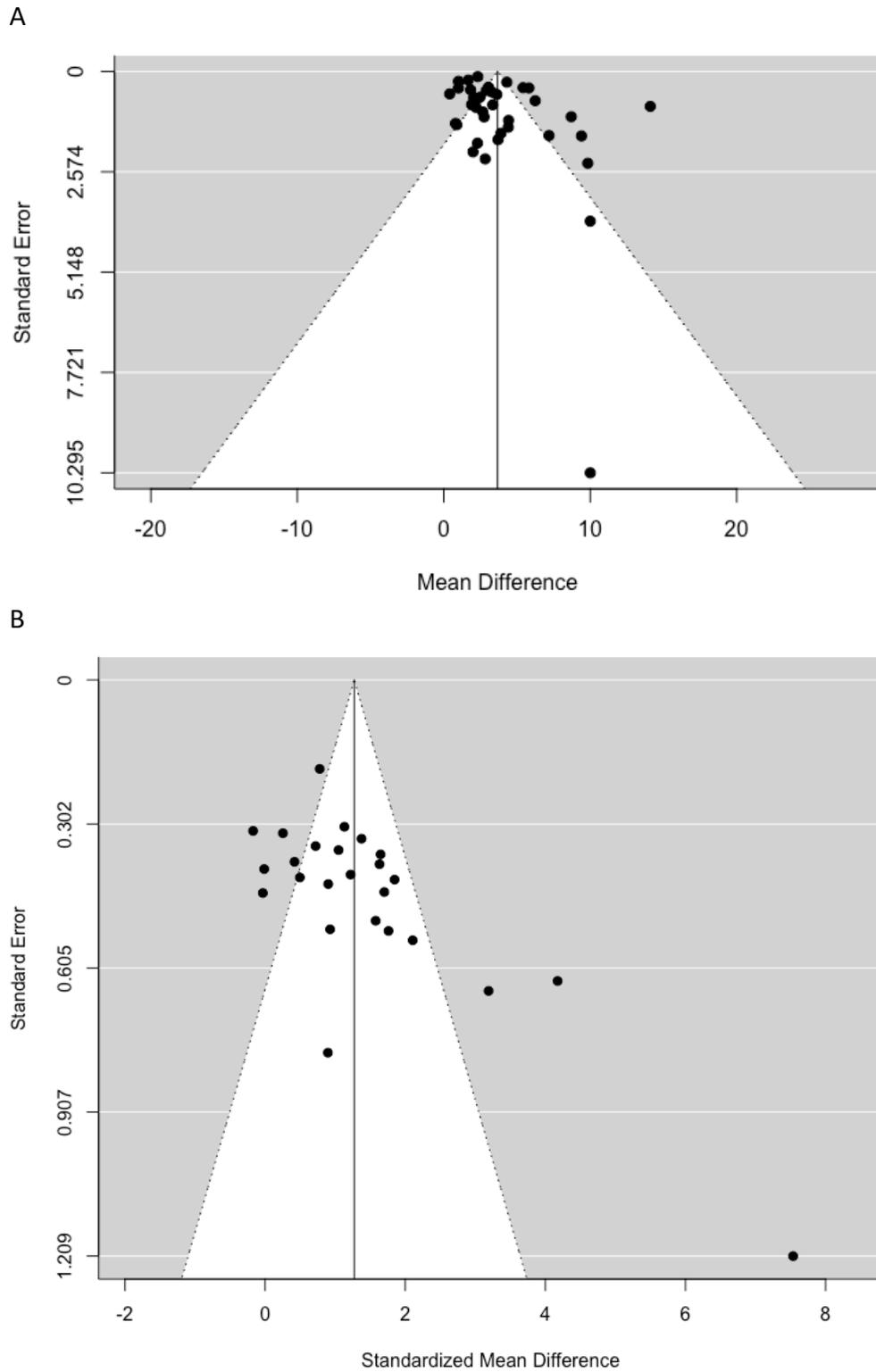


Figure 5-S1. Detection of publication bias following evaluation of funnel plot asymmetry in data for **(A)** macrovascular and **(B)** microvascular endothelium-dependent reactivity in those with type 2 diabetes.

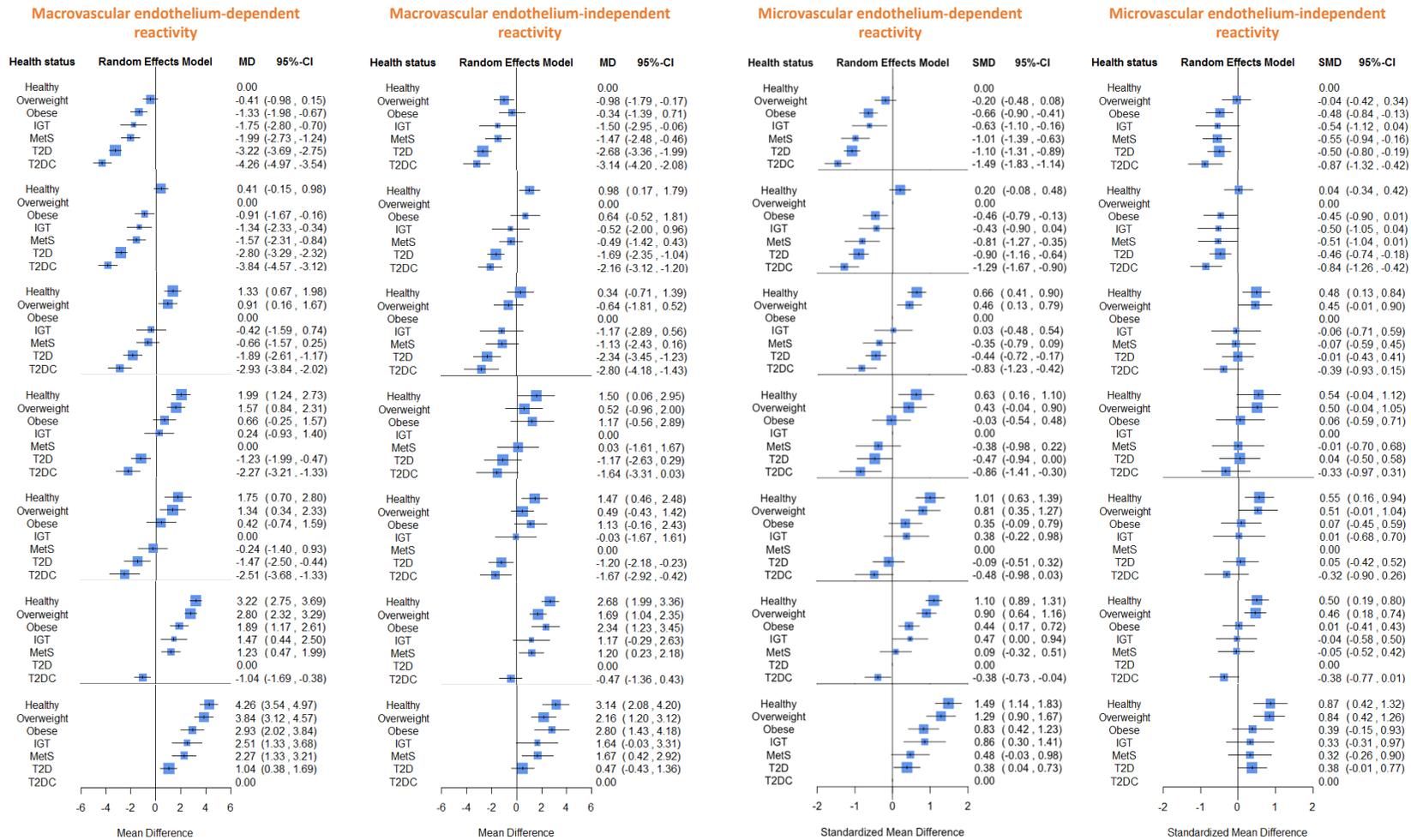
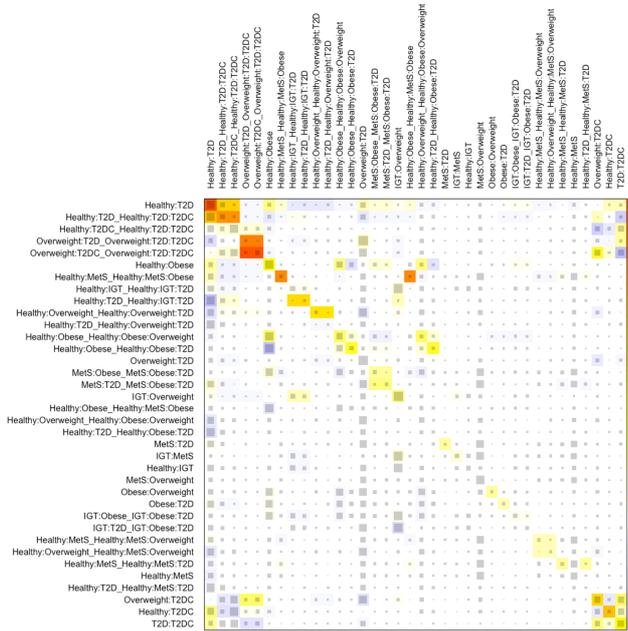
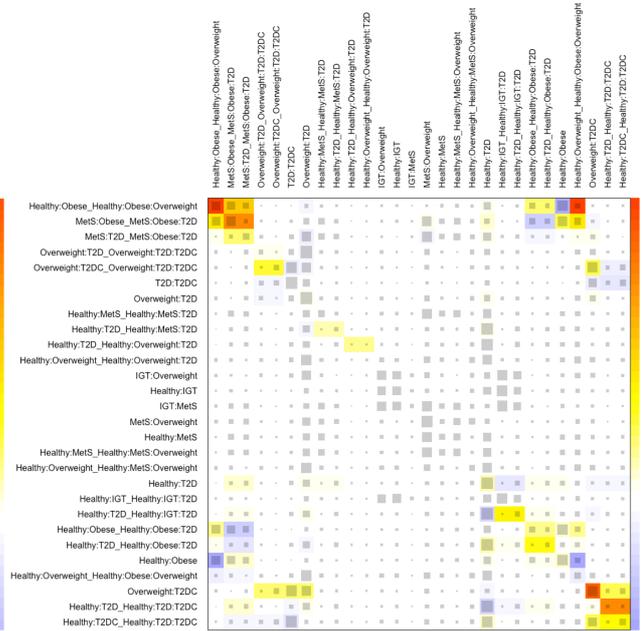


Figure 5-S2. Forest plots of the mean difference (MD) in macrovascular reactivity and standardized mean difference (SMD) in microvascular reactivity between each health group included in the network meta-analyses, using each health group’s vascular data as the reference. CI, confidence interval; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

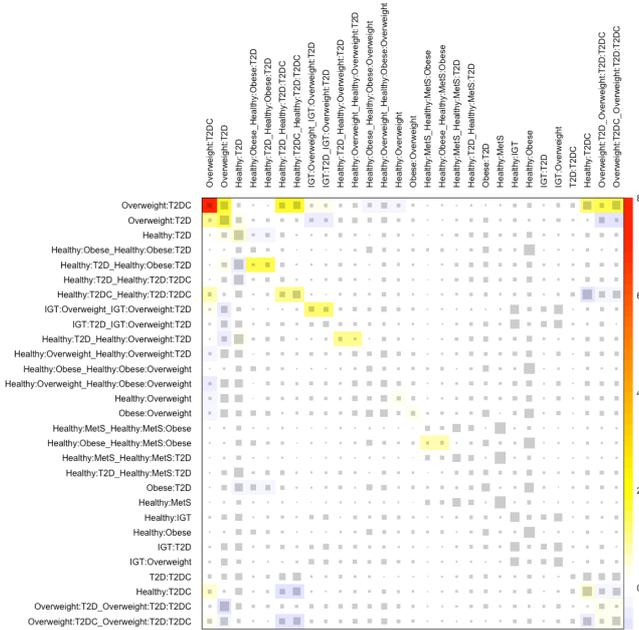
Macrovascular endothelium-dependent reactivity



Macrovascular endothelium-independent reactivity



Microvascular endothelium-dependent reactivity



Microvascular endothelium-independent reactivity

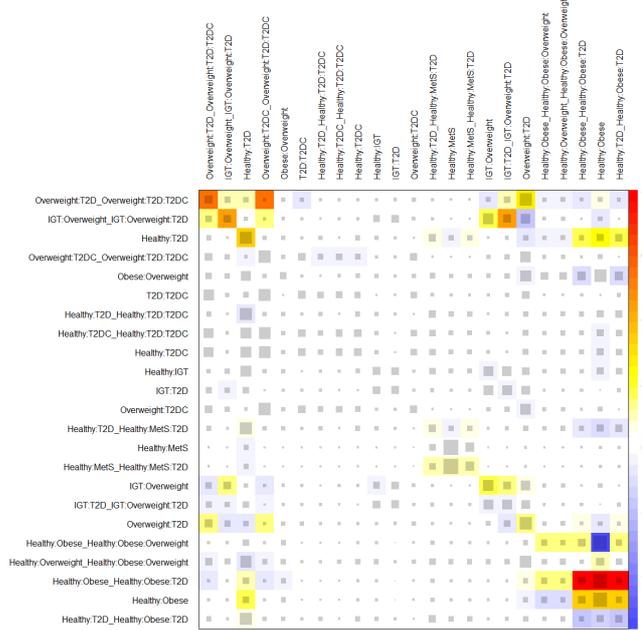
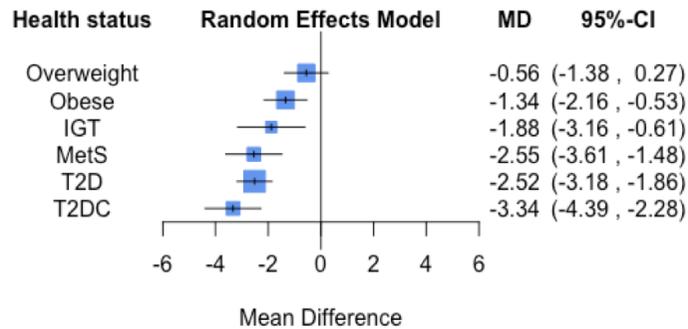
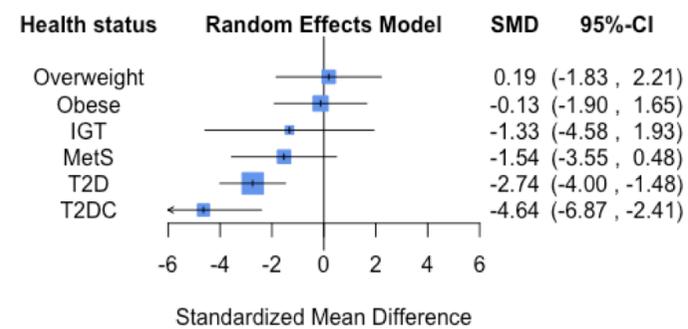


Figure 5-S3. A netheat plot illustrating the consistency of the direct and indirect comparisons. Grey squares indicate the contribution of the direct estimate (shown in the columns) to the network estimate (shown in the rows). The colours are associated with the change in inconsistency between direct and indirect evidence. Blue colours indicate an increase and warm colours indicate a decrease; with the stronger the intensity of the colour, the stronger the change. IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

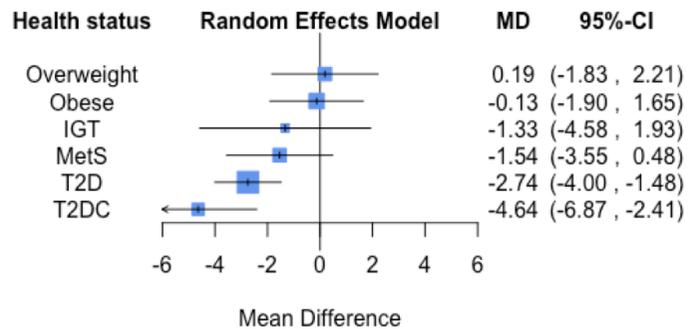
Macrovascular endothelium-dependent reactivity



Microvascular endothelium-dependent reactivity



Macrovascular endothelium-independent reactivity



Microvascular endothelium-independent reactivity

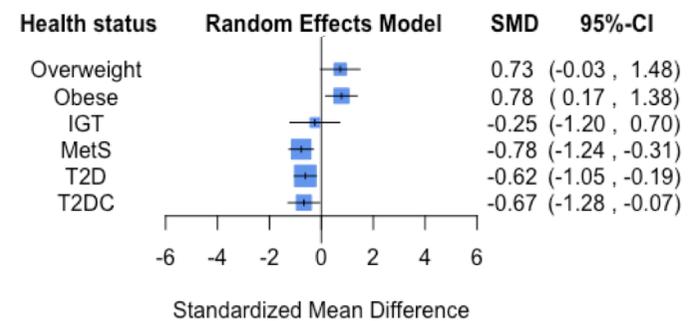


Figure 5-S4. Sensitivity analysis: Forest plots of the mean difference (MD) in macrovascular reactivity and the standardized mean difference (SMD) in microvascular reactivity between each health group with a classification of overweight, obesity or cardiometabolic disease as compared to the healthy group, including only blinded studies, in the network meta-analysis. CI, confidence interval; IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

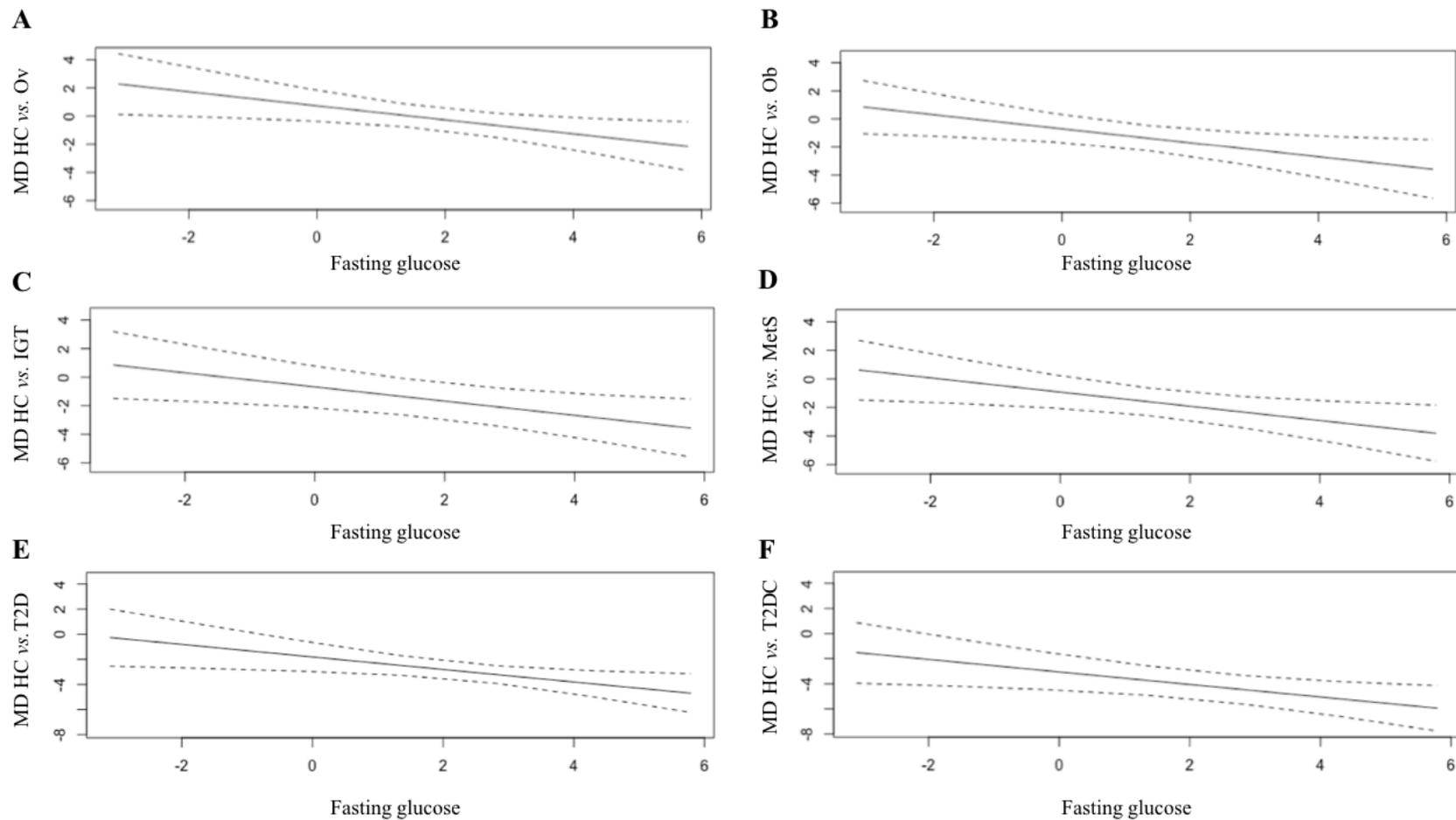


Figure 5-S5. The results from meta-regressions analyses demonstrating the correlation between flow-mediated dilation and fasting blood glucose data; with the healthy group plotted against each health group classified as **A**) overweight (Ov) or **B**) obese (Ob), or those with **C**) impaired glucose tolerance (IGT), **D**) metabolic syndrome (MetS), **E**) type 2 diabetes (T2D) or **F**) type 2 diabetes and complications (T2DC). MD, mean difference.

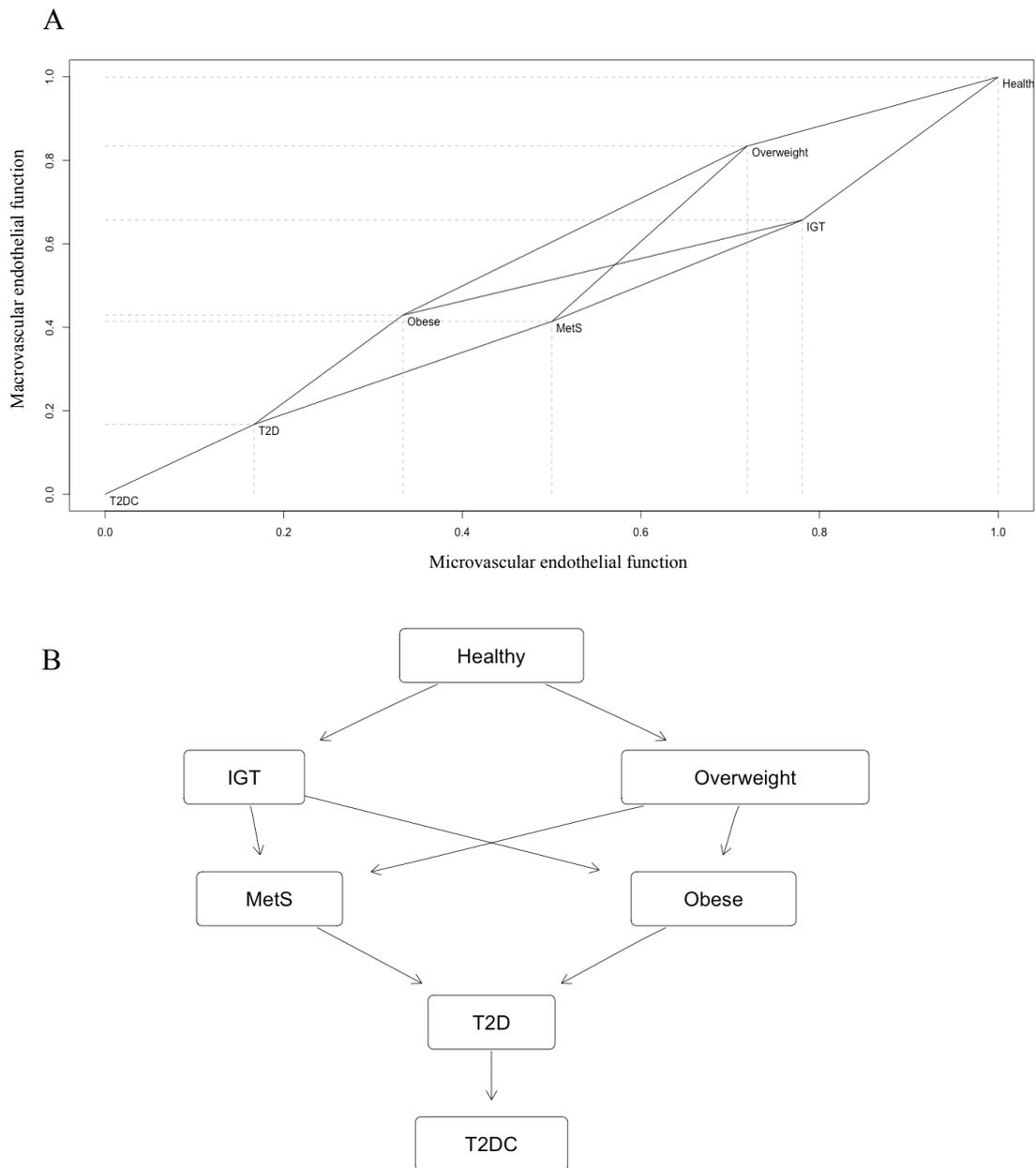


Figure 5-S6. (A) The ranking plot and (B) Hasse diagram demonstrate the impairment of microvascular and macrovascular reactivity throughout the pathogenesis of type 2 diabetes (T2D). IGT, impaired glucose tolerance; MetS, metabolic syndrome; T2DC, type 2 diabetes with complications.

5.7.3 Supplemental tables

Table 5-S1. The search criteria used in MEDLINE and EMBASE for this systematic review and network meta-analysis.

Field 1 (Title, abstract and subject headings)
obes* OR pre-diabet* OR prediabet* OR metabolic syndrome OR metS OR diabet* OR T2D
AND
Field 2 (Title, abstract and subject headings)
flow-mediated dilation OR FMD OR nitrate-mediated dilation OR NMD OR brachial artery ultrasound OR post-occlusive reactive hyper* OR PORH OR iontophoresis OR skin microdialysis OR intradermal injection* OR pressure-induced vasodilation OR PIV OR local thermal hyper* OR LTH OR current-induced vasodilation OR CIV OR nerve-axon reflex OR laser Doppler* OR LDF OR LDPI OR LDPM OR LDI OR LDSI OR laser speckle* OR LSCI OR LASCA OR Doppler wires OR strain gauge plethysmography OR venous occlusion plethysmography

Field 1 and Field 2 were combined by the Boolean operator “AND”. Terms within each field were combined with the Boolean operator “OR”.

Table 5-S2. The main characteristics of each study included in the systematic review and network meta-analysis

Study, year of publication	Quality Score (0-17)	Risk of Bias	Health Groups (n =)	Tests of vascular reactivity	
				Microvascular	Macrovascular
Joris et al, ²⁵⁷ 2017	15	Low	H (25); Ob (23); MetS (26)		FMD
Blum et al, ²⁵⁸ 2016	12.5	Moderate	Ov (23); T2D (25); T2DC (48)		FMD
Dimassi et al, ²⁵⁹ 2016	15	Low	H (46); Ob (69)	LDF - LTH, ACh	
Dow et al, ²⁶⁰ 2016	13.5	Low	H (11); Ob (10); MetS (11)	SGP - ACh	
Fakhrzadeh et al, ²⁶¹ 2016	17	Low	Ov (103); T2D (105)		FMD
Fetterman et al, ²⁶² 2016	16	Low	Ov (98); T2D (74); T2DC (48)		FMD; NMD
Francois et al, ²⁶³ 2016	11.5	Moderate	Ov (12); T2D (12)		FMD
Jahn et al, ²⁶⁴ 2016	11.5	Moderate	H (16); MetS (18)		FMD
Kovamees et al, ²⁶⁵ 2016	14	Moderate	H (12); T2D (12)	LDF - ACh	
Nasr et al, ³¹ 2016	15	Low	H (211); Ob (183)	LDF - ACh	
Park et al, ²⁶⁶ 2016	8	High	H (16); T2DC (20)	LDI - PORH, LTH	
Patik et al, ²⁶⁷ 2016	12.5	Low	H (13); Ob (14)	LDF - SNP	
Roustit et al, ³⁰ 2016	16	Low	Ov (59); IGT (75)		FMD
Shimabukuro et al, ²⁶⁸ 2016	12.5	Low	H (18); MetS (19)	SGP - ACh, SNP	
Sorensen et al, ²⁶⁹ 2016	16	Low	Ov (863); IGT (254)	LDF - LTH	
Zeng et al, ²⁷⁰ 2016	13.5	Low	H (40); T2D (40)		FMD; NMD
Antonopoulos et al, ²⁷¹ 2015	15	Low	H (196); T2D (214); T2DC (70)		FMD
Diaw et al, ²⁷² 2015	14.5	Low	H (14); T2D (14)		FMD
Greyling et al, ²⁷³ 2015	13	Low	Ov (10); T2D (10)		FMD
Heidari et al, ²⁷⁴ 2015	17	Low	Ov (101); T2D (107)		FMD
Ito et al, ²⁷⁵ 2015	16	Low	H (95); T2DC (161)		FMD
Janczura et al, ²⁷⁶ 2015	16	Low	Ov (126); MetS (109)		FMD
Kramer-Aguiar et al, ²⁷⁷ 2015	15.5	Low	H (9); Ov (11); Ob (60)	SGP - ACh, SNP	
Lind, ²⁷⁸ 2015	15	Low	H and MetS (totals unclear)	SGP - ACh, SNP	FMD
Lu et al, ²⁷⁹ 2015	13	Low	H (148); T2D (245)		FMD
Madsen et al, ²⁸⁰ 2015	17	Low	Ob (13); T2D (10)		FMD
Regensteiner et al, ²⁸¹ 2015	13.5	Low	Ov (34); T2D (29)	SGP - PORH	
Schinzari et al, ²⁸² 2015	15	Moderate	H (56); Ob (34)	SGP - ACh	

Schreuder et al, ²⁸³ 2015	13.5	Moderate	Ov (10); T2D (13)		FMD; NMD
Siasos et al, ²⁸⁴ 2015	12	Moderate	H (100); T2D (108); T2DC (92)		FMD
Walther et al, ²⁸⁵ 2015	16	Low	H (40); MetS (53); T2D (25)	LDF - ACh, SNP	FMD; NMD
Alatab et al, ²⁸⁶ 2014	15	Low	Ov (153); T2D (125)		FMD
Fernandes et al, ²⁸⁷ 2014	13.5	Moderate	H (10); MetS (21)		FMD
Franklin et al, ²⁸⁸ 2014	16	Low	H (8); Ob (9)		FMD
Hallmark et al, ²⁸⁹ 2014	13.5	Moderate	H (14); Ob (14)		FMD
Kinouchi et al, ²⁹⁰ 2014	17	Low	Ov (153); T2D (167)		FMD
Liao et al, ²⁹¹ 2014	16.5	Low	H (13); T2D (22)		FMD; NMD
Mahfouz et al, ²⁹² 2014	14	Low	Ov (78); IGT (92)		FMD
Martin-Rodriguez et al, ²⁹³ 2014	16	Low	H (30); Ob (25)	LDF - PORH	
Nguyen et al, ²⁹⁴ 2014	16	Low	H (25); Ob (30); T2D (118)		FMD
Pienaar et al, ²⁹⁵ 2014	15.5	Low	H (21); IGT (16)	LDI - ACh	
Ramakumari et al, ²⁹⁶ 2014	16	Low	H (42); T2D (42)		FMD; NMD
Schreuder et al, ²⁹⁷ 2014	13.5	Low	Ov (9); T2D (12)		FMD; NMD
Shimabukuro et al, ²⁹⁸ 2014	14	Moderate	IGT (5); MS (6)		FMD; NMD
Xiang et al, ²⁹⁹ 2014	17	Low	H (52); T2D (55)		FMD; NMD
Zhang et al, ³⁰⁰ 2014	14	Moderate	H (30); T2D (36)		FMD
Fonseca et al, ³⁰¹ 2013	14	Low	H (19); Ov (35); Ob (34)		FMD; NMD
Jan et al, ³⁰² 2013	10.5	Moderate	Ov (8); T2DC (18)	LDF - PORH, LTH	
Lupattelli et al, ³⁰³ 2013	14	Low	Ov (28); Ob (65)		FMD
Palazzo et al, ³⁰⁴ 2013	16	Low	Ov (18); T2D (26)		FMD
Rajapakse et al, ³⁰⁵ 2013	12.5	Low	H (11); T2D (9)	SGP - SNP	
Rossi et al, ³⁰⁶ 2013	15.5	Low	H (78); Ob (113)	SGP - PORH	
Yiu et al, ³⁰⁷ 2013	14	Moderate	H (150); T2D (100)		FMD
Aellen et al, ³⁰⁸ 2012	14.5	Moderate	H (21); MetS (25); T2D (21)	LDI - LTH	
Biere-Rafi et al, ³⁰⁹ 2012	14.5	Low	H (50); Ob (42)		FMD
Bruno et al, ³¹⁰ 2012	16	Low	Ov (257); T2D (84)		FMD; NMD
Dosi et al, ³¹¹ 2012	13.5	Moderate	H (40); T2D (40)		FMD; NMD
Shemyakin et al, ³¹² 2012	13.5	Low	Ov (16); T2DC (16)	SGP - SNP	
Sogawa et al, ³¹³ 2012	14	Low	H (21); T2D (30); T2DC (41)		FMD

Tian et al, ³¹⁴ 2012	14.5	Moderate	H (59); T2D (116)		FMD; NMD
Al-Tahami et al, ³¹⁵ 2011	12.5	Low	H (36); Ob (36)	LDF - ACh, SNP	
Anderson et al, ³¹⁶ 2011	13.5	Moderate	Ov (19); T2DC (26)		FMD; NMD
Ayer et al, ³¹⁷ 2011	17	Low	H (19); Ob (19)		FMD; NMD
Bruno et al, ³¹⁸ 2011	14.5	Low	Ov (76); T2D (32)		FMD; NMD
Ceriello et al, ¹⁶³ 2011	13	Low	Ov (12); T2D (16)		FMD
Chen et al, ³¹⁹ 2011	16	Low	H (25); T2D (33)		FMD; NMD
Doupis et al, ³²⁰ 2011	16	Low	H (40); Ob (37)	LDI - ACh, SNP	FMD; NMD
Mah et al, ³²¹ 2011	13	Low	H (8); Ob (8)		FMD
Rossi et al, ³²² 2011	13	Moderate	H (28); Ob (19), T2D (18)	LDF - PORH	
Taslipinar et al, ³²³ 2011	14	Low	T2D (30); T2DC (25)		FMD; NMD
Tian et al, ³²⁴ 2011	11.5	Moderate	H (83); T2D (87)		FMD
Tomiyama et al, ³²⁵ 2011	16	Low	H (1879); MetS (257)		FMD
Vinet et al, ²²³ 2011	15.5	Low	H (16); Ob (16)		FMD; NMD
Yim-Yeh et al, ³²⁶ 2011	17	Low	H (14); Ob (33); T2D (68)	LDF - ACh, SNP	FMD; NMD
Zhang et al, ³²⁷ 2011	13	Moderate	H (483); T2D (512)		FMD
Ayer et al, ³²⁸ 2010	17	Low	H (11); Ob (11)	SGP - PORH	FMD; NMD
Barutcuoglu et al, ³²⁹ 2010	15.5	Low	H (10); Ov (18); T2D (26)		FMD; NMD
Biasucci et al, ³³⁰ 2010	16	Low	H (13), Ob (58)		FMD
Buscemi et al, ³³¹ 2010	14.5	Low	Ob (30); MetS (23); T2D (22)		FMD; NMD
Chousos et al, ³³² 2010	15.5	Low	Ov (24); T2D (20)	LDF - ACh, SNP	
Czernichow et al, ³³³ 2010	16.5	Low	H (130); Ov (120)	LDF - LTH, ACh	
de Matthaëis et al, ³³⁴ 2010	16	Low	Ov (137); MetS (43)		FMD; NMD
Eyileten et al, ³³⁵ 2010	17	Low	Ov (33); T2D (32)		FMD
Feng et al, ³³⁶ 2010	17	Low	H (15); T2D (35)		FMD
Jelic et al, ³³⁷ 2010	16	Low	H (5); Ov (11); Ob (17)		FMD
Kizhakekuttu et al, ³³⁸ 2010	13.5	Moderate	Ov (14); T2D (29)		FMD; NMD
Liao et al, ¹⁰³ 2010	17	Low	H (51); T2D (46)		FMD; NMD
Quinn et al, ³³⁹ 2010	17	Low	Ov (24); IGT (40)		FMD
Ran et al, ³⁴⁰ 2010	13.5	Low	T2D (18); T2DC (32)		FMD; NMD
Schinzari et al, ³⁴¹ 2010	13	Low	H (13); MetS (14)	SGP - ACh, SNP	

Yilmaz et al, ³⁴² 2010	17	Low	Ov (38); T2DC (78)		FMD
Aizawa et al, ³⁴³ 2009	16	Low	Ov (44); MetS (45)		FMD; NMD
Fahs et al, ³⁴⁴ 2009	14	Low	H (36); Ov (37); Ob (37)	SGP - PORH	
Lind et al, ³⁴⁵ 2009	8.5	Moderate	H (19); Ob (19)		FMD
McLellan et al, ³⁴⁶ 2009	9.5	Moderate	H (30); T2D (15)	LDF - LTH	
Rousseau et al, ³⁴⁷ 2009	17	Low	H (10); T2D (20)	LDF - PORH, ACh, SNP	
Yeh et al, ³⁴⁸ 2009	13	Low	Ov (52); T2D (213)		FMD; NMD
Brooks et al, ³⁴⁹ 2008	13.5	Moderate	Ov (20); T2D (20); T2DC (20)	LDF - ACh, SNP	
Donald et al, ³⁵⁰ 2008	9.5	Moderate	H (25); T2D (25)		FMD
Esposito et al, ³⁵¹ 2008	17	Low	Ov (44); T2D (30)		FMD
Ghiadoni et al, ³⁵² 2008	14	Moderate	Ov (78); MetS (29)		FMD; NMD
Hamburg et al, ³⁵³ 2008	16	Low	Ov (1361); MetS (762)		FMD
Irace [A] et al, ³⁵⁴ 2008	16	Low	Ov (65); T2D (118)		FMD; NMD
Irace [B] et al, ³⁵⁵ 2008	14	Moderate	Ov (30); T2D (66)		FMD; NMD
Maenaka et al, ³⁵⁶ 2008	11	Moderate	H (20); T2D (20)	SGP - PORH	
Makino et al, ³⁵⁷ 2008	14	Low	T2D (121); T2DC (96)		FMD; NMD
Malecki et al, ³⁵⁸ 2008	17	Low	T2D (111); T2DC (71)		FMD
Mizia-Stec et al, ³⁵⁹ 2008	16	Low	H (16); Ov (18); Ob (22)		FMD; NMD
Natali et al, ¹⁸⁰ 2008	17	Low	Ov (20); IGT (16); T2D (117)	SGP - ACh, SNP	
Petrofsky et al, ³⁶⁰ 2008	12.5	Low	H (30); T2D (15)	LDI - LTH	
Plantinga et al, ³⁶¹ 2008	14	Moderate	H (166); Ov (260); MetS (131)		FMD; NMD
Quattrini et al, ³⁶² 2008	12.5	Moderate	H (12); T2D (12); T2DC (41)	LDF - ACh, SNP	
Su et al, ³⁶³ 2008	14	Low	H (46); IGT (99); T2D (44)		FMD; NMD
Title et al, ³⁶⁴ 2008	14	Moderate	Ov (1150); MetS (267)		FMD; NMD
Van Guilder et al, ³⁶⁵ 2008	13	Low	H (42); Ob (44)	SGP - ACh	
Voidonikola et al, ³⁶⁶ 2008	15	Low	H (154); Ob (155)		FMD
Xiang et al, ¹⁹² 2008	17	Low	H (26); IGT (21)		FMD; NMD
Yilmaz et al, ³⁶⁷ 2008	17	Low	Ov (38); T2D (85)		FMD; NMD
Acree et al, ³⁶⁸ 2007	15	Low	H (30); Ov (28), Ob (29)	SGP - PORH	
Bahia et al, ³⁶⁹ 2007	12.5	Moderate	H (8); MetS (18)	SGP - ACh, SNP	
Cangemi et al, ³⁷⁰ 2007	13.5	Moderate	H (30); MetS (18)		FMD

Esposito et al, ³⁷¹ 2007	17	Low	Ov (20); T2D (30)		FMD
Jin et al, ³⁷² 2007	15.5	Low	H (13); T2DC (19)	LDF - LTH	
Karabag [A] et al, ³⁷³ 2007	14	Low	Ov (40); T2D (70)		FMD; NMD
Karabag [B] et al, ³⁷⁴ 2007	14	Low	Ov (16); T2D (59)		FMD; NMD
Krishnan et al, ³⁷⁵ 2007	12.5	Moderate	H (12); T2DC (12)	LDF - LTH	
Schmiedel et al, ³⁷⁶ 2007	16.5	Low	Ov (10); T2D (12); T2DC (17)	LDF - LTH, ACh, SNP	
Suetsugu et al, ³⁷⁷ 2007	13.5	Moderate	H (20); T2D (59); T2DC (43)		FMD
Waring et al, ³⁷⁸ 2007	14	Moderate	Ov (10); T2D (10)	SGP - ACh, SNP	
Bahia et al, ³⁷⁹ 2006	12.5	Moderate	H (9); MetS (18)	SGP - ACh, SNP	
Baykan et al, ³⁸⁰ 2006	12.5	Low	T2D (39); T2D (29)		FMD
Chan et al, ³⁸¹ 2006	15.5	Low	H (52); T2D (32); T2DC (26)		FMD; NMD
Colberg et al, ³⁸² 2006	13.5	Low	Ob (9); T2D (10)	LDF - LTH	
Esposito [A] et al, ³⁸³ 2006	14	Moderate	Ov (60), MetS (100)		FMD
Esposito [B] et al, ³⁸⁴ 2006	17	Low	H (40); MetS (41)		FMD; NMD
Esposito [C] et al, ³⁸⁵ 2006	17	Low	Ov (50); MetS (100)		FMD
Hermann et al, ³⁸⁶ 2006	9.5	Moderate	H (15); T2D (15)	SGP - SNP	
Madsen et al, ³⁸⁷ 2006	9.5	Low	H (33); T2D (13)		FMD
Olson et al, ³⁸⁸ 2006	16	Low	H (18); Ov (22); Ob (19)		FMD; NMD
Pulerwitz et al, ³⁸⁹ 2006	16	Low	H (130); Ov (278); Ob (146)		FMD
Shemyakin et al, ³⁹⁰ 2006	12.5	Moderate	H (8); IGT (12)	SGP - ACh, SNP	
Tretjakovs et al, ³⁹¹ 2006	16.5	Low	Ov (10); T2DC (20)	LDF - PORH	
van Wijk et al, ³⁹² 2006	13.5	Moderate	Ov (14); T2D (13)		FMD; NMD
Colberg et al, ³⁹³ 2005	12.5	Low	Ob (10); T2D (9)		
Gooding et al, ³⁹⁴ 2005	14	Low	Ov (20); T2D (17)	LDI - ACh, SNP	
Rodriguez et al, ³⁹⁵ 2005	16.5	Low	Ov (484); IGT (95)		FMD
Skilton et al, ³⁹⁶ 2005	15	Low	H (15); Ov (15); T2D (15)	SGP - PORH	FMD
Strain et al, ³⁹⁷ 2005	17	Low	Ov (100); Ob (88); T2D (117)	LDF - PORH, LTH	
Tesauro et al, ³⁹⁸ 2005	10.5	Moderate	H (12); MetS (12)	SGP - ACh, SNP	
Williams et al, ³⁹⁹ 2005	15.5	Low	H (24); Ov (15)		FMD; NMD
de Jongh [A] et al, ⁴⁰⁰ 2004	13.5	Low	H (16); Ob (12)	LDF - ACh, SNP	
de Jongh [B] et al, ⁴⁰¹ 2004	16.5	Low	H (16); Ob (12)	LDF - ACh, SNP	

Economides et al, ⁴⁰² 2004	15.5	Low	H (7); T2D (38)	LDI - ACh, SNP	FMD; NMD
Henry et al, ⁴⁰³ 2004	16	Low	Ov (246); IGT (135)		FMD; NMD
Krishnan et al, ⁴⁰⁴ 2004	13.5	Moderate	H (20); T2D (20); T2DC (18)	LDF - LTH	
Krishnan and Rayman, ⁴⁰⁵ 2004	14.5	Low	H (12); T2D (12); T2DC (12)	LDI - LTH	
Nair et al, ⁴⁰⁶ 2004	16	Low	T2D (23); T2DC (23)		FMD
Nystrom [A] et al, ⁴⁰⁷ 2004	10.5	Moderate	H (10); T2D (12)		FMD; NMD
Nystrom [B] et al, ⁴⁰⁸ 2004	12.5	Moderate	H (10); Ov (10); T2D (12)		FMD; NMD
Park et al, ⁴⁰⁹ 2004	7.5	High	H (13); T2D (11)		FMD
Seywert et al, ⁴¹⁰ 2004	14.5	Low	H (16); Ob (16)	LDI - PORH	
Shimabukuro et al, ⁴¹¹ 2004	11.5	Low	H (12); MetS (22)	SGP - PORH	NMD
Strey et al, ⁴¹² 2004	13	Moderate	Ov (10); T2D (19)	SGP - PORH, ACh	
Bhargava et al, ⁴¹³ 2003	13	Low	H (67); T2D (38); T2DC (35)		FMD
Caballero et al, ⁴¹⁴ 2003	16	Low	T2D (27); T2DC (31)	LDI - ACh, SNP	FMD; NMD
Ching et al, ⁴¹⁵ 2003	15.5	Low	H (27); Ov (23); T2D (15)	SGP - PORH	
Colberg et al, ⁴¹⁶ 2003	13.5	Low	Ob (13); T2D (17)	LDF - LTH	
de Kreutzenberg et al, ⁴¹⁷ 2003	13.5	Low	Ov (10); Ob (10)	SGP - SNP	
Hamdy et al, ⁴¹⁸ 2003	14	Low	Ob (8); IGT (8); T2D (8)		FMD
Regensteiner et al, ⁴¹⁹ 2003	13.5	Low	H (10); T2D (10)	SGP - PORH	FMD
Rizzoni et al, ⁴²⁰ 2003	13.5	Low	Ov (15); MetS (15); T2D (30)		FMD
Colberg et al, ⁴²¹ 2002	12.5	Low	Ob (8); T2D (8)	LDF - PORH, LTH	
Ihlemann et al, ⁴²² 2002	17	Low	Ov (23); T2D (23)		FMD; NMD
van Etten et al, ⁴²³ 2002	14	Low	Ov (21); T2D (23)	SGP - SNP	
Woodman et al, ⁴²⁴ 2002	16.5	Low	Ov (17); T2D (29)		FMD; NMD
Hamdy et al, ⁴²⁵ 2001	12	Moderate	Ov (69); T2D (42)	LDF - ACh, SNP	
Heitzer et al, ⁴²⁶ 2001	10.5	Moderate	H (11); T2D (39)	SGP - ACh, SNP	
Kimura et al, ⁴²⁷ 2001	15	Low	H (12); T2D (15)		FMD; NMD
Rask-Madsen et al, ⁴²⁸ 2001	16.5	Low	H (31); T2DC (28)	SGP - ACh	
Ghiadoni et al, ⁴²⁹ 2000	16.5	Low	H (10); T2D (8)		FMD; NMD
Heitzer et al, ⁴³⁰ 2000	10	Moderate	H (12); T2D (23)	SGP - ACh, SNP	
Preik et al, ⁴³¹ 2000	16.5	Low	Ov (12); IGT (12); T2D (20)	SGP - ACh, SNP	
Watts et al, ⁴³² 2000	10.5	Moderate	Ov (19); Ob (20)	SGP - PORH	

Bornmyr et al, ⁴³³ 1999	8.5	Moderate	H (80); T2D (50)	LDI - LTH	
Caballero et al, ⁴³⁴ 1999	15.5	Low	Ov (30); IGT (32)	LDI - ACh, SNP	FMD
Hogikyan et al, ⁴³⁵ 1999	13.5	Moderate	H (6); T2D (11)	SGP - SNP	
Lim et al, ⁴³⁶ 1999	12.5	Low	Ov (20); T2D (45); T2DC (14)	LDI - ACh, SNP	
Stansberry et al, ⁴³⁷ 1999	16.5	Low	Ob (10); T2D (10)	LDF - PORH, LDF	
Hashimoto et al, ⁴³⁸ 1998	17	Low	H (23); Ob (15)		FMD; NMD
Hogikyan et al, ⁴³⁹ 1998	15.5	Low	Ov (20); T2D (17)	SGP - ACh, SNP	
Jaap et al, ⁴⁴⁰ 1997	17	Low	H (24); IGT (24)	LDF - LTH	
McVeigh et al, ⁴⁴¹ 1992	17	Low	H (21); T2D (29)	SGP - ACh	
Tenembaum et al, ⁴⁴² 1981	9.5	Moderate	H (19); T2D (20)	SGP - PORH	

Study quality was assessed using a modified systematic appraisal for observational research. Risk of bias was assessed according to the guidelines for evaluating observational studies presented by the Grading of Recommendations Assessment, Development and Evaluation. H, Healthy; Ov, overweight; Ob, Obese; MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications; SGP, strain-gauge plethysmography; LDI, laser Doppler imaging; LDF, laser Doppler flowmetry; ACh, acetylcholine; SNP, sodium nitroprusside; PORH, post-occlusive reactive hyperemia; FMD, flow-mediated dilation; NMD, nitrate-mediated dilation.

Table 5-S3. Quality of evidence for each outcome in macrovascular endothelium-dependent reactivity when comparing overweight or obese populations, or populations with cardiometabolic disease, to a healthy population

Health Group (Outcome)	No. of Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations*	Quality of Evidence (GRADE)
Overweight (Impaired)	9	Not detected	Serious†	Not detected	Not detected	None	Very low
Obese (Impaired)	22	Not detected	Serious†	Not detected	Not detected	None	Very low
IGT (Impaired)	2	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low
MetS (Impaired)	9	Not detected	Serious†	Not detected	Not detected	None	Very low
T2D (Impaired)	38	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low
T2DC (Impaired)	7	Not detected	Not detected	Not detected	Not detected	None	Low

Due to the nature of this present study's research question, all studies included in the network meta-analyses are considered observational and, thus, the quality of evidence for all outcomes begin as low before down-grading or up-grading according to the GRADE (Grading of Recommendations Assessment, Development and Evaluation). H, Healthy; Ov, overweight; Ob, Obese; MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

*Large-magnitude of effect, dose-response, plausible biases decreasing the magnitude of effect, or publication bias.

†Large I^2 and/or point estimates vary widely across studies indicating that macrovascular endothelium-dependent reactivity is impaired in those considered overweight or obese, or in those with impaired glucose tolerance.

‡Publication bias is likely as the evidence consists of a small number of studies or funnel plot asymmetry was detected.

Table 5-S4. Quality of evidence for each outcome in macrovascular endothelium-independent reactivity when comparing overweight or obese populations, or populations with cardiometabolic disease, to a healthy population

Health Group (Outcome)	No. of Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations*	Quality of Evidence (GRADE)
Overweight (No Effect)	6	Not detected	Serious†	Not detected	Serious‡	None	Very low
Obese (No Effect)	10	Not detected	Serious†	Not detected	Serious‡	None	Very low
IGT (Impaired)	2	Not detected	Not detected	Not detected	Not detected	Publication bias likely	Very low
MetS (Impaired)	3	Not detected	Not detected	Not detected	Not detected	Publication bias likely	Very low
T2D (Impaired)	19	Not detected	Serious†	Not detected	Not detected	None	Very low
T2DC (Impaired)	1	Not detected	Not detected	Not detected	Not detected	Publication bias likely	Very low

Due to the nature of this present study's research question, all studies included in the network meta-analyses are considered observational and, thus, the quality of evidence for all outcomes begin as low before down-grading or up-grading according to the GRADE (Grading of Recommendations Assessment, Development and Evaluation) criteria. H, Healthy; Ov, overweight; Ob, Obese; MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

*Large-magnitude of effect, dose-response, plausible biases decreasing the magnitude of effect, or publication bias.

†Large I^2 and/or point estimates vary widely across studies indicating that macrovascular endothelium-independent reactivity is impaired and unaffected in those considered overweight or obese, or in those with type 2 diabetes.

‡The 95 % confidence interval includes both negative and positive effects of an overweight or obese state on macro-VSM reactivity.

||Publication bias is likely as the evidence consists of a small number of studies or funnel plot asymmetry was detected.

Table 5-S5. Quality of evidence for each outcome in microvascular endothelium-dependent reactivity when comparing overweight or obese populations, or populations with cardiometabolic disease, to a healthy population

Health Group (Outcome)	No. of Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations*	Quality of Evidence (GRADE)
Overweight (No Effect)	6	Not detected	Not detected	Not detected	Not detected	None	Low
Obese (Impaired)	20	Not detected	Serious†	Not detected	Not detected	None	Very low
IGT (No Effect)	3	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low
MetS (Impaired)	10	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low
T2D (Impaired)	25	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low
T2DC (Impaired)	9	Not detected	Not detected	Not detected	Not detected	Publication bias likely‡	Very low

Due to the nature of this present study's research question, all studies included in the network meta-analyses are considered observational; and, thus, the quality of evidence for all outcomes begin as low before down-grading or up-grading according to the GRADE (Grading of Recommendations Assessment, Development and Evaluation) criteria. H, Healthy; Ov, overweight; Ob, Obese; MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

*Large-magnitude of effect, dose-response, plausible biases decreasing the magnitude of effect, or publication bias.

†Large I^2 and point estimates vary widely across studies indicating that microvascular endothelium-dependent reactivity is impaired and unaffected in those considered obese.

‡Publication bias is likely as the evidence consists of a small number of studies or funnel plot asymmetry was detected.

Table 5-S6. Quality of evidence for each outcome in microvascular endothelium-independent reactivity when comparing overweight or obese populations, or populations with cardiometabolic disease, to a healthy population

Health Group (Outcome)	No. of Studies	Risk of Bias	Inconsistency	Indirectness	Imprecision	Other Considerations*	Quality of Evidence (GRADE)
Overweight (No Effect)	1	Not detected	Not detected	Not detected	Not detected	Publication bias likely	Very low
Obese (Impaired)	9	Not detected	Serious‡	Not detected	Not detected	None	Very low
IGT (No Effect)	1	Serious†	Not detected	Not detected	Not detected	Publication bias likely	Very low
MetS (Impaired)	7	Not detected	Not detected	Not detected	Not detected	None	Low
T2D (Impaired)	10	Not detected	Serious‡	Not detected	Not detected	None	Very low
T2DC (Impaired)	3	Not detected	Not detected	Not detected	Not detected	Publication bias likely	Very low

Due to the nature of this present study's research question, all studies included in the network meta-analyses are considered observational; and, thus, the quality of evidence for all outcomes begin as low before down-grading or up-grading according to the GRADE (Grading of Recommendations Assessment, Development and Evaluation) criteria. H, Healthy; Ov, overweight; Ob, Obese; MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

*Large-magnitude of effect, dose-response, plausible biases decreasing the magnitude of effect, or publication bias.

†Failure to adequately control for confounding factors including differences in clinical characteristics and medication status.

‡Large I^2 and/or point estimates vary widely across studies indicating that microvascular endothelium-independent reactivity is impaired and unaffected in those considered obese or in those with type 2 diabetes.

||Publication bias is likely as the evidence consists of a small number of studies or funnel plot asymmetry was detected.

Table 5-S7. Results of the node-splitting method assessing the evidence from direct and indirect comparisons, as well as the discrepancies between direct and indirect results, in macrovascular endothelium-dependent reactivity

Comparison	k	prop	nma	95%-CI	direct	95%-CI	indir.	95%-CI	Diff	95%-CI	z	p-value
Healthy:Overweight	9	0.23	-0.41	(-0.98, 0.15)	-1.24	(-2.43, -0.05)	-0.17	(-0.82, 0.48)	-1.07	(-2.43, 0.28)	-1.55	0.121
Healthy:Obese	22	0.77	-1.33	(-1.98, -0.67)	-0.87	(-1.61, -0.12)	-2.87	(-4.24, -1.50)	2	(0.45, 3.56)	2.52	0.0116
Healthy:IGT	2	0.26	-1.75	(-2.80, -0.70)	-1.18	(-3.22, 0.86)	-1.96	(-3.18, -0.74)	0.78	(-1.60, 3.16)	0.64	0.5212
Healthy:MetS	9	0.5	-1.99	(-2.73, -1.24)	-1.75	(-2.80, -0.69)	-2.23	(-3.28, -1.17)	0.48	(-1.01, 1.97)	0.63	0.5289
Healthy:T2D	38	0.73	-3.22	(-3.69, -2.75)	-3.59	(-4.14, -3.03)	-2.23	(-3.14, -1.33)	-1.35	(-2.41, -0.29)	-2.5	0.0123
Healthy:T2DC	8	0.44	-4.26	(-4.97, -3.54)	-3.13	(-4.22, -2.05)	-5.12	(-6.08, -4.17)	1.99	(0.54, 3.43)	2.69	0.0071
Overweight:Obese	7	0.32	0.91	(0.16, 1.67)	1.63	(0.28, 2.98)	0.58	(-0.33, 1.49)	1.05	(-0.58, 2.68)	1.26	0.2064
Overweight: IGT	6	0.59	1.34	(0.34, 2.33)	2.09	(0.79, 3.38)	0.26	(-1.29, 1.81)	1.82	(-0.19, 3.84)	1.77	0.0762
Overweight: MetS	9	0.54	1.57	(0.84, 2.31)	1.43	(0.43, 2.42)	1.75	(0.67, 2.83)	-0.32	(-1.79, 1.15)	-0.43	0.6675
Overweight:T2D	34	0.66	-2.8	(-3.29, -2.32)	-2.83	(-3.42, -2.24)	-2.75	(-3.58, -1.93)	-0.08	(-1.09, 0.94)	-0.14	0.8852
Overweight:T2DC	8	0.32	-3.84	(-4.57, -3.12)	-3.87	(-5.14, -2.60)	-3.83	(-4.71, -2.95)	-0.04	(-1.59, 1.50)	-0.05	0.9564
Obese:IGT	1	0.12	0.42	(-0.74, 1.59)	-0.4	(-3.82, 3.02)	0.53	(-0.70, 1.77)	-0.93	(-4.57, 2.71)	-0.5	0.6156
Obese:MetS	2	0.19	0.66	(-0.25, 1.57)	-0.75	(-2.85, 1.34)	0.99	(-0.02, 2.00)	-1.74	(-4.07, 0.58)	-1.47	0.1421
Obese:T2D	5	0.26	-1.89	(-2.61, -1.17)	-1.39	(-2.80, 0.03)	-2.07	(-2.90, -1.23)	0.68	(-0.96, 2.32)	0.81	0.4162
Obese:T2DC	0	0	-2.93	(-3.84, -2.02)	.	.	-2.93	(-3.84, -2.02)
IGT:MetS	1	0.09	-0.24	(-1.40, 0.93)	-1.75	(-5.65, 2.15)	-0.09	(-1.31, 1.13)	-1.66	(-5.75, 2.43)	-0.8	0.426
IGT:T2D	2	0.22	-1.47	(-2.50, -0.44)	-2.76	(-4.96, -0.57)	-1.1	(-2.27, 0.07)	-1.66	(-4.15, 0.82)	-1.31	0.1897
IGT:T2DC	0	0	-2.51	(-3.68, -1.33)	.	.	-2.51	(-3.68, -1.33)
MetS:T2D	3	0.16	-1.23	(-1.99, -0.47)	0.14	(-1.76, 2.04)	-1.5	(-2.33, -0.66)	1.64	(-0.44, 3.71)	1.55	0.1217
MetS:T2DC	0	0	-2.27	(-3.21, -1.33)	.	.	-2.27	(-3.21, -1.33)
T2D:T2DC	18	0.71	-1.04	(-1.69, -0.38)	-1.69	(-2.46, -0.91)	0.53	(-0.67, 1.74)	-2.22	(-3.66, -0.79)	-3.03	0.0024

k, number of studies providing direct evidence; prop, direct evidence proportion; nma, estimated treatment effect (md) in the network meta-analysis; direct, estimated treatment effect (md) derived from direct evidence; indir., estimated treatment effect (md) derived from indirect evidence; diff, difference between direct and indirect treatment estimates; z, z-value of test for disagreement (direct versus indirect); p-value, p-value of test for disagreement (direct versus indirect); MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-S8. Results of the node-splitting method assessing the evidence from direct and indirect comparisons, as well as the discrepancies between direct and indirect results, in macrovascular endothelium-independent reactivity

Comparison	k	prop	nma	95%-CI	direct	95%-CI	indir.	95%-CI	Diff	95%-CI	z	p-value
Healthy:Overweight	6	0.26	-0.98	(-1.79, -0.17)	-1.19	(-2.77, 0.39)	-0.91	(-1.85, 0.03)	-0.29	(-2.13, 1.55)	-0.3	0.7606
Healthy:Obese	10	0.71	-0.34	(-1.39, 0.71)	-1.4	(-2.65, -0.16)	2.29	(0.33, 4.24)	-3.69	(-6.01, -1.37)	-3.12	0.0018
Healthy:IGT	2	0.6	-1.5	(-2.95, -0.06)	-0.91	(-2.77, 0.96)	-2.4	(-4.68, -0.12)	1.49	(-1.46, 4.43)	0.99	0.3222
Healthy:MetS	3	0.42	-1.47	(-2.48, -0.46)	-1.39	(-2.94, 0.15)	-1.53	(-2.86, -0.20)	0.14	(-1.90, 2.18)	0.13	0.8957
Healthy:T2D	19	0.72	-2.68	(-3.36, -1.99)	-2.25	(-3.05, -1.45)	-3.79	(-5.09, -2.49)	1.54	(0.01, 3.06)	1.97	0.0484
Healthy:T2DC	1	0.14	-3.14	(-4.20, -2.08)	-4.4	(-7.25, -1.55)	-2.94	(-4.08, -1.79)	-1.46	(-4.53, 1.61)	-0.93	0.3515
Overweight:Obese	4	0.26	-0.64	(-1.81, 0.52)	0.61	(-1.65, 2.88)	-1.09	(-2.45, 0.26)	1.71	(-0.93, 4.34)	1.27	0.2047
Overweight: IGT	1	0.32	0.52	(-0.96, 2.00)	0.83	(-1.77, 3.43)	0.38	(-1.42, 2.17)	0.45	(-2.71, 3.62)	0.28	0.778
Overweight: MetS	5	0.63	0.49	(-0.43, 1.42)	0.35	(-0.82, 1.51)	0.74	(-0.79, 2.27)	-0.39	(-2.31, 1.53)	-0.4	0.6901
Overweight:T2D	19	0.62	-1.69	(-2.35, -1.04)	-2.1	(-2.94, -1.27)	-1.03	(-2.10, 0.03)	-1.07	(-2.42, 0.29)	-1.55	0.122
Overweight:T2DC	4	0.46	-2.16	(-3.12, -1.20)	-1.09	(-2.51, 0.32)	-3.08	(-4.39, -1.77)	1.99	(0.06, 3.92)	2.02	0.0438
Obese:IGT	0	0	1.17	(-0.56, 2.89)	.	.	1.17	(-0.56, 2.89)
Obese:MetS	1	0.29	1.13	(-0.16, 2.43)	3.2	(0.81, 5.59)	0.29	(-1.25, 1.82)	2.91	(0.07, 5.75)	2.01	0.0445
Obese:T2D	2	0.35	-2.34	(-3.45, -1.23)	-5.06	(-6.95, -3.18)	-0.89	(-2.27, 0.48)	-4.17	(-6.50, -1.84)	-3.51	0.0005
Obese:T2DC	0	0	-2.8	(-4.18, -1.43)	.	.	-2.8	(-4.18, -1.43)
IGT:MetS	1	0.04	0.03	(-1.61, 1.67)	-1.87	(-9.89, 6.15)	0.11	(-1.56, 1.79)	-1.98	(-0.18, 6.21)	-0.47	0.6351
IGT:T2D	1	0.34	-1.17	(-2.63, 0.29)	-0.33	(-2.85, 2.19)	-1.6	(-3.39, 0.20)	1.27	(-1.83, 4.36)	0.8	0.4217
IGT:T2DC	0	0	-1.64	(-3.31, 0.03)	.	.	-1.64	(-3.31, 0.03)
MetS:T2D	2	0.29	-1.2	(-2.18, -0.23)	-1.85	(-3.68, -0.03)	-0.94	(-2.10, 0.22)	-0.91	(-3.08, 1.25)	-0.83	0.4083
MetS:T2DC	0	0	-1.67	(-2.92, -0.42)	.	.	-1.67	(-2.92, -0.42)
T2D:T2DC	8	0.7	-0.47	(-1.36, 0.43)	-1.26	(-2.33, -0.18)	1.39	(-0.26, 3.03)	-2.64	(-4.61, -0.68)	-2.64	0.0083

k, number of studies providing direct evidence; prop, direct evidence proportion; nma, estimated treatment effect (md) in the network meta-analysis; direct, estimated treatment effect (md) derived from direct evidence; indir., estimated treatment effect (md) derived from indirect evidence; diff, difference between direct and indirect treatment estimates; z, z-value of test for disagreement (direct versus indirect); p-value, p-value of test for disagreement (direct versus indirect); MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-S9. Results of the node-splitting method assessing the evidence from direct and indirect comparisons, as well as the discrepancies between direct and indirect results, in microvascular endothelium-dependent reactivity

Comparison	k	prop	nma	95%-CI	direct	95%-CI	indir.	95%-CI	Diff	95%-CI	z	p-value
Healthy:Overweight	6	0.33	-0.2	(-0.48, 0.08)	0	(-0.49, 0.49)	-0.3	(-0.64, 0.05)	0.3	(-0.30, 0.90)	0.98	0.3295
Healthy:Obese	20	0.75	-0.66	(-0.90, -0.41)	-0.69	(-0.97, -0.41)	-0.56	(-1.06, -0.07)	-0.13	(-0.69, 0.44)	-0.43	0.6642
Healthy:IGT	3	0.41	-0.63	(-1.10, -0.16)	-0.73	(-1.46, -0.01)	-0.56	(-1.17, 0.05)	-0.18	(-1.13, 0.77)	-0.37	0.7115
Healthy:MetS	10	0.90	-1.01	(-1.39, -0.63)	-1.03	(-1.42, -0.63)	-0.84	(-2.06, 0.38)	-0.19	(-1.47, 1.10)	-0.29	0.7752
Healthy:T2D	25	0.66	-1.1	(-1.31, -0.89)	-1.17	(-1.43, -0.90)	-0.98	(-1.34, -0.61)	-0.19	(-0.64, 0.26)	-0.82	0.4118
Healthy:T2DC	9	0.56	-1.49	(-1.83, -1.14)	-1.23	(-1.69, -0.77)	-1.81	(-2.34, -1.29)	0.58	(-0.12, 1.28)	1.63	0.1026
Overweight:Obese	4	0.30	0.46	(0.13, 0.79)	0.64	(0.04, 1.23)	0.39	(0.00, 0.77)	0.25	(-0.46, 0.96)	0.69	0.4876
Overweight: IGT	3	0.52	0.43	(-0.04, 0.90)	0.3	(-0.35, 0.95)	0.57	(-0.11, 1.25)	-0.27	(-1.21, 0.67)	-0.56	0.5763
Overweight: MetS	0	0.00	0.81	(0.35, 1.27)	.	.	0.81	(0.35, 1.27)
Overweight:T2D	15	0.67	-0.9	(-1.16, -0.64)	-0.6	(-0.92, -0.28)	-1.51	(-1.97, -1.06)	0.91	(0.36, 1.47)	3.23	0.0013
Overweight:T2DC	5	0.36	-1.29	(-1.67, -0.90)	-1.83	(-2.47, -1.19)	-0.98	(-1.46, -0.50)	-0.85	(-1.65, -0.04)	-2.07	0.0387
Obese:IGT	0	0.00	-0.03	(-0.54, 0.48)	.	.	-0.03	(-0.54, 0.48)
Obese:MetS	1	0.11	0.35	(-0.09, 0.79)	-0.26	(-1.60, 1.08)	0.42	(-0.04, 0.89)	-0.68	(-2.10, 0.73)	-0.95	0.3441
Obese:T2D	8	0.38	-0.44	(-0.72, -0.17)	-0.73	(-1.17, -0.28)	-0.27	(-0.62, 0.08)	-0.45	(-1.02, 0.11)	-1.56	0.1179
Obese:T2DC	0	0.00	-0.83	(-1.23, -0.42)	.	.	-0.83	(-1.23, -0.42)
IGT:MetS	0	0.00	-0.38	(-0.98, 0.22)	.	.	-0.38	(-0.98, 0.22)
IGT:T2D	2	0.29	-0.47	(-0.94, 0.00)	-0.49	(-1.36, 0.38)	-0.46	(-1.02, 0.09)	-0.03	(-1.06, 1.01)	-0.05	0.9620
IGT:T2DC	0	0.00	-0.86	(-1.41, -0.30)	.	.	-0.86	(-1.41, -0.30)
MetS:T2D	2	0.26	-0.09	(-0.51, 0.32)	-0.1	(-0.91, 0.72)	-0.09	(-0.58, 0.39)	0	(-0.95, 0.95)	-0.01	0.9927
MetS:T2DC	0	0.00	-0.48	(-0.98, 0.03)	.	.	-0.48	(-0.98, 0.03)
T2D:T2DC	7	0.56	-0.38	(-0.73, -0.04)	-0.22	(-0.68, 0.24)	-0.59	(-1.11, -0.07)	0.37	(-0.33, 1.07)	1.05	0.2958

k, number of studies providing direct evidence; prop, direct evidence proportion; nma, estimated treatment effect (md) in the network meta-analysis; direct, estimated treatment effect (md) derived from direct evidence; indir., estimated treatment effect (md) derived from indirect evidence; diff, difference between direct and indirect treatment estimates; z, z-value of test for disagreement (direct versus indirect); p-value, p-value of test for disagreement (direct versus indirect); MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-S10. Results of the node-splitting method assessing the evidence from direct and indirect comparisons, as well as the discrepancies between direct and indirect results, in microvascular endothelium-independent reactivity

Comparison	k	prop	nma	95%-CI	direct	95%-CI	indir.	95%-CI	Diff	95%-CI	z	p-value
Healthy:Overweight	10	0.74	-0.50	(-0.80, -0.19)	-0.31	(-0.66, 0.05)	-1.02	(-1.61, -0.43)	0.72	(0.03, 1.41)	2.04	0.0411
Healthy:Obese	1	0.06	-0.04	(-0.42, 0.34)	-0.14	(-1.66, 1.38)	-0.03	(-0.42, 0.36)	-0.11	(-1.68, 1.46)	-0.14	0.8919
Healthy:IGT	7	0.95	-0.55	(-0.94, -0.16)	-0.58	(-0.97, -0.18)	-0.07	(-1.78, 1.63)	-0.50	(-2.25, 1.25)	-0.56	0.574
Healthy:MetS	9	0.85	-0.48	(-0.84, -0.13)	-0.57	(-0.96, -0.18)	0.00	(-0.91, 0.92)	-0.57	(-1.57, 0.42)	-1.13	0.2596
Healthy:T2D	3	0.29	-0.87	(-1.32, -0.42)	-1.11	(-1.95, -0.27)	-0.78	(-1.31, -0.24)	-0.33	(-1.33, 0.66)	-0.65	0.5142
Healthy:T2DC	0	0	-0.01	(-0.70, 0.68)	.	.	-0.01	(-0.70, 0.68)
Overweight:Obese	1	0.2	-0.01	(-0.43, 0.41)	-1.33	(-2.27, -0.39)	0.31	(-0.15, 0.78)	-1.64	(-2.69, -0.59)	-3.07	0.0022
Overweight: IGT	2	0.52	0.04	(-0.50, 0.58)	-0.15	(-0.89, 0.60)	0.25	(-0.53, 1.03)	-0.40	(-1.48, 0.68)	-0.72	0.4717
Overweight: MetS	1	0.25	0.05	(-0.42, 0.52)	-0.00	(-0.95, 0.95)	0.07	(-0.47, 0.61)	-0.07	(-1.16, 1.02)	-0.13	0.8973
Overweight:T2D	4	0.57	-0.84	(-1.26, -0.42)	-1.02	(-1.57, -0.46)	-0.60	(-1.24, 0.03)	-0.41	(-1.26, 0.43)	-0.96	0.3384
Overweight:T2DC	5	0.7	-0.38	(-0.77, 0.01)	-0.22	(-0.69, 0.24)	-0.73	(-1.44, -0.03)	0.51	(-0.33, 1.36)	1.19	0.2351
Obese:IGT	2	0.58	0.50	(-0.04, 1.05)	0.40	(-0.32, 1.11)	0.66	(-0.19, 1.50)	-0.26	(-1.37, 0.85)	-0.46	0.6452
Obese:MetS	0	0	0.51	(-0.01, 1.04)	0.51	(-0.01, 1.04)	.	.
Obese:T2D	0	0	-0.39	(-0.93, 0.15)	-0.39	(-0.93, 0.15)	.	.
Obese:T2DC	11	0.81	-0.46	(-0.74, -0.18)	-0.45	(-0.76, -0.13)	-0.51	(-1.15, 0.14)	0.06	(-0.66, 0.78)	0.16	0.8704
IGT:MetS	0	0	0.06	(-0.59, 0.71)	0.06	(-0.59, 0.71)	.	.
IGT:T2D	0	0	-0.33	(-0.97, 0.31)	-0.33	(-0.97, 0.31)	.	.
IGT:T2DC	0	0	0.07	(-0.45, 0.59)	0.07	(-0.45, 0.59)	.	.
MetS:T2D	0	0	-0.32	(-0.90, 0.26)	-0.32	(-0.90, 0.26)	.	.
MetS:T2DC	2	0.32	0.45	(-0.01, 0.90)	0.51	(-0.30, 1.32)	0.42	(-0.13, 0.97)	0.09	(-0.89, 1.07)	0.18	0.8586
T2D:T2DC	1	0.22	-0.54	(-1.12, 0.04)	-0.79	(-2.03, 0.45)	-0.47	(-1.13, 0.19)	-0.32	(-1.73, 1.09)	-0.45	0.6558

k, number of studies providing direct evidence; prop, direct evidence proportion; nma, estimated treatment effect (md) in the network meta-analysis; direct, estimated treatment effect (md) derived from direct evidence; indir., estimated treatment effect (md) derived from indirect evidence; diff, difference between direct and indirect treatment estimates; z, z-value of test for disagreement (direct versus indirect); p-value, p-value of test for disagreement (direct versus indirect); MetS, metabolic syndrome; IGT, impaired glucose tolerance; T2D, type 2 diabetes; T2DC, type 2 diabetes with complications.

Table 5-S11. Results of the meta-regressions for each potential effect modifier of flow-mediated dilation

Covariate	Available data (% of the total)	Mean	95% Credibility Interval
Age	122 (92%)	-0.47	(-1.54; 0.58)
Resting brachial artery diameter	64 (48%)	-0.48	(-2.42; 1.34)
Body mass index	117 (89%)	0.80	(-0.47; 2.15)
Systolic blood pressure	102 (77%)	-0.24	(-1.32; 0.87)
Diastolic blood pressure	100 (76%)	0.74	(-0.40; 1.88)
Total cholesterol	105 (80%)	0.13	(-1.37; 1.60)
High-density lipoproteins	101 (77%)	-0.02	(-1.16; 1.13)
Low-density lipoproteins	88 (67%)	0.31	(-0.99; 1.63)
Triglycerides	99 (75%)	-0.25	(-1.49; 0.98)
Fasting glucose	100 (76%)	-1.76	(-3.18; -0.35)
HbA1c	69 (52%)	-0.66	(-2.65; 1.18)

Significant results are in bold font.

Chapter 6 – Study four: Assessing cutaneous microvascular function with iontophoresis: Avoiding non-specific vasodilation

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6.1 Linking paragraph

As outlined in the literature review, chapter 2.2.2, of this thesis, there is an array of techniques available for researchers to assess microvascular reactivity. One of the most commonly used techniques to assess microvascular reactivity in the research included in studies one and three was iontophoresis of a vasoactive agent; a methodology that, within itself, presents a range of varying protocols for researchers to choose from, with no standardization of the methods or data expression. In addition to lack of standardization amongst the variety of techniques used to assess microvascular reactivity, the considerable heterogeneity observed in the microvascular data from studies one and three may have also been influenced by non-specific vasodilatory effects that can be induced by the iontophoresis technique. Indeed, there is no consensus as to which protocols of iontophoresis are free of non-specific vasodilatory effects. Considering this, study four aimed to examine the reliability of several published protocols of acetylcholine, sodium nitroprusside and insulin iontophoresis, including those developed by our research team and utilized in study two, by evaluating each for evidence of non-specific vasodilatory effects. Acetylcholine and sodium nitroprusside are the most commonly utilized vasoactive agents in the literature, while interest in insulin iontophoresis is growing due to its status in those with impaired glucose tolerance and T2D. Overall, this study also aimed to provide updated recommendations for the collection and treatment of microvascular data acquired with iontophoresis.

6.2 Abstract

Aim

Iontophoresis of vasoactive agents is commonly used to assess cutaneous microvascular reactivity. However, it is known that iontophoresis can be limited by confounding non-specific vasodilatory effects. Despite this, there is still no standardization of protocols or data expression. Therefore, this study evaluated commonly used protocols of iontophoresis by assessing each for evidence of non-specific vasodilatory effects and examined the reproducibility of those protocols that are free of non-specific responses.

Approach and results

Twelve healthy participants were administered doses of acetylcholine 1-2 % and sodium nitroprusside 1 %, diluted in sodium chloride 0.9 % or deionized water, and insulin 100 U/mL in a sterile diluent using iontophoresis coupled with LSCI. Increases in blood flux at a control electrode, containing the diluent only, indicated a non-specific response. Reproducibility of iontophoresis protocols that were free of non-specific vasodilatory effects were subsequently compared to that of PORH, used as a standard, in 20 healthy participants. Iontophoresis of acetylcholine or sodium nitroprusside in sodium chloride (0.02 mA for 200 and 400 s, respectively) and acetylcholine in deionized water (0.1 mA for 30 s) mediated the least non-specific vasodilatory effects. Microvascular responses to insulin were mediated mainly by non-specific effects. Compared to PORH, the intraday and interday reproducibility for iontophoresis of acetylcholine and sodium nitroprusside (0.02 mA for 200 and 400 s, respectively) with LSCI was weaker, but still deemed good to excellent when data was expressed, in PU or CVC, as the absolute peak blood flux response to the vascular reactivity test or as the change in blood flux between peak and baseline values.

Conclusion

This study provides updated recommendations for assessing cutaneous microvascular reactivity with iontophoresis.

6.3 Introduction

Cardiovascular disease remains the single leading cause of death representing an estimated 31 % of the global mortality rate.²⁰⁹ In recent decades it has become widely accepted that vascular dysfunction is a primary mechanism in the pathogenesis of CVD, occurring long before atherosclerotic remodelling of the vascular network or even the onset of obesity, MetS or diabetes.²¹⁴ Indeed, vascular dysfunction may also contribute to the initial development and adverse progression of obesity and such cardiometabolic diseases.¹⁷ Interestingly, emerging evidence suggests that coronary microvascular disease may explain the occurrence of myocardial ischemia, heart failure and CVD mortality following myocardial infarction without apparent coronary macrovascular disease,²³³ highlighting the microcirculation, which represents most of the arterial vascular network and exerts dominant control over local blood flow to the nutritive network, as an important concentration in the study of vascular function. Increased focus in the field of microvascular research warrants the need to comprehensively evaluate the reliability of existing methods that are used to assess microvascular function in order to further determine their potential clinical and prognostic applications.

Microvascular function is often assessed at the cutaneous microcirculation due, mostly, to its accessibility and its potential role as a surrogate marker of systemic microvascular function,⁴⁴³ using common tests of vascular reactivity that include iontophoresis and PORH coupled with laser-based technologies, such as LSCI, LDF and LDI.³⁸ Iontophoresis involves the delivery of a vasoactive agent to the cutaneous microcirculation using a low-intensity electrical current and, in microvascular research, is typically used to administer acetylcholine or insulin to assess endothelium-dependent microvascular reactivity, or sodium nitroprusside that examines endothelium-independent microvascular reactivity.⁴⁴⁴ However, it is known that iontophoresis may induce non-specific vasodilatory effects that are influenced by variations in the type of diluent used for each vasoactive agent, administration of a higher intensity current or a higher total iontophoretic current density; or by the method of electrical current delivery (e.g. continuous or multiple pulses).²²⁰ Ultimately, non-specific vasodilatory effects confound the microvascular response to the vasoactive agent being studied, limiting the

technique and the overall interpretation of the microcirculatory data, as well as any subsequent conclusions. Despite this, there are still no standardized protocols for iontophoresis; and, consequently, there is an array of methods for iontophoresis being utilized in the literature with no consensus as to which are free of non-specific vasodilatory effects.

Therefore, the primary objective of this present study was to examine the reliability of several published protocols for iontophoresis of acetylcholine, sodium nitroprusside and insulin by evaluating each for evidence of non-specific vasodilatory effects. The second objective of this study was to compare the reproducibility of those protocols that were found to be free of non-specific vasodilatory effects to the excellent reproducibility of PORH,⁶⁶ as observed when performed in conjunction with LSCI.

6.4 Methods

This study was comprised of two separate protocols. As explained in detail below, Protocol A evaluated the reliability of several published protocols of iontophoresis by testing each method for evidence of non-specific vasodilatory effects. Protocol B was then conducted to compare the intraday and interday (morning and afternoon) reproducibility of iontophoresis protocols that were found to be free of non-specific vasodilatory effects in Protocol A to the excellent reproducibility of PORH,⁶⁶ when performed in conjunction with LSCI.

6.4.1 Participants and screening

Healthy male and female students were recruited through email and poster advertisements to participate in one of, or both Protocol A and Protocol B (Table 6-1). Participants were excluded if they had any history of CVD, cigarette smoking, or current use of any vasoactive medications. The Human Research Ethics Committee at the Australian Catholic University approved this study and written informed consent was obtained from all participants prior to commencing each protocol.

Table 6-1. Basal characteristics of each separate study population as measured at trial one of each Protocol A and Protocol B

	Protocol A	Protocol B
n	12	20
Gender (Male/Female)	6/6	10/10
Age (years)	25.9 ± 3.4	22.9 ± 3.2
BMI (kg/m ²)	23.4 ± 2.4	23.7 ± 3.0
Fasting blood glucose (mmol/L)	5.2 ± 0.5	4.9 ± 0.4
Heart rate (beats.min ⁻¹)	59.8 ± 10.4	63.4 ± 11.2
Blood pressure (mmHg)		
SBP	109.9 ± 8.8	113.9 ± 11.5
DBP	64.1 ± 6.1	67.7 ± 7.3
MAP	79.4 ± 5.6	83.1 ± 7.7

Values are mean ± SD. *n*, sample size; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

6.4.2 Study design

All clinical and vascular measurements were performed in a temperature controlled ($23.1 \pm 1.7^{\circ}\text{C}$) room and all five testing sessions included in Protocol A were conducted separately to the four testing sessions that comprised Protocol B. From the 12 participants who completed Protocol A, a total of four participants also participated in Protocol B. To standardize the responses to the tests of cutaneous microvascular reactivity, participants were instructed to abstain from strenuous exercise for 48 hours, alcohol consumption for 24 hours, and caffeine consumption for 12 hours preceding each testing session; and to maintain their current level of physical activity throughout each protocol. Additionally, participants were instructed to present to each testing session in Protocol A following overnight fasting in order to minimize postprandial hyperglycemia-mediated variations in vascular reactivity. However, given that testing sessions in Protocol B were conducted in the morning and afternoon, participants were instructed to present only four hours fasted. Clinical measurements assessing each participant's height and mass were performed at the beginning of the first testing session for both Protocol A and B, while fasting blood glucose, blood pressure, resting heart rate, and skin temperature measurements were conducted at all testing sessions following 20 minutes of acclimatization in a supine position; immediately prior to the beginning of cutaneous microvascular measurements.

6.4.2.1 Protocol A – Examining published protocols of iontophoresis for evidence of non-specific vasodilatory effects

Ten experiments, each assessing a unique published protocol of iontophoresis, were conducted over five testing sessions that were separated by no less than 48 hours; with two separate experiments being performed at each testing session. Cutaneous microvascular reactivity was first assessed on the participant's right arm (experiment one) before the LSCI and iontophoresis equipment was immediately moved and aligned with the participant's left arm. Cutaneous microvascular reactivity and another unique protocol of iontophoresis was then assessed on the participant's left arm (experiment two). Participants remained rested in a supine position throughout microvascular assessment and the repositioning of the equipment from right to left arm alignment. This sequence of testing was repeated at the next four testing sessions, in which, the remaining eight unique protocols of iontophoresis were assessed.

6.4.2.2 Protocol B – The intraday and interday reproducibility of iontophoresis and PORH coupled with LSCI

To assess the variability between LSCI measurements when performed in conjunction with iontophoresis and PORH, each participant's cutaneous microvascular reactivity was assessed during testing session one and testing session two in the morning and afternoon, respectively, of day zero. This sequence of testing was then repeated seven days later with testing sessions three (morning) and four (afternoon) allowing for evaluation of intraday reproducibility on day zero and interday reproducibility (morning and afternoon) between day zero and seven measurements. Morning and afternoon testing sessions were separated by five hours. The sequence of clinical and vascular measurements remained the same for each testing session.

6.4.3 Clinical measurements

Height and mass were measured using a stadiometer and calibrated scale, respectively. Body mass index was calculated as mass (kg) divided by height² (m). Fasting blood glucose concentrations were assessed with a handheld blood glucose monitoring system (Freestyle Optium, Abbott Diabetes Care Ltd, UK). Systolic and diastolic blood pressure, heart rate, and skin temperature were measured using a digital sphygmomanometer (Dinamap, GE Medical Systems, Milwaukee, USA). Blood pressure variables were expressed as mean arterial pressure (mmHg) calculated by $[(2 \times \text{diastolic blood pressure}) + \text{systolic blood pressure}] / 3$.

6.4.4 Cutaneous microvascular assessment

Changes in cutaneous microvascular blood flux, in response to iontophoresis and PORH, were quantified by LSCI using a 70mW system (PeriCam PSI System[®], Perimed, Järfälla, Sweden) with a laser wavelength of 785 nm. The laser head working distance, laser measurement area and image acquisition rate were set at 15 cm, 100 cm² (10 cm X 10 cm), and 10 images/s, respectively. Briefly, the LSCI head unit emits and detects light in the tissue that is partially backscattered by moving blood cells, causing a change in frequency from which variations in microvascular blood flux are calculated.²¹⁹ Laser speckle measurements were recorded continuously at a frequency of 18 Hz using an interfaced computer with data acquisition software (PimSoft 1.2.2.0[®], Perimed, Järfälla, Sweden). In preparation for each iontophoresis experiment, two adhesive drug delivery electrodes (LI 611, Perimed, Järfälla, Sweden) with

administration areas of 1.54 cm² were installed approximately 5 cm apart on the ventral surface of the forearm, avoiding any hair, broken skin, areas of increased skin pigmentation and visible veins. Dispersive electrodes (PF 384, Perimed, Järfälla, Sweden) were then positioned at the elbow and wrist approximately 10 cm from each drug delivery electrode to complete the electrical current circuit. Basal cutaneous microvascular blood flow was measured with LSCI for two minutes before iontophoresis was performed using PF 751 PeriIont systems (Perimed, Järfälla, Sweden). Changes in blood flow were then recorded for 18-22 minutes from the beginning of iontophoresis. Electrical cutaneous resistance was also recorded by the PeriIont systems simultaneously to delivery of the electrical current.

6.4.4.1 Protocol A – Protocol for detecting non-specific vasodilatory effects in response to iontophoresis

Ten unique published protocols of iontophoresis that administer doses of acetylcholine, sodium nitroprusside, or insulin were assessed for evidence of non-specific vasodilatory effects across 10 separate experiments (Table 6-2). For each experiment, the vasoactive agent dissolved in its specified diluent was pipetted into the proximal electrode; while to detect evidence of non-specific vasodilatory effects, the diluent only was injected into the distal (control) electrode. The iontophoresis protocol being assessed was administered simultaneously to the proximal and distal electrodes. Anodal and cathodal currents were used to administer acetylcholine (Sigma-Aldrich, Australia) and sodium nitroprusside (Sigma-Aldrich, Australia), respectively. Additionally, a cathodal current was used to administer insulin (Humulin R[®] - 100 U/mL, Lilly, Australia) and its control, a sterile insulin-specific diluent (Sterile Diluent for Humulin R[®], Lilly, Australia). The sterile diluent had the same composition as insulin Humulin R[®], but did not contain insulin molecules. Increases in cutaneous microvascular blood flux from baseline values in the control electrode during and following iontophoresis indicated that the protocol induced non-specific vasodilatory effects.

Table 6-2. The 10 experiments conducted that assessed published protocols of iontophoresis for evidence of non-specific vasodilatory effects

Vasoactive agent (concentration)	Diluent (concentration)	Iontophoretic electrical current strength and duration	Current density (mA/cm ²)	Total iontophoretic charge density (mC/cm ²)	Vasoactive agent (concentration)	REF
ACh (2 %*)	NaCl (0.9 % **)	0.02 mA for 200 s	0.01	2.60	ACh (2 %*)	256
ACh (1 %***)	Deionized H ₂ O	7 pulses of 0.1 mA for 20 s each; 60 s intervals	0.06	9.09	ACh (1 %***)	229
ACh (2 %)	Deionized H ₂ O	0.1 mA for 30 s	0.06	1.95	ACh (2 %)	445
ACh (2 %)	Deionized H ₂ O	Pulses of 0.03, 0.06, 0.09, 0.12, 0.15, and 0.18 mA for 10 s each; 60 s intervals	0.02, 0.04, 0.06, 0.08, 0.10, and 0.12	4.09	ACh (2 %)	446
SNP (1 %**)	NaCl (0.9 %)	0.02 mA for 400 s	0.01	5.19	SNP (1 %**)	256
SNP (1 %)	Deionized H ₂ O	9 pulses of 0.2 mA for 20 s each; 90 s intervals	0.13	23.38	SNP (1 %)	229
SNP (1 %)	Deionized H ₂ O	0.1 mA for 30 s	0.06	1.95	SNP (1 %)	445§
SNP (1 %)	Deionized H ₂ O	Pulses of 0.03, 0.06, 0.09, 0.12, 0.15, and 0.18 mA for 10 s each; 60 s intervals	0.02, 0.04, 0.06, 0.08, 0.10, and 0.12	4.09	SNP (1 %)	446§
INS (100 U/mL)	Sterile diluent [†]	12 pulses of 0.2 mA for 20 s each; 90 s intervals	0.13	31.17	INS (100 U/mL)	447
INS (100 U/mL)	Sterile diluent	6 pulses of 0.1 mA for 20 s each; 40 s intervals	0.06	7.79	INS (100 U/mL)	448

ACh, acetylcholine; H₂O, water; INS, insulin (Humulin R[®]); NaCl, sodium chloride; REF, reference; SNP, sodium nitroprusside. *2 % = 0.02 g/mL. **0.9 % = 0.009 g/mL. ***1 % = 0.01 g/mL. [†]The sterile diluent had the same composition as insulin Humulin R[®], but did not contain insulin molecules. [§]The protocol was originally used in the cited publication for acetylcholine iontophoresis only, but it was also tested for iontophoresis of sodium nitroprusside in this present study.

6.4.4.2 Protocol B – Iontophoresis and PORH protocols for reproducibility assessment

The reproducibility of iontophoresis coupled with LSCI was conducted for protocols that did not present any non-specific vasodilatory effects in Protocol A. The methods for performing iontophoresis remained unchanged between Protocol A and Protocol B. Post-occlusive reactive hyperemia was performed immediately following the conclusion of iontophoresis. In brief, a sphygmomanometer cuff was positioned around the upper arm, ipsilateral to iontophoresis measurements, 2-3 cm proximal to the antecubital fossa. Following two minutes of basal measurements, the cuff was inflated to 50 mmHg above resting systolic blood pressure, occluding the forearm circulation for three minutes. The cuff was then rapidly deflated inducing PORH that was recorded for a further five minutes.⁶⁶ Cutaneous microvascular blood flow was measured with LSCI throughout the baseline, occlusion, and PORH periods.

6.4.5 Data analyses

The PimSoft data acquisition software was used to set regions of interest at 30 mm² for both iontophoresis and PORH. These regions of interest were adjusted retrospectively to find the area of maximal blood flux in response to iontophoresis. All data were exported to Microsoft Excel and coded by a researcher (CL) not involved in data collection or analyses in order to blind the investigator. Cutaneous microvascular blood flux values were averaged for the 30 seconds immediately prior to the beginning of iontophoresis or occlusion in PORH for baseline and for the five seconds at every two-minute interval following the beginning of iontophoresis in Protocol A; and for the five seconds at the maximal plateau in response to iontophoresis or during peak PORH in Protocol B. The vascular response to iontophoresis was expressed as the absolute blood flux in PU at each two-minute interval from the beginning of iontophoresis in Protocol A. In Protocol B, the vascular response to iontophoresis and PORH was expressed as the absolute peak blood flux response, the change in blood flux between peak and baseline values, the percentage increase from baseline to peak values and the area under the curve. Absolute data were expressed as PU or CVC, which is the blood flux in PU divided by the mean arterial pressure (mmHg), to account for variations in blood pressure between testing sessions in Protocol B.³⁸

6.4.6 Statistical analyses

Protocols of iontophoresis were analysed for non-specific vasodilatory effects using the two-way ANOVA for repeated measures, which assessed the similarity of the microvascular response to iontophoresis between each the electrode containing the vasoactive agent and the control electrode; and by using the one-way ANOVA for repeated measures with Sidak post-hoc analyses, which detected increases in blood flux from baseline measurements at each electrode.²²⁰ The intraday and interday reproducibility of iontophoresis or PORH with LSCI were assessed using the within-subjects coefficients of variation with <35 % deemed acceptable; and the intra-class correlation of coefficients, ranging from 0 to 1 with values of <0.40, 0.40 to 0.75 and >0.75 representing poor, fair to good and excellent agreements, respectively.^{66,68} Quantitative data were reported as mean \pm standard deviation, unless stated otherwise. Statistical comparisons were performed using SPSS (version 23; IBM Corp., Armonk, NY, USA) and Microsoft Excel. A P-value of <0.05 was considered statistically significant.

6.5 Results

6.5.1 Non-specific vasodilatory effects in published protocols of iontophoresis

6.5.1.1 Acetylcholine

In each acetylcholine protocol, there was a rapid significant increase in cutaneous microvascular blood flux from baseline values within two minutes from beginning the administration of the anodal electrical current (Figure 6-1). When each protocol was performed at the control electrode containing the diluent only (i.e. sodium chloride 0.9 % or deionized water), the change in blood flux was not similar to the response to iontophoresis at the electrode containing both the vasoactive agent and diluent. However, in a protocol that administered a total iontophoretic charge density of 9.09 mC/cm² (Figure 6-1B), the peak blood flux response to iontophoresis at the control electrode, containing deionized water, was nearly significantly greater than baseline values (44.45 ± 19.38 vs. 17.81 ± 3.21 PU, $P=0.057$). The peak response to iontophoresis of acetylcholine occurred later (between 8-10 minutes) in each protocol that administered the electrical current using multiple pulses (Figures 6-1B and 6-1D), compared to a faster peak response (between 2-4 minutes) in those that used a continuous method. Interestingly, the rate of return for blood flux towards basal conditions was also slower in the protocols that used multiple pulses to deliver the electrical current. Indeed, protocols using continuous delivery of 0.02 mA for 200 s and of 0.1 mA for 30 s, delivering total iontophoretic charge densities of 2.60 mC/cm² and 1.95 mC/cm², respectively, induced minimal non-specific vasodilatory effects as demonstrated by only a slight peak increase in blood flux from baseline values (6.82 ± 3.81 ΔPU and 2.98 ± 2.92 ΔPU, respectively) at the control electrode (Figure 6-1A and 6-1C).

6.5.1.2 Sodium nitroprusside

The cutaneous microvascular blood flux responses to iontophoresis of sodium nitroprusside and of the diluent alone were different for all protocols (Figure 6-2). However, in addition to mediating a significant increase in blood flux following iontophoresis of sodium nitroprusside, three protocols that used deionized water as a diluent, detailed in Figures 6-2B, 6-2C and 6-2D, also induced a significant increase in

blood flux at the control electrode within six to eight minutes from the beginning of administering the cathodal electrical currents; when a total iontophoretic charge density of 10.39 mC/cm², 1.95 mC/cm² and 4.09 mC/cm², respectively, had been delivered in conjunction with current densities >0.013 mA/cm². Indeed, in each of these protocols the peak blood flux response was greater than baseline values at the control electrode (79.13 ± 27.70 vs. 23.64 ± 3.75 PU; 62.77 ± 25.66 vs. 23.20 ± 3.84 PU; and 83.34 ± 22.76 vs. 26.60 ± 4.42 PU, respectively, P<0.01). Iontophoresis of sodium nitroprusside in sodium chloride 0.9 %, using a low cathodal electrical current charge (0.02 mA) for a period of 400 s, induced a peak blood flux response greater than baseline values (92.28 ± 21.25 vs. 30.65 ± 4.34 PU, P<0.01), but did not mediate any significant non-specific vasodilatory effects when delivered to the control electrode. Of note, this maximal blood flux plateau occurred ~16 minutes after the beginning of iontophoresis.

6.5.1.3 Insulin

In contrast to the acetylcholine and sodium nitroprusside protocols, the cutaneous microvascular blood flux responses to iontophoresis of insulin in the insulin specific diluent and at the control electrode were similar for each protocol (Figure 6-3). Indeed, there were simultaneous increases in blood flux at both the electrode containing insulin and the control electrode for each protocol. Each significant increase in blood flux occurred after delivering a total charge density of 10.39 mC/cm² in the first protocol (Figure 6-3A) and 5.19 mC/cm² in the second protocol (Figure 6-3B).

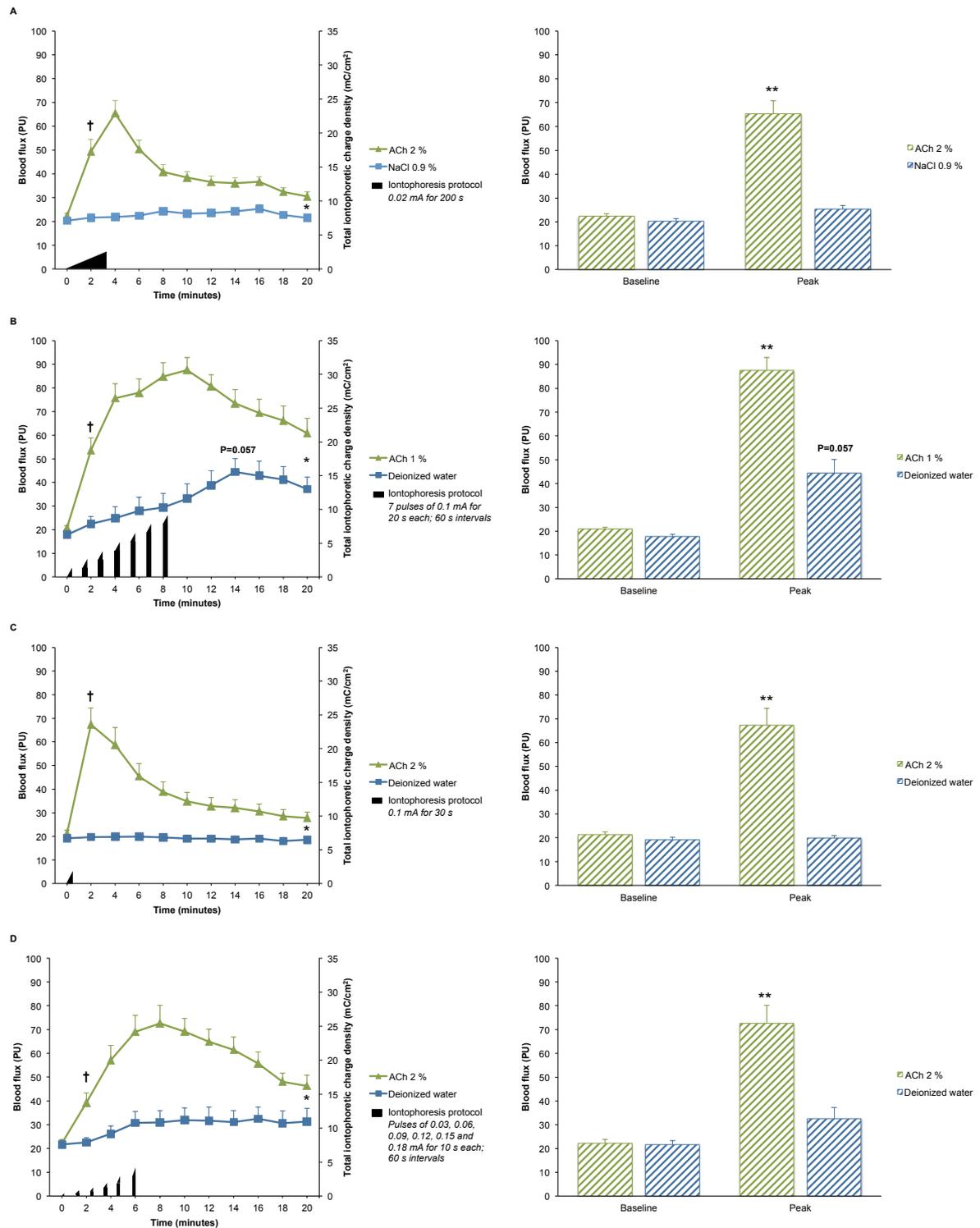


Figure 6-1. Changes in cutaneous microvascular blood flux in response to iontophoresis of the vasoactive substance, acetylcholine (ACh), in its diluent, sodium chloride (NaCl) or deionized water; and in response to iontophoresis of the diluent alone (control). Significant increases in cutaneous microvascular blood flux from baseline measurements (0 minutes) in response to iontophoresis of the diluent alone indicates that the protocol induces non-specific vasodilatory effects. Data are expressed as mean \pm SEM. PU, perfusion units. * $P < 0.01$ ACh vs. control; † $P < 0.05$ first significant increase from baseline; ** $P < 0.01$ vs. baseline.

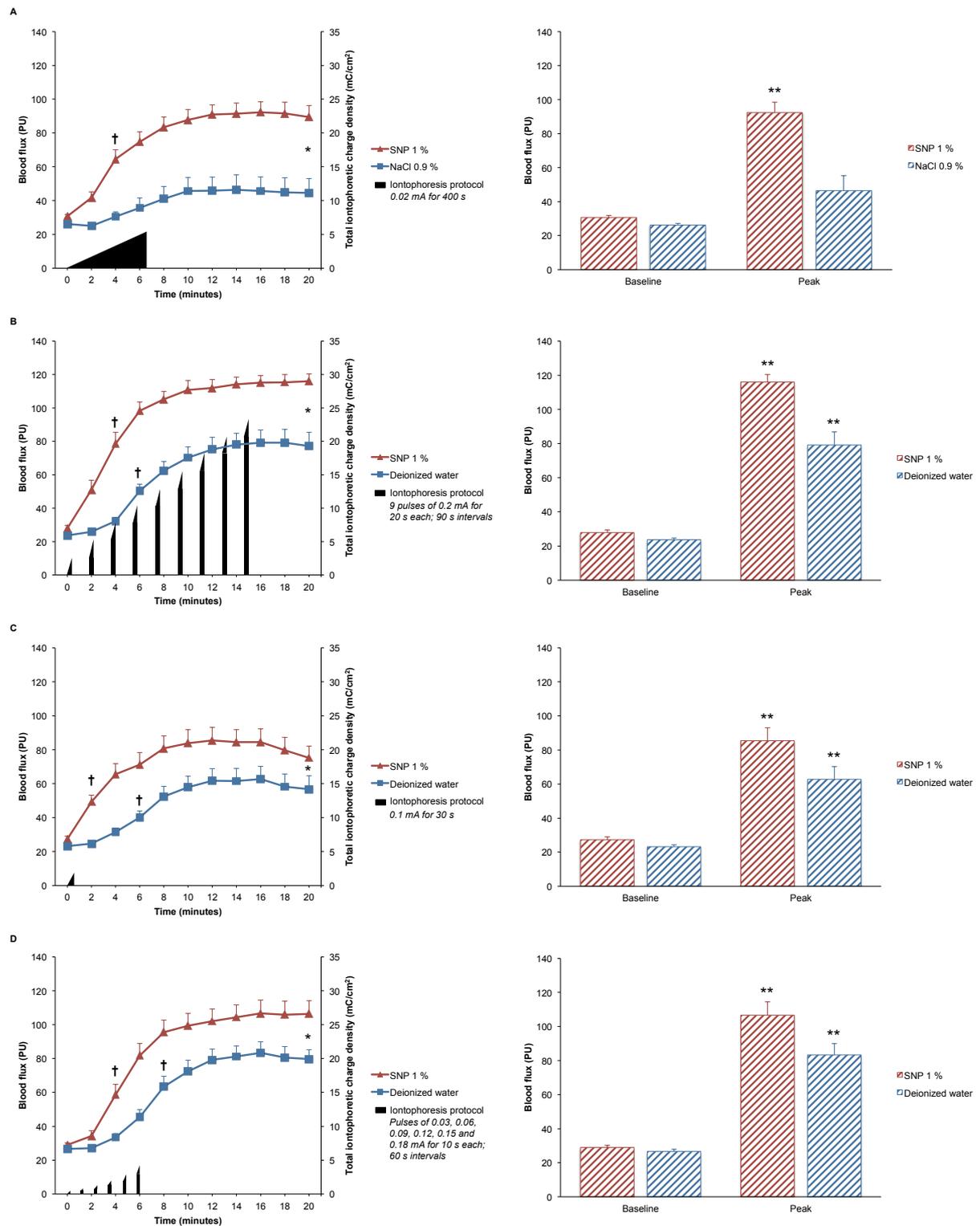


Figure 6-2. Changes in cutaneous microvascular blood flux in response to iontophoresis of the vasoactive substance, sodium nitroprusside (SNP), in its diluent, sodium chloride (NaCl) or deionized water; and in response to iontophoresis of the diluent alone (control). Significant increases in cutaneous microvascular blood flux from baseline measurements (0 minutes) in response to iontophoresis of the diluent alone indicates that the protocol induces non-specific vasodilatory effects. Data are expressed as mean \pm SEM. PU, perfusion units. * $P < 0.01$ SNP vs. control; † $P < 0.05$ first significant increase from baseline; ** $P < 0.01$ vs. baseline.

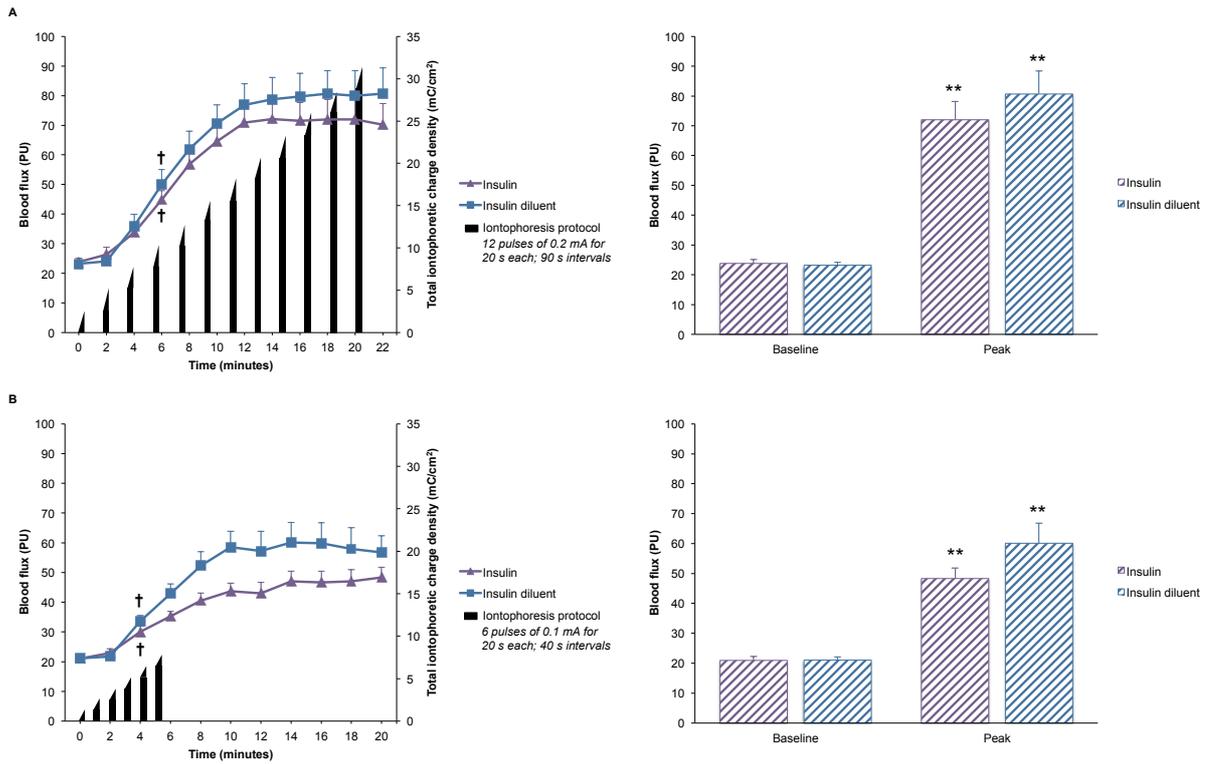


Figure 6-3. Changes in cutaneous microvascular blood flux in response to iontophoresis of the vasoactive substance, insulin, in insulin diluent; and in response to iontophoresis of the insulin diluent alone (control). Significant increases in cutaneous microvascular blood flux from baseline measurements (0 minutes) in response to iontophoresis of the insulin diluent alone indicates that the protocol induces non-specific vasodilatory effects. Data are expressed as mean \pm SEM. PU, perfusion units. * $P < 0.01$ insulin vs. insulin diluent; † $P < 0.05$ first significant increase from baseline; ** $P < 0.02$ vs. baseline.

6.5.2 The intraday and interday reproducibility of iontophoresis and PORH with LSCI

Considering that previous research has investigated some aspects of LSCI reproducibility when measuring the cutaneous microvascular response to a single pulse of acetylcholine 2 % iontophoresis that delivers an electrical current of 0.1 mA for 30 s,^{68,445,449} this present study evaluated the reproducibility of continuous delivery of 0.02 mA for 200 s coupled with LSCI that, when assessed in Protocol A of this present study, induced similar blood flux responses with no significant non-specific vasodilatory effects. Indeed, protocols that used multiple pulses to deliver the electrical current in acetylcholine iontophoresis induced a vascular response that may be more influenced by non-specific vasodilatory effects than that in protocols that used a continuous method as indicated by a delayed peak and a slower rate of return for blood flux towards basal conditions. Additionally, given that it was the only sodium nitroprusside protocol that was free of significant non-specific vasodilatory effects in Protocol A, Protocol B used continuous delivery of 0.02 mA for 400s when assessing the reproducibility of sodium nitroprusside iontophoresis with LSCI. Given that each insulin protocol was found to induce significant non-specific vasodilatory effects in Protocol A, the reproducibility of insulin iontophoresis with LSCI was not evaluated in this present study.

The intraday and interday (morning and afternoon) reproducibility of LSCI with PORH was excellent when data was expressed, in PU or CVC, as the absolute peak blood flux response during reactive hyperemia and as the change in blood flux between peak and baseline values (Tables 6-3 and 6-4). The intraday and interday (morning measurements only) reproducibility of acetylcholine and sodium nitroprusside iontophoresis were also good to excellent when evaluating the same outcome measures. However, there was greater variation between trials of iontophoresis than that between assessments of PORH. In contrast, the interday reproducibility of iontophoresis was only poor to fair when performed in the afternoon; acknowledging that there was no difference in electrical cutaneous resistance, measured during iontophoresis of acetylcholine and sodium nitroprusside, between any of the trials. Finally, LSCI data was most reproducible when expressed as the absolute peak blood flux response, in either PU or CVC, for all reactivity tests. Indeed, the reproducibility of LSCI data was poor or

deemed not acceptable when data was expressed as the percentage increase from baseline measurements or area under the curve.

Table 6-3. The intraday reproducibility of acetylcholine and sodium nitroprusside iontophoresis and PORH when assessed with LSCI on the forearm

	AM	PM	CV (%)	ICC
ACh Peak (PU)	67.59 ± 15.44	66.99 ± 15.25	17.37	0.73 [0.30 - 0.89]
ACh Peak (CVC)	0.82 ± 0.18	0.84 ± 0.20	18.51	0.65 [0.10 - 0.86]
ACh Δ (PU)	47.12 ± 15.23	45.12 ± 14.27	23.73	0.80 [0.48 - 0.92]
ACh Δ (CVC)	0.57 ± 0.18	0.56 ± 0.19	25.05	0.75 [0.34 - 0.90]
ACh %Δ	241.46 ± 90.00	229.15 ± 132.00	34.37	0.60 [-0.04 - 0.84]
ACh AUC	467.89 ± 240.57	404.62 ± 205.07	63.32	0.57 [-0.48 - 0.88]
SNP Peak (PU)	86.40 ± 16.73	93.71 ± 21.16	14.65	0.73 [0.34 - 0.89]
SNP Peak (CVC)	1.05 ± 0.23	1.18 ± 0.30	15.14	0.74 [0.32 - 0.90]
SNP Δ (PU)	63.24 ± 15.48	69.00 ± 21.94	19.23	0.76 [0.42 - 0.91]
SNP Δ (CVC)	0.77 ± 0.22	0.87 ± 0.29	20.61	0.74 [0.37 - 0.90]
SNP %Δ	292.84 ± 124.09	295.82 ± 134.61	35.81	0.41 [-0.56 - 0.77]
SNP AUC	809.23 ± 226.34	801.18 ± 327.91	28.31	0.83 [0.55 - 0.94]
PORH Peak (PU)	87.71 ± 12.77	86.79 ± 11.13	8.24	0.77 [0.42 - 0.91]
PORH Peak (CVC)	1.06 ± 0.14	1.08 ± 0.14	8.95	0.76 [0.39 - 0.90]
PORH Δ (PU)	55.08 ± 9.84	55.00 ± 8.72	13.02	0.67 [0.16 - 0.87]
PORH Δ (CVC)	0.66 ± 0.11	0.69 ± 0.12	12.78	0.71 [0.28 - 0.88]
PORH %Δ	171.14 ± 31.92	175.74 ± 32.85	30.02	0.25 [-0.97 - 0.71]
PORH AUC	134.37 ± 30.13	139.99 ± 35.99	17.26	0.66 [0.15 - 0.87]

Data are presented as the mean ± standard deviation and reported as the peak blood flux and the change (Δ) in blood flux between peak and baseline values, expressed in perfusion units (PU) and cutaneous vascular conductance (CVC); as well as the percentage change (%Δ) from baseline measurements and the area under the curve (AUC). Coefficients of variation (CV) of <35 % were deemed acceptable and intra-class correlation coefficient (ICC) values of <0.40, 0.40 to 0.75 and >0.75 represented poor, fair to good and excellent agreements, respectively. ACh, acetylcholine; PORH, post-occlusive reactive hyperemia; SNP, sodium nitroprusside.

Table 6-4. The interday reproducibility of acetylcholine and sodium nitroprusside iontophoresis and PORH when assessed with LSCI on the forearm in the morning and afternoon of day zero and day seven

	Day 0 - AM	Day 7 - AM	CV (%)	ICC	Day 0 - PM	Day 7 - PM	CV (%)	ICC
ACh Peak (PU)	67.59 ± 15.44	70.04 ± 18.31	21.21	0.55 [-0.16 - 0.82]	66.99 ± 15.25	61.76 ± 16.51	25.72	0.27 [-0.81 - 0.71]
ACh Peak (CVC)	0.82 ± 0.18	0.86 ± 0.25	21.75	0.59 [-0.03 - 0.84]	0.84 ± 0.20	0.78 ± 0.20	25.43	0.31 [-0.72 - 0.72]
ACh Δ (PU)	47.12 ± 15.23	47.77 ± 17.09	28.24	0.63 [0.03 - 0.86]	45.12 ± 14.27	41.73 ± 14.71	36.14	0.31 [-0.77 - 0.73]
ACh Δ (CVC)	0.57 ± 0.18	0.59 ± 0.23	28.30	0.66 [0.13 - 0.87]	0.56 ± 0.19	0.52 ± 0.18	36.26	0.35 [-0.66 - 0.74]
ACh %Δ	241.46 ± 90.00	219.05 ± 78.82	31.61	0.58 [-0.05 - 0.83]	229.15 ± 132.00	215.78 ± 83.53	37.14	0.64 [0.07 - 0.86]
ACh AUC	467.89 ± 240.57	462.80 ± 182.11	40.79	0.66 [-0.27 - 0.91]	404.62 ± 205.07	371.08 ± 182.18	66.66	0.07 [-2.87 - 0.75]
SNP Peak (PU)	86.40 ± 16.73	91.90 ± 22.88	13.75	0.79 [0.49 - 0.92]	93.71 ± 21.16	91.12 ± 16.99	18.25	0.54 [-0.18 - 0.82]
SNP Peak (CVC)	1.05 ± 0.23	1.13 ± 0.28	14.39	0.80 [0.49 - 0.92]	1.18 ± 0.30	1.15 ± 0.22	21.14	0.39 [-0.60 - 0.76]
SNP Δ (PU)	63.24 ± 15.48	67.15 ± 22.12	19.55	0.77 [0.42 - 0.91]	69.00 ± 21.94	67.02 ± 15.69	25.23	0.55 [-0.16 - 0.83]
SNP Δ (CVC)	0.77 ± 0.22	0.83 ± 0.27	20.96	0.76 [0.41 - 0.90]	0.87 ± 0.29	0.84 ± 0.19	28.26	0.40 [0.57 - 0.77]
SNP %Δ	292.84 ± 124.09	277.82 ± 97.10	36.97	0.19 [-1.14 - 0.69]	295.82 ± 134.61	288.99 ± 85.98	32.22	0.55 [-0.17 - 0.83]
SNP AUC	809.23 ± 226.34	870.30 ± 272.04	22.40	0.80 [0.48 - 0.92]	801.18 ± 327.91	814.64 ± 203.80	40.39	0.53 [-0.30 - 0.83]
PORH Peak (PU)	87.71 ± 12.77	90.05 ± 9.59	10.19	0.49 [-0.30 - 0.80]	86.79 ± 11.13	83.91 ± 16.27	10.45	0.81 [0.53 - 0.92]
PORH Peak (CVC)	1.06 ± 0.14	1.10 ± 0.13	9.92	0.59 [0.01 - 0.84]	1.08 ± 0.14	1.06 ± 0.22	12.57	0.78 [0.44 - 0.91]
PORH Δ (PU)	55.08 ± 9.84	56.96 ± 8.81	11.69	0.66 [0.16 - 0.87]	55.00 ± 8.72	51.67 ± 15.51	21.81	0.66 [0.16 - 0.86]
PORH Δ (CVC)	0.66 ± 0.11	0.70 ± 0.12	11.67	0.69 [0.24 - 0.87]	0.69 ± 0.12	0.65 ± 0.20	23.59	0.67 [0.17 - 0.87]
PORH %Δ	171.14 ± 31.92	178.08 ± 43.46	20.49	0.45 [-0.42 - 0.78]	175.74 ± 32.85	163.99 ± 52.16	31.39	0.31 [-0.74 - 0.73]
PORH AUC	134.37 ± 30.13	138.52 ± 40.17	29.80	0.05 [-1.56 - 0.63]	139.99 ± 35.99	129.87 ± 34.14	24.45	0.56 [-0.07 - 0.83]

Data are presented as the mean ± standard deviation and reported as the peak blood flux and the change (Δ) in blood flux between peak and baseline values, expressed in perfusion units (PU) and cutaneous vascular conductance (CVC); as well as the percentage change (%Δ) from baseline measurements and the area under the curve (AUC). Coefficients of variation (CV) of <35 % were deemed acceptable and intra-class correlation coefficient (ICC) values of <0.40, 0.40 to 0.75 and >0.75 represented poor, fair to good and excellent agreements, respectively. ACh, acetylcholine; PORH, post-occlusive reactive hyperemia; SNP, sodium nitroprusside.

6.6 Discussion

Currently, there are no standardized methods for iontophoresis in the literature and, consequently, researchers are using an array of protocols that may be at risk of inducing non-specific vasodilatory effects; confounding the overall interpretation of the microvascular data and any subsequent conclusions. Considering this, this study aimed to provide updated recommendations for using iontophoresis to assess cutaneous microvascular reactivity; assessing the reliability of several published protocols by evaluating each for evidence of non-specific vasodilatory effects. Following this, the reproducibility of those protocols that were free of non-specific vasodilatory effects was compared to the excellent reproducibility of PORH.⁶⁶ The findings of this study reveal that of the ten protocols of iontophoresis that were assessed, only three protocols, which delivered continuous electrical currents with low total iontophoretic charge densities to administer doses of acetylcholine 2 % (0.02 mA for 200 s, diluted in sodium chloride 0.9 %; and 0.1 mA for 30 s, diluted in deionized water) and sodium nitroprusside 1 % (0.02 mA for 400 s, diluted in sodium chloride 0.9 %), were found to have no evidence of non-specific vasodilatory effects. Indeed, although no protocol of acetylcholine iontophoresis induced a significant non-specific increase in blood flux at the control electrode, protocols that used multiple pulses to deliver the electrical current may have mediated a vascular response that is more influence by non-specific vasodilatory effects than that which delivers continuous stimulation; indicated by a delayed peak blood flux response that coincides with increased non-specific vasodilatory responses at the control electrode and a slower rate of return to basal blood flux conditions. Interestingly, all protocols for iontophoresis of insulin in its insulin-specific diluent and sodium nitroprusside in deionized water induced non-specific vasodilatory effects. Considering these results, reproducibility assessments for iontophoresis of acetylcholine and sodium nitroprusside coupled with LSCI were then performed using the protocols that delivered the lowest continuous electrical current charge of 0.02 mA for 200 and 400 s, respectively. Compared to PORH, the intraday and interday (morning measurements only) reproducibility for iontophoresis of acetylcholine and sodium nitroprusside with LSCI was weaker, but still deemed good to excellent when data was expressed, in PU or CVC, as the absolute peak blood flux

response to the vascular reactivity test or as the change in blood flux between peak and baseline values.

In recent decades, several studies have brought further understanding to the mechanisms and variables that mediate non-specific vasodilatory effects in iontophoresis.^{220,446,450–456} In one such study, it was found that non-specific vasodilatory effects can be prevented by limiting the current density and the total iontophoretic charge density to $<0.013 \text{ mA/cm}^2$ and $<7.80 \text{ mC/cm}^2$, respectively, for both anodal and cathodal iontophoresis.²²⁰ Despite these recommendations, it is still not entirely clear as to which protocols of iontophoresis from the literature should be utilized to accurately assess cutaneous microvascular function without confounding non-specific vasodilatory effects. The results of this present study were consistent with those from Droog *et al.*,²²⁰ demonstrating that non-specific vasodilatory effects were induced in several protocols for cathodal iontophoresis of insulin and sodium nitroprusside after total iontophoretic charge densities of 7.79 mC/cm^2 and 10.39 mC/cm^2 were administered. The importance of limiting the total iontophoretic charge density was further highlighted by the results of anodal iontophoresis of acetylcholine, which, although being generally less susceptible, still induced considerable non-specific vasodilatory effects when a total charge density of 9.09 mC/cm^2 was delivered. Non-specific vasodilatory responses were also induced in protocols of sodium nitroprusside iontophoresis that administered total iontophoretic charge densities of just 1.95 mC/cm^2 and 4.09 mC/cm^2 , but delivered current densities that were $>0.013 \text{ mA/cm}^2$; supporting recommendations to administer electrical currents with a low current density. The results from this study also demonstrate that the cutaneous microvascular response to iontophoresis of insulin can be attributed primarily to non-specific vasodilatory effects. These findings contrast those of a previous study, in which, the increase in microvascular blood flux at the control electrode was not similar to that observed in response to iontophoresis of insulin; when assessing the efficacy of the same protocol (12 pulses of 0.2 mA for 20 s each with 90 s interval between each pulse) as that examined in this present study and when using a similar insulin-specific diluent as the control solution.⁴⁴⁷ These varied results may be explained by differences in the laser-based technologies used to estimate skin blood flux between the two studies. Whereas this study used LSCI at depths of $\sim 300 \mu\text{m}$, de Jongh *et al* used single point LDF,⁴⁴⁷ which penetrates to $\sim 1\text{-}1.5 \text{ mm}$.³⁸ Indeed, the non-specific increases in blood flux

observed using LSCI might be less apparent in the deeper cutaneous microvasculature that is assessed by LDF.

In addition to higher current densities and total iontophoretic charges applied during iontophoresis, the use of protocols that deliver multiple pulses of an electrical current may also contribute to inducing non-specific vasodilating effects. This study assessed protocols for iontophoresis of acetylcholine 2 % diluted in deionized water using either a continuous dose (0.1 mA for 30 seconds) or multiple pulses (single pulses of 0.03, 0.06, 0.09, 0.12, 0.15 and 0.18 mA for 10 s each with 60 s interval between each pulse) of an electrical current, each delivering a total iontophoretic charge density within the recommended limits and a current density outside the recommended limits. Despite these similarities between each protocol, the rate of return from peak blood flux towards basal flux appeared to be decreased following delivery multiple pulses of the electric current, relative to rate of return in the continuous protocols. Indeed, such prolonged elevation in blood perfusion may be explained by the influence of non-specific vasodilatory effects. It must be acknowledged that previous research has revealed that this exact protocol for iontophoresis of acetylcholine, which uses multiple pulses of an electrical current, can induce non-specific increases in blood flux.⁴⁴⁶ This study also reaffirms the importance of diluent selection in minimizing non-specific vasodilatory effects with the results demonstrating that, aside from one acetylcholine protocol (0.1 mA for 30 s, diluted in deionized water), non-specific increases in blood flux were observed in each protocol that used deionized water as a diluent; contrasting those protocols using sodium chloride as a diluent. Indeed, sodium chloride may suppress non-specific vasodilatory effects by enhancing the permeability of the skin (i.e. minimizing the resistance of the circuit) and, thus, allowing for application of a lower potential to drive the electrical current.^{454,455} Although the type of diluent is an important variable to consider when minimizing interference to the physiological effect of the vasoactive agent, it has been suggested that non-specific vasodilatory effects can be attributed entirely to application of the iontophoretic electrical current.⁴⁵⁷ The mechanisms underlying the non-specific vasodilatory response vary between anodal and cathodal electrical currents. Application of an anodal electrical current may hyperpolarize the cell membrane, resulting in a local increase in positive charges or a decrease in negative charges in the extracellular fluid that decreases the flow of Ca^{2+} into the VSM cell; whereas, when a cathodal electrical current is applied, the cell

membrane may depolarize as a result of a decrease in positive charges and an increase in negative charges in the extracellular fluid.⁴⁵⁵ Indeed, application of higher iontophoretic electric currents may depolarize nociceptive C-afferent nerve fibres and induce vasodilation via a local nerve-axon reflex;⁴⁵⁷ providing important information about the health of vascular sensory nerves and their capacity to modulate cutaneous microvascular function.

Previously, the microvascular responses to tests of vascular reactivity have been quantified mostly using laser-based technologies including LDF or LDI. More recently, non-invasive LSCI has emerged as a more reliable tool for quantifying cutaneous microvascular function due, in part, to improvements in design that decrease the spatial and temporal variability typically associated with the LDF and LDI technologies, respectively.^{65,218} Therefore, after determining which protocols of iontophoresis were free of non-specific vasodilatory effects, this study compared the reproducibility of two protocols for iontophoresis of acetylcholine 2 % (0.02 mA for 200 s, diluted in sodium chloride 0.9 %) and sodium nitroprusside 1 % (0.02 mA for 400 s, diluted in sodium chloride 0.9 %) to the reproducibility of PORH, when performed in conjunction with LSCI. Notably, this present study was the first to assess the reproducibility of a protocol for iontophoresis of sodium nitroprusside coupled with LSCI; as well as the first to evaluate the intraday reproducibility of LSCI and the interday reproducibility with respect to the timing of assessment (e.g. morning or afternoon). Importantly, the results of this study support those in previous research, finding that the reproducibility of PORH coupled with LSCI is excellent for assessing cutaneous microvascular function.^{66,67,449} Compared to the excellent reproducibility of PORH with LSCI, the reproducibility of acetylcholine and sodium nitroprusside iontophoresis was decreased, but still good to excellent for intraday and interday (morning) measurements only. Indeed, this level of reliability was consistent with previous research, which assessed the interday reproducibility for iontophoresis of acetylcholine 1 % in deionized water using an anodal current of 0.1 mA for 30 s.⁶⁸ Interestingly, the interday reproducibility of performing iontophoresis with LSCI in the afternoon was only poor to fair, while the reproducibility of PORH with LSCI was slightly decreased. Noting that although participants were instructed to present to morning measurements of microvascular function at least four hours fasted, most presented to morning assessments following complete overnight fasting and to afternoon assessments exactly four hours fasted, out

of convenience to their daily schedule. Considering these differences in durations of fasting between morning and afternoon measurements, as well as the increased variation in afternoon measurements of cutaneous microvascular function, collectively, reaffirms the importance of applying a standardized duration of overnight fasting to the design of any study that is assessing vascular function. Finally, consistent with previous research,⁶⁶⁻⁶⁸ LSCI data was most reproducible when expressed as the absolute peak blood flux response and as the change in blood flux between peak and baseline values, in either PU or CVC, for all reactivity tests. In addition to emphasizing the poor or unacceptable reproducibility of LSCI data expressed as the percentage increase from baseline measurements, this study, for the first time, demonstrates that LSCI data is unacceptable when expressed as area under the curve. Considering all results from this present study, a brief set of recommendations for performing iontophoresis, without confounding non-specific vasodilatory effects, is presented in Table 6-5.

There are several inherent limitations to this present study that must be addressed. As stated previously, the imaging depth of LSCI is less to that of LDF;³⁸ and, thus, it cannot be confirmed if the non-specific vasodilatory effects, occurring in response to the numerous published protocols of iontophoresis assessed by this study, also occur in deeper regions of the cutaneous microcirculation. It must also be acknowledged that although this study indicates which protocols of iontophoresis induce non-specific vasodilatory effects, it does not reveal exactly what percentage of the increase in cutaneous microvascular blood flux induced by iontophoresis is attributed to the vasoactive agent, itself, or to the mediators of non-specific vasodilatory responses such as the iontophoretic electrical current. Indeed, when the vasoactive agent is not present in the diluent, the ionic composition of the solution is changed, altering how the electrical current is carried towards the skin and, possibly, how the cutaneous microcirculation responds.⁴⁴⁴ Given that this and previous research has examined the reliability of LSCI in predominantly Caucasian populations or has not defined the population of interest, findings may not be automatically be extrapolated to all ethnicities as differences in skin pigmentation may effect blood flux measurements. Considering this, future research should further explore the efficacy of using LSCI with common tests of vascular reactivity to measure cutaneous microvascular blood flux, validating its use across a range of ethnicities. Furthermore, those studies using iontophoresis with other laser-based technologies such as LDF or LDI to measure

cutaneous microvascular function should demonstrate that all protocols utilized are free of non-specific vasodilatory effects. Moreover, noting that peak blood flux responses to iontophoresis of sodium nitroprusside occurred ~16 minutes after beginning the application of the iontophoretic electrical current, all future studies should report the duration of microvascular assessment; ensuring that recording periods are long enough in duration to detect the maximal microvascular response to the vasoactive agent.

In conclusion, this research demonstrates a lack of a consensus in the literature; highlighting that many studies of microvascular function may be utilizing protocols of iontophoresis that induce non-specific vasodilatory effects. Furthermore, this study provides additional data that demonstrates the excellent reproducibility of LSCI. Altogether, this study provides new recommendations that minimize the influence of non-specific vasodilatory effects during iontophoresis, ensuring that vascular data more reflects the microvascular response to the vasoactive agent being studied; ultimately, improving the understanding of how underlying mechanisms of microvascular function change throughout the pathogenesis of cardiometabolic and CVD.

Table 6-5. Recommendations for using iontophoresis with LSCI to assess cutaneous microvascular function based on findings from this present study

Assessment of endothelium-dependent vascular reactivity				
Vasoactive agent (concentration)	Diluent (concentration)	Iontophoretic electrical current intensity	Duration of iontophoretic electrical current	Recommended duration of vascular assessment*
ACh (2 %)	NaCl (0.9 %)	0.02 mA	200 s	10 mins
ACh (2 %)	Deionized H ₂ O	0.1 mA	30 s	10 mins
INS (100 U/mL)	Results indicate that current protocols for iontophoresis of INS, coupled with LSCI, are not suitable for assessing cutaneous microvascular insulin-mediated endothelial function. Measurements more specific to the vascular response may be acquired using LDF; noting that its reproducibility is poor when compared to LSCI.			

Assessment of endothelium-independent vascular reactivity				
Vasoactive agent (concentration)	Diluent (concentration)	Iontophoretic electrical current strength (mA)	Duration of iontophoretic electrical current	Recommended duration of vascular assessment*
SNP (1 %)	NaCl (0.9 %)	0.02 mA	400 s	20 mins

Expression of LSCI data when using iontophoresis	
Expression	Reproducibility**
Absolute peak blood flux response (PU)	Good to excellent – recommended
Absolute peak response (CVC)	Good to excellent – recommended
Change between baseline and peak blood flux (PU)	Good to excellent – recommended
Change between baseline and peak blood flux (CVC)	Good to excellent – recommended
Percentage increase from baseline	Poor – not recommended
Area under the curve	Poor – not recommended

ACh, acetylcholine; CVC, cutaneous vascular conductance; H₂O, water; INS, insulin (Humulin R[®]); LSCI, laser speckle contrast imaging; NaCl, sodium chloride; PU, perfusion units; SNP, sodium nitroprusside. *Duration of vascular assessment from the beginning of iontophoresis. **Based on recommendations that microvascular assessments are most reproducible when performed in the morning, following overnight fasting. **Note:** recommendations are based on results acquired using an iontophoretic electrode with a drug delivery surface area of 1.54 cm².

Chapter 7 – General discussion, limitations and conclusion

The primary objective of this program of research was to evaluate the extent and nature of vascular dysfunction across the spectrum of cardiometabolic health, from healthy populations, to those with obesity, MetS, impaired glucose tolerance, T2D and T2D with complications. To our best knowledge, this research is the first to provide evidence that there is a progressive impairment in endothelium-dependent and endothelium-independent reactivity throughout the pathogenesis of T2D and its complications; and that this continuum of impairment may be adversely influenced by excess sugar consumption, such as that in the form of SSB; which induces transient endothelial dysfunction in those considered healthy or obese and in those with cardiometabolic disease. Furthermore, this research highlights major flaws in many studies of microvascular function, demonstrating that they may be utilizing protocols of iontophoresis that induce non-specific vasodilatory effects and that, subsequently, confound the interpretation of their vascular data. Importantly, this research responds by providing new recommendations that minimize the influence of these non-specific effects and, ultimately, improve the capacity to understand how microvascular function changes throughout the pathogenesis of obesity, cardiometabolic disease and CVD.

This data demonstrates that chronic vascular dysfunction begins early in the decline of cardiometabolic health, presenting in those considered overweight, long before the clinical onset of obesity, MetS or T2D. The impact of chronic vascular dysfunction on the pathogenesis of CVD, even at this early stage, may be significant with data showing that the risk of coronary heart disease is increased in those who are metabolically healthy, but are considered obese.²⁵⁴ A slight increase in endothelium-independent vascular reactivity can be observed in obese patients after an initial impairment in those considered overweight. This increase in endothelium-independent vascular reactivity following an initial impairment may be chance, or a local protective mechanism, exerted by the perivascular adipose tissue.⁴⁵⁸ Indeed, the perivascular adipose tissue is not only a structural support surrounding the arteries, but also a source of vasoactive molecules that affect the underlying vascular cells.⁴⁵⁹ Such protective mechanisms in the decline of cardiometabolic health may explain the obesity paradox, which suggests that mildly obese patients with CVD have better outcomes than in their leaner

counterparts.⁴⁶⁰ Interestingly, data from this research program contradicts popular hypotheses that microvascular dysfunction precedes macrovascular dysfunction;²³³ demonstrating that, when compared to water consumption, a single 600 mL SSB impaired both microvascular and macrovascular endothelial function even in a healthy population. Importantly, in contrast to many previous studies of vascular function, this research accounted for differences in vascular shear rate between assessments of macrovascular function, confirming that in addition to microvascular dysfunction, acute hyperglycemia induces a macrovascular dysfunction that is explained by the disruption of vasoactive mechanisms intrinsic to the macrocirculation.^{34,58} Considering this finding, it may be more suitable at this time to use FMD or NMD of the brachial artery to assess vascular reactivity, procedures that are more standardized and easy to perform in a routine clinical setting; rather than using methods of evaluating microvascular reactivity that lack the same consensus as that in macrovascular techniques.

Notably, meta-regression analyses of FMD data and potential effect modifiers found one significant, negative correlation, that between FMD and fasting blood glucose concentration; indicating that as fasting blood glucose concentration increases by 1 mmol/l, FMD decreases by 0.52%. Indeed, hyperglycemia is one of several other factors, including insulin resistance, obesity and low-grade inflammation, which may influence vascular function throughout the decline of cardiometabolic health. Current evidence suggests that acute hyperglycemia induces transient endothelial dysfunction, even in those considered healthy, by mediating an abnormal elevation in oxidative stress that disturbs normal underlying mechanisms of NO synthesis.^{235,236} While it is important to acknowledge that other signalling pathways of vasomotion may be also be affected by oxidative stress, reduced NO bioavailability is considered a strong predictor of CVD outcomes.⁷ In the postprandial state, oxidative metabolism initiates oxidative phosphorylation of adenosine triphosphate at the electron transport chain of the mitochondria, which via the phenomenon of electron leakage causes superoxide generation of ROS.⁸⁵ Moreover, it has been clearly reported that increased glycemia is responsible for the activation of NADPH oxidase, which also contributes to production of the superoxide anion.²⁴ Although these ROS are normally readily detoxified, elevated activity within this mechanism such as that following excess sugar consumption increases production to a rate beyond the suppressive capabilities of the

antioxidant systems.⁸⁶ Importantly, the applicability of this oxidative stress-dependent mechanism to acute hyperglycemia-mediated endothelial dysfunction in living organisms was also demonstrated in this research, which revealed for the first time in an *in vivo* experimental rat model that antioxidant treatment also fully restores cutaneous microvascular endothelial function during acute hyperglycemia. This research also found that eNOS^{ser1177} and the eNOS dimer/monomer ratios remained preserved during acute hyperglycemia, while the concentration of nitrites was decreased. Such experimental findings provide evidence that acute hyperglycemia-mediated endothelial dysfunction is due, at least in part, to decreased bioavailability of NO that is not caused by a disruption to the synthesis of NO via the eNOS pathway. Indeed, the reaction between NO and free radical superoxide results in the formation of peroxynitrite, a potent cytotoxic molecule.²³⁷ This nitro-oxidative stress could be a primary mechanism responsible for the decrease in NO bioavailability that was observed in our model of acute hyperglycemic stress. Although the effects of acute hyperglycemia are initially transient, habitual excess sugar consumption promotes the development of obesity, dysglycemia, insulin resistance and low-grade inflammation. Indeed, elevated generation of ROS appears to be the unifying pathway between these factors and chronic vascular dysfunction, driving the progressive impairment of vascular reactivity across the spectrum of cardiometabolic health.⁷ Obesity and cardiometabolic diseases are characterized by abnormally frequent hyperglycemic excursions, which may also induce VSM cell proliferation by disrupting VSM cell apoptosis.²⁵⁵ Furthermore, hyperglycemia may enhance the production of AGE and collagen cross-linking.²⁵⁵ Collectively, these mechanisms stiffen the arterial wall; possibly explaining why endothelium-independent vascular reactivity may be reduced in those with obesity, impaired glucose tolerance, MetS and T2D. Ultimately, this increase in myogenic tone elevates the risk of injury to the endothelial wall; an event that is significant in the pathogenesis of CVD.

There are several inherent limitations to this research that must be addressed. Given that previous studies have demonstrated differences in vascular function between ethnicities,²¹⁰ it must be acknowledged that this research focussed on Caucasian populations or assessed data from studies in which ethnicity was poorly reported. Furthermore, some of this research focussed on healthy male populations. Therefore, the findings of this data cannot be automatically extrapolated across all ethnicities,

genders and health populations. Although this research confirmed that excess sugar consumption transiently impairs endothelial function, it must also be considered that ingredients other than sugar that comprise commercial SSB were not evaluated individually in this research and, therefore, it is not known how they may contribute to the observed acute hyperglycemia-mediated endothelial dysfunction. Additionally, it was not possible to blind participants to the intervention by using a sugar-free placebo such as a commercial diet soda due to previous research suggesting that even artificial sweeteners may interact with taste receptors stimulating insulin secretion, which may induce a vascular response.²⁴¹ Many studies included in the network meta-analysis used control groups that had no specific health classification. Considering this, mean clinical data from all included studies assessing health groups not defined as T2D, with or without complications, was checked and, if need be, redefined according to the World Health Organization guidelines for classification of overweight and obesity;²⁴⁹ the joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention, harmonizing the criteria for defining MetS;²⁵⁰ and the American Diabetes Association criteria for impaired glucose tolerance and diabetes.²⁵¹ It also must be acknowledged that most studies did not include parameters that define MetS and are known to have an influence on vascular function, such as insulin resistance; and, therefore, the effect of such factors on the development of vascular function could not be accounted for in this analysis. Additionally, while there is a large amount of data focussing on macrovascular endothelium-dependent vascular reactivity in studies of vascular function, the amount of research that has assessed endothelium-independent vascular reactivity and microvascular endothelium-dependent reactivity is limited. Finally, study four demonstrated that a majority of the studies included in the reviews of this research have utilized methodology that induces non-specific effects and confounds vascular data. Therefore, while the direction of the effect may be accurate, the magnitude of the microvascular impairment during acute hyperglycemia or across the spectrum of cardiometabolic health may not yet be fully revealed.

Acknowledging the findings and limitations of this study, it is still not clear where in the pathophysiological continuum that transient endothelial dysfunction becomes chronic vascular dysfunction. Indeed, this present study demonstrates that vascular reactivity may be impaired early, even in those considered overweight; while previous research from our laboratory has demonstrated that acute hyperglycemia, induced by

excess sugar consumption, transiently decreases endothelial function, even in those considered healthy.²⁵⁶ Considering this, further research is needed to assess if transient impairments in endothelial function, mediated by several dietary and lifestyle factors, develop into chronic vascular dysfunction before, when someone is still considered clinically healthy, or after the clinical onset of an overweight state or insulin resistance. Furthermore, given that there may be differences in vascular reactivity between ethnicities,²¹⁰ future research may also assess this continuum of vascular impairment, as well as the mechanisms that underlie any dysfunction (e.g. disruption of the NO, PGI₂ or EDHF pathways) in varying health populations from a range of ethnic backgrounds. Before further vascular research is performed, several methodological issues should be addressed. Future studies should ensure that assessments of macrovascular reactivity with FMD and ultrasound of the brachial artery account for changes in shear stress between assessments, ensuring that intrinsic function of the macrocirculation is being examined. Furthermore, future vascular studies would also benefit highly from research that standardizes protocols for assessing microvascular function, ensuring that the impact of obesity and cardiometabolic disease, as well as dietary and lifestyle habits, on vascular function is interpreted with greater accuracy. While it is important to understand the physiological effects of poor lifestyle, a multidisciplinary approach should be used to also understand the psychology that drives sedentary behaviours and poor diet, such as excess sugar consumption, in both adults and children. Indeed, comprehensive understanding of these multifactorial systems will provide the foundation for developing mechanisms, which may enhance cardiovascular function, and interventions that promote healthy lifestyles and reduce the incidence of obesity and cardiometabolic disease.

In conclusion, this research program demonstrates that there is a progressive impairment in vascular reactivity across the spectrum of cardiometabolic health, from healthy populations, to those with obesity, MetS, impaired glucose tolerance, T2D and T2D with complications. This research program also confirmed that excess sugar consumption, including that in the form of SSB, induces transient impairments in endothelial function, which may contribute to the initial and ongoing development of chronic vascular dysfunction, obesity, MetS, T2D and CVD. It is still unclear where in the continuum that transient endothelial dysfunction develops into chronic vascular impairment. Therefore, further research is needed to assess if transient impairments in

vascular reactivity, mediated by several dietary and lifestyles factors, develop into chronic vascular dysfunction before, when the individual is still considered clinically healthy, or after the clinical onset of an overweight state. However, several issues within the current methodology for assessing vascular function should be addressed before further research is completed. Regardless, the findings of this research program demonstrate and further inform public health policy that transient and chronic impairments of vascular reactivity, which are representative of critical events in the pathogenesis of CVD and that are associated with CVD mortality,⁴⁶¹ occur long before the clinical onset of obesity, MetS and T2D. Furthermore, they provide data that may enhance the timing and, subsequent, effectiveness of treatment or preventative strategies that aim to improve vascular health and outcomes in CVD.

Chapter 8 – References

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Chapter 9 – Appendices

Appendix I: Research Portfolio

Study one

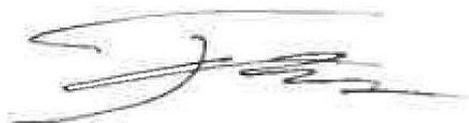
Loader J, Montero D, Lorenzen C, Watts R, Meziat C, Reboul C, Stewart S, Walther G. Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: systematic review and meta-analysis. *Arter Thromb Vasc Biol.* 2015;35(9):2060-72. Doi: 10.1161/ATVBAHA.115.305530.

Contribution statement: GW was responsible for the concept and design of the study. Acquisition of data was performed by JL, DM, CL and GW. JL, DM and GW analysed and interpreted the data. Drafting of the manuscript was completed by JL, DM and GW. JL, DM, CL, RW, CM, CR, SS and GW critically revised the manuscript for important intellectual content. DM provided statistical expertise and JL, CL, RW, SS and GW provided administrative, technical or material support. All authors approved the final version of the manuscript.

Approximate percentage contributions:

Loader J, 60 %; Montero D, 10 %; Lorenzen C, 10 %; Watts R, 2.5 %; Meziat C, 2.5 %; Reboul C, 2.5 %; Stewart S, 2.5 %; Walther G, 10 %.

I acknowledge that my contribution to the above publication is 60 % (6/10).



Jordan Luke Loader

Date: 29/09/2017

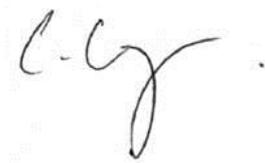
I acknowledge that my contribution to the above publication is 10 %.



David Montero

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 10 %.



Christian Lorenzen

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Rani Watts

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Cindy Meziat

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Cyril Reboul

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.

A handwritten signature in black ink, appearing to be 'S. Stewart', written in a cursive style.

Simon Stewart

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 10 %.

A handwritten signature in black ink, appearing to be 'Guillaume Walther', written in a cursive style.

Guillaume Walther

Date: 29/09/2017

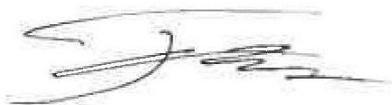
Study two

Loader J*, Meziat C*, Watts R, Lorenzen C, Sigauco-Roussel D, Stewart S, Reboul C, Meyer G, Walther G. Effects of sugar-sweetened beverage consumption on microvascular and macrovascular function in a healthy population. *Arter Thromb Vasc Biol.* 2017;37(6):1250-60. Doi: 10.1161/ATVBAHA.116.308010.

Contribution statement: GW was responsible for the concept and design of the study. CM, GM and GW performed data acquisition. JL exported and blinded the data. CM and GW analysed the data. GW provided statistical expertise. JL, CM, CR and GW interpreted the data. JL, CM and GW performed drafting of the manuscript. JL, CM, RW, CL, DSR, SS, CR, GM and GW provided administrative, technical or material support; and critically revised the manuscript for important intellectual content. All authors approved the final version of the manuscript.

Approximate percentage contributions: Loader J, 50 %; Meziat C, 40 %; Watts R, 0.5 %; Lorenzen C, 0.5 %; Sigauco-Roussel D, 0.5 %; Stewart S, 0.5 %; Reboul C, 1.5 %; Meyer G, 1.5 %; Walther G, 5 %.

I acknowledge that my contribution to the above publication is 50 % (5/10).



Jordan Luke Loader

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 40 %.



Cindy Meziat

Date: 29/09/2017

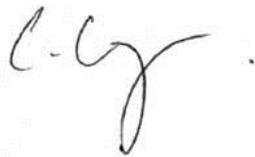
I acknowledge that my contribution to the above publication is 0.5 %.



Rani Watts

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 0.5 %.



Christian Lorenzen

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 0.5 %.



Dominique Sigauco-Roussel

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 0.5 %.



Simon Stewart

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 1.5 %.



Cyril Reboul

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 1.5 %.



Gregory Meyer

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 5 %.



Guillaume Walther

Date: 29/09/2017

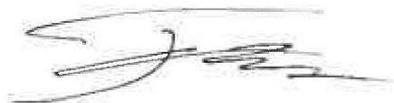
Study three

Loader J, Khouri C, Taylor F, Stewart S, Lorenzen C, Walther G, Roustit M.
Manuscript to be submitted.

Contribution statement: JL was responsible for the concept and design of the study. JL, FT, GW and MR acquired the data. JL, CK, GW and MR analysed and interpreted the data; and drafted the manuscript. JL, CK, FT, SS, CL, GW and MR provided administrative, technical or material support; and critically revised the manuscript for important intellectual content. All authors approved the final version of the manuscript.

Approximate percentage contributions: Loader J, 50 %; Khouri C, 20 %; Taylor F, 2.5 %; Stewart S, 2.5 %; Lorenzen C, 2.5 %; Walther G, 2.5 %; Roustit M, 20 %.

I acknowledge that my contribution to the above publication is 50 %.



Jordan Luke Loader

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 20 %.



Charles Khouri

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Frances Taylor

Date: 29/09/2017

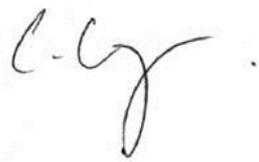
I acknowledge that my contribution to the above publication is 2.5 %.



Simon Stewart

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Christian Lorenzen

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Guillaume Walther

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 20 %.



Matthieu Roustit

Date: 29/09/2017

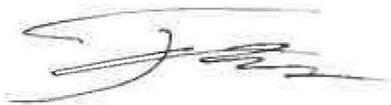
Study four

Loader J, Roustit M, Taylor F, MacIsaac RJ, Stewart S, Lorenzen C, Walther G. Assessing cutaneous microvascular function with iontophoresis: Avoiding non-specific vasodilation. *Microvascular Research*. 2017;113:29-39. Doi: 10.1016/j.mvr.2017.04.006.

Contribution statement: JL, CL and GW were responsible for the concept and design of the study. JL performed data acquisition, analysed the data and interpreted the data. JL performed drafting of the manuscript. JL, MR, FT, RJM, SS, CL and GW provided administrative, technical or material support. All authors critically revised the manuscript for important intellectual content and approved the final version of the manuscript.

Approximate percentage contributions: Loader J, 75 %; Roustit M, 5 %; Taylor F, 2.5 %; MacIsaac RJ, 2.5 %; Stewart S, 2.5 %; Lorenzen C, 2.5 %; Walther G, 10 %.

I acknowledge that my contribution to the above publication is 75 % (7.5/10).



Jordan Luke Loader

Date: 29/09/2017

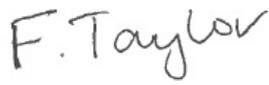
I acknowledge that my contribution to the above publication is 5 %.



Matthieu Roustit

Date: 29/09/2017

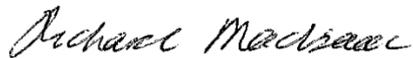
I acknowledge that my contribution to the above publication is 2.5 %.



Frances Taylor

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Richard J. MacIsaac

Date: 29/09/2017

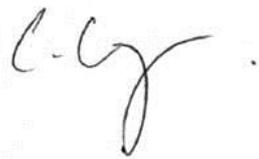
I acknowledge that my contribution to the above publication is 2.5 %.



Simon Stewart

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 2.5 %.



Christian Lorenzen

Date: 29/09/2017

I acknowledge that my contribution to the above publication is 10 %.



Guillaume Walther

Date: 29/09/2017

Appendix II: Published papers that form Chapter 3

Loader J, Montero D, Lorenzen C, Watts R, Meziat C, Reboul C, Stewart S, Walther G. Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: systematic review and meta-analysis. *Arter Thromb Vasc Biol.* 2015;35(9):2060-72. Doi: 10.1161/ATVBAHA.115.305530

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<http://atvb.ahajournals.org/content/35/9/2060>

Appendix III: Published papers that form Chapter 4

Loader, J*, Meziat, C*, Watts, R, Lorenzen, C, Sigaud-Roussel, D, Stewart, S, Reboul, C, Meyer, G, Walther, G. Effects of sugar-sweetened beverage consumption on microvascular and macrovascular function in a healthy population. *Arter Thromb Vasc Biol.* 2017;37(6):1250-60. Doi: 10.1161/ATVBAHA.116.308010.

Due to copyright restrictions, the published version of this journal article is not available here. The published version can be viewed online at:

<http://atvb.ahajournals.org/content/37/6/1250>

Appendix IV: Published papers that form Chapter 6

Loader J, Roustit M, Taylor F, MacIsaac RJ, Stewart S, Lorenzen C, Walther G. Assessing cutaneous microvascular function with iontophoresis: Avoiding non-specific vasodilation. *Microvascular Research*. 2017;113:29-39. Doi: 10.1016/j.mvr.2017.04.006.

Due to copyright restrictions, the published version of this journal article is not available here. The published version can be viewed online at:

<http://www.sciencedirect.com/science/article/pii/S0026286217300717?via%3Dihub>

Appendix V: Ethics approvals, letters to participants and consent forms

Study 2: Letter to participants and consent form

ACU Human Ethics Committee Approval ID: 2014 02V

PARTICIPANT INFORMATION LETTER

PROJECT TITLE: Sugar-sweetened beverage consumption in healthy, type 1 and type 2 diabetic adults

PRINCIPAL INVESTIGATORS:

Dr. Rani Watts (rani.watts@acu.edu.au)

Dr. Guillaume Walther (guillaume.walther@univ-avignon.fr)

STUDENT RESEARCHER: Mr. Jordan Loader (jordan.loader@acu.edu.au)

Dear Participant,

You are invited to participate in the research project described below.

What is the project about?

A non-healthy diet is commonly accompanied by consumption of sugar-sweetened beverages (SSBs), reported to provide little (if any) nutritional benefit. The 2010 National Nutrition Survey in Australia found that 58% of young adults drink an average of 2.1 cans (800 mL) of a SSB per day, which provide the main daily source of sugar. Several studies in children and adults have reported strong associations between SSB consumption and the risk of developing type 2 diabetes, due to its potential adverse effects on weight gain and glucose metabolism.

SSB contain between 70-120 g of sugar per litre and lead to a peak in blood glucose. Long-term SSB consumption is reported to be a precursor of insulin resistance but may also negatively impact the vascular system (blood vessels). The *endothelium* is a thin layer of cells lining the blood vessels and the heart and helps to control blood pressure through vasodilation (widening) and vasoconstriction (narrowing). *Very few studies have investigated the underlying mechanisms involved in the dysfunction of the endothelium in response to blood glucose peaks following SSB consumption.*

Endothelial dysfunction, in this context, has been shown to impair the capacity of blood vessels to vasodilate (widen). This is likely associated with a reduction in nitric oxide (NO) synthesis and its bioavailability. Nitric oxide (NO) induces a healthy widening of the blood vessels. We have previously reported data in healthy rats that implicates endothelial dysfunction immediately following a peak in blood glucose. Dysfunction was characterized by oxidative stress and lowered activity of nitric oxide synthase (eNOS; important for the synthesis of NO) within the microcirculation of blood vessels. ***The aim of this research study is to explore the effect of SSB consumption on endothelial function in blood vessels of healthy and type 2 diabetic individuals, with a particular focus on the underlying mechanisms of the NO pathway.***

Who is undertaking the project?

This project is being conducted by Dr. Rani Watts and Jordan Loader (PhD candidate) from the School of Exercise Science at Australian Catholic University in collaboration with Dr. Guillaume Walther from the University of Avignon, France.

What will I be asked to do?

To be eligible to take part in the study you will need to be (i) male or female; (ii) aged between 18 and 60 years old; (iii) diabetic (type 1 or type 2) or non-diabetic (healthy); and (iv) to agree to refrain from exercise the day prior to the testing being undertaken. You will be required to visit the Exercise Science Research Laboratory, in the Daniel Mannix Building on ACU's Fitzroy campus, for three sessions of approximately 1 to 1.5 hours in duration. During each session, you will be required to remain in a supine position (lying down) and we will test your blood glucose and microvascular responses with non-invasive, painless devices that use ultrasound and laser light technology. Your microvascular responses will be checked before and following the consumption of a SSB (600 mL of the Solo brand lemon drink). At two of the three sessions, you will be asked to swallow a capsule containing either 10 mg/kg⁻¹⁰ salmeterol dihydrochloride (BH4) or 500 mg N-acetylcysteine (NAC). At each of these sessions, dilute concentrations of insulin (100 U/ml), acetylcholine (a 2% solution) and sodium nitroprusside (a 2% solution) will be applied to the skin of the forearm during the assessment of your microvascular response to the SSB. Very small droplets of blood will be collected from the end of your finger using a simple finger-prick technique (5 times per session). These small droplets of blood (less than 1/20th of 1ml) will be used to measure blood glucose. You are asked to abstain from strenuous exercise in the 24 hours leading up to the study day and arrive at the laboratory first thing in the morning without eating or drinking i.e. in the fasted state. Following testing you will be provided with breakfast.

Are there any risks associated with participating in this project?

This project primarily uses non-invasive techniques (ultrasound and laser light technology). While all techniques will be performed by well-trained and experienced operators, some risk, although minimal, is associated with participation in this study. The researchers take any risk in participating in the study seriously and will do everything within their capacity to minimise risk and maximise comfort. Details of (i) these risks, (ii) how they can be minimised and (iii) how they will be managed in the unlikely event that they occur are described as follows:

- 1) *Minor infection at the site of blood sampling.* Finger-prick sampling will be performed to assess your blood glucose response to SSB consumption. The small risk of infection from finger-prick sampling will be significantly reduced by swabbing the area with alcohol and using a sterile single-use lancet. In the unlikely event of infection, you will be advised to consult your medical practitioner.
- 2) *Temporary hypoglycaemia (low blood sugar).* You may experience symptoms of temporary hypoglycaemia (e.g. weakness, sweating, intense hunger) following the overnight fast. All testing will start early (approximately 8 am) to prevent the risk of hypoglycaemia. Analysis of blood glucose undertaken as part of the study will permit researchers to monitor and stop the testing if blood glucose levels fall below 3.0 mmol/L. In the unlikely event that clinical hypoglycaemia occurs, you will be supplied with water, coffee or tea and breakfast. Researchers will continue to monitor your blood

glucose levels until they return to acceptable levels before allowing you to leave the research venue. For safety, it is recommended that you organise for a friend or family member to accompany you or drive you home. Alternative arrangements will be made by researchers to get you home if this is not possible.

- 3) *Temporary hyperglycaemia (high blood sugar)*. A temporary elevation in blood glucose levels is expected following the consumption of SSB. However, it is unlikely to be of a duration that places participants at risk of producing any symptoms. Should blood glucose levels remain elevated beyond 11.1 mmol/L at the end of testing we will advise you to seek advice from your medical practitioner.
- 4) *Headache in response to nitronal-glyceryl trinitrate (NMD) administration*. Infrequently, headache occurs following sublingual (below the tongue) administration of nitronal-glyceryl trinitrate. In the unlikely event of a headache, researchers will recommend that you respond to this in your usual way e.g. self-medicate. If headache persists, the researchers will recommend you seek advice from your medical practitioner.
- 5) *Temporary reddening, allergic reaction or sensation of 'pins and needles' of the skin at the site of iontophoresis*. For the iontophoresis (microcirculation) procedure, three vasoactive substances will be transdermally administered (by placing the substance into the small chambers situated on the electrodes) on to the skin of the forearm: insulin (100 U/ml), acetylcholine (2% solution) and sodium nitroprusside (2% solution). Microvascular endothelium-dependent and independent vasodilation will then be assessed using laser light technology. The predictable risk for iontophoresis is the possibility of temporary red patches at the placement of the electrodes, regardless of which compound is placed into the chamber of the electrode. A prolonged allergic reaction is very unlikely and has never been observed in any of our previous studies. The researcher will verbally confirm that you have not experienced an allergy to transdermal administration of insulin, acetylcholine or sodium nitroprusside in the past. If your skin shows excessive reaction to these substances testing will immediately cease. You may also feel 'pins and needles' at or around the iontophoresis application zone (at the electrodes). This sensation will only occur during iontophoresis period (up to 2.5 minutes per visit) and is not painful. In the past, the sensation of 'pins and needles' has occurred in only approximately 10% of participants and no one has yet asked to stop the iontophoresis procedure. Nevertheless, the testing will be stopped in the case of your expressed discomfort.
- 6) *Side effects from oral medication administration*. Two substances (plus one placebo) are to be orally administered: saproterin dihydrochloride (BH4; 10 mg/kg⁻¹⁰) and N-acetylcysteine (NAC; 500 mg).
The 10 mg/kg⁻¹⁰ dose of BH4 to be given in this study represents a common starting concentration given for treatment in diseases such as phenylketonuria. Patients frequently tolerate higher daily doses of 20 mg/kg⁻¹⁰ without side effects. However, side effects of this medication may include headache, diarrhoea, abdominal pain and nausea, but again these have only been reported for chronic use. Previous studies in humans reported excellent acute tolerance with higher plasma BH4 levels.
The 500 mg dose of NAC given in the present study is the dose reported in clinical literature to generate an acute effect in humans. Several previous studies have used this antioxidant treatment to study the impact of oxidative stress. NAC is safe for adults, when used as a prescription medication. However, side effects of this medication may

include nausea, vomiting, diarrhoea or constipation. Rarely, it can cause rashes, fever, headache, drowsiness, low blood pressure, and liver problems, although reports of this nature are usually associated with oral solutions and less in studies using capsules (as proposed in our study). Moreover, previous works reported on doses of 2400 mg per day or more. In the unlikely event of experiencing side effects in response to either substance, you will be advised to consult your medical practitioner.

- 7) *Temporary soreness of finger at the site of blood sampling.* Finger-prick sampling will be undertaken five times during each visit. This method is associated with temporary discomfort with the possibility of a short period (~1-2 days) of soreness at the site where the finger is pricked, however is a far more well-tolerated and less invasive procedure for measuring your blood glucose responses than standard catheterisation or venepuncture.

What are the benefits of the research project?

While there are no immediate benefits to you for participating in this study, we will provide you with information about your blood sugar response following SSB consumption. This study will allow us to investigate the microvascular response to the intake of a single SSB and provide a better understanding of the mechanisms involved in endothelial dysfunction of the microcirculation.

Can I withdraw from the study?

Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw from the study at any time without adverse consequences.

Will anyone else know the results of the project?

We intend to present aggregated data from this study at scientific conferences and publish it in a scientific journal. It will also be used in a PhD thesis. Your data will remain confidential and known only to the researchers and yourself.

What if I have a complaint or any concerns?

The study has been reviewed by the Human Research Ethics Committee at Australian Catholic University (approval number 2014 02V). If you have any complaints or concerns about the conduct of the project, you may write to the Chair of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research):

Manager, Ethics. C/o Office of the Deputy Vice Chancellor (Research)
Australian Catholic University; North Sydney Campus; PO Box 968
NORTH SYDNEY, NSW 2059
Email: res.ethics@acu.edu.au

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

Yours sincerely,

Dr. Rani Watts
Principal investigator



CONSENT FORM

Copy for Researcher/Copy for Participant to Keep

TITLE OF PROJECT: Sugar-sweetened beverage consumption in adults with and without type 2 diabetes

(NAME OF) PRINCIPAL INVESTIGATOR: Dr. Rani Watts

I (*the participant*) have read (*or, where appropriate, have had read to me*) and understood the information provided in the Letter to Participants. Any questions I have asked, have been answered to my satisfaction. I agree to participate in this project involving overnight fasting, three x one-hour visits to test the blood and blood pressure responses (using ultrasound and light technology) to drinking a sugar-sweetened drink (600 mL of Solo Lemon drink). I understand that a small, albeit safe, amount of drugs (oral or transdermal application) will be used during each visit. I realise that I can withdraw my consent at any time without adverse consequences. I agree that research data collected for the study will be password protected and may be published or may be provided to other researchers in a form that does not identify me in any way.

NAME OF PARTICIPANT:

SIGNATURE DATE

SIGNATURE OF PRINCIPAL INVESTIGATOR:

DATE:.....

Study 4: Letters to participants and consent forms

Study 4 is comprised of 2 separate protocols, which were approved by the local ethics committee following receipt of 2 separate applications; and, thus, 2 separate letters to participants and 2 separate consent forms are presented below.

ACU Human Ethics Committee Approval IDs: 2013 238V & 2015-18H

PARTICIPANT INFORMATION LETTER

PROJECT TITLE: Intraday and interday reproducibility of skin blood flow measurement using laser speckle imaging.

PRINCIPAL INVESTIGATOR: Dr. Christian Lorenzen

STUDENT RESEARCHER: Jordan Loader

Dear Participant,

You are invited to participate in the research project described below.

What is the project about?

The research project investigates the repeatability of a piece of equipment that measures blood flow and small blood vessel function. The blood flow and function of small blood vessels are thought to be early indicators of cardiovascular health. The PeriMed Laser Speckle Contrast Imager is a new laser imaging device that can measure blood flow and small blood vessel function quickly with little discomfort for patients. Presently, repeatability of measurement of this device is still not completely understood (i.e. whether the device can deliver the same information regarding blood flow when the conditions of testing remain similar). The aim of this study is therefore to assess repeatability of blood flow measurement with the PeriMed. This research is important as it will provide important information for clinicians and researchers who wish to assess blood flow with the PeriMed. If the measurements are repeatable, clinicians and researchers may use the PeriMed to assess the effectiveness of interventions (e.g. pharmaceuticals, physical activity) in vascular health.

Who is undertaking the project?

Dr. Christian Lorenzen and Jordan Loader (PhD candidate) from the Australian Catholic University are conducting this project.

Who is eligible to participate?

We are seeking healthy male and female participants who are over the age of 18 years. Those with any significant medical history for chronic cardiovascular disease and the use of any medications affecting microcirculation are not eligible to participate.

Are there any risks associated with participating in this project?

The only foreseeable risk will be discomfort when a blood pressure cuff is pumped 50 mmHg above your normal resting systolic blood pressure. The pressure from the cuff will be similar to what you experience when you visit the Doctor, although for this study, pressure will be maintained for three minutes, in which you may feel a numb tingling feeling in your forearm and hand. If the pressure is too uncomfortable you will be free to ask the researcher to deflate the cuff immediately. Medications are

administered safe dose levels and will remain in the local application area, not spreading around the body. A finger prick for blood glucose testing may cause momentary discomfort, and possibly slight bruising at the puncture site.

What will I be asked to do?

You will be requested to attend the Cardiovascular Research Laboratory in the School of Exercise Science, Fitzroy, on four occasions, over a seven day period. You will be required to abstain from caffeine, nicotine and alcohol for at least 4 hours before testing to avoid their effects on microcirculation. Test one will be conducted on the morning of day one, and test two in the afternoon of day one. Tests three and four will be conducted approximately 7 days later, in the morning and afternoon respectively. Each test will last about 60 minutes, and all testing procedures will be the same from test to test. The step by step procedure for each test will be:

1. You will lay on a bench for 20 minutes in a temperature controlled room for acclimatization.
2. A blood pressure cuff placed will be placed on your upper arm, on the same side of the blood flow recordings.
3. Resting blood pressure assessed.
4. Baseline blood glucose levels assessed using a finger prick lancet and blood glucose monitor.
5. Two medication-delivery electrodes (acetylcholine and sodium nitroprusside) will be placed on your inner forearm.
6. Two weak electrical currents will be administered simultaneously to move the medication to your skin blood vessels. Laser imaging will occur throughout.
7. You will rotate your arm so that the palm of your hand is flat on the table.
8. Two minute baseline measurement of blood flow.
9. Blood pressure cuff inflated for 3 minutes.
10. Immediately after blood pressure cuff is deflated blood flow will be recorded for a 5-minute period. Laser imaging will occur throughout.

How much time will the project take?

You will be requested to attend four 60 minute sessions over a one week period.

What are the benefits of the research project?

There will be little direct benefit to you for this study; however, your contribution may contribute to community health in the future. The information in this study may help clinicians and researchers interpret data from the Perimed, enabling them to determine the success of an intervention (e.g. pharmaceutical or physical activity) more confidently and identify at-risk patients.

Can I withdraw from the study?

Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw from the study at any time without adverse consequences.

Will anyone else know the results of the project?

It is the researcher's intention to publish the results of this study in an international research journal. You will not be identifiable in this publication as only aggregated data will be presented. During the study, your data will be de-identified and coded so

only the Principal Investigator can determine that it is you, and will be stored on a password protected computer in the Cardiovascular Research Laboratory in the School of Exercise Science, ACU. At the completion of the study, data will be kept on a password protected computer in the Principal Investigators officer.

Will I be able to find out the results of the project?

On completion of the study, the Principal Investigator will send you via mail a summary of the findings, and your individual results.

Who do I contact if I have questions about the project?

Should you have any questions regarding this project, please contact the Principal Investigator:

Dr Christian Lorenzen
(03) 9953 3849
School of Exercise Science
ACU, St Patrick's Campus, 115 Victoria Parade, Fitzroy, VIC 3065

What if I have a complaint or any concerns?

The study has been approved by the Human Research Ethics Committee at Australian Catholic University (approval number 2013 238V). If you have any complaints or concerns about the conduct of the project, you may write to the Chair of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research).

Research Ethics Manager (ResEthics.Manager@acu.edu.au)
Office of the Deputy Vice-Chancellor (Research)
Australian Catholic University
North Sydney Campus
PO Box 968
North Sydney, NSW 2059.

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

I want to participate! How do I sign up?

If you wish to participate in this study, contact the Principal Investigator Dr. Christian Lorenzen at christian.lorenzen@acu.edu.au. You should sign both copies of the provided Consent Form. Please retain one copy for your records and return the other copy to the Principal Investigator.

Yours sincerely,

Dr. Christian Lorenzen
Principal Investigator

CONSENT FORM

Copy for Researcher / Copy for Participant to Keep

TITLE OF PROJECT: Intraday and interday reproducibility of skin blood flow measurement using laser speckle imagery

PRINCIPAL INVESTIGATOR: Dr. Christian Lorenzen

STUDENT RESEARCHER: Jordan Loader

I have read and understood the information provided in the Letter to Participants. Any questions I have asked, have been answered to my satisfaction. I agree to participate in this study assessing skin blood flow and blood vessel function, and understand that I will be requested to attend the Cardiovascular Research Laboratory in the School of Exercise Science, Fitzroy, on four occasions, over a seven day period, realising that I can withdraw my consent at any time (without adverse consequences). I agree that research data collected for the study may be published or may be provided to other researchers in a form that does not identify me in any way.

NAME OF PARTICIPANT:

SIGNATURE DATE

SIGNATURE OF PRINCIPAL INVESTIGATOR:

DATE:.....

If you become distressed, alarmed or disadvantaged while participating in the study, it is recommended that you call Lifeline Melbourne on 13 11 14, which is a 24 hour telephone counselling service for people over 18 years. If you believe you have experienced any negative physical, you are advised to contact your general practitioner.

PARTICIPANT INFORMATION LETTER

PROJECT TITLE: Improving techniques that assess cardiovascular health

PRINCIPAL INVESTIGATOR: Dr. Christian Lorenzen

STUDENT RESEARCHER: Jordan Loader (PhD student)

Dear Participant,

You are invited to participate in the research project described below.

What is the project about?

The blood flow and function of small blood vessels are thought to be early indicators of cardiovascular health. Transdermal iontophoresis, which uses a weak electrical current that moves a medication across the skin, is a non-invasive technique that can measure blood vessel function quickly with little discomfort for patients. Presently, it isn't completely understood if widening of the blood vessels (vasodilation) and increased blood flow is only responding to the medication or partly due the electrical current stimulation (i.e. non-specific vasodilation). The aim of this study is therefore to determine what non-specific vasodilation, if any, is induced using several commonly published iontophoresis protocols. This research is important, as it will provide a reliable testing protocol for clinicians and researchers who wish to assess blood vessel function with transdermal iontophoresis. Furthermore, the development of a reliable testing protocol free of non-specific vasodilation will allow clinicians and researchers to more confidently and non-invasively assess the effectiveness of interventions (e.g. pharmaceuticals, physical activity) in vascular health.

Who is undertaking the project?

Dr. Christian Lorenzen and Jordan Loader (PhD candidate) from the Australian Catholic University are conducting this project.

Who is eligible to participate?

We are seeking healthy male and female participants who are aged 18 years and over. Potential participants with any significant medical history of chronic cardiovascular disease and regularly using any medications affecting microcirculation are not eligible to participate.

Are there any risks associated with participating in this project?

Medications are administered safe dose levels and will remain in the local application area, not spreading around the body. A small brief tingling sensation (i.e. pins and needles feeling) and/or skin rash may be experienced locally to the medication application area, but any sensation leaves within minutes and any rash leaves within 24 hours. The effects will be minimized by having a trained and experienced researcher administering the tests. A finger prick for blood glucose testing may cause momentary discomfort, and possibly slight bruising at the puncture site. You may also feel some

discomfort from lying down for an extended period of time or need to leave the testing session (e.g. you may need to use the bathroom). If for any reason you feel uncomfortable and wish to cease the test, you will be welcome to do so. In the unlikely event that you experience any physical discomfort beyond what is described above, you will be advised to contact your General Practitioner. The cost of your visit to your General Practitioner will be covered by the School of Exercise Science, Australian Catholic University.

What will I be asked to do?

You will be requested to attend the Cardiovascular Research Laboratory in the School of Exercise Science, Fitzroy, on five occasions, over a two week period. Each trial will be separated by at least 48 hours. You will be required to abstain from caffeine, nicotine and alcohol for at least 4 hours before testing to avoid their effects on microcirculation. Each testing session will be separated by at least 2 days. Each test will last about 70 minutes, and all testing procedures will be the same from test to test. However, the medication and current strength will vary. The step-by-step procedure for each test will be:

1. You will lie on a bench for 20 minutes in a temperature controlled room for acclimatization.
2. A blood pressure cuff placed will be placed on your upper left arm.
3. Resting blood pressure will be taken.
4. Resting blood glucose levels assessed using a finger prick device and blood glucose monitor.
5. Two medication-delivery electrodes (containing acetylcholine, sodium nitroprusside, insulin, insulin diluent, deionized water or saline) will be placed on each of your inner left and right forearms.
6. Two weak electrical currents will be administered simultaneously to move the medications to your skin blood vessels on your right forearm. Laser imaging will occur throughout.
7. Following 20 minutes of laser imaging, the process at step 6 will be repeated on your left arm for 20 minutes in order to assess another iontophoresis protocol.

How much time will the project take?

As stated above you will be requested to attend five 70-minute sessions over a two week period.

What are the benefits of the research project?

You will receive a fruit and drink refreshment after each testing session. Your participation may also contribute to community health in the future. The information in this study may develop protocols enabling clinicians and researchers to more confidently assess before and after an intervention (e.g. pharmaceutical or lifestyle) and identify at-risk patients for developing cardiovascular disease.

Can I withdraw from the study?

Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw from the study at any time without adverse consequences.

Will anyone else know the results of the project?

It is the researchers' intention to publish the results of this study in a scientific journal and PhD thesis. You will not be identifiable in this publication, as only averaged data will be presented. During the study, your data will be deidentified and coded so only the Principal Investigator can determine that it is you, and will be stored on a password protected computer in the Cardiovascular Research Laboratory in the School of Exercise Science, at ACU. At the completion of the study, data will be kept on a password-protected computer in the office of the Head of School.

Will I be able to find out the results of the project?

On completion of the study, the Principal Investigator will send you an email with a summary of the findings, and your individual results.

Who do I contact if I have questions about the project?

Should you have any questions regarding this project, please contact the Principal Investigator:

Dr Christian Lorenzen
(03) 9953 3849
School of Exercise Science
ACU, St Patrick's Campus, 115 Victoria Parade, Fitzroy, VIC 3065

What if I have a complaint or any concerns?

This study has been approved by the Human Research Ethics Committee at Australian Catholic University (approval number 0000019039). If you have any complaints or concerns about the conduct of the project, you may write to the Chair of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research).

Research Ethics Manager (ResEthics.Manager@acu.edu.au)
Office of the Deputy Vice-Chancellor (Research)
Australian Catholic University
North Sydney Campus
PO Box 968
North Sydney, NSW 2059.

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

I want to participate! How do I sign up?

If you wish to participate in this study, contact the Principal Investigator Dr. Christian Lorenzen at christian.lorenzen@acu.edu.au. You should sign both copies of the provided Consent Form. Please retain one copy for your records and return the other copy to the Principal Investigator.

Yours sincerely,

Dr. Christian Lorenzen
Principal Investigator



CONSENT FORM

Copy for Researcher / Copy for Participant to Keep

TITLE OF PROJECT: Improving techniques that assess cardiovascular health

PRINCIPAL INVESTIGATOR: Dr. Christian Lorenzen

STUDENT RESEARCHER: Jordan Loader

I have read and understood the information provided in the Letter to Participants. Any questions I have asked, have been answered to my satisfaction. I agree to participate in this study assessing skin blood flow and blood vessel function, and understand that I will be requested to attend the Cardiovascular Research Laboratory in the School of Exercise Science, Fitzroy, on five occasions, over 2 week period. I understand the associated risks with participating in this study, as outlined in the Letter to Participants, realising that I can withdraw my consent at any time (without adverse consequences). I agree that research data collected for the study may be published or may be provided to other researchers in a form that does not identify me in any way.

NAME OF PARTICIPANT:

SIGNATURE DATE

SIGNATURE OF PRINCIPAL INVESTIGATOR:

DATE:.....

If you believe you have experienced any negative physical reactions as a result of participating in this study, you are advised to contact your general practitioner and the cost of your visit will be covered by the Australian Catholic University.

END OF DOCUMENT