Uncovering the Interaction between Undercarboxylated Osteocalcin and Vascular Function in Normoglycaemic and Hyperglycaemic Environments

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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~ 2020 ~

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Abstract

Endothelial dysfunction is the initiating process in the development of atherosclerosis and cardiovascular disease (CVD) and is a significant predictor of future adverse cardiovascular events. Increasing evidence suggests a link between vascular function and the skeleton, an association that may be mediated by bone-derived proteins such as osteocalcin (OC). OC is an osteoblast-derived, vitamin K-dependent protein that primarily exists in two biological forms. Carboxylated osteocalcin (cOC) is involved in bone formation and undercarboxylated osteocalcin (ucOC) is suggested to be the bioactive form of the protein responsible for regulating energy metabolism and glucose homeostasis. As such, ucOC may be targeted as a therapeutic treatment for metabolic diseases such as diabetes.

In humans, the association of OC with endothelial dysfunction and CVD is conflicting, with some suggesting that OC is associated with beneficial effects in the vasculature and others reporting adverse effects. Research in animals suggest that *in vivo* OC treatment improves vascular function. However, corresponding improvements in metabolic outcomes suggest that the improvements in vascular function may occur indirectly, due to improvements in energy metabolism. As such, the primary aim of this thesis is to investigate if ucOC has a direct biological effect on vascular function in normoglycaemic and hyperglycaemic environments in preclinical models and humans. This was examined in four studies.

Study 1: Hyperglycaemia is a pathological condition that has a toxic effect on blood vessels and is a major risk factor for atherosclersosis and CVD. However, it is unclear whether the dysfunction caused by hyperglycaemia is blood vessel specific and whether

the dysfunction is exacerbated by an atherogenic diet. It was important to identify which blood vessels developed dysfunction for subsequent studies to assess the vasoactive role of ucOC. Abdominal aorta, iliac and mesenteric arteries were dissected from male New Zealand White Rabbits following either a four week normal or atherogenic diet ($n = 6 – 12$ per group). The arteries were incubated *ex vivo* in normal or high glucose solutions (20 mM or 40 mM) for 2 h and isometric tension myography was used to determine endothelial-dependent vasodilation. The atherogenic diet reduced blood vessel relaxation, as measured by area under the curve (AUC), by 25% ($p < 0.05$) in the aorta, 17% ($p = 0.06$) in the iliac artery and 40% ($p = 0.07$) mesenteric artery. In the aorta of the atherogenic diet-fed rabbits the 20 mM glucose incubation altered EC_{50}, thereby reducing the potency of acetylcholine ($p < 0.05$), and tended to reduce E_{max} and AUC in the normal diet-fed rabbits. Incubation of the iliac artery from atherogenic diet-fed rabbits in 40 mM glucose also altered EC_{50}, reducing the potency of acetylcholine ($p < 0.05$). No dysfunction occurred in the mesentery with high glucose incubation following either the normal or atherogenic diet. High glucose-induced endothelial dysfunction appears to be blood vessel specific; the aorta may be the optimal artery to study potential therapeutic treatments of hyperglycaemia-induced endothelial dysfunction.

Study 2: In Study 1, we established that acute high glucose incubations and an atherogenic diet cause endothelial dysfunction in rabbit aorta. As such, this study examined the biological effect of ucOC on blood vessel function in rabbit aorta *ex vivo*, as well as determining the effect of ucOC on markers of endothelial function in human cells *in vitro*. Isometric tension and immunohistochemistry techniques were used on the aorta of male New Zealand White Rabbits and human aortic endothelial cells (HAEC) were cultured to assess the effect of ucOC in normal and high glucose environments.

Overall, ucOC, both 10 ng/ml and 30 ng/ml, did not significantly alter acetylcholine-induced blood vessel relaxation in rabbits ($p > 0.05$). The ucOC treatment did not cause any significant changes in the immunoreactivity of cellular signalling markers (endothelial nitric oxide synthase, protein kinase B, mammalian target of rapamycin and nitrotyrosine) in rabbit aorta ($p > 0.05$). In HAEC, ucOC did not attenuate endothelin 1, interleukin 6, vascular adhesion molecule 1, monocyte chemoattractant protein 1 or lactate dehydrogenase, all of which were increased in response to high glucose treatment ($p > 0.05$). In conclusion, the results of this study suggest that ucOC has no direct influence on endothelial function in rabbit aorta *ex vivo* or in human endothelial cells *in vitro*.

Study 3: In this study we examined whether ucOC is related to blood pressure and vascular function in older adults and whether ucOC has a direct effect on endothelial function in the carotid artery of rabbits. To undertake the study, we used perfusion myography, which allows for the examination of whole vessel segments with pulsatile flow and pressure that mimics an endogenous environment. In older adults, ucOC, blood pressure, pulse wave velocity (PWV) and brachial artery flow mediated dilation (BAFMD) were measured ($n = 38$, 26 post-menopausal women and 12 men, mean age 73 ± 1 years). In male New Zealand White Rabbits, the vasoactivity of the carotid artery was assessed following a four week normal or atherogenic diet. An ucOC dose response curve (0.3 – 45 ng/ml) was administered following incubation of the arteries for 2 h in either normal or high glucose conditions. The concentration of ucOC was higher in normotensive older adults compared to those with stage 2 hypertension (34%, $p < 0.05$), particularly in women (43%, $p < 0.01$), but not men ($p > 0.05$). In all participants, higher ucOC was also associated with lower PWV ($p < 0.05$), but not BAFMD ($p > 0.05$). In

rabbits, ucOC at any dose did not cause an alteration in the vasoactivity of the carotid artery, following either a normal or atherogenic diet ($p > 0.05$). In conclusion, ucOC is associated with vascular function in older adults, exclusively in post-menopausal women, but it has no direct effect on endothelial function in rabbit carotid arteries.

Study 4: Vitamin K is a regulator of OC carboxylation, with higher vitamin K intake known to reduce circulating levels of ucOC. As ucOC was associated with vascular function in adults in **Study 3**, we tested the hypothesis that a suppression of ucOC following an increase in dietary vitamin K1 would exhibit a relative worsening of cardiometabolic risk factors. Men ($n = 20$) and women ($n = 10$) aged 62 ± 10 years participated in a randomised, controlled, crossover study. Participants were split into high and low responder subgroups following a four week high vitamin K1 diet (HK) of increased leafy green vegetables. High and low responders were defined based on the median percent reduction (30%) in ucOC following the HK diet. Blood pressure (resting and 24 h), arterial stiffness, plasma glucose and lipid concentrations, and serum OC forms were assessed. Following the HK diet, ucOC and ucOC/tOC were suppressed more ($p < 0.01$) in high responders (41% and 29% respectively) than in low responders (12% and 10% respectively). The reductions in ucOC and ucOC/tOC were not associated with changes in blood pressure, PWV, plasma glucose or lipid concentrations in the high responders ($p > 0.05$). The results from this study suggest that the suppression of ucOC via consumption of leafy green vegetables has no negative effects on cardiometabolic health, perhaps, in part, due to compensatory mechanisms, such as increased nitric oxide.

General conclusions: Overall, the results of this thesis suggest that ucOC does not have a direct biological role in the regulation of endothelial function in rabbit arteries and human endothelial cells. In humans there is some association between ucOC and vascular

function but the suppression of circulating ucOC does not influence vascular function or cardiovascular risk factors. As ucOC was not found to have a detrimental effect on vascular function, it may be targeted as a therapeutic treatment for metabolic diseases, such as T2DM, without a risk of adverse effects on the vasculature.

Student Declaration

I, Alexander Tacey, declare that the PhD thesis entitled **Uncovering the interaction between undercarboxylated osteocalcin and vascular function in normoglycaemic and hyperglycaemic environments** is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

Signature: 

Date: 17/12/2020

Acknowledgements

This thesis would not have been possible without the commitment and dedication of my supervisory team. In particular, I would like to acknowledge my principle supervisor Prof Itamar Levinger who always made himself available when I needed anything and was incredibly supportive every step of the way. The commitment you have to all of your students is second to none and for that I am extremely grateful. To A/Prof Anthony Zulli, thank you for welcoming me into your lab, taking the time to teach me new techniques and encourage new thinking. To Prof Alan Hayes, thank you for providing sensible and timely words of advice when it was needed.

Thank you to all of the colleagues I have worked with throughout my journey, for your assistance, ideas and for making it an enjoyable experience. Thank you to everyone in my research group, especially my fellow osteocalcin buddy Cassandra Smith for your assistance and discussions along the way. A big shout out to my desk mate Rhiannon Patten for sharing many long days, but also for your encouragement and opinions (of which there were many) even when we didn't always agree.

To everyone at Victoria University, the technical staff at both Werribee and Footscray, in particular, Anne Luxford the Animal Facilities Supervisor, as well the research and administrative staff, thank you for your assistance.

I want to add my thanks to my editor, Dr Jon Rosalky, for his copy-editing of my thesis and his many helpful comments about aspects of English grammar and plain English. Any remaining errors in the text are mine.

An important acknowledgment to all of the participants who contributed to the research within this thesis for without them this research would not have been possible.

Finally, to all of my family and friends (even the SBs), who have asked on many occasions what it was that I was researching, thank you for expressing your interest and for your encouragement every step of the way.

Publications and Presentations

Details of included publications: thesis with publication

Chapter No.	Publication Title	Publication Status	Publication Details
2	Potential role for osteocalcin in the development of atherosclerosis and blood vessel disease	Published	**Tacey, A**. et al. (2018). Potential Role for Osteocalcin in the Development of Atherosclerosis and Blood Vessel Disease. Nutrients, 10(10), 1426. (Scimago rank - Q1).
3	The effect of an atherogenic diet and acute hyperglycaemia on endothelial function in rabbits is artery specific	Published	**Tacey, A**. et al. (2020). The Effect of an Atherogenic Diet and Acute Hyperglycaemia on Endothelial Function in Rabbits is Artery Specific. Nutrients, 12(7), 2108. (Scimago rank - Q1).
4	Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells	Published	**Tacey, A**. et al. (2020). Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells. J. Cell. Physiol., 236(4), 2840-2849. (Scimago rank - Q1).
5	Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery ex vivo	Published	**Tacey, A**. et al. (2020). Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery ex vivo. PLoS One, 15(11). (Scimago rank - Q1).
6	Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet	Published	**Tacey, A**. et al. (2020). Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet. Ann. Nutr. Metab., 76(5), 361-367. (Scimago rank Q1).

Declaration by: Alexander Tacey **Signature:** Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 **Date:** 16/12/2020

Details of publications not included in this thesis

Publication title	Publication status	Publication details
The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review	Published	Smith, C., **Tacey**, **A**., Mesinovic, J., Scott, D., Lewis, J. R., Duque, G., Levinger, I. The effects of acute exercise on bone turnover markers in middle-aged and older adults: A systematic review. *Bone*, 115766. (Scimago rank - Q1).
The potential actions of angiotensin eonverting Enzyme II (ACE2) activator diminazene aceturate (DIZE) in various diseases	Published	Qaradakhi, T., Gadanec, L. K., McSweeney, K. R., **Tacey**, **A**., Apostolopoulos, V., Levinger, I., Rimarova, K., Egom, E., Rodrigo, L., Kruzliak, P., Kubatka, P., Zulli, A. (2020). The Potential Actions of Angiotensin Converting Enzyme II (ACE2) Activator Diminazene Aceturate (DIZE) in various Diseases. *Clin. Exp. Pharmacol. Physiol.*, 47(5), 751. (Scimago rank - Q2).
Single-dose prednisolone alters endocrine and haematologic responses and exercise performance in men	Published	**Tacey, A**., Parker, L., Yeap, B,B., Joseph, J., Lim, E, M., Garnham, A., Hare, D., Brennan-Speranza, T, C., Levinger, I. (2019). Single-dose prednisolone alters endocrine and haematologic responses and exercise performance in men. *Endocr. Connect.* 8(2), 111. (Scimago rank - Q2).
The effect of recombinant undercarboxylated osteocalcin on endothelial dysfunction	Published	Qaradakhi, T., Gadanec, L. K., **Tacey**, **A**., Hare, D. L., Buxton, B. F., Apostolopoulos, V., Levinger, I., & Zulli, A. (2019). The Effect of Recombinant Undercarboxylated Osteocalcin on Endothelial Dysfunction. *Calcif. Tissue Int.*, *105*(5), 546. (Scimago rank - Q1).
Mas-related G-protein coupled receptor type D (MrgD) antagonism produces splanchnic vasculature-specific effects, leading to a large reduction in portal pressure in cirrhotic animals	Published	Gunarathne, L., Zulli, A., Qaradakhi, T., Smith, R., **Tacey**, **A**., Angus, P., & Herath, C. (2018). Mas-related G-protein coupled receptor type D (MrgD) antagonism produces splanchnic vasculature-specific effects, leading to a large reduction in portal pressure in cirrhotic animals. *J. Hepatol.*, *68*, S117. (Scimago rank - Q1).

Publication title	Publication status	Publication details
The effect of acute and short term glucocorticoid administration on exercise capacity and metabolism	Published	**Tacey, A**., Parker, L., Garnham, A., Brennan-Speranza, T, C., Levinger, I. (2017). The effect of acute and short term glucocorticoid administration on exercise capacity and metabolism. *J Sci Med Sport,* 20(6), 543. (Scimago rank - Q1).
Osteocalcin and vascular function, is there a cross-talk?	Published	**Tacey, A**., Hayes, A., Zulli, A., Levinger, I. (2021). Osteocalcin and vascular function, is there a cross-talk? *Mol Metab,* 49, 101205. (Scimago rank - Q1)
The evolution of technology and physical inactivity: the good, the bad, and the way forward	Under review	Woessner, M. N., **Tacey**, **A**., Levinger-Limor, A., Parker, A. G., Levinger, P., Levinger, I. The evolution of technology and physical inactivity: the good, the bad, and the way forward.
Distinct effects of a high fat diet on bone in skeletally mature and developing male C57BL/6J mice	Under Review	Ross, D., Yeh, T., King, S., Mathers, J., Neist, E., Rybchyn, M., Cameron, M., **Tacey, A**., et al. Distinct effects of a high fat diet on bone in skeletally mature and developing male C57BL/6J mice.
Association of serum osteocalcin levels with energy metabolism and bone structure in elderly women	In preparation	Brennan-Speranza, T., Prince, R., Byrnes, E., Levinger, I., Sim, M., Blekkenhorst, L., Smith, C., **Tacey**, **A**., et al. Association of serum osteocalcin levels with energy metabolism and bone structure in elderly women.

Presentations arising from data in this thesis

Year	Presentation title	Presentation type	Presentation location
2020	The influence of undercarboxylated osteocalcin on endothelial function in normal and high glucose conditions	Oral	American Society for Bone and Mineral Research (ASBMR), virtual conference
2020	The influence of undercarboxylated osteocalcin on endothelial function in normal and high glucose conditions	Oral	Australian Institute for Musculoskeletal Science (AIMSS), virtual symposium
2020	The influence of undercarboxylated osteocalcin on endothelial function in normal and high glucose conditions	Poster	Australia and New Zealand Bone and Mineral Society (ANZBMS), virtual meeting
2019	The effect of undercarboxylated osteocalcin on hyperglycaemia-induced blood vessel dysfunction	Oral	Australian Physiological Society (AUPS), Canberra Aus
2019	Undercarboxylated osteocalcin and vascular function in normal and high glucose environments	Oral	Victoria University HDR conference, Melbourne Aus
2019	The potential role of undercarboxylated osteocalcin as a therapeutic target for blood vessel disease	Poster	Australian Atherosclerosis Society (AAS), Melbourne Aus
2019	The potential role of undercarboxylated osteocalcin as a therapeutic target for blood vessel disease	Poster	Australia and New Zealand Bone and Mineral Society (ANZBMS), Darwin Aus
2019	The potential role of undercarboxylated osteocalcin as a therapeutic target for blood vessel disease	Poster	Western Health Research and Best Care conference, Melbourne Aus
2017	Uncovering the effects of undercarboxylated osteocalcin in blood vessel dysfunction induced by hyperglycaemia	Poster	Victoria University HDR conference, Melbourne Aus

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List of Abbreviations

AA: arachidonic acid

AAC: abdominal aortic calcification

ACE: angiotensin converting enzyme

ACh: acetylcholine

ACS: aortic calcification score

AD: atherogenic diet

AGEs: advanced glycation end products

Akt: protein kinase B

ALP: alkaline phosphatase

Ang I: angiotensin I

Ang II: angiotensin II

ANOVA: analysis of variance

ApoE: apolipoprotein E

ATP: adenosine triphosphate

AUC: area under the curve

BAFMD: brachial artery flow mediated dilation

baPWV: brachial artery pulse wave velocity

BGP/BGLAP: bone gamma-carboxyglutamic acid protein / γ-carboxyglutamic acid-containing protein of bone

BH_4: tetrahydrobiopterin

BP: blood pressure

bpm: beats per minute

Ca^{2+}: calcium

CABG: coronary artery bypass graft

$CaCl_2$: calcium chloride

CACS: coronary artery calcification score

CAD: coronary artery disease

CaM: calmodulin

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

C-IMT: carotid intima-media thickness

CKD: chronic kidney disease

cm: centimetre

CO_2: carbon dioxide

cOC: carboxylated osteocalcin

con: control

COX: cyclooxygenase

CT: computed tomography

CVD: cardiovascular disease

d: Cohen's d

DAB: Diaminobenzidine

DAG: diacylglycerol

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

EC_{50}: Half the maximal effective concentration

ECLIA: electrochemiluminescence immunoassay

EIA: enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

EDHF: endothelial derived hyperpolarizing factor

eGFR: estimated glomerular filtration rate

E_{max}: maximum response

eNOS: endothelial nitric oxide synthase

ER: endoplasmic reticulum

ERK: extracellular signalling regulated kinase

Esp: enterococcal surface protein

ET-1: endothelin 1

ET_A: endothelin receptor type A

ET_B: endothelin receptor type B

FMD: flow mediated dilation

g: gram

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GGCX: γ-glutamyl carboxylase

GLA: glutamic acid

GPR158: G protein-coupled receptor 158

GPRC6A: G protein-coupled receptor class C group 6 member A

GTP: guanosine triphosphate

H_2O_2: non-radical hydrogen peroxide

HAEC: human aortic endothelial cells

HASMC: human aortic smooth muscle cells

HbA1c: haemoglobin A1c

HDL: high-density lipoprotein

HFD: high fat diet

HIF-1α: hypoxia-inducible factor 1α

HK: high vitamin K1 intake

h: hour/s

HUVEC: human umbilical vein endothelial cells

ICAM-1: intracellular adhesion molecule 1

IgG: immunoglobulin G

IGT: impaired glucose tolerance

IHC: immunohistochemistry

IHD: ischaemic heart disease

IL-1: interleukin 1

IL-6: interleukin 6

IL-8: interleukin 8

IMT: intima media thickness

iNOS: inducible nitric oxide synthase

IRβ: insulin receptor β

IRMA: immunoradiometric assay

IRS-1: insulin receptor substrate 1

K^+: potassium

K_{ca}: calcium dependent potassium channels

KCI: potassium chloride

kDa: kilodalton

kg: kilogram

KH_2PO_4: Monopotassium phosphate

LDH: lactate dehydrogenase

LDL: low-density lipoprotein

LDLR: low-density lipoprotein receptor

LK: low vitamin-K1 intake

Log: logarithm

L-NAME: N^G-nitro-$_L$-arginine methyl ester

LSD: least significant difference

M: molar

m: metre

MAP: mean arterial pressure

MAPK: mitogen-activated protein kinase

MCP-1: monocyte-chemoattractant protein 1

MGP: matrix glutamic acid protein

$MgSO_4$: magnesium sulfate

min: minute/s

ml: millilitre

mm: millimetre

mM: millimolar

mmHg: millimetre of mercury

mmol/L: millimoles per litre

MMP-3: matrix metalloproteinase 3

MOVAS: mouse vascular smooth muscle cells

MRI: magnetic resonance imaging

mRNA: messenger ribonucleic acid

MS: metabolic syndrome

m/s: metres per second

mTOR: mammalian target of rapamycin

n: number

NaCl: sodium chloride

NAD^+: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

$NaHCO_3$: sodium bicarbonate

NCD: normal chow diet

ND: normal diet

NF-κβ: nuclear factor kappa-light-chain-enhancer of active β cells

ng: nanogram

NGT: normal glucose tolerance

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOS: nitric oxide synthase

n/s: not significant

NT: nitrotyrosine

O_2: oxygen

O_2^-: superoxide

OC: osteocalcin

$^{\cdot}OH$: hydroxyl radicals

$ONOO^-$: peroxynitrite

OPG: osteoprotegerin

PAI-1: plasminogen activator inhibitor 1

PARP-1: poly [ADP-ribose] polymerase 1

PGI_2: prostacyclin

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PKG: protein kinase G

PM: post-menopausal

PWV: pulse wave velocity

RAGE: receptor for advanced glycation end products

RIA: radioimmunoassay

RNA: ribonucleic acid

ROS: reactive oxygen species

Runx2: runt-related transcription factor 2

s: second

SEM: standard error of the mean

Ser: serine

SMC: smooth muscle cells

SNP: sodium nitroprusside

STZ: streptozotocin

T2DM: type 2 diabetes mellitus

TGF-β: transforming growth factor beta

Thr: threonine

TNF-α: tumor necrosis factor-α

tOC: total osteocalcin

Tris HCl: tris (hydroxymethyl) aminomethane hydrochloride

TXA_2: thromboxane A_2

ucOC: undercarboxylated osteocalcin

UDP-GlcNAc: uridine diphosphate *N*-acetylglucoseamine

ug/d: microgram per day

μm: micrometre

VCAM-1: vascular adhesion molecule 1

VEC: vascular endothelial cells

VEGF: vascular endothelial growth factor

VIABP: Vegetable Intake and Blood Pressure

Vit D: vitamin D

Vit K: vitamin K

VKDP: vitamin K-dependent proteins

VRI: vascular reactivity index

VSMC: vascular smooth muscle cells

Chapter 1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in Australia and throughout the world, accounting for approximately 30% of global death (Nichols et al., 2016, Organization, 2019). One of the most prevalent forms of CVD is atherosclerosis, a complex, chronic inflammatory disease of the medium to large arteries. Endothelial dysfunction is a critical initiating stage in the development of atherosclerosis and is a major predictor of future adverse cardiovascular events (Lerman and Zeiher, 2005). One of the major risk factors for endothelial dysfunction and atherosclerosis is diabetes and the associated hyperglycaemia (Hadi et al., 2005). Hyperglycaemia, which is characterised by elevated levels of circulating blood glucose, often has a major adverse effect on the microvasculature and macrovasculature, in part via the production of reactive oxygen species (ROS) (Potenza et al., 2009). The excess ROS promotes oxidative stress which causes tissue damage via a complex pathological signalling cycle that reduces the availability of vasoactive factors, promoting endothelial dysfunction (Ceriello, 2008). Therefore, exploring novel therapeutic approaches to target hyperglycaemia-induced endothelial dysfunction is of major clinical importance.

Traditionally, bone functions as a site of muscle attachment and a mineral reservoir. In addition, it is now known that bone mediates a number of endocrine functions throughout the body (Kirk et al., 2020). The osteoblast-derived protein osteocalcin (OC) is suggested as one of the major mediators of the interaction between bone and target tissue. OC exists in two main forms: carboxylated osteocalcin (cOC), which has a high affinity for hydroxyapatite within the bone matrix, and undercarboxylated osteocalcin (ucOC) which is detected predominantly in the circulation and is thought to be the biologically active form of OC outside of bone (Li et al., 2016).

The primary biological effect attributed to ucOC is the regulation of energy metabolism, particularly glucose homeostasis (Lin et al., 2020b). Increasing evidence also suggests that ucOC may regulate functions in other organs, including the vasculature (Levinger et al., 2017). Importantly, ucOC has been suggested as a future therapeutic treatment for metabolic diseases such as type 2 diabetes mellitus (T2DM) (Villafan-Bernal et al., 2011). Therefore, investigating its biological effect, particularly any negative off-target effects on other tissue is of interest.

Currently, the exact role of ucOC within the vasculature is conflicting and unclear (D'Onofrio et al., 2019). In humans, a number of studies have identified that OC is associated with a reduced risk of atherosclerosis and cardiovascular disease outcomes (Confavreux et al., 2013, Zhang et al., 2015), while others report an increased risk with higher OC levels (Liu et al., 2019, Okura et al., 2010). Interestingly, experimental studies in animals suggest that ucOC administration may improve vascular function (Dou et al., 2014, Huang et al., 2017). However, these studies identified simultaneous improvements in metabolic outcomes such as glucose regulation, insulin sensitivity and body composition. As such, whether ucOC is exerting a direct impact on the vasculature or whether the biological effect is regulated indirectly via improvements in energy metabolism is currently unknown (Tacey et al., 2018).

The primary aim of this thesis was to identify whether ucOC directly influences vascular function in physiological and pathological conditions, and if so, to explore the potential mechanisms. This thesis includes four studies (**Chapters 3 – 6**). **Chapter 3** (**Study 1**) examines the effect of acute hyperglycaemia and an atherogenic diet on the development of endothelial dysfunction in various rabbit arteries. **Chapters 4** and **5** (**Studies 2** and **3**) examine the direct biological effect of ucOC on endothelial function in

rabbit arteries *ex vivo* and human vascular cells *in vitro* in normoglycaemic and hyperglycaemic conditions. **Chapter 5** (**Study 3**) examines the association of ucOC with vascular function in older adults and **Chapter 6** (**Study 4**) examines the influence of dietary-induced reductions in ucOC bioavailability on vascular function.

Chapter 2. Literature review

This literature review comprises four main sections. **Section 2.1** discusses the role of endothelial dysfunction in the development of atherosclerosis. **Section 2.2** describes osteocalcin and its biological functions. **Section 2.3** describes the interaction of osteocalcin with endothelial dysfunction and atherosclerosis. **Section 2.4** outlines the aims of this PhD thesis.

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Declaration of co-authorship and co-contribution

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS

Title of Paper/Journal/Book:	Potential Role for Osteocalcin in the Development of Atherosclerosis and Blood Vessel Disease

Surname:	Tacey	First name:	Alexander
Institute:	Institute for Health and Sport	Candidate's Contribution (%):	70

Status:

Accepted and in press:	☐	Date:	
Published:	✔	Date:	4/10/2018

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 09:46:03+11'00'	16/12/2020
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

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1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
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Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
Tawar Qaradakhi	5	Editing of manuscript	[redacted]	16/12/20
Tara C. Brennan-Speranza	5	Editing of manuscript		16/12/20
Alan Hayes	5	Editing of manuscript		16/12/20
Anthony Zulli	5	Editing of manuscript		16/12/20
Itamar Levinger	10	Editing of manuscript		16/12/20

Tacey A, Qaradakhi T, Brennan-Speranza T, Hayes A, Zulli A, Levinger I. Potential Role for Osteocalcin in the Development of Atherosclerosis and Blood Vessel Disease. Nutrients. 2018; 10 (10):1426. https://doi.org/10.3390/nu10101426

2.1 Endothelial dysfunction and atherosclerosis

2.1.1 Atherosclerosis background and epidemiology

Cardiovascular disease (CVD) is the umbrella term for a group of pathological diseases of the myocardium and vasculature. Accounting for approximately 30% of global mortality, CVD is the leading cause of death in the world (Organization, 2019, Nichols et al., 2016). Atherosclerosis, otherwise known as atherosclerotic vascular disease, is the most common form of CVD and develops in large conduit blood vessels including the carotid, cerebral, iliac, coronary and aortic arteries (Ross and Glomset, 1973, Beckman et al., 2002). In the coronary arteries, atherosclerosis manifests clinically as ischaemic heart disease (IHD) and can contribute to a myocardial infarction. In the cerebral and carotid arteries, atherosclerosis manifests as cerebrovascular disease and can be a major cause of a cerebrovascular accident. IHD and cerebrovascular disease are the first and third leading causes of mortality, respectively in Australia and throughout the world, accounting for approximately 85% of all CVD related deaths ((ABS), Benjamin et al., 2019, Collaborators, 2018, Barquera et al., 2015).

Atherosclerosis is a complex and chronic disease that is characterised by inflammation of the medium to large arteries that develops over many decades (Ross, 1999). The development of atherosclerosis, a process otherwise termed atherogenesis, generally occurs in several specific stages: initially endothelial dysfunction followed by plaque formation and growth, ultimately ending in vascular calcification. The development of endothelial dysfunction is a critical initiating stage in atherogenesis and will be the focus of this thesis. Atherosclerosis often begins in childhood, or even in utero, but does not present clinically until mid to late adulthood and is often exacerbated by an

unhealthy lifestyle and a high number of risk factors (Bentzon et al., 2014, Skilton et al., 2005, Hopkins et al., 2013).

Due to improved medical care and pharmacological treatments, there has been a reduction in the mortality rate from atherosclerosis related deaths in developed countries over recent decades (Herrington et al., 2016, Hadi et al., 2005). However, the decline has not occurred in developing countries, where the prevalence is expected to increase in the future (Herrington et al., 2016, Barquera et al., 2015). This is attributable to the ageing population, changes in food systems in low and middle income countries and a shift from infectious diseases to non-communicable diseases (Barquera et al., 2015, Libby et al., 2016). Overall, the burden of death and disability attributable to atherosclerosis makes it one of the most important public health problems that exist today (Libby et al., 2016). Consequently, developing novel therapeutics that aim to reduce atherosclerosis and its risk factors is of major importance.

2.1.2 Atherosclerosis risk factors

A significant number of modifiable and non-modifiable risk factors contribute to the development of endothelial dysfunction leading to atherosclerosis (**Table 2.1)** (Hadi et al., 2005, Mubanga et al., 2017, Barquera et al., 2015, Bonetti et al., 2003, Jensen and Mehta, 2016, Lacey et al., 2017, Sena et al., 2013, Cosselman et al., 2015, Schultz et al., 2018, Keyes, 2004, Boggild and Knutsson, 1999, Elderon and Whooley, 2013, Kelishadi and Poursafa, 2014). Metabolic risk factors such as hyperglycaemia and insulin resistance, which occur in patients with diabetes, are major risk factors for the development of atherosclerosis (Bornfeldt and Tabas, 2011). This is due, at least in part, to the fact that diabetes and atherosclerosis share common risk factors and often progress simultaneously (Del Turco et al., 2013). The likelihood of developing atherosclerosis may

rise with an increased number and severity of the risk factors present (Bonetti et al., 2003). Importantly, a reduction or improvement in modifiable risk factors may prevent or delay the development of atherosclerosis (Barquera et al., 2015).

Table 2.1 Non-modifiable and modifiable risk factors for the development of endothelial dysfunction and atherosclerosis.

Non-modifiable risk factors		
Age	Genetics	Family History
Menopause status	Ethnicity	Sex
Modifiable risk factors		
Diabetes - Hyperglycaemia - Insulin resistance	Sedentary lifestyle - Lack of physical activity - Sedentary behaviour	Environmental risk factors - Low SES - Air pollutants - lack of pets
Smoking	Obesity	Hypertension
Hyperlipidaemia - Elevated total cholesterol - Elevated LDL - Reduced HDL - Elevated triglycerides	Poor dietary choices - High sodium - Regular alcohol - High saturated fat - High sugar	Mental health illnesses - Depression - Anxiety - Chronic stress
Infections	Irregular sleep patterns	

Abbreviations - HDL; high-density lipoprotein, LDL; low-density lipoprotein, SES; socioeconomic status.

2.1.3 Endothelial function and dysfunction physiology

An alteration to the physiological function of endothelial cells, which is described as endothelial dysfunction, is the initiating stage in the development of atherosclerosis. This section will describe the physiological functions of the endothelium and the pathological underpinnings that occur during the development of endothelial dysfunction.

2.1.3.1 Normal endothelial function

Blood vessels consist of three layers in which each layer features distinct morphology and functions. The vascular endothelium is a cellular monolayer and the predominant feature of the tunica intima. Endothelial cells create a barrier between the components of blood and the interstitial compartments of the body and regulate

homeostatic control in the vasculature (Rubanyi, 1993). The human body comprises of approximately 60 million endothelial cells that make up around 1.5% of our total body mass (Aird, 2006, Cines et al., 1998, Rubanyi, 1993). On average endothelial cells are 20 – 40 µm long, 10 – 15 µm wide and 0.1 – 0.5 µm thick (Cahill and Redmond, 2016). The internal elastic lamina encloses the endothelium, forming the remainder of the tunica intima. The tunica media, the middle layer of the arterial wall, predominantly contains smooth muscle cells (SMCs) that function to regulate vascular tone (Brown et al., 2017). The tunica externa or adventitia, which is separated from the media by the external elastic lamina, consists predominantly of connective tissue; collagen and proteoglycans (Brown et al., 2017) (**Figure 2.1**).

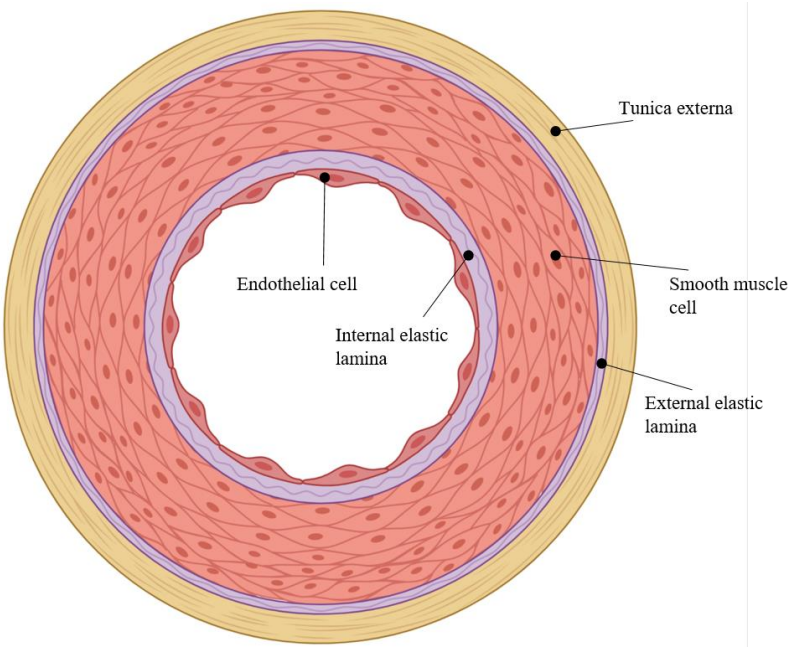

Figure 2.1 Cross sectional view of an artery and its morphological structure. The tunica intima consists of endothelial cells and the internal elastic lamina. The tunica media contains smooth muscle cells and the external elastic lamina. The tunica externa predominantly consists of connective tissue such as collagen. Created with BioRender.com.

The endothelium serves not only as a barrier but functions as a receptor-effector structure that senses physical and chemical mediators in the circulation and can respond

by producing a number of vasoactive substances (Esper et al., 2006). The homeostatic functions include degradation and synthesis of bioactive molecules, the transport of molecules between the circulation and the vascular SMCs and the secretion and remodelling of extracellular matrix components (Gimbrone and Garcia-Cardena, 2016). In a physiological environment the endothelium has a number of anti-atherogenic functions including the maintenance of vascular tone, anti-inflammatory effects and the inhibition of platelet aggregation, smooth muscle cell proliferation and leukocyte adhesion (Bonetti et al., 2003, Jensen and Mehta, 2016). The endothelium regulates these physiological effects by stimulation from humoral factors in the circulation (acetylcholine) and mechanical and haemodynamic forces (shear stress) exerted on endothelial cells (Traub and Berk, 1998).

The importance of the endothelium in maintaining vascular function was first reported in several seminal papers published in the 1980s (Furchgott and Zawadzki, 1980, Furchgott et al., 1981, Furchgott, 1983). These studies established that vasodilation of arteries is endothelium-dependent and attributed this function to an endothelial-derived relaxing factor, which would later be termed nitric oxide (NO). Further evidence on the importance of the endothelium in regulating vascular function can be seen in pathological environments when endothelium-dependent relaxation of blood vessels is reduced but relaxation to endothelium-independent vasodilation is unaltered (Zhao et al., 2015). It is now well established that the endothelium is an active paracrine, endocrine and autocrine organ, responsible for the homeostatic regulation of the vasculature (Bonetti et al., 2003, Brown et al., 2017).

2.1.3.2 Endothelial dysfunction

Endothelial dysfunction is defined as an alteration in the phenotypical function of endothelial cells (Gimbrone and Garcia-Cardena, 2016). It is the first detectable stage of atherogenesis and a strong predictor of future adverse cardiovascular events (Bonetti et al., 2003, Lerman and Zeiher, 2005). The development of endothelial dysfunction involves an increase in pathological mechanisms including: oxidative stress, local and systemic inflammation, an increase in noxious stimuli within circulation, altered haemodynamic forces and as yet unknown factors (**Figure 2.2**) (Jensen and Mehta, 2016, Gimbrone and Garcia-Cardena, 2016).

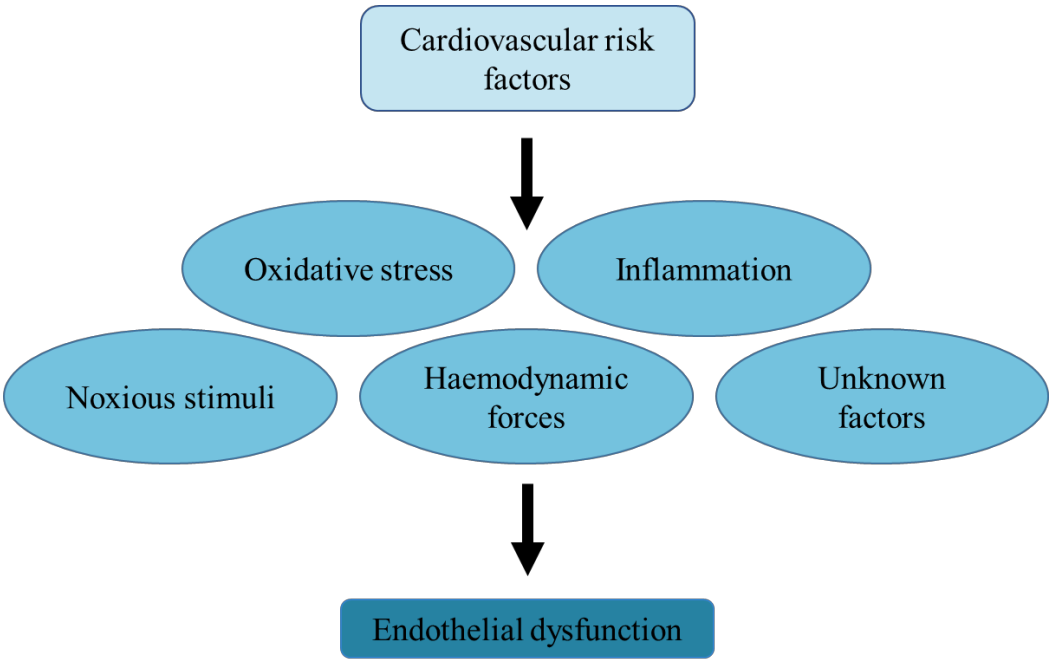

Figure 2.2 The pathological mechanisms activated by cardiovascular risk factors that promote endothelial dysfunction.

The presence of these pathological mechanisms causes a reduction in the production and release of atheroprotective factors such as NO (**Section 2.1.3.2.1**) and an increase in atherogenic factors such as endothelin 1 (ET-1) and angiotensin II (Ang II) (**Section 2.1.3.2.2**). The persistent imbalance between atheroprotective and atherogenic

factors results in the inability of the endothelium to vasodilate effectively, leading to a chronic state of vasoconstriction (Cahill and Redmond, 2016, Lerman and Zeiher, 2005). A reduction in endothelium-dependent vasodilation is a hallmark and one of the earliest indicators of endothelial dysfunction (Bornfeldt and Tabas, 2011, Cosentino and Luscher, 2001). In addition to an alteration in vascular tone, endothelial dysfunction is characterised by an increase in inflammation, apoptosis, platelet adhesion, oxidant activity and thrombotic factors (Esper et al., 2006, Jensen and Mehta, 2016, Gradinaru et al., 2015) (**Figure 2.3**).

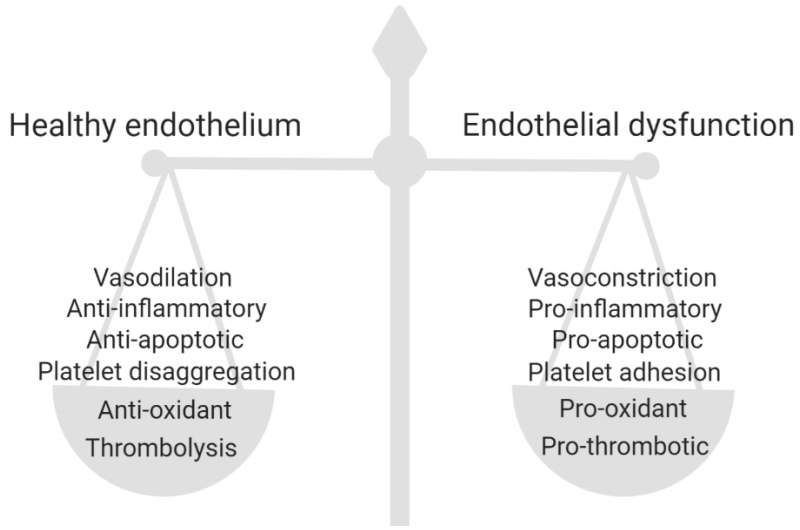

Figure 2.3 The balance between the biological functions of the endothelium that maintain homeostasis versus the pathological functions that promote atherogenesis. Adapted from (Esper et al., 2006). Created with BioRender.com.

An alteration in haemodynamic forces such as shear stress is an important factor in the development of endothelial dysfunction (Traub and Berk, 1998). Shear stress is the frictional force created by blood flow. It causes endothelial cells to respond acutely to changes in blood flow via activation of integrin receptors (Lu and Kassab, 2011). Endothelial dysfunction and atherogenesis often develop in lesion prone segments of arteries, such as curvatures or bifurcations where the endothelium is exposed to low shear

stress and disturbed flow (Tabas et al., 2015, Traub and Berk, 1998). These altered flow patterns increase the atherogenic phenotype by reducing vasodilation and increasing cell permeability, proliferation and cell adhesion molecules (Cahill and Redmond, 2016). This noxious environment promotes the adhesion of low density lipoprotein (LDL) and leukocytes to the sub-endothelial space, an important event in the development of an atherosclerotic lesion (Cahill and Redmond, 2016). Pro-inflammatory and pro-thrombotic factors such as nuclear factor kappa-light-chain-enhancer of activated β cells (NF-κβ) released from lesion prone sites further stimulate endothelial dysfunction (Tabas et al., 2015). NF-κβ is thought to regulate several effector molecules including vascular adhesion molecule 1 (VCAM-1) and monocyte-chemoattractant protein 1 (MCP-1), the latter of which is a pro-coagulant chemokine that mediates leukocyte adhesion (Gimbrone and Garcia-Cardena, 2016). VCAM-1 and intracellular adhesion molecule 1 (ICAM-1) function as markers of leukocyte upregulation and endothelial dysfunction (Gimbrone and Garcia-Cardena, 2016, Linton et al., 2019). Endothelial dysfunction is a present and contributing factor throughout the lifecycle of an atherosclerotic plaque and a major clinical risk factor for future adverse cardiovascular events (Gradinaru et al., 2015). This evidence highlights the importance of identifying novel therapeutic targets to counteract endothelial dysfunction and prevent atherosclerosis.

2.1.3.2.1 Anti-atherogenic regulators of endothelial function

The endothelium produces several factors which function to protect the vasculature from the development of endothelial dysfunction. These factors are stimulated by receptor (acetylcholine (ACh) and bradykinin) and non-receptor (shear stress) mediated agonists acting on endothelial cells (Gimbrone and Garcia-Cardena, 2016, Davignon and Ganz, 2004, Vanhoutte, 2018). The primary anti-atherogenic factors

produced in the endothelium are NO (discussed in **Section 2.1.3.2.1.1**), prostacyclin (PGI_2) and endothelial-derived hyperpolarizing factor (EDHF) (**Figure 2.4**) (Jamwal and Sharma, 2018, Ozkor et al., 2011). While these substances have an array of protective effects in the vasculature, the focus of this discussion is on their vasodilatory effects.

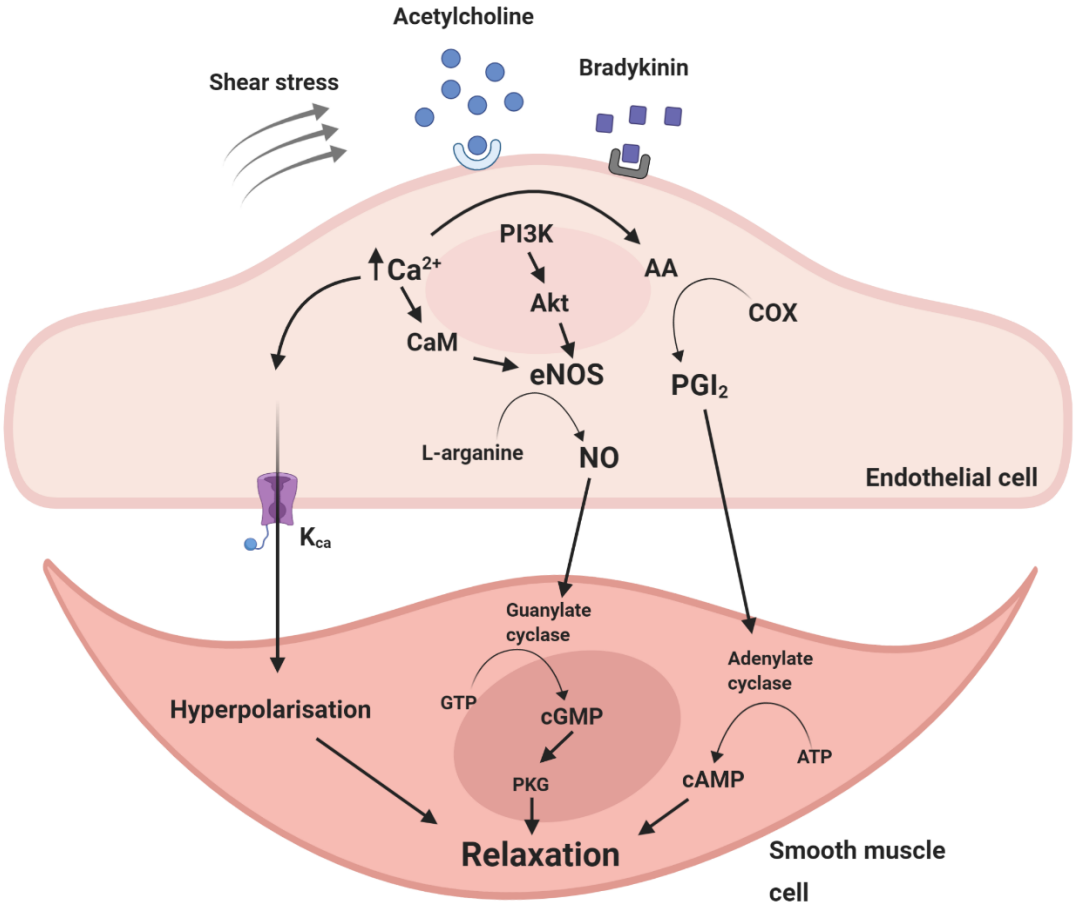

Figure 2.4 Signalling pathways that mediate endothelium-dependent vasodilation. Agonists cause the activation of endothelium-derived substrates which function to produce relaxation of smooth muscle cells and protect against atherosclerosis development. Created with BioRender.com

Abbreviations - AA: arachidonic acid, Akt: protein kinase B, ATP: adenosine triphosphate, Ca^{2+}: calcium, CaM: calmodulin, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, COX: cyclooxygenase, eNOS: endothelial nitric oxide synthase, GTP: guanosine triphosphate, K_{ca}: calcium dependent potassium channels, NO: nitric oxide, PGI_2: prostacyclin, PI3K: phosphoinositide 3-kinase, PKG: protein kinase G.

PGI_2 is an endothelium-derived prostaglandin that functions similarly to NO (Mitchell et al., 2008). PGI_2 is synthesised in endothelial cells by cyclooxygenase (COX), via an increase in arachidonic acid (AA) metabolism stimulated by intracellular calcium (Ca^{2+}) (Mitchell et al., 2008). Under normal physiological conditions PGI_2 causes smooth muscle cell relaxation by activating adenylate cyclase and increasing the production of cyclic adenosine monophosphate (cAMP) via adenosine triphosphate (ATP) (**Figure 2.4**) (Antman et al., 2005, Vanhoutte, 1997). However, the vasodilatory effects of PGI_2 were not identified until the vasodilatory factors NO and EDHF were inhibited (Triggle et al., 2012), suggesting its contribution to vasodilation is limited. In addition, high concentrations of PGI_2 may also contribute to vasoconstriction by activating thromboxane receptors (Triggle et al., 2012). Given PGI_2 likely has a limited role in vasodilation, its primary anti-atherogenic functions are thought to be as an inhibitor of platelet aggregation and deposition (Cines et al., 1998). This would contribute indirectly to a reduction in vasoconstriction by reducing the risk of thrombosis and vascular smooth muscle cell remodelling (Mitchell et al., 2008).

Rather than representing a specific substrate, EDHF represents a number of processes which contribute to the hyperpolarization and subsequent relaxation of vascular SMCs (Feletou and Vanhoutte, 2009, Ledoux et al., 2006). This primarily occurs via the increase in intracellular calcium within endothelial cells, resulting in the opening of calcium-dependent potassium channels (K_{ca}) and the efflux of potassium (K^+) (Feletou and Vanhoutte, 2009). The efflux of K^+ from endothelial cells results in hyperpolarization of myocyte-endothelial cell gap junctions, subsequently inducing hyperpolarization of smooth muscle cells and vasodilation (**Figure 2.4**) (Quyyumi and Ozkor, 2006). Previous research has suggested that EDHF may have a greater vasoactive function in resistance

blood vessels than conduit blood vessels (Ozkor et al., 2011, Shimokawa et al., 1996). This is likely due to the greater proportion of myocyte-endothelial gap junctions in smaller vessels compared to larger vessels (Feletou and Vanhoutte, 2009, Ozkor et al., 2011, Sena et al., 2013). As such, smaller blood vessels may have a reduced reliance on NO and an increased reliance on EDHF to induce relaxation (Shimokawa et al., 1996).

2.1.3.2.1.1 Nitric oxide (NO)

Originally termed endothelial-derived relaxing factor, NO is a gaseous free radical that functions systemically (Kolluru et al., 2010). In the vasculature, NO is a key factor in the maintenance of optimal vascular health, primarily responsible for mediating endothelium-dependent vasodilation and preventing the development of endothelial dysfunction and atherosclerosis (Barbato and Tzeng, 2004). In addition, NO regulates a diverse range of cellular processes including: endothelial cell migration, proliferation, angiogenesis and extracellular matrix degradation (Zhu et al., 2016).

NO is produced by several NO synthase (NOS) enzymes; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Barbato and Tzeng, 2004). In the vasculature eNOS, also known as NOS-3, is the predominant NOS enzyme responsible for the production of NOS (Barbato and Tzeng, 2004). Activation of eNOS occurs via Ca^{2+}-dependent and Ca^{2+}-independent pathways in endothelial cells (Zhao et al., 2015). In the Ca^{2+}-dependent pathways, agonists (ACh, bradykinin and shear stress) increase intracellular Ca^{2+} which binds to calmodulin (CaM) and activates the CaM binding domain of eNOS (Kolluru et al., 2012, Vanhoutte et al., 2017) (**Figure 2.4**). In addition, circulating factors such as insulin, vascular endothelial growth factor (VEGF) and adiponectin increase eNOS independently of intracellular Ca^{2+} via phosphorylation (Vanhoutte, 2018, Forstermann and Li, 2011). Protein phosphorylation of eNOS can

occur at multiple sites, the primary sites include Serine (Ser)1177 and Ser633 which are activators of eNOS and Threonine (Thr)495, which is an inhibitory site (Kolluru et al., 2012, Kolluru et al., 2010, Li et al., 2007). For example, insulin activates the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway to phosphorylate eNOS at Ser1177 (Janus et al., 2016, Muniyappa et al., 2008, Muniyappa et al., 2007), while at the same time reducing the phosphorylation of eNOS at Thr495 (Muniyappa and Quon, 2007, Zhu et al., 2016). NO is synthesised from eNOS when L-arginine converts to L-citrulline (Zhu et al., 2016). The synthesis of NO from eNOS is also dependent on the availability of essential cofactors and co-substrates including tetrahydrobiopterin (BH_4) and nicotinamide adenine dinucleotide phosphate (NADPH) (Vanhoutte, 2018, Davignon and Ganz, 2004).

NO has a very short half-life (approximately 4 s), before it is metabolised into nitrite and nitrate and subsequently excreted in urine (Sena et al., 2013). When released from endothelial cells, NO regulates its biological effects through a number of diverse signalling pathways. The major pathway in the vasculature is via the activation of guanylyl cyclase which produces cyclic guanosine monophosphate (cGMP) via guanosine triphosphate (GTP) (Barbato and Tzeng, 2004, Rask-Madsen and King, 2007). The production of cGMP promotes an increase in protein kinase G (PKG) which reduces intracellular Ca^{2+} in SMCs leading to the interruption of actin-myosin cross-bridges and the subsequent relaxation of SMCs and vasodilation of arteries (**Figure 2.4**) (Zhao et al., 2015, Vanhoutte, 2018).

Under physiological conditions there is a well maintained balance between NO synthesis and breakdown which maintains vascular homeostasis and prevents vasoconstriction (Wever et al., 1998). In a pathological environment, there is a reduction

in the bioavailability of NO resulting in vasoconstriction and endothelial dysfunction (Cai and Harrison, 2000). One of the primary ways in which NO bioavailability is reduced is via oxidative stress and reactive oxygen species (ROS), which promote NO degradation and enhance eNOS production of superoxide (O_2^-) at the expense of NO (discussed in **Section 2.1.5.1.1**) (Zhu et al., 2016, Kolluru et al., 2012, Sena et al., 2013).

2.1.3.2.2 Pro-atherogenic regulators of endothelial function

In a normal physiological environment the balance between pro- and anti-atherogenic factors maintains homeostasis within endothelial cells and reduces the risk of atherosclerosis (Jamwal and Sharma, 2018). The loss or reduction in vasodilatory factors and/or the increase in vasoconstrictive substances upsets the balance and promotes endothelial dysfunction (Vanhoutte et al., 2017). The primary endothelial-derived constriction factors are ET-1, Ang II and vasoconstrictor prostanoids. These pro-atherogenic regulators are often increased in response to noxious stimuli, such as those caused by cardiovascular risk factors (Sena et al., 2013).

ET-1 is the most potent vasoconstrictor and also the most abundant factor in the cardiovascular system (Vanhoutte et al., 2017). The expression of ET-1 occurs via the conversion of big ET-1 into ET-1 by endothelin converting enzymes (Potenza et al., 2009). Insulin can also regulate ET-1 secretion in the endothelium, via the mitogen-activated protein kinase (MAPK) pathway (Muniyappa et al., 2007, Muniyappa and Quon, 2007). A state of insulin resistance promotes the increase in ET-1 and a reduction in NO, leading to vasoconstriction (Muniyappa and Sowers, 2013). ET-1 acts on smooth muscle cells via endothelin receptor type A (ET_A) or B (ET_B) receptors and causes an increase in intracellular Ca^{2+} and subsequently vasoconstriction (Tang and Vanhoutte, 2010, Cines et al., 1998). On top of its vasoactive function, ET-1 increases inflammation,

leukocyte adhesion and ROS, further promoting a state of endothelial dysfunction (Sandoo et al., 2010).

Ang II is produced from angiotensin I (Ang I), a process which is often dependent on angiotensin converting enzyme (ACE) at the endothelial surface in the lungs, but Ang II production can also occur within endothelial cells (Rubanyi, 1993). When synthesised, Ang II mediates vasoconstriction via activation of Ang II type 1 receptor and increases the activation of NADPH and ROS, affecting NO availability and reducing vasodilation (Triggle et al., 2012, Su, 2015). Furthermore, Ang II is also thought to enhance ET-1 production, further increasing vasoconstriction and endothelial dysfunction (Barton and Haudenschild, 2001).

An increase in Ca^{2+} by ET-1 can influence the synthesis of vasoconstrictor prostanoids such as thromboxane A_2 (TXA_2) and prostaglandins (Tang and Vanhoutte, 2010). AA-induced COX activation promotes the release of these factors which increase vasoconstriction and the oxidative stress response (Tang and Vanhoutte, 2010, Wong and Vanhoutte, 2010). TXA_2 and prostaglandins are synthesised through a similar pathway to PGI_2 and likely have opposing biological effects (Triggle et al., 2012, Sandoo et al., 2010).

2.1.4 Atherosclerosis progression beyond endothelial dysfunction

A chronically uncontrolled state of endothelial dysfunction can result in the initiation of atherogenesis, characterised by intimal thickening, fatty streak and lesion formation. In the initial stage lipid and macrophage deposits combine into foam cells within the arterial wall, T-cells and lipids begin to accumulate and smooth muscle cell migration to the lesion site occurs (Szmitko et al., 2003, Stary et al., 1994, Ross, 1999).

This stage of atherogenesis is not generally associated with adverse cardiovascular events and it is possible for lesions to regress if interventions are introduced (Stary, 2000).

The later stages of atherogenesis are associated with the progression of the lesion into a plaque, also known as an atheroma, which is often characterised by the development of a necrotic lipid core (Stary, 1992, Stary et al., 1995). A fibrous cap often begins to develop over the lipid core, the fibrous structure consisting of smooth muscle cells with infiltration of leukocytes and collagen (Virmani et al., 2000, Sakakura et al., 2013). The clinically adverse side effects occur during these stages when the plaques are most vulnerable to haemorrhage and thrombosis (Stary, 1992, Stary, 2000). Haemorrhage generally occurs in areas with a thin fibrous cap, spilling the components of the plaque into the lumen which can result in thrombosis (Ross, 1999, Sakakura et al., 2013).

Vascular calcification is associated with the final stages of atherosclerotic plaque development. This is characterised by the accumulation of calcium in patchy, non-continuous formations, causing the stiffening of the blood vessels and a reduction in vessel compliance (Shroff and Shanahan, 2007, Farzaneh-Far et al., 2001). The presence of calcification is a strong predictor of future adverse cardiovascular events, increasing the risk of mortality or an adverse event by up to four times (Karwowski et al., 2012, Rennenberg et al., 2009). The extent of mineralisation within the vasculature can be examined by magnetic resonance imaging (MRI), electron beam computed tomography (CT) (reported as coronary artery calcification score (CACS)), angiography and B-mode ultrasonography (carotid intima-media thickness (C-IMT)) (Higgins et al., 2005, Wang et al., 2018). Interestingly, imaging techniques have demonstrated that the structure of mineralised vasculature is similar to the composition of bone within the skeleton (Shanahan et al., 2000, Tyson et al., 2003, Duer et al., 2008, Yamanouchi et al., 2012). In

a normal physiological environment smooth muscle cells have an inhibitory effect on mineralisation (Evrard et al., 2015). However, in the presence of pathological stresses such as inflammation, oxidative stress and hormonal imbalances, vascular smooth muscle cells can differentiate into an osteoblast-like phenotype. These cells are capable of expressing and releasing bone-derived proteins present and active during mineralisation and calcification of the skeleton (Evrard et al., 2015, Tyson et al., 2003). These proteins include matrix glutamic acid (GLA) protein (MGP), bone morphogenetic protein, osteonectin, osteoglycin and osteocalcin (OC) (Shanahan et al., 1998, Escobar Guzman et al., 2020).

2.1.5 Diabetes and CVD

T2DM has reached epidemiological levels. The worldwide prevalence of diabetes is 7 – 10% of the adult population (N C D Risk Factor Collaboration, 2016). The prevalence of diabetes is expected to increase further, to around 15% of the adult population in the coming decades (Guariguata et al., 2014). T2DM is a major risk factor for cardiovascular complications, including both microvascular and macrovascular diseases and has been extensively reviewed (Del Turco et al., 2013, Janus et al., 2016, Muniyappa and Sowers, 2013, Ormazabal et al., 2018, Paneni et al., 2015, Tallapragada et al., 2015, Beckman et al., 2002, Bornfeldt and Tabas, 2011, Ceriello and Genovese, 2016, DeFronzo, 2010, Fiorentino et al., 2013, King et al., 2016, Rask-Madsen and King, 2013, Sena et al., 2013, Sowers, 2013, Saad et al., 2015). The devastating effect of T2DM to the cardiovascular system is caused in part by hyperglycaemia (Creager et al., 2003, Potenza et al., 2009). Indeed, the glucose toxicity that is associated with hyperglycaemia reduces NO bioavailability and adversely affects endothelial function (**Figure 2.5**) (Saad et al., 2015). In fact, CVD, is the leading cause of death in patients with diabetes (Tancredi

et al., 2015, Creager et al., 2003, Schalkwijk and Stehouwer, 2005, Rask-Madsen and King, 2013), with mortality rates being 2 – 4 times higher in people with diabetes than those without (Benjamin et al., 2017). As such, the development of novel pharmacological treatments that restore blood glucose levels to normal are clinically important.

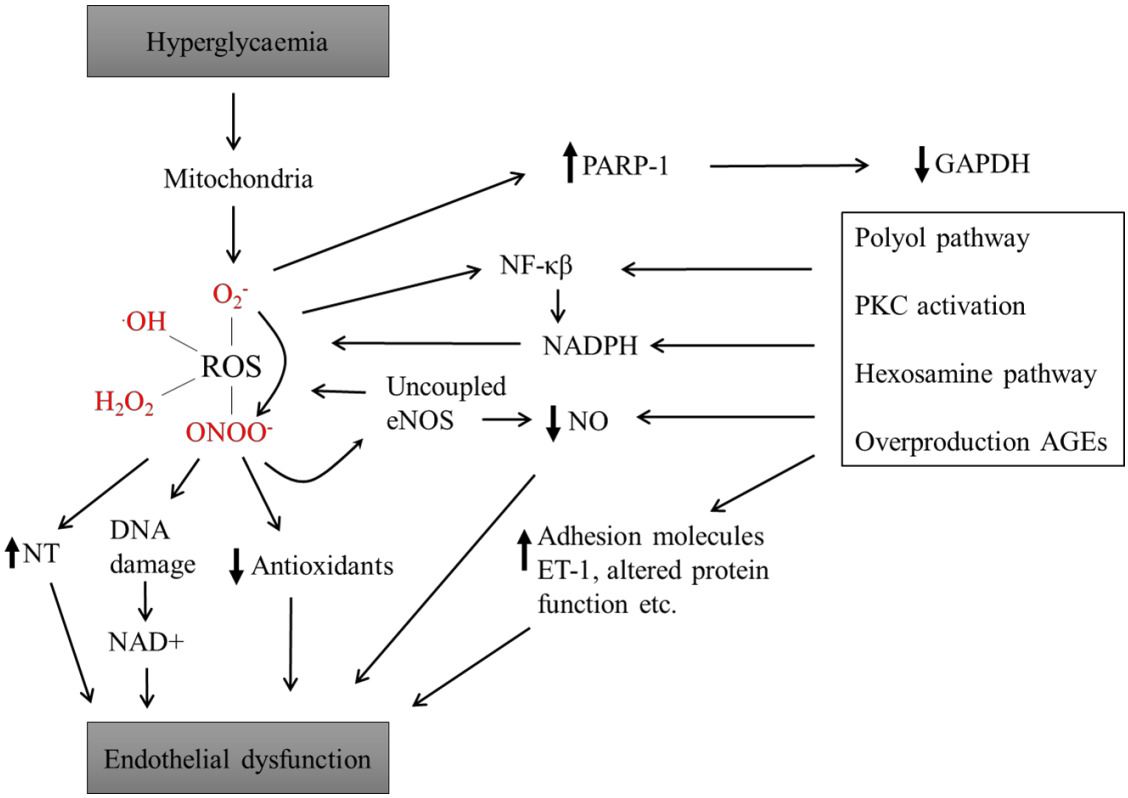

Figure 2.5 Pathological processes that promote hyperglycaemia-induced endothelial dysfunction. Mitochondrial-induced O_2^- overproduction promotes $ONOO^-$ formation and oxidative stress; this is characterised by eNOS uncoupling, DNA damage, a reduction in antioxidants and the formation of NT, which is a marker of oxidative stress. O_2^- overproduction also causes the activation of the polyol pathway, PKC activation, the hexosamine pathway and AGE overproduction, all of which promote endothelial dysfunction and further oxidative stress.

Abbreviations - AGEs: advanced glycation end products, eNOS: endothelial nitric oxide synthase, ET-1: endothelin 1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, H_2O_2: non-radical hydrogen peroxide, NADPH: nicotinamide adenine dinucleotide phosphate, NAD+: nicotinamide adenine dinucleotide, NF-κβ: nuclear factor κβ, NO: nitric oxide, NT: nitrotyrosine, O_2^-:

superoxide, ·OH: hydroxyl radicals, $ONOO^-$: peroxynitrite, PARP-1: poly [ADP-ribose] polymerase 1, PKC: protein kinase C, ROS: reactive oxygen species.

2.1.5.1 Hyperglycaemia and endothelial dysfunction

Normal circulating blood glucose levels are defined by a fasting blood glucose ≤ 5.5 mmol/L. Glucose levels between 5.6 mmol/L and 6.9 mmol/L indicate impaired fasting glucose and ≥ 7 mmol/L suggests the presence of diabetes (Choi et al., 2008, Organization, 2013). During hyperglycaemia, all cells within the body are exposed to toxic levels of circulating blood glucose. Most cells are able to reduce the transport of glucose into the cell, maintaining homeostatic intracellular glucose levels (Brownlee, 2005). However, endothelial cells as well as cells within the retina, kidney and peripheral nerves cannot reduce their intake of glucose, causing elevated intracellular glucose levels (Brownlee, 2005). This results in altered intracellular function and the development of microvascular diseases within the eye, kidney and peripheral vasculature, while in the macrovasculature it leads to the development of endothelial dysfunction and atherosclerosis (Zimering, 2011, Brownlee, 2005).

Chronically elevated plasma glucose levels as assessed via haemoglobin A1c (HbA1c) are associated with endothelial dysfunction (Ceriello, 2003). Yet fluctuations in circulating blood glucose, such as the spikes that occur in the post-prandial period have also been identified as an important risk factor in CVD development (Ceriello and Genovese, 2016, Wright et al., 2006). A number of studies in humans have reported that acute hyperglycaemia is correlated with a reduction in endothelial function in people with diabetes, as well as those who are metabolically healthy (Hu et al., 2010, Kim et al., 2003, Loader et al., 2015, Williams et al., 1998, Kawano et al., 1999, Ceriello et al., 2008). This phenomenon has also been observed in animals (Tesfamariam et al., 1990, Tesfamariam and Cohen, 1992). The cellular mechanisms that promote acute hyperglycaemia-induced

endothelial dysfunction are triggered by one defining process: the development of ROS and the presence of oxidative stress (**Figure 2.5**). However, it is not clear whether this occurs in all conduit blood vessels. **Chapter 3** (**Study 1**) will investigate the effect of acute hyperglycaemia on endothelial function in several different blood vessels.

2.1.5.1.1 The role of oxidative stress and reactive oxygen species (ROS) in hyperglycaemia

Under normal physiological conditions, oxidants, or ROS, are produced as a by-product of aerobic metabolism (Esper et al., 2006). Antioxidants, such as superoxide dismutase, glutathione peroxidase and catalase, neutralise ROS and prevent oxidative damage (Esper et al., 2006). However, when oxidants are produced more quickly than they can be removed, a state of imbalance occurs and oxidative stress develops (Siti et al., 2015, Sies, 1997). The elevated levels of ROS cause an alteration in cellular deoxyribonucleic acid (DNA), impairing cellular and tissue function (Bayraktutan, 2002, Siti et al., 2015).

The development of hyperglycaemia-induced oxidative stress in the endothelium is driven by ROS-induced alterations in NO bioavailability (Creager et al., 2003, Zimering, 2011). Elevated levels of glucose increase intracellular glucose transport and oxidation, resulting in the overproduction of O_2^- by the electron transport chain (Giacco and Brownlee, 2010, Ceriello, 2005, Brownlee, 2001, Shah and Brownlee, 2016), a process which is suggested as the common mechanism from which all the pathological effects from hyperglycaemia develop (Ceriello, 2008). The elevated levels of O_2^- oxidize available NO, to form peroxynitrite ($ONOO^-$), a powerful and toxic ROS (Ceriello, 2008, Wright et al., 2006). NO bioavailability is further reduced by $ONOO^-$-induced eNOS uncoupling which promotes oxidative stress (Bakker et al., 2009). $ONOO^-$ also increases

nitrotyrosine (NT) via the nitration of protein tyrosine residues. NT is a commonly used marker of $ONOO^-$ and ROS formation (Wright et al., 2006, Ceriello et al., 2002, Shishehbor et al., 2003, Ceriello, 2002). Other common forms of ROS include hydroxyl radicals ($^{\cdot}OH$) and the non-radical hydrogen peroxide (H_2O_2). The formation of ROS often acts in a pathological cycle, with increases in NF-κβ promoting further O_2^- production via NADPH (Wright et al., 2006, Ceriello and Motz, 2004) (**Figure 2.5**).

The overproduction of O_2^- activates poly [ADP-ribose] polymerase 1 (PARP-1), which causes the inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Du et al., 2003, Pircher et al., 2016). With a reduction in GAPDH there is an increase in glycolytic intermediaries and the activation of four downstream signalling pathways which promote hyperglycaemia-induced damage (Giacco and Brownlee, 2010, Shah and Brownlee, 2016). These pathways are:

- The polyol pathway
- The hexosamine pathway
- Activation of protein kinase C (PKC)
- Overproduction of advanced glycation end products (AGEs)

In a pathological loop, these processes promote further ROS accumulation via uncoupled of eNOS and NADPH oxidase, further increasing the effects of hyperglycaemia-induced oxidative stress (Son, 2012, Fiorentino et al., 2013, Giacco and Brownlee, 2010, Incalza et al., 2018). Each of these pathways will be discussed in brief in **Section 2.1.5.1.2**.

2.1.5.1.2 O_2^- overproduction promotes endothelial dysfunction

The polyol pathway is the first pathway activated by O_2^- overproduction. The increase in intracellular glucose causes polyol pathway flux, where aldose reductase converts glucose to sorbitol via NADPH, sorbitol dehydrogenase then converts sorbitol to fructose via nicotinamide adenine dinucleotide (NAD^+) (Saad et al., 2015, Shah and Brownlee, 2016) (**Figure 2.6**). The utilisation of NADPH by the polyol pathway reduces glutathione peroxidase, a key antioxidant (Rask-Madsen and King, 2013, Brownlee, 2001) (**Figure 2.5**). The polyol pathway does not directly promote endothelial dysfunction, but contributes to the process indirectly.

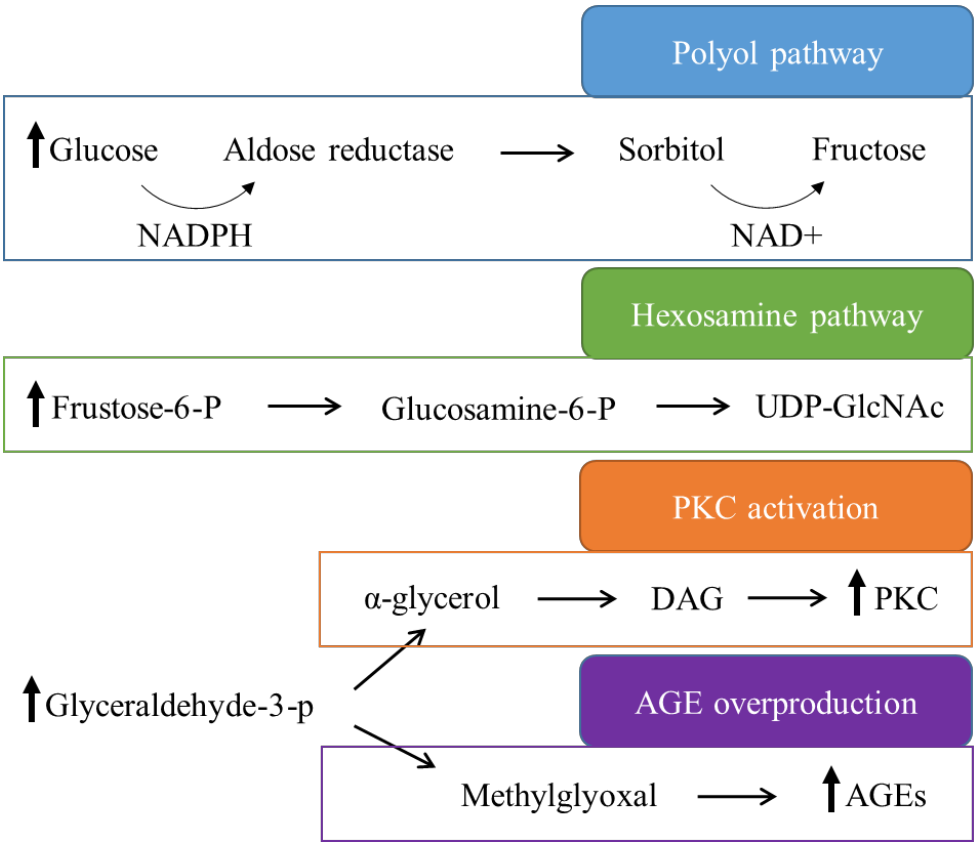

Figure 2.6 Pathways promoting endothelial dysfunction by hyperglycaemia. An increase in intracellular glucose causes superoxide overproduction and promotes the polyol pathway, hexosamine pathway, PKC activation and AGE overproduction.

Abbreviations - AGEs: advanced glycation end products, DAG: diacylglycerol, NAD+: nicotinamide adenine dinucleotide, NADPH: nicotinamide adenine dinucleotide phosphate, PKC: protein kinase C, UDP-GlcNAc: uridine diphosphate N-acetylglucosamine.

The hexosamine pathway involves the conversion of fructose-6-phosphate into glucosamine-6-phophate, which is further converted to uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) (Saad et al., 2015, Shah and Brownlee, 2016) (**Figure 2.6**). The hexosamine pathway causes alterations to endothelial function by altering gene expression and protein function and decreasing NO availability (Brownlee, 2001, Brownlee, 2005, Saad et al., 2015) (**Figure 2.5**). Increases in plasminogen activator inhibitor 1 (PAI-1) and transforming growth factor beta (TGF-β), and inhibition of eNOS phosphorylation at Ser1177 are characteristic of enhanced hexosamine pathway activation (Saad et al., 2015, Shah and Brownlee, 2016).

The diversion of glyceraldehyde-3-phosphate to α-glycerol and diacylglycerol (DAG) promotes PKC activation (Schalkwijk and Stehouwer, 2005, Shah and Brownlee, 2016) (**Figure 2.6**). PKC is a family of at least twelve isoforms; the β, α and δ isoforms are primarily activated in hyperglycaemia (Aronson and Rayfield, 2002, Brownlee, 2005). Activation of these PKC isoforms inhibits the expression of eNOS and subsequent production of NO, as well as increasing ET-1 and adhesion molecules (Brownlee, 2001, Potenza et al., 2009). PKC also activates NADPH and NF-κβ, further enhancing ROS production and oxidative stress (Potenza et al., 2009, Brownlee, 2001) (**Figure 2.5**).

The overproduction of AGEs has similar pathological effects to PKC activation, inhibiting NO production and eNOS expression, as well as enhancing ET-1 expression and increasing adhesion molecules (Saad et al., 2015, Potenza et al., 2009, Rojas and Morales, 2004, Aronson and Rayfield, 2002). Binding of AGEs to their receptor (RAGE)

promotes further O_2^- production via an increase in NADPH and NF-κβ (Potenza et al., 2009, Rojas and Morales, 2004, Brownlee, 2001) (**Figure 2.5**). AGEs can be formed in several ways, via the Maillard reaction which involves formation of a Schiff base and Amadori products (Ott et al., 2014, Rojas and Morales, 2004). Yet the most prominent way in which AGEs are formed in hyperglycaemia is by the activation of the glycolytic intermediary glyceraldehyde-3-phosphate to methylglyoxal (Schalkwijk and Stehouwer, 2005, Ott et al., 2014) (**Figure 2.6**).

2.1.6 Assessment of endothelial function and atherosclerosis

In a clinical setting, endothelial function can be examined by a number of invasive and non-invasive techniques (Zimering, 2011). Some of the most commonly utilised non-invasive techniques are flow mediated dilation (FMD), pulse wave velocity (PWV), finger plethysmography and cardiac magnetic resonance imaging (Arrebola-Moreno et al., 2012, Gokce, 2011, Higashi, 2015). Clinically, FMD is the gold standard non-invasive method of endothelial function assessment (Frolow et al., 2015, Donald et al., 2006). It is often undertaken in the brachial artery (BAFMD) and utilises shear stress to induce NO-mediated vasodilation. Ultrasound is then used to assess the change in the diameter of the arterial wall (Arrebola-Moreno et al., 2012, Bruyndonckx et al., 2013). Impairment of BAFMD is common in those with atherosclerosis and correlates highly with dysfunction in the coronary arteries (Flammer et al., 2012). PWV is a commonly used clinical technique which measures arterial stiffness (Frolow et al., 2015, Kim and Kim, 2019). It involves analysing the velocity of a pulse waveform travelling along the arteries and commonly uses a pulse wave from the femoral to carotid arteries (Frolow et al., 2015). PWV is higher in a stiffened artery which is suggestive of blood vessel dysfunction and increased clinical risk (Kim and Kim, 2019). The gold standard invasive test of

endothelial function is coronary artery angiography, which involves guiding a catheter into the coronary arteries and the infusion of vasodilative substances to assess endothelial relaxation (Deanfield et al., 2005). In healthy arteries, stimulation of NO causes vasodilation, but in diseased arteries, when endothelial cells are damaged, there is a lack of vasodilation and even an increase in vasoconstriction (Matsuzawa and Lerman, 2014).

The ability to examine endothelial function directly in humans is difficult and so *ex vivo* animal models are commonly used to assess the effect of pharmacological stimulants directly on the vasculature. In organ bath experiments, isolated blood vessels are simulated by vasoactive agents to assess the vasodilation or vasoconstriction response of arteries (Ding and Triggle, 2005). This was in fact the method used four decades ago when the vasoactive role of endothelial cells was discovered (Furchgott and Zawadzki, 1980). Studies within this thesis used both clinical and organ bath techniques to examine endothelial function and the vasoactive effect of a novel therapeutic target.

2.1.7 Therapies

Therapeutic treatments for endothelial dysfunction and atherosclerosis vary in their purpose and target. Common targets of pharmaceutical treatment are hyperlipidaemia, hypertension and thrombosis (Weber and Noels, 2011, Ademi et al., 2009). One of the most common classes of pharmaceutical therapeutics in the treatment of CVD are statins, which act as an anti-inflammatory, reduce lipid content and improve endothelial function (Park and Park, 2015). The LDL lowering effect of statins means they function in both the prevention and treatment of atherosclerosis (Davies et al., 2016). In conjunction with statins, niacin treatment works to increase high-density lipoprotein (HDL) cholesterol and reduce the risks of cardiovascular events (Duggal et al., 2010). Anti-hypertensive therapeutics include beta-blockers, ACE inhibitors, diuretics and

calcium channel blockers (Beckman et al., 2002). ACE inhibitors improve vasodilation by inhibiting ACE-induced production of Ang II and reducing oxidative stress (Park and Park, 2015, Forstermann and Li, 2011). ACE inhibitors also improve endothelial function by reducing the production of ROS and promoting bradykinin-induced production of NO and PGI_2 (Su, 2015). Anti-platelet aggregators, of which aspirin is the most common, reduce platelet adhesion and coagulation (Baigent et al., 2009, Beckman et al., 2002). However, given that aspirin functions to improve blood flow and reduce coagulation, there is an increased risk of haemorrhage in people who are receiving aspirin therapy (McNeil et al., 2018).

Non-pharmaceutical therapeutics such as physical activity, smoking cessation, diet control and weight control are important prevention and treatment strategies of atherosclerosis and CVD (Park and Park, 2015). Physical activity is an important strategy that can improve endothelial function and reduce cardiovascular risk independent of changes in other risk factors (Muniyappa et al., 2008). Further, increasing dietary antioxidant intake can prevent the development of oxidative stress and improve endothelial function (Su, 2015). Unfortunately, these strategies are often underutilised and a high reliance is placed on pharmaceutical interventions to treat CVD. Overall, the development of novel therapeutic strategies and approaches are constantly occurring and there is an important need for further alternatives to reduce the global burden of CVD (Incalza et al., 2018, Weber and Noels, 2011). **Chapters 4**, **5** and **6** (**Studies 2**, **3** and **4**) investigate the vasoactive role of a novel target to treat endothelial dysfunction and prevent the development of atherosclerotic CVD disease.

2.2 Osteocalcin

2.2.1 Bone as an endocrine organ

Traditionally, the skeleton has been recognised to have a role in the protection of vital organs, as a site of muscular attachment to enable movement and as a mineral reservoir (Brotto and Bonewald, 2015). However, increasing evidence suggests that bone is not a static tissue, but a biologically active organ. The skeleton is in a constant state of remodelling and the cellular machinery responsible is a set of cells called osteoclasts (bone resorption cells) and osteoblasts (bone formation cells), which require tight energy regulation (Ducy, 2011, Karsenty and Oury, 2010). Further, bone can intuitively adapt to changes in load, such as the reduction in bone mass that occurs with extended bed rest and in astronauts who spend extended time without gravity (Robling et al., 2006). Conversely, increases in bone mass and strength can occur if the skeleton is regularly loaded, for instance in people who regularly complete weight bearing exercise or in those who are obese (Robling et al., 2006). The fact that bone requires constant and adaptive metabolic regulation led some to theorise that it may act as an endocrine organ, actively involved in energy regulation systemically (Brotto and Johnson, 2014, Ansari and Sims, 2020, Kirk et al., 2020).

In 2007, the Karsenty group reported for the first time a link between bone and targeted tissue via the osteoblast-derived protein osteocalcin (OC). OC is the most abundant non-collagenous protein in the human body, making up approximately 15 – 25% of the bone matrix (Wolf, 1996, Price, 1989, Hauschka et al., 1989, Brown et al., 1984) and was first identified more than four decades ago in chicken and bovine bone (Hauschka et al., 1975, Price et al., 1976). OC was originally termed bone gamma-carboxyglutamic acid protein, which can be otherwise written as γ-carboxyglutamic acid-containing protein of bone or bone GLA protein (BGP or BGLAP) (Price et al., 1980,

Delmas et al., 1983). Early research on OC in animals and humans established that circulating concentrations of OC reflect bone formation (Brown et al., 1984, Price et al., 1980, Ducy et al., 1996).

2.2.2 Osteocalcin structure and production

In its mature form, human OC contains 49 residue amino acid proteins (**Figure 2.7**). It consists of three helical regions and has a low molecular weight of 5.7 kDa (Wolf, 1996, Gundberg et al., 2012). OC production is influenced by external factors including age, diurnal variations, bone diseases, lifestyle factors, medications and physical stimuli such as exercise (Hauschka et al., 1989, Lian and Gundberg, 1988). These factors activate the OC gene (BGLAP gene) in osteoblasts via vitamin D (vit D), hormones (leptin, glucocorticoids and insulin) and cytokines (TGF-β, bone morphogenetic protein and interleukin 1 (IL-1)) (Villafan-Bernal et al., 2011). Subsequently, OC messenger ribonucleic acid RNA (mRNA) is translated into the rough endoplasmic reticulum (ER) and undergoes proteolysis where the mature form of OC is created (**Figure 2.8**) (Villafan-Bernal et al., 2011). The residues 17, 21 and 24 are GLA residues that allow for post-translational γ-carboxylation by vitamin K-dependent γ-glutamyl carboxylase (GGCX) (**Figure 2.7**) (Zoch et al., 2016, Hauschka et al., 1989). Alterations in vitamin K (vit K) intake and availability influence the degree of OC carboxylation and subsequently the isoform of OC that is synthesised (Binkley et al., 2009, Bolton-Smith et al., 2007, Booth et al., 2008).

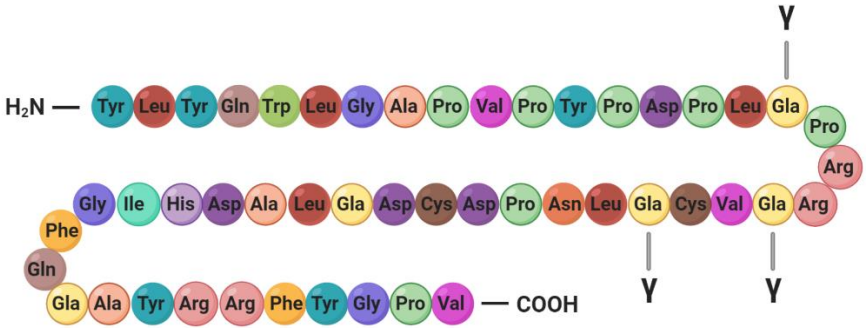

Figure 2.7 Amino acid sequence of OC. Adapted from Zoch et al. (2016). Created with BioRender.com

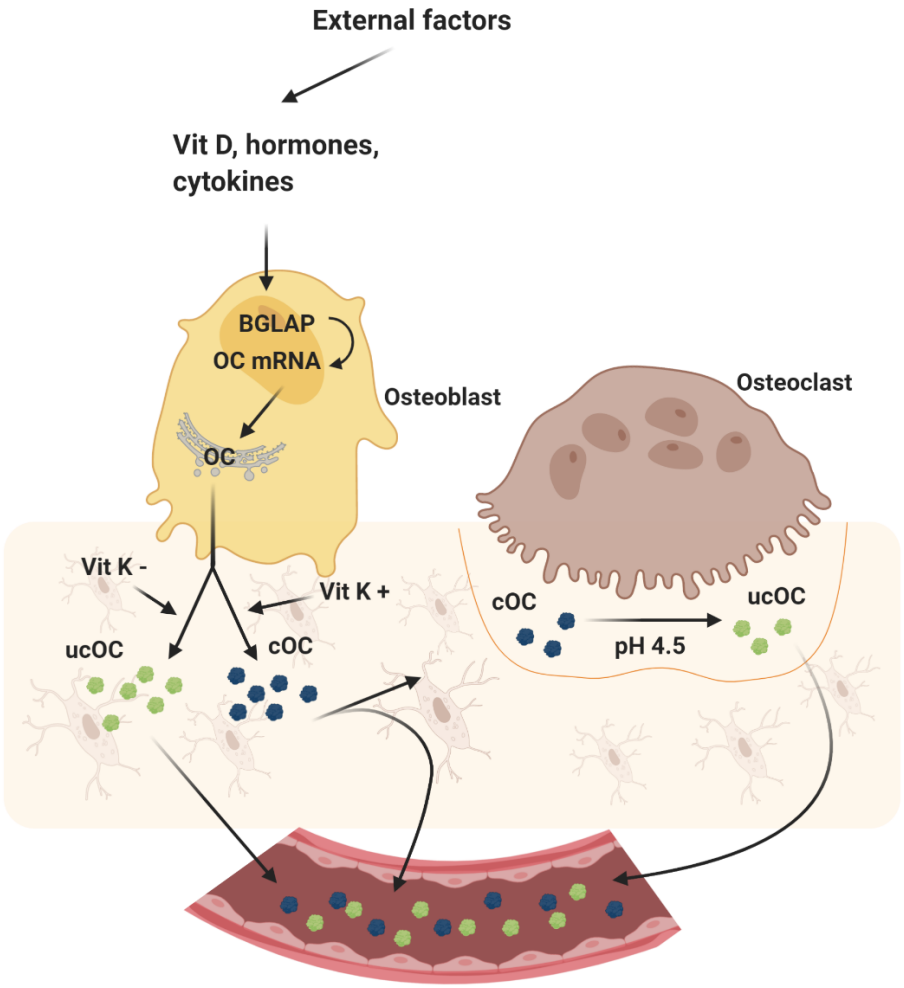

Figure 2.8 Physiological production of ucOC and cOC in bone. The BGLAP gene promotes the development of OC in osteoblasts, where the isoform of OC produced depends on the availability of vit K, lower levels of vit K promoting ucOC production. cOC is found predominantly in the bone matrix whereas ucOC moves into blood circulation. cOC is also converted to ucOC in the acidic environment produced by osteoclasts during bone resorption. Created with BioRender.com

Abbreviations - BGLAP: bone GLA protein, cOC: carboxylated osteocalcin, OC: osteocalcin, ucOC: undercarboxylated osteocalcin, Vit D: vitamin D, vit K: vitamin K.

Carboxylation of all three residues produces carboxylated OC (cOC), also known as GLA OC (Li et al., 2016). Once released into the extracellular space cOC binds to Ca^{2+} and has a high affinity for hydroxyapatite, and is thus predominantly located in the bone matrix (Zoch et al., 2016, Booth et al., 2013). The remainder of the cOC (about one-third) does not bind to hydroxyapatite and is released into blood circulation (Hauschka et al., 1989). If there is a shortage of GGCX, fewer than three glutamic acid residues undergo carboxylation, leading to undercarboxylated OC (ucOC), or Glu OC. Decarboxylation of cOC also produces ucOC, a process which occurs in the resorption lacuna, an acidic environment produced by osteoclasts during bone resorption (Li et al., 2016). In the bone matrix ucOC has a low affinity for hydroxyapatite and as a result is predominantly released into blood circulation (**Figure 2.8**) (Li et al., 2016, Hauschka et al., 1989). Interestingly, ucOC is heterogeneous and represents carboxylation at one or two carboxyl residues and even no carboxyl residues (uncarboxylated OC) (Belfiore and LeRoith, 2018, Rehder et al., 2015). Whether the different degrees of ucOC carboxylation may have divergent physiological functions, and whether the biological function of ucOC changes depending on the site of carboxylation are still unknown (Li et al., 2016).

2.2.3 Osteocalcin concentration in the circulation

The concentration of circulating OC in each of its forms varies greatly and the standard physiological concentrations are difficult to establish. Generally, circulating total OC (tOC), which is a combination of both ucOC and cOC, is reported at concentrations between 10 – 40 ng/ml in humans (Belfiore and LeRoith, 2018, Hiam et al., 2019, Zhou et al., 2009, Levinger et al., 2016, Xu et al., 2019, Yeap et al., 2015b). However, it has also been reported below 5 ng/ml and over 100 ng/ml in different

populations (Massera et al., 2018, Szulc et al., 1993, Seppa et al., 2019, van Summeren et al., 2007). The circulating levels of ucOC usually make up 40 – 60% of circulating tOC (Gundberg et al., 2012), often reported between 5 – 10 ng/ml and up to 30 ng/ml (Millar et al., 2019b, Tanaka et al., 2020, Yeap et al., 2015b). The large variability in OC concentrations is likely attributable to the numerous physiological processes that regulate OC synthesis, one of the most important of which is age. A recent study identified a U-shape distribution in all OC forms across the lifespan of adult men, which is thought to reflect the bone remodelling occurring at each stage of life (Smith et al., 2020). Several earlier studies have reported similar findings in women also (Sokoll and Sadowski, 1996, Gundberg et al., 2002, Hannemann et al., 2013, Khosla et al., 1998, Hu et al., 2013). Women generally have a large spike in OC levels during menopause, consistent with increased bone remodelling and higher osteoclast activity throughout that stage of life (Gundberg et al., 2002, Sokoll and Sadowski, 1996, Hu et al., 2013). Furthermore, the ratio of ucOC to tOC (ucOC/tOC) has been identified as a strong marker of vit K status and an important consideration when assessing risk of disease (Yeap et al., 2015a, Yeap et al., 2015b).

In combination with the physiological factors that influence circulating OC, inconsistent quantification methods confound the variability in the reporting of OC concentrations (Ducy, 2020). A number of different methods of measuring tOC and its forms in circulation exist, each with varying specificity and reliability (Ducy, 2020). Several new methods for measuring ucOC, each of which aims to be more accurate than previous techniques, have recently been developed (Lacombe et al., 2020, Arponen et al., 2020). In addition, the circulating concentrations of OC differ between species. For example, the circulating concentration of OC is 5 – 10 times higher in mice than in

humans (Al Rifai et al., 2020, Mera et al., 2016a). This is an important consideration when examining experimental models of OC biology and translating findings to human populations.

2.2.4 Osteocalcin functions outside of the skeleton

In little over a decade, a plethora of research has examined the potential biological functions of OC in organs outside of the skeleton. Over several years the Karsenty group published a number of ground-breaking studies that demonstrated novel biological functions of OC (Ferron et al., 2008, Mera et al., 2016b, Oury et al., 2011, Lee et al., 2007). The majority of the evidence suggests that ucOC is the bioactive form of OC (Karsenty and Mera, 2017, Lin et al., 2020b) and that it is implicated in the regulation of energy metabolism, male fertility, cognition, skeletal muscle and perhaps vascular function and calcification (**Figure 2.9**) (Lin et al., 2018a, Han et al., 2018, Moser and van der Eerden, 2018, Nakamura et al., 2020, Obri et al., 2018, De Toni et al., 2020, Oury, 2012, Bonewald, 2019, Colaianni et al., 2020). The following section discusses in detail the available evidence regarding the potential biological functions of OC in energy metabolism and glucose regulation.

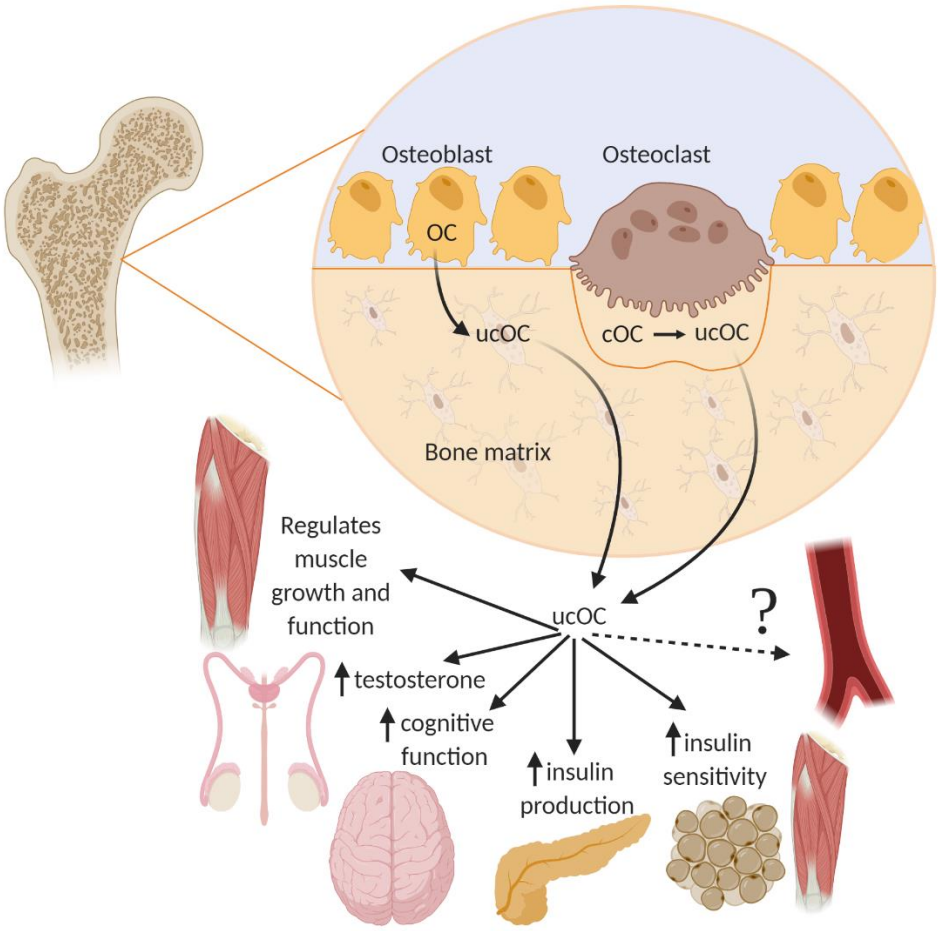

Figure 2.9 Proposed biological functions of ucOC outside of the skeleton. ucOC is released into the blood stream where it is suggested to regulate biological processes in a number of tissues such as skeletal muscle, testes, brain, pancreas and adipose tissue. Growing evidence also suggests it is involved in the vasculature but this requires further investigation. Created with BioRender.com.

Abbreviations - cOC: carboxylated osteocalcin, ucOC: undercarboxylated osteocalcin.

2.2.4.1 OC and energy metabolism

The initial evidence that linked OC with biological functions outside of the skeleton occurred by chance. Genetically modified OC deficient mice were originally developed with the hypothesis that OC was involved in bone extra-cellular matrix mineralisation (Karsenty, 2017). The OC -/- mice exhibited increased bone formation, cortical thickness and strength but there was no change in bone mineralization (Ducy et al., 1996). However, in the process of completing these experiments, it was observed that

the genetically modified animals had several endocrine abnormalities in comparison to their wild type counterparts. The mice exhibited increased blood glucose levels, reduced insulin sensitivity, increased fat pad weight and reduced adiponectin (Lee et al., 2007). This finding was further supported by a genetically modified knockout model of enterococcal surface protein (Esp), a gene expressed in osteoblasts, with this model exhibiting a phenotype opposite to that of the OC -/- mouse, because OC is overexpressed. The Esp -/- mouse was protected from metabolic abnormalities such as insulin resistance, obesity and glucose intolerance and importantly demonstrated increased OC activity (Lee et al., 2007). This seminal discovery was the first to demonstrate that OC acts as a hormone and has been the foundation of all the subsequent research.

Numerous studies have since corroborated these findings and have established that OC, in particular ucOC, is involved in the regulation of energy metabolism (Ferron et al., 2008, Ferron et al., 2012, Mizokami et al., 2014, Mizokami et al., 2013, Zhou et al., 2016, Zhou et al., 2013b, Lin et al., 2017, Lin et al., 2018b). For example, glucose metabolism, insulin sensitivity, adiposity and triglyceride levels were improved in mice administered ucOC (Mizokami et al., 2013, Mizokami et al., 2014, Ferron et al., 2008). Additionally, the *ex vivo* administration of ucOC directly increased muscle glucose uptake in mice, an effect that was muscle specific (Lin et al., 2017, Lin et al., 2018b). Furthermore, a positive feed-forward loop has also been proposed between the pancreas and the skeleton. Insulin receptor signalling in osteoblasts has been found to promote OC production, suggesting a bi-directional cross-talk between OC and energy metabolism (Ducy, 2011, Ferron et al., 2010, Fulzele et al., 2010).

However, recent studies have led some researchers to question the extent of the findings from the OC -/- mice used by the Karsenty group (Manolagas, 2020, Komori,

2020). Two new OC -/- mouse models exhibit normal blood glucose and insulin levels and normal body composition compared to control mice (Diegel et al., 2020, Moriishi et al., 2020). A potential reason for the differences in OC -/- mouse phenotypes may be due to the genetic background from which they were bred (Berezovska et al., 2019). Further, a genetically modified rat model has also exhibited a phenotype with no changes in body composition and improvements in glucose regulation (Lambert et al., 2016). These studies challenge previous findings and suggest that OC may not be as biologically active outside of the skeleton as originally proposed.

OCs role in energy metabolism in humans is difficult to establish as an inherent limitation of cross-sectional studies is that they are correlational in nature and do not describe cause and effect. In middle-aged and older men and women, it is commonly reported that higher levels of tOC and ucOC are associated with a reduced risk of diabetes, metabolic syndrome, insulin resistance and risk factors for metabolic diseases (Yeap et al., 2015b, Yeap et al., 2010, Kanazawa et al., 2009, Massera et al., 2018, Bezerra dos Santos Magalhaes et al., 2013, Kanazawa et al., 2011b, Kindblom et al., 2009, Iki et al., 2012, Urano et al., 2018). This was confirmed in a meta-analysis which reported that lower levels of tOC are present in those with T2DM and metabolic syndrome than in those with normal glucose control (Kunutsor et al., 2015). Several interventional studies have attempted to alter OC levels indirectly to determine an association with glucose homeostasis. The acute administration of prednisolone, a glucocorticoid, caused the suppression of ucOC as well as an increase in fasting glucose levels and insulin resistance (Parker et al., 2019, Tacey et al., 2019). Acute glucose loading caused a reduction in circulating tOC in pre- and post-menopausal women and reduced ucOC in the post-menopausal women (Levinger et al., 2016). Conflictingly, tOC and ucOC were not altered

by insulin infusions during a hyperinsulinemia-euglycaemic clamp in diabetic and non-diabetic individuals (Basu et al., 2011).

Overall, the cross-talk between OC and energy metabolism has been extensively reviewed and it is generally accepted that ucOC is involved in the regulation of energy metabolism (**Figure 2.9**) (Lin et al., 2018a, Bonnet, 2017, Karsenty, 2017, Wei and Karsenty, 2015, Confavreux, 2011, Confavreux et al., 2009, Han et al., 2018, Patti et al., 2013, Mera et al., 2018, Neve et al., 2013, Lee and Karsenty, 2008, Manolagas, 2020, Sherk et al., 2020, Razzaque, 2011, Komori, 2020, Dirckx et al., 2019, Brennan-Speranza and Conigrave, 2015, Kunutsor et al., 2015, Liu et al., 2015a, Liu et al., 2015b, Lin et al., 2020b, Desentis-Desentis et al., 2020, Ducy, 2020). Given this interaction, it is suggested that ucOC may be a target for future drug development to combat metabolic diseases, such as diabetes (Villafan-Bernal et al., 2011, De Toni et al., 2020). However, for the development of novel therapeutics it is crucial to determine that ucOC does not have debilitating off-target effects on other systems in the body. This is particularly important for the cardiovascular system given the close link between energy metabolism and vascular health and function. As such, this thesis investigates the bioactive role of ucOC on vascular function in **Chapters 4**, **5** and **6** (**Studies 2**, **3** and **4**).

2.2.5 GPRC6A: the putative OC receptor

G-protein-coupled receptors are a group of widely prevalent membrane receptors that regulate a large number of cellular responses (Rosenbaum et al., 2009). G protein-coupled receptor class C group 6 member A (GPRC6A) is suggested to be the putative receptor for OC in several tissues (Pi et al., 2016, Pi et al., 2017, Pi et al., 2011). Research has identified GPRC6A as the OC receptor in the testes (Oury et al., 2011), β-cells (Wei et al., 2014) and skeletal muscle (Mera et al., 2016a, Mera et al., 2016b) of mice.

Furthermore, research using a GPRC6A -/- mouse model suggested that the receptor may be involved in the regulation of energy metabolism (Pi et al., 2020b). In support of these findings, a genetically modified GPRC6A gain of function mouse model exhibited improved glucose tolerance, and OC administration promoted an increase in circulating insulin levels (Pi et al., 2020a). In humans, GPRC6A has been identified as the OC receptor in the testes (De Toni et al., 2016, Oury et al., 2013). However, several studies also report that ucOC does not activate this G-protein (Jacobsen et al., 2013, Rueda et al., 2016). Since G protein-coupled receptor 158 (GPR158) is the putative OC receptor in the brain (Khrimian et al., 2017a, Khrimian et al., 2017b, Kosmidis et al., 2018), OC likely have multiple receptors (Jorgensen and Brauner-Osborne, 2020).

There is increasing evidence regarding the role of GPRC6A as the receptor for OC in the vasculature. GPRC6A is present in the vasculature of rats (Harno et al., 2008) and recent evidence has identified the receptor in human aortic endothelial cells (HAEC) and human aortic smooth muscle cells (HASMC) (Millar et al., 2019a). Furthermore, immunohistochemistry detection has identified that GPRC6A is present in human and rabbit arteries (Qaradakhi et al., 2019) but whether OC interacts with this receptor in the vasculature is presently unknown. Although not the aim of the current thesis, it is of interest to further investigate whether there is an interaction between OC and GPRC6A in the vasculature in future research.

2.3 The potential role for OC in the development of endothelial dysfunction and atherosclerosis

Parts of the section below have been published in *Nutrients* as follows:

Tacey, **A**., Qaradakhi, T., Brennan-Speranza, T., Hayes, A., Zulli, A., & Levinger, I. (2018). Potential Role for Osteocalcin in the Development of Atherosclerosis and Blood Vessel Disease. *Nutrients*, *10*(10), 1426. (Scimago rank - Q1).

The published study is included in **Appendix A**.

2.3.1 Association between OC and atherosclerosis outcomes

Despite no specific evidence of an interaction with its putative receptor, investigation into the cross-talk between OC and metabolic outcomes led some researchers to investigate whether OC is also involved in CVD development. However, it remains unclear as to whether OC is biologically active in the vasculature (Levinger et al., 2017, Li et al., 2016). A number of studies have reported the association between tOC and clinically adverse CVD events, with conflicting findings (Holvik et al., 2014, Yamashita et al., 2013, Zhang et al., 2018, Barbarash et al., 2019, Fahrleitner-Pammer et al., 2008, Lerchbaum et al., 2014, Lerchbaum et al., 2013, Yeap et al., 2012). Additionally, the association of OC with markers of endothelial function and atherosclerosis have been extensively investigated and this will be the focus of the discussion below.

Over 30 studies have examined the association between circulating OC and outcomes related to endothelial function and atherosclerosis in humans (**Table 2.2**). Several different methods have been used to assess endothelial function and atherosclerosis, including aortic calcification score (ACS), BAFMD, coronary angiography, PWV and IMT. Overall, the results are conflicting. Twelve studies report that higher OC in circulation is associated with beneficial vascular outcomes including a reduced prevalence of diseased vessels, plaque formation and calcification. In addition, eight studies associate higher OC with adverse outcomes including increased levels of plaque and calcification, seven studies report mixed results (conflicting results within the same study) and eight studies report no association between OC and vascular outcomes (**Table 2.2**).

In the studies which reported that higher concentrations of tOC or ucOC were associated with adverse vascular function and atherosclerosis outcomes, the participants all had some form of chronic disease (i.e. hypertension, T2DM, chronic kidney disease (CKD) or osteoporosis) (Barbarash et al., 2016, Janda et al., 2013, Kanazawa et al., 2011a, Liu et al., 2019, Montalcini et al., 2004, Okura et al., 2010, Lin et al., 2020a, Lv et al., 2020). This suggests that the positive association reported by these studies may be a result of the presence of other disease states and not necessarily an association with vascular outcomes. However, there were also a number of studies which reported improved outcomes or no association in those with similar chronic diseases (Golovkin et al., 2016, Sheng et al., 2013, Zhang et al., 2015, Zhang et al., 2010, Iba et al., 2004).

Opposing results between men and women was the primary reason that mixed findings were reported within individual studies. Several studies identified an association of tOC with vascular function and atherosclerosis in men, but not in women (Choi et al., 2015, Kanazawa et al., 2009, Ogawa-Furuya et al., 2013). However, one study found the opposite, describing an association in women, but not in men (Reyes-Garcia et al., 2012). Further investigation into the sex differences between men and women are important and will be examined in **Chapter 5** (**Study 3**) of this thesis.

There is limited research on the association of OC with vascular function specifically. Three studies have examined the association between OC and PWV; one reporting that higher tOC is associated with lower PWV in men, but not women (Kanazawa et al., 2009). Similarly, when adjusted for confounding factors, higher tOC was associated with lower PWV in middle-aged and older men, but not women (Yun et al., 2016). Whereas, ucOC was not associated with PWV in older adults with CKD or aged-matched controls (Millar et al., 2020). One study reported the association between

OC and endothelial function, as measured by BAFMD in post-menopausal women, but found no association (Sumino et al., 2007). Finally, a recent study has reported the association between tOC and vascular reactivity index (VRI) which was measured by digital thermal monitoring in people who received kidney transplants (Lin et al., 2020a). Higher levels of tOC were associated with lower VRI, suggesting poorer endothelial function in those with the highest circulating levels of tOC. As described previously (**Section 2.1.3.2)**, endothelial dysfunction is an important stage in the development of atherosclerosis and CVD. Consequently, further studies are needed to investigate whether OC is associated with and has a direct effect on endothelial dysfunction. This is examined in **Chapters 4**, **5** and **6** (**Studies 2**, **3** and **4**) of this thesis.

The conflicting outcomes of the studies discussed above may be related to a number of limitations. First, tOC was reported in the majority of the studies, whereas each isoform of OC was minimally reported. This is a major limitation as each form of OC likely has distinct biological functions (Villafan-Bernal et al., 2011, Zoch et al., 2016). Furthermore, different techniques were used to analyse circulating OC:

- Enzyme-linked immunosorbent assay (ELISA)
- Electrochemiluminescence immunoassay (ECLIA)
- Immunoradiometric assay (IRMA)
- Radioimmunoassay (RIA)

Each technique can produce different results (Gundberg et al., 2012, Rossi et al., 2019). Finally, a number of different methods were used to assess vascular function and atherosclerosis outcomes. Given the different analysis methods, inconsistent reporting of OC and different functional measures, the association of OC with endothelial function

and atherosclerosis is unclear. The data suggests that OC may be a marker of vascular diseases but whether it has a regulatory function requires further investigation. Consequently, the remainder of this review focuses on experimental studies that report the effect or expression of OC.

Table 2.2 Association of OC with endothelial function and atherosclerosis outcomes in humans.

Reference	Participant characteristics (men/women)	Measurement of vascular function and OC	Results	Association of OC with endothelial function / atherosclerosis
(Awan et al., 2010)	**n:** 19 (unclear) **Age:** 49 **Health status:** LDLR gene mutation	**Vascular:** ACS **tOC:** ECLIA	Higher tOC associated with lower ACS	↓
(Bao et al., 2011)	**n:** 181 men **Age:** 65 **Health status:** Community dwelling	**Vascular:** Coronary angiography **tOC:** ECLIA	Higher tOC in those without CAD	↓
(Barbarash et al., 2016)	**n:** 112 men **Age:** 60 **Health status:** Angina	**Vascular:** CACS **tOC:** ELISA	Higher tOC in those with higher CACS	↑
(Choi et al., 2015)	**n:** 162 (114/48) **Age:** 49 – 62 **Health status:** Healthy	**Vascular:** CACS **tOC:** ECLIA **ucOC:** ELISA	Higher ucOC and %ucOC/tOC associated with increased calcification risk in men No association in women	↑,↔
(Confavreux et al., 2013)	**n:** 774 men **Age:** 65 **Health status:** Community dwelling	**Vascular:** AAC **tOC:** IRMA	Higher tOC associated with lower AAC progression rate	↓
(Golovkin et al., 2016)	**n:** 112 men **Age:** 60 **Health status:** Angina	**Vascular:** CACS **tOC:** ELISA	Higher tOC found in those with mild CACS compared to those with severe CACS	↓
(Gu et al., 2014)	**n:** 84 men **Age:** 59 **Health status:** NGT, IGT or T2DM	**Vascular:** C-IMT **tOC:** EIA	Higher tOC associated with lower C-IMT in those with IGT or T2DM, but not NGT	↓
(Iba et al., 2004)	**n:** 130 PM women **Age:** 72	**Vascular:** AAC	No differences in tOC in those with aortic calcification compared to those without	↔

Reference	Participant characteristics (men/women)	Measurement of vascular function and OC	Results	Association of OC with endothelial function / atherosclerosis
	Health status: Osteoporosis	**tOC:** EIA		
(Janda et al., 2013)	**n:** 67 (36/31) **Age:** 53 **Health status:** CKD	**Vascular:** C-IMT **tOC:** ELISA	Higher tOC associated with higher C-IMT	↑
(Kanazawa et al., 2009)	**n:** 328 (179/149 PM) **Age:** 65 – 67 **Health status:** T2DM	**Vascular:** PWV and C-IMT **tOC:** RIA	Higher tOC associated with lower PWV and lower C-IMT in men No association in women	↑,↓,↔
(Kanazawa et al., 2011a)	**n:** 50 (28/22) **Age:** 65 **Health status:** T2DM	**Vascular:** Plaque score **tOC:** RIA	Higher tOC associated with higher plaque score	↑
(Kang, 2016)	**n:** 98 (24/74) [53 PM] **Age:** 54 **Health status:** Community dwelling	**Vascular:** coronary angiography **tOC:** ECLIA	No difference in tOC in those with CAD compared to those without	↔
(Kim et al., 2012)	**n:** 769 women (577 PM) **Age:** 65 **Health status:** Community dwelling	**Vascular:** ACS **tOC:** ECLIA	Higher tOC associated with lower aortic calcification	↓
(Kim et al., 2016)	**n:** 122 men **Age:** 59 – 62 **Health status:** CABG surgery and matched controls	**Vascular:** CACS **ucOC:** ELISA **cOC:** ELISA	No association between ucOC or cOC with CACS	↔

Reference	Participant characteristics (men/women)	Measurement of vascular function and OC	Results	Association of OC with endothelial function / atherosclerosis
(Lin et al., 2020a)	**n:** 68 (34/34) **Age:** 47 **Health status:** Kidney transplant	**Vascular:** VRI **tOC:** ELISA	Higher tOC associated with lower VRI	↑
(Ling et al., 2018)	**n:** 227 PM women **Age:** 66 **Health status:** Community dwelling	**Vascular:** coronary angiography **tOC:** ECLIA	No difference in tOC in those with CAD compared to those without	↔
(Liu et al., 2019)	**n:** 1571 (780/791) **Age:** 64 – 69 **Health status:** Referred for angiography	**Vascular:** carotid and coronary angiography **tOC:** unclear	Higher tOC in those with calcified plaque compared to those without	↑
(Luo et al., 2015)	**n:** 476 (155/321) [120 PM] **Age:** 48 **Health status:** Healthy	**Vascular:** C-IMT **tOC:** ECLIA	No association between tOC and C-IMT	↔
(Lv et al., 2020)	**n:** 326 (166/160) **Age:** 59 **Health status:** T2DM with or without atherosclerotic plaque	**Vascular:** F-IMT **tOC:** ECLIA	Higher tOC in those with plaque compared to those without	↑
(Ma et al., 2014)	**n:** 1077 men **Age:** 61 **Health status:** Community dwelling	**Vascular:** C-IMT and presence of plaque **tOC:** ECLIA	No association between tOC and C-IMT in whole population In subgroup analysis of NGT participants, higher tOC was associated with lower risk of plaque	↔,↓
(Millar et al., 2020)	**n:** 48 (28/20) **Age:** 76 **Health status:** CKD and controls	**Vascular:** PWV and CS **tOC:** Multiplex assay	No association between tOC and PWV or CS in those with CKD or controls	↔

Reference	Participant characteristics (men/women)	Measurement of vascular function and OC	Results	Association of OC with endothelial function / atherosclerosis
(Montalcini et al., 2004)	**n:** 157 PM women **Age:** 56 **Health status:** Community dwelling	**Vascular:** C-IMT **tOC:** IRMA	Higher tOC associated with higher C-IMT in PM women with low BMD	↑
(Ogawa-Furuya et al., 2013)	**n:** 218 (118/100 PM) **Age:** 64 – 65 **Health status:** T2DM	**Vascular:** ACS **tOC:** IRMA **ucOC:** ECLIA	Higher tOC and ucOC in men with lower ACS No association between tOC or ucOC with ACS in women	↓,↔
(Okura et al., 2010)	**n:** 92 (53/39) **Age:** 61 **Health status:** Hypertension	**Vascular:** C-IMT **ucOC:** ELISA	Higher ucOC in those with higher C-IMT	↑
(Parker et al., 2010)	**n:** 363 PM women **Age:** 72 **Health status:** Community dwelling	**Vascular:** AAC **tOC:** IRMA	No association between tOC and AAC	↔
(Pennisi et al., 2004)	**n:** 66 (35/31 PM) **Age:** 63 **Health status:** Presence of atherosclerosis and controls	**Vascular:** C-IMT **tOC:** ELISA	Lower tOC in those with atherosclerosis than in controls	↓
(Prats-Puig et al., 2014)	**n:** 99 (50/49) **Age:** 8 **Health status:** Offspring of family with MS	**Vascular:** C-IMT **tOC:** ELISA **ucOC:** EIA	Higher ucOC and % ucOC/tOC associated with increased C-IMT No association of tOC with C-IMT	↑,↔
(Reyes-Garcia et al., 2012)	**n:** 78 (43/35) **Age:** 58 **Health status:** T2DM	**Vascular:** C-IMT and plaques **tOC:** RIA	Higher tOC associated with increased C-IMT and plaques in women No association between tOC or C-IMT in men	↑,↔

Reference	Participant characteristics (men/women)	Measurement of vascular function and OC	Results	Association of OC with endothelial function / atherosclerosis
(Sheng et al., 2013)	**n:** 817 (382/435 PM) **Age:** 61 – 62 **Health status:** T2DM	**Vascular:** C-IMT and presence of plaque **tOC:** RIA	Higher tOC associated with lower C-IMT and plaque	↓
(Sumino et al., 2007)	**n:** 85 PM women **Age:** 58 **Health status:** Community dwelling	**Vascular:** BAFMD **tOC:** IRMA	No association between tOC and BAFMD	↔
(Xu et al., 2018a)	**n:** 618 **Age:** 59 **Health status:** Community dwelling	**Vascular:** C-IMT **tOC:** ECLIA	Higher tOC associated with lower C-IMT	↓
(Yang et al., 2013)	**n:** 1319 PM women **Age:** 57 **Health status:** Community dwelling	**Vascular:** C-IMT **tOC:** ECLIA	Higher tOC associated with lower C-IMT	↓
(Yun et al., 2016)	**n:** 3604 (1691/1913) **Age:** 51 **Health status:** Community dwelling	**Vascular:** baPWV **tOC:** ECLIA	Higher tOC associated with lower PWV in men No association in women after adjustment for confounding variables	↑,↔
(Zhang et al., 2010)	**n:** 461 (299/162) **Age:** 62 – 63 **Health status:** Angina	**Vascular:** Coronary Angiography **tOC:** ECLIA	Higher tOC associated with a reduced number of diseased blood vessels	↓
(Zhang et al., 2015)	**n:** 240 (151/ 89 PM) **Age:** 62 **Health status:** CKD	**Vascular:** C-IMT and plaques **ucOC:** ELISA	Higher ucOC associated with less plaques and lower C-IMT	↓

Association of OC with endothelial function and atherosclerosis outcomes: ↑ Higher OC associated with adverse vascular function, ↓ Lower OC associated with adverse function, ↔ no correlation between OC and vascular function.

Abbreviations - AAC: abdominal aortic calcification, ACS: aortic calcium score, BAFMD: brachial artery flow mediated dilation, baPWV: brachial artery pulse wave velocity, CABG: coronary artery bypass graft, CACS: coronary artery calcium score, CAD: coronary artery disease, C-IMT: carotid intima-media thickness, CKD: chronic kidney disease, ECLIA: electrochemiluminescence immunoassay, EIA: enzyme immunoassay, ELISA: enzyme-linked immunosorbent assay, F-IMT: femoral intima-media thickness, IGT: impaired glucose tolerance, IRMA: immunoradiometric assay, LDLR: Low-density lipoprotein receptor, MS: metabolic syndrome, NGT: normal glucose tolerance, OC: osteocalcin, PM: post-menopausal, RIA: radioimmunoassay, tOC: total osteocalcin, T2DM: type 2 diabetes mellitus, ucOC: undercarboxylated osteocalcin, VRI: vascular reactivity index.

2.3.2 Osteocalcin and endothelial function

2.3.2.1 *In vivo* OC treatment and vascular function in animals

The direct biological effects of OC in the vasculature are not fully understood. A limited number of studies have examined the effect of tOC administration *in vivo* on the vascular system in animal models of disease. For instance, apolipoprotein E-deficient (ApoE -/-) mice that were treated with daily injections of tOC (30 ng/g) for 12 weeks were protected from high fat diet-induced elevations in diastolic and mean blood pressure (BP) by 7 mmHg and 5 mmHg, respectively, compared to the high fat diet alone group (Dou et al., 2014). PWV was increased (by 6%) in a rat model fed a high fat diet and induced with diabetes via streptozotocin (STZ) injection. Following 12 weeks of a high fat diet, daily intraperitoneal injections of tOC (30 ng/g) reversed the alteration in PWV, albeit by a small magnitude. BP, heart rate, and mean arterial pressure (MAP) were not altered by the high fat diet or the OC injections (Huang et al., 2017) (**Table 2.3**). However, these animals also exhibited reductions in body weight and fasting glucose levels and an improved glucose tolerance, circulating lipids and markers of inflammation following OC treatment (Dou et al., 2014, Huang et al., 2017). As such, whether the observed improvements in BP and PWV were due to OC acting directly on the vasculature or an indirect effect via the improvement in the animals' metabolic profile is unclear.

Table 2.3 Studies examining the effects of *in vivo* OC treatment on vascular function outcomes in animals.

Reference	Experimental Overview	Measurement of Vascular Function	Results
(Dou et al., 2014)	ApoE -/- mice received NCD or HFD and daily treatment of vehicle or tOC (30 ng/g) for 12 weeks	BP, heart rate, and isometric myography	*In vivo*: mean and diastolic BP normalized by tOC treatment in HFD group, no change in systolic BP or heart rate. *Ex vivo*: 20% improvement in relaxation in tOC-treated mice on HFD
(Huang et al., 2017)	Sprague Dawley rats induced with diabetes via STZ injection and received NCD or HFD, daily treatment of vehicle or tOC (30 ng/g) for 12 weeks	BP, PWV, heart rate, pulse pressure, and mean arterial pressure	PWV normalized in tOC-treated rats with diabetes compared to diabetic rats treated with vehicle, no change in BP, heart rate, mean arterial pressure, and pulse pressure
(Kondo et al., 2016)	Wild type C57BL/6 mice received HFD and treated 5 times a week for 10 weeks with vehicle or ucOC (30 ng/g)	NO production	Increased NO concentration in ucOC-treated mice compared to vehicle-treated mice
(Zhou et al., 2013a)	C57BL/6J mice received NCD or HFD for 8 weeks with daily injections of vehicle or ucOC (30 ng/g)	Autophagy and ER stress	Autophagy and ER stress attenuated in mice receiving ucOC

Abbreviations - ApoE: apolipoprotein E, BP: blood pressure, ER: endoplasmic reticulum, HFD: high fat diet, NCD: normal chow diet, NO: nitric oxide, OC: osteocalcin, PWV: pulse wave velocity, STZ: streptozotocin, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin.

2.3.2.2 *In vivo* OC treatment and markers of atherosclerosis risk in animals

Isometric myography is an *ex vivo* technique used to examine endothelial function directly, independent of factors such as sheer stress and circulating hormones. This technique was used to examine the function of the thoracic aorta in ApoE -/- mice following 12 weeks on a high fat diet, receiving simultaneous daily injections of tOC (30 ng/g) or vehicle (Dou et al., 2014). Vehicle-treated mice had a reduction in endothelial function by 20%, a pathological effect that was attenuated in the mice receiving OC treatment. An examination of the mechanisms revealed that co-incubation with N^G-nitro-$_L$-arginine methyl ester (L-NAME), an inhibitor of NOS, blocked the endothelium-dependent relaxation. However, co-incubation with sodium nitroprusside (SNP), an endothelium-independent NO donor, resulted in a similar relaxation between tOC-treated and non-treated tissue. The relaxation of all vessels to SNP demonstrates that the high fat diet or tOC does not affect the ability of vascular smooth muscle cells to respond to NO (Dou et al., 2014). Thus, tOC appears to have a protective effect on endothelial function that may assist in the prevention of atherosclerosis.

Whether one or both forms of OC were responsible for this effect is unclear. As a result, each form of OC was administered to female wild type C57BL/6 mice, to determine the effect on NO availability. Treatment with ucOC (30 ng/g) but not an equivalent dose of cOC increased serum NO, providing further evidence that ucOC is the bioactive form of the protein, at least in mice (Kondo et al., 2016). In addition, ucOC (30 ng/g) was administered via intraperitoneal injection five times per week for 10 weeks into mice fed an atherogenic diet. The diet did not induce the development of atherosclerotic plaques but it did increase total cholesterol, LDL and LDL/HDL ratio, all

of which are associated with an increased risk of atherosclerosis. Administration of ucOC significantly lowered all the lipid markers and produced a 1.7-fold increase in serum NO bioavailability compared to saline-treated mice (Kondo et al., 2016). The improvement in lipid markers and serum NO availability would likely assist in the prevention of atherosclerosis development.

Furthermore, eight weeks of daily ucOC (30 ng/g) treatment following a high fat diet produced an improvement in insulin signalling and a reduction in autophagy and ER stress in the aorta of C57 black 6J mice (Zhou et al., 2013a). Several markers of autophagy (autophagy related 7, ubiquitin binding protein p62, and light chain 3) and ER stress (protein kinase-like endoplasmic reticulum kinase and eukaryotic initiation factor 2α) were increased in the high fat diet-fed mice. However, the administration of ucOC following the high fat diet attenuated the pathologic autophagy and ER stress marker response. The improvement in insulin signalling in ucOC-treated mice was related to an increase in the phosphorylation of IRβ subunit and Akt Ser-473, demonstrating that ucOC rescues high fat diet-induced insulin resistance in mouse aorta (Zhou et al., 2013a).

In summary, *in vivo* ucOC treatment protects vascular function and pathological disease markers that often contribute to or are involved in the development of atherosclerosis (**Table 2.3**). However, the protective effects of ucOC on the vasculature are often associated with improved metabolic outcomes, such as improvements in insulin signalling or lipid markers. As such, *in vitro* studies are needed to confirm (1) whether OC and its forms are acting directly on vascular tissue, and (2) that ucOC is the active form of OC mediating these effects.

2.3.2.3 *In vitro* OC treatment in human cells

The endothelium has an important role in the maintenance of vascular homeostasis as it mediates the release of a number of regulatory factors (Rubanyi, 1993). Molecular signalling mechanisms that regulate endothelial function have been examined in several studies to determine if there is a direct link between OC and atherosclerosis (**Table 2.4**).

Human umbilical vein endothelial cells (HUVEC) cultured with tOC (10 – 150 ng/mL) displayed a dose-dependent upregulation of Akt and eNOS phosphorylation—up to 100 ng/mL of tOC (Dou et al., 2014). Akt is a common protein kinase involved in numerous cellular signalling pathways, including the phosphorylation of eNOS via ser1177 (Barbato and Tzeng, 2004). When treated with 100 ng/mL of tOC, Akt and eNOS phosphorylation increased, peaking at 1 h and 2 h following treatment, respectively (Dou et al., 2014). Of note, the properties of HUVECs are not ubiquitous to all endothelial cells and therefore the findings cannot be directly associated with adult endothelial function (O'Donnell et al., 2000). Despite this, similar results have been reported in HAECs. HAECs incubated with ucOC or cOC for 30 min resulted in an increase in eNOS phosphorylation in cells treated with 25 ng/ml and 100 ng/mL of ucOC by 1-fold and 2.5-fold respectively. However, equivalent doses of cOC had no effect, suggesting it does not influence NO production (Kondo et al., 2016). Similarly, eNOS phosphorylation and NO secretion were increased in a dose-dependent manner between 0.3 – 30 ng/mL of ucOC treatment in HAECs (Jung et al., 2013). Mechanistic investigation demonstrated that the phosphorylation of Akt/eNOS by ucOC was inhibited by the addition of wortmannin, an inhibitor of PI3K, which is the protein kinase responsible for phosphorylating Akt (Jung et al., 2013).

In a recent study, ucOC treatment (0.5, 1 and 10ng/ml) for 72 h increased HAEC proliferation, but this effect was blocked by inclusion of Akt and extracellular signalling regulated kinase (ERK) inhibitors, suggesting that ucOC may cause proliferation via Akt and ERK pathways. However, the phosphorylation of Akt, ERK and mammalian target of rapamycin (mTOR) were unaltered in HAECs treated with 10 ng/ml of ucOC for up to 30 min. Furthermore, ucOC treatment (10 ng/ml) did not affect angiogenesis, permeability or adhesion markers (Millar et al., 2019a). It was also reported that ucOC (10 ng/ml) treatment did not attenuate the increase in interleukin 6 (IL-6), interleukin 8 (IL-8), ICAM-1, VCAM-1 or MCP-1 following acute (24 h) inflammation in HAECs or HASMCs (Millar et al., 2019c). In HAECs and HASMCs exposed to inflammation for up to 144 h, ucOC treatment did not influence markers of cellular inflammation and dysfunction (Millar et al., 2019c).

Table 2.4 Cell culture studies examining the effects of *in vitro* osteocalcin treatment in human and animal vascular cells.

Reference	Experimental overview	Outcomes	Results
(Dou et al., 2014)	HUVECs incubated with tOC (10 – 150 ng/ml) for 15 min and up to 2 h Descending aorta of ApoE -/- mice, previously treated with tOC, incubated with LY294002 (10 µmol/L) and Akt inhibitor V (5 µmol/L)	eNOS, Akt and PI3K phosphorylation and expression	Max phosphorylation of eNOS & Akt with 100 ng/ml of tOC. Max phosphorylation of eNOS & Akt occurred after 1 h and 2 h, respectively In aorta, PI3K, Akt and eNOS phosphorylation and expression increased, inhibited with LY294002 and Akt inhibitor V
(Guo et al., 2017)	HUVECs incubated with ucOC (5 ng/ml for 4 h), tunicamycin (5 µg/ml for 4 h), insulin (10 nM for 10 min), wortmannin and Akti-1/2 (10 µM for 4 h)	Insulin resistance, ER stress	ucOC blocked ER stress and insulin resistance, which was inhibited by wortmannin and Akti-1/2
(Jung et al., 2013)	HAECs incubated with ucOC (0.3 – 30 ng/ml), linoleic acid (100 µmol/L for 16 h), wortmannin (100 nmol/L for 15 min)	NO concentration, eNOS and Akt phosphorylation and apoptosis	ucOC increased eNOS and Akt phosphorylation and NO concentration, which was inhibited by wortmannin. UcOC attenuated linoleic acid-induced apoptosis
(Kondo et al., 2016)	HAECs incubated with ucOC (5, 25 and 100 ng/ml) and cOC (25 and 100 ng/ml) for 30 min	eNOS phosphorylation	Incubation of ucOC increased eNOS phosphorylation in dose dependent manner, cOC had no effect
(Millar et al., 2019a)	HAECs and HASMCs incubated with ucOC (0.1 – 50 ng/ml) for up to 72 h	Vascular permeability, proliferation, angiogenesis, migration	ucOC increased cell proliferation, but had no effect on migration, permeability or angiogenesis

Reference	Experimental overview	Outcomes	Results
(Millar et al., 2019c)	HAECs and HASMCs induced with acute (24 h) or chronic (144 h) inflammation with or without treatment with ucOC (10 ng/ml)	Markers of cellular inflammation	ucOC does not influence markers of acute or chronic inflammation in HAECs or HASMCs
(Zhou et al., 2013a)	Mouse VECs and VSMCs incubated with tunicamycin (5 µg/ml for 4 h), ucOC (5 ng/ml for 0 h, 2 h, 4 h and 8 h), Akti-1/2 (10 µM for 4 h) and rapamycin (10 nM for 4 h)	Autophagy and ER stress	ucOC attenuates autophagy and ER stress in mouse VECs and VSMCs, which was inhibited by Akti-1/2 and rapamycin

Abbreviations - Akt: protein kinase B, ApoE: apolipoprotein E, cOC: carboxylated osteocalcin, eNOS: endothelial nitric oxide synthase, ER: endoplasmic reticulum, HAEC: human aortic endothelial cells, HASMC: human aortic smooth muscle cell, HUVEC: human umbilical vein endothelial cells, NO: nitric oxide, PI3K: phosphoinositide 3-kinase, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin, VEC: vascular endothelial cells, VSMC: vascular smooth muscle cells.

In addition to the loss of NO bioavailability, pathological mechanisms such as apoptosis and ER stress promote the development of endothelial dysfunction and atherosclerosis. Treatment with ucOC (30 ng/mL) prior to the administration of linoleic acid, which acts as a free fatty acid, inhibited the induction of apoptosis in HAECs via the PI3K/Akt pathway (Jung et al., 2013). Additionally, HUVECs exhibited ER stress and insulin resistance when incubated with tunicamycin; however, co-incubation with ucOC (5 ng/mL) for 4 h reduced the ER stress and increased the phosphorylation of insulin receptor substrate 1 (IRS-1), a molecule involved in insulin signal transduction (Guo et al., 2017). The co-incubation of wortmannin and Akti-1/2 (an Akt inhibitor) blocked the insulin sensitizing effect of ucOC. However, U0126 (an MAPK inhibitor) did not block the effect of ucOC. Additionally, NF-κβ, a key cellular signalling molecule, which was suppressed by tunicamycin, exhibited a normalization when co-incubated with ucOC. Furthermore, the inhibition of NF-κβ signalling and the silencing of the NF-κβ p65 gene confirmed that NF-κβ was involved in the regulation of ER stress and insulin signalling by ucOC (Guo et al., 2017). The results from this study suggest that ucOC suppresses ER stress via the PI3K/Akt/NF-κβ signalling pathway and that improved insulin sensitivity initiates this response.

Taken together, these results indicate that ucOC may elicit an atheroprotective effect in human endothelial cells. The protective effects of ucOC often occurred through improved insulin signalling or in the presence of high lipid content. However, ucOC also produced a protective effect in endothelial cells without the presence of any metabolic mediators, suggesting that OC may have a direct bioactive influence in human endothelial cells. Conversely, several recent studies have suggested that ucOC did not mediate functions in human vascular cells. Overall, ucOC may have a protective function in the

vasculature, independent from its influence on metabolic outcomes (**Figure 2.10**). However, further studies are required to confirm this. The direct protective role of ucOC in the vasculature is investigated in **Chapters 4**, **5** and **6** (**Studies 2**, **3** and **4**).

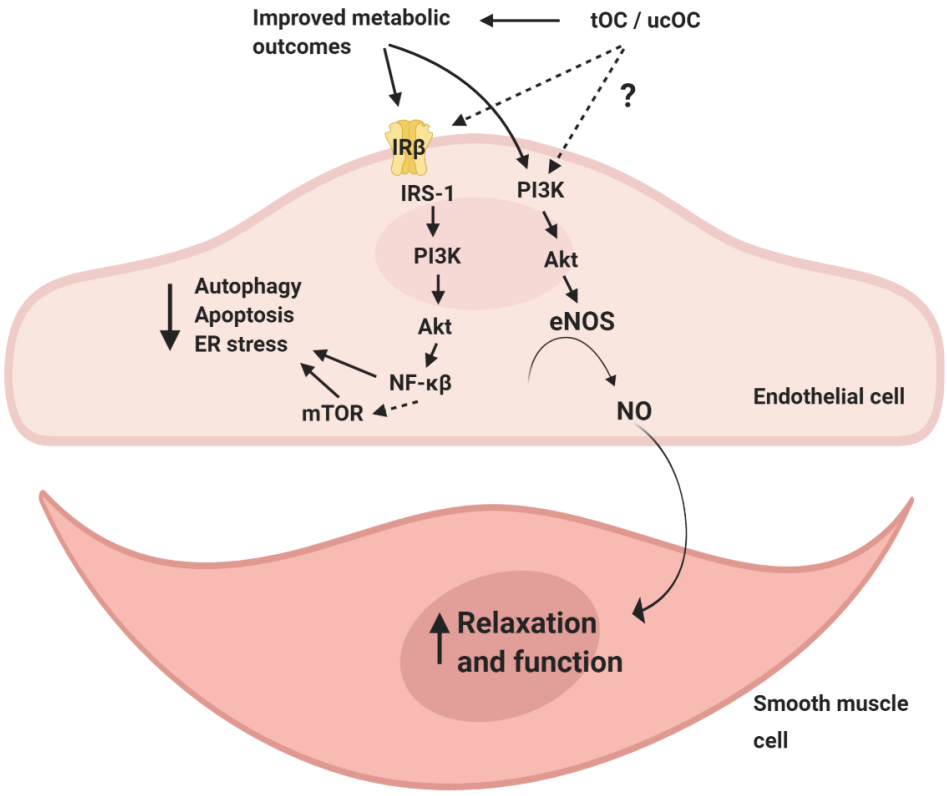

Figure 2.10 The proposed mechanism through which tOC and ucOC have been reported to elicit atheroprotective functions in vascular cells. By improving metabolic outcomes, tOC and ucOC reduce pathological mechanisms, including autophagy, apoptosis, and ER stress, through the β-subunit of the insulin receptor (IRβ) and via the IRS-1/PI3K/Akt/NF-κβ/mTOR signalling pathway. Vascular function is improved via the PI3K/Akt/eNOS signalling pathway which stimulates NO in the smooth muscle cells. Solid lines: known effect, dashed lines: proposed biological effect. Created with BioRender.com

Abbreviations - Akt: protein kinase B, eNOS: endothelial nitric oxide synthase, ER: endoplasmic reticulum, IRβ: insulin receptor β, IRS-1: insulin receptor substrate 1, mTOR: mammalian target of rapamycin, NO: nitric oxide, NF-κβ: nuclear factor kappa β, PI3K: phosphoinositide 3-kinase, ucOC: undercarboxylated osteocalcin

2.3.2.4 *In vitro* OC treatment and markers of atherosclerosis risk in animal cells

In addition to the use of human cells, animal studies have been completed to examine the effect of OC in vascular tissue and cells. Cultured aortic strips obtained from ApoE -/- mice revealed that tOC treatment increased the phosphorylation and expression of PI3K, Akt, and eNOS. Furthermore, the phosphorylation of Akt and eNOS was blocked by the co-incubation of LY294002 and Akt inhibitor V, which inhibit the signalling of PI3K and Akt respectively (Dou et al., 2014). Incubation of mouse vascular endothelial cells (VEC) and vascular smooth muscle cells (VSMC) in ucOC (5 ng/mL) were protected against tunicamycin-induced autophagy and ER stress. The protective effect was mediated through the Akt/mTOR signalling pathway as a result of NF-κβ activation (Zhou et al., 2013a).

The results in animal tissues support the work from human cells and suggest that ucOC protects against the development of atherosclerosis through the activation of several signalling pathways (**Table 2.4**). NO is likely increased via the activation of the PI3K/Akt/eNOS signalling pathway, which would result in an improvement in endothelial function. However, increased eNOS expression can also enhance endothelial dysfunction by increasing eNOS uncoupling and oxidative stress (Forstermann and Li, 2011, Zhao et al., 2015). Further studies are needed to determine if the upregulation of eNOS prevents or enhances endothelial dysfunction. To examine the molecular signalling pathways activated by ucOC, the expression of eNOS, Akt and mTOR are examined in **Chapter 4** (**Study 2**).

2.3.3 Osteocalcin and vascular calcification

Advanced atherosclerotic plaques are characterised by the development of vascular calcification, a pathological process which increases the risk of adverse cardiovascular events (Demer and Tintut, 2008, Giachelli, 2004). The development of vascular calcification promotes a phenotypical switch in VSMCs into osteoblast-like cells (Evrard et al., 2015). This process is regulated, at least to some degree, by runt-related transcription factor 2 (Runx2) and is characterised by a loss of SMC proteins and an increase in the expression of osteogenic proteins, including OC (Maddaloni et al., 2020, Byon et al., 2008, Johnson et al., 2006). Since the early 1980s, studies have detected OC in calcified plaques and aortic valves (Levy et al., 1983, Levy et al., 1980, Fleet and Hock, 1994, Rashdan et al., 2019). Yet, despite the presence of OC in calcifying vascular tissue, in cultured human VSMCs there is minimal evidence to support the role of OC as a regulator of calcification (Millar et al., 2020, Proudfoot et al., 2002, Severson et al., 1995). In contrast, numerous studies in animals have demonstrated a link between OC and vascular calcification (Akiyoshi et al., 2016, Morony et al., 2008, Idelevich et al., 2011, Rashdan et al., 2019). However, other animal studies have not demonstrated this link (Murshed et al., 2004). In OC -/- mouse VSMCs there was a reduction in calcium deposition, Runx2 and glucose uptake (Rashdan et al., 2019), whereas in mouse VSMCs OC overexpression and treatment increased glucose uptake via hypoxia-inducible factor 1α (HIF-1α) and increased vascular calcification (Idelevich et al., 2011). These results suggest that OC influences glycolytic pathways to regulate osteochondrogenic differentiation and to promote vascular calcification.

Overall, OC expression appears to be increased in calcified lesions throughout the intimal and medial layers of the vascular wall and may be involved in calcification via

glycolytic pathways, at least in animals (**Table 2.5**). Whether OC mediates calcification in humans or is increased as a result of the differentiation of VSMCs into an osteogenic phenotype requires further examination. It is important that future studies examine the role of each individual form of OC in the development of vascular calcification, in particular cOC, as cOC is the form of OC that is predominantly found within the mineralised matrix in bone. While not a major focus of this thesis, consideration of how OC is involved in the development of vascular calcification would assist in understanding the role of OC in vascular function and CVD development.

Table 2.5 Studies examining interaction between osteocalcin and calcification in human and animal tissue and cells.

Reference	Experimental overview	Outcomes	Results
(Akiyoshi et al., 2016)	Thoracic aorta of C57BL/6 mice cultured to induced calcification	OC expression	OC expression increased in calcified thoracic aortas
(Fleet and Hock, 1994)	Human aortic tissue	OC mRNA levels	OC mRNA increased in calcified aorta and plaque compared to non-calcified aorta
(Idelevich et al., 2011)	Cultured MOVAS cells-induced with calcification and overexpressed with OC. Sprague Dawley rats-induced with calcification	Mineralisation, OC mRNA, metabolic signalling pathways	*In vitro* - overexpression of OC in MOVAS cells associated with mineralisation and upregulation of insulin signalling *In vivo* - OC mRNA is increased in calcified vasculature and associated with activation of metabolic signaling pathways
(Levy et al., 1983)	Human aortic and valve tissue	OC and Gla levels	OC and Gla levels higher in calcified tissue than in non-calcified tissue
(Levy et al., 1980)	Human aortic and valve tissue	Gla levels	Higher Gla levels in calcified aorta and valves than non-calcified tissue
(Millar et al., 2020)	Cultured VSMCs in normal or mineralization inducing media with or without ucOC (10 ng/ml + 30 ng/ml)	ALP, Runx2, calcium, MMP-3, IL-1	ucOC co-treatment had no effect on markers of mineralisation and osteoblast differentiation in VSMCs
(Morony et al., 2008)	LDLR -/- mice fed HFD for 5 months and treated with OPG	Calcification, OC mRNA and circulating levels	OC mRNA levels were unchanged, circulating OC increased over the 5 months, which was associated with calcification
(Murshed et al., 2004)	MGP -/- mice inter-crossed with pSM22α-OC	Mineralisation of aorta	OC gain of function model did not inhibit the mineralisation of mouse aorta

Reference	Experimental overview	Outcomes	Results
(Pal et al., 2010)	OPG +/+ and OPG -/- mice	Calcification and Mononuclear cells expressing OC	Increased calcification in OPG -/- mice which was associated with an increased percentage of OC positive mononuclear cells
(Proudfoot et al., 2002)	Cultured human aortic VSMCs with lipid content modification	OC expression	OC expression increased in calcified cells compared to non-calcified cells, which was altered with the modification of lipid content
(Rashdan et al., 2019)	Human carotid artery plaques, OC -/- mice and murine VSMCs	Immunostaining for OC, OC expression and calcium deposition	OC present in calcified areas of human plaque and absent in non-calcified areas OC expression increased in calcified VSMCs and in VSMCs from OC -/- mice calcium was reduced
(Severson et al., 1995)	Cultured human aortic VSMCs	Immunostaining for OC	Minimal presence of OC in mineralised VSMCs
(Tyson et al., 2003)	Human aortic and carotid tissue	OC expression	Calcified vessels had an increase in expression of OC

Abbreviations - ALP: alkaline phosphatase, HFD: high fat diet, IL-1: interleukin 1, LDLR: low-density lipoprotein receptor, MGP: matrix glutamic acid protein, MMP-3: matrix metalloproteinase 3, MOVAS: mouse vascular smooth muscle cells, mRNA: messenger ribonucleic acid, OPG: osteoprotegerin, OC: osteocalcin, Runx2: runt-related transcription factor 2, VSMC: vascular smooth muscle cells

The current evidence examining the role of OC in the development of endothelial dysfunction and atherosclerosis is unclear. In humans, the association between OC and vascular outcomes is highly conflicting. *In vitro* and *in vivo* evidence suggests that OC, and in particular ucOC, may function to protect endothelial dysfunction. However, whether this occurs directly, rather than via improvements in metabolic outcomes requires further investigation. It is clinically important to understand the role of ucOC in the vasculature, as ucOC may be a therapeutic target for metabolic diseases in the future. As such, this thesis will investigate the direct biological effect of ucOC on endothelial function in animal models *ex vivo* and cell culture *in vitro* in both normoglycaemic and hyperglycaemic conditions. In addition, this thesis will include cross-sectional and intervention studies in humans to determine if the results obtained in the preclinical studies translate to humans. The research completed in this thesis provides a thorough examination into the influence of ucOC on endothelial function and investigated if it has a direct biological effect.

2.4 Aims and hypotheses

The overall aim of this thesis is to investigate if ucOC has a direct biological effect on vascular function in normoglycaemic and hyperglycaemic environments in preclinical models and humans. This was investigated in four studies. The specific hypothesis and aims of each study are listed below.

Study 1 (Chapter 3)

1. To determine whether acute *ex vivo* high glucose incubations would impair endothelial function in rabbit arteries in an artery specific manner and if this would be exacerbated by an atherogenic diet. This is based on the hypothesis that high

glucose incubations would cause dysfunction in each artery and the effect would be exacerbated by an atherogenic diet.

2. To investigate the molecular mechanisms of dysfunction caused by acute high glucose incubation.

Study 2 (Chapter 4)

1. To determine whether ucOC has a beneficial effect on endothelium-dependent and endothelium-independent vasodilation in rabbit aorta following incubations in normal and high glucose solutions. The hypothesis is that ucOC will improve the vasodilation of rabbit aorta *ex vivo*.
2. To investigate the molecular mechanisms in endothelial cells altered by the ucOC treatment.
3. To determine whether the treatment of human aortic endothelial cells with ucOC alters endothelial cell homeostasis following incubation in high glucose media.

Study 3 (Chapter 5)

1. To investigate the association of circulating ucOC levels with endothelial function, arterial stiffness and BP in older adults.
2. To examine the effect of ucOC on endothelial function of rabbits *ex vivo* in near physiological conditions. This is based on the hypothesis that ucOC will improve the vasodilation of rabbit aorta *ex vivo*.

Study 4 (Chapter 6)

1. To determine if a large reduction in ucOC is associated with alterations in cardiometabolic risk factors such as BP, arterial stiffness, blood glucose and lipid concentrations. The hypothesis is that the reduction in ucOC will cause an

improvement or have no effect on blood vessel function and the cardiometabolic risk factors examined in the study.

Chapter 3. The Effect of an Atherogenic Diet and Acute Hyperglycaemia on Endothelial Function in Rabbits is Artery Specific

This chapter was published as a study in *Nutrients* as follows:

Tacey, **A**., Qaradakhi, T., Smith, C., Pittappillil, C., Hayes, A., Zulli, A., Levinger, I. (2020). The Effect of an Atherogenic Diet and Acute Hyperglycaemia on Endothelial Function in Rabbits is Artery Specific. *Nutrients*, 12(7), 2108. (Scimago rank - Q1)

The published study is included in **Appendix B**.

Published version available from: https://doi.org/10.3390/nu12072108

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Declaration of co-authorship and co-contribution

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS

Title of Paper/Journal/Book:	The Effect of an Atherogenic Diet and Acute Hyperglycaemia on Endothelial Function in Rabbits is Artery Specific

Surname:	Tacey	First name:	Alexander
Institute:	Institute for Health and Sport	Candidate's Contribution (%):	60

Status:

Accepted and in press:	☐	Date:	
Published:	✔	Date:	16/7/2020

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 09:46:03+11'00'	16/12/2020
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Victoria University

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
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Alan Hayes	5	Editing of manuscript		16/12/20
Anthony Zulli	5	Editing of manuscript		16/12/20
Itamar Levinger	5	Editing of manuscript		16/12/20

3.1 Introduction

Type 2 diabetes mellitus (T2DM) is a major risk factor for cardiovascular complications including atherosclerosis and IHD (Beckman et al., 2002, Creager et al., 2003). While T2DM and atherosclerosis can occur independently, diabetes often accelerates atherosclerosis development, increasing the risk of adverse cardiovascular events such as myocardial infarction (Beckman et al., 2002). The devastating effect of T2DM on the vasculature is caused, in part, by hyperglycaemia, which is characterised by toxic levels of circulating blood glucose (Bornfeldt and Tabas, 2011, Saad et al., 2015).

Endothelial dysfunction is the first detectable sign of atherogenesis and is a significant predictor of future cardiovascular events (Davignon and Ganz, 2004, Gimbrone and Garcia-Cardena, 2016). The impairment of NO mediated endothelium-dependent vasodilation is a hallmark and one of the earliest indications of endothelial dysfunction (Gimbrone and Garcia-Cardena, 2016). Hyperglycaemia promotes endothelial dysfunction via a number of pathways, each of which are associated with a common link, the generation of reactive oxygen species (ROS), and oxidative/nitrative stress (Fiorentino et al., 2013). Specifically, hyperglycaemia-induced mitochondrial electron transport chain overproduction of superoxide binds with NO to produce peroxynitrite, reducing the bioavailability of NO and promoting endothelial dysfunction (Brownlee, 2001, Son, 2012).

Acute elevations in circulating blood glucose, such as that which occurs in the post-prandial state, are a major risk factor for diabetes-induced endothelial dysfunction (Ceriello and Genovese, 2016, Yamagishi et al., 2007), perhaps more so than fasting blood glucose and haemoglobin A1c (HbA1c) (Bonora et al., 2001). A number of studies have reported that acute (2 – 6 h) *ex vivo* high glucose incubations can reduce endothelial-

dependent vasodilation in arteries of rabbits (Tesfamariam et al., 1991, Tesfamariam et al., 1990, Tesfamariam and Cohen, 1992) and rats (Qian et al., 2010, Qian et al., 2006, Salheen et al., 2015, Taylor and Poston, 1994). However, no previous studies have completed high glucose incubations following a diet that mimics an atherosclerotic milieu, which is important to understand the effects of acute hyperglycaemia in a disease state. Furthermore, a study from our laboratory has shown that different vascular beds (thoracic aorta, renal, carotid, and iliac arteries) respond differently to hormonal stimulus, indicating that vascular beds are not homogeneous in their responses (Habiyakare et al., 2014).

As such, the aim of this study was to determine if acute *ex vivo* high glucose incubations would impair endothelial function in aorta, iliac, and mesenteric arteries and whether the impairment would be exacerbated by an atherogenic diet. We hypothesised that high glucose incubations would reduce endothelium-dependent relaxation and that the impairment in endothelial function would be aggravated following an atherogenic diet.

3.2 Methods

3.2.1 Ethical approval

This study was approved by the Victoria University Animal Ethics Committee (#14/005) and complied with the Australian National Health and Medical Research Council code for the care and use of animals for scientific purposes (8th edition).

3.2.2 Animal model

Male New Zealand White Rabbits (n = 6 – 12) at three months of age were randomly allocated into two groups and fed a normal chow diet (Specialty Feeds, Glen Forrest, WA, Australia) or an atherogenic diet (a normal diet combined with 1%

methionine, 0.5% cholesterol, and 5% peanut oil; SF00-218, Specialty Feeds, Glen Forrest, WA, Australia) for four weeks (Zulli and Hare, 2009). The animals were housed in separate cages on a 12 h light/dark cycle at a constant temperature of 21ºC. Food and water were supplied *ad libidum*.

3.2.3 Isometric tension myography

Following the four week diet, the rabbits were sedated with medetomidine (0.25 mL/kg), anaesthetised with 4% isoflurane, and exsanguinated via severing the inferior vena cava. The arterial system was immediately flushed with ice cold Krebs (118 mM NaCl; 4.7 mM KCl; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 25 mM $NaHCO_3$; 1.25 mM $CaCl_2$ and 11.7 mM glucose). The abdominal aorta (2 – 3 cm below the diaphragm), external iliac artery (immediately after the aortic bifurcation), and main mesenteric artery were excised, cleaned of connective tissue and fat, and cut into 3 mm rings. Blood vessel reactivity was measured via an isometric tension organ bath system (Zultek Engineering, Melbourne, Australia), as previously described (El-Hawli et al., 2017, Smith et al., 2019). Briefly, each vessel was incubated in physiological Krebs solution warmed to 37ºC and bubbled with 95% O_2 and 5% CO_2. Following 30 min acclimatisation, the rings were strung up between two metal hooks attached to a force transducer to measure the tension of the vessel. Each vessel was passively stretched to a tension comparative to its size: the abdominal aorta to 2 g, the iliac artery to 1 g, and the mesenteric artery to 0.5 g. After 30 min, the vessels were again stretched to their respective tension for a further 30 min. Subsequently, the vessels were incubated in Krebs (11 mM glucose) or high glucose Krebs (20 mM or 40 mM glucose). Vasodilation of blood vessels in 11 mM glucose has previously been shown to cause relaxation equivalent to incubation in 5 mM glucose (Tesfamariam et al., 1990). The respective Krebs solutions

were refreshed every 30 min and incubated for a total of 2 h. Following the incubation, blood vessels were pre-contracted with 3×10^{-7} M phenylephrine (aorta and iliac artery) or 3×10^{-7} M cirazoline (mesenteric artery). Once the contraction reached a plateau, endothelium-dependent vasodilation was determined via a cumulative dose response curve to acetylcholine (ACh) in half-log increments (10^{-8} M to 10^{-5} M). Maximal relaxation (E_{max}) was determined as the maximal dilation below the phenylephrine/cirazoline plateau. The log dose of ACh that produced half the maximal relaxation was reported as EC_{50}. The area under the curve (AUC) was determined as the total area of relaxation below the phenylephrine/cirazoline plateau. Endothelial dysfunction was considered when there was an alteration to one or a combination of E_{max}, EC_{50}, and AUC that represented a reduction in the vasodilation of the blood vessels. All chemicals and reagents were supplied by Sigma Aldrich, St. Louis, MO, USA unless otherwise specified.

3.2.4 Immunohistochemistry

The blood vessel rings were placed into 4% paraformaldehyde, left overnight, and then transferred into 1× phosphate buffered saline at 4ºC. This was followed by paraffin processing (Microm STP120, Thermo Scientific, Waldorff, Germany) and embedding in paraffin blocks. Sections were cut at 5 µm, deparaffinised in xylene, rehydrated, and blocked with 1% goat serum in 10mM Tris HCl (pH 7.4) for 20 min. Primary mouse monoclonal anti-bodies Anti-3-Nitrotyrosine [39B6] (Abcam 61392) and eNOS type III (BD Biosciences 610296) at 1:100 dilution were applied overnight. A no primary antibody control was completed to detect non-specific protein binding. Samples were subsequently incubated with anti-mouse IgG for 1 h (Immpress HRP reagent kit, MP-7452 Vector laboratories). Diaminobenzidine (DAB) (BD Biosciences 550880) was

applied as a chromogen before counterstaining with hematoxylin, dehydration, and mounting in Dibutylphthalate Polystyrene Xylene (Arora et al., 2012).

3.2.5 Immunohistochemistry quantification

Images of each vessel were taken at 40× magnification (Leica DFC 450F, Leica Microsystems, Wetzlar, Germany). The endothelium was traced and the degree of staining (brown from DAB) was quantified using the MCID programme (MCID 7.0, Interfocus, Linton, UK). Researchers were blinded to the samples for quantification, using methods previously established (Qaradakhi et al., 2017, Zulli et al., 2008, Zulli et al., 2006a, Zulli et al., 2006b, Zulli et al., 2014, Zulli et al., 2009). The proportional intensity (arbitrary unit) of staining was calculated as a ratio of colour intensity to proportional area, normalised to the no primary antibody control. Finally, the immunoreactivity of each protein was calculated based on a fold change from the respective control vessel (the control ring from the normal diet or atherogenic diet groups).

3.2.6 Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). Unpaired Student's t-test was used for comparison between the diets. A one-way analysis of variance (ANOVA) was used to analyse the comparison between glucose incubations and Post-hoc analysis was completed using Fisher's least significance difference (LSD) test to identify the differences between groups. Data was analysed in Graphpad prism (version 7.1, Graphpad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant, trends were reported when $p = 0.05 – 0.099$, and > 0.099 was considered not significant (n/s). Effect sizes are commonly used to study the clinical relevance of an intervention and show the magnitude of the effect that it is producing (Maylor et al., 2019, Rodevand et al., 2019, Silva et al., 2019). The Cohen's d (d) equation was used to examine

the magnitude of the effect of the high glucose incubations on blood vessel relaxation and immunohistochemistry results. A large effect is considered when d is > 0.8, a medium effect between 0.5 – 0.79, and a small effect between 0.2 – 0.49 (Cohen, 2013).

3.3 Results

3.3.1 Endothelial function following the normal diet vs atherogenic diet

The atherogenic diet significantly reduced the relaxation of the abdominal aorta as measured by AUC (25%, $p < 0.05$) and EC_{50} ($p < 0.05$) compared to the normal diet (**Figure 3.1 A + B**). In the iliac artery, the atherogenic diet reduced EC_{50} ($p < 0.05$) and there was a strong trend for a reduction in AUC (17%, $p = 0.06$) compared to the normal diet (**Figure 3.1 C + D**). Similarly, in the mesenteric artery, the atherogenic diet shifted EC_{50} to the right ($p < 0.05$) and there was a strong trend for a reduction in AUC (40%, $p = 0.07$) (**Figure 3.1 E + F**).

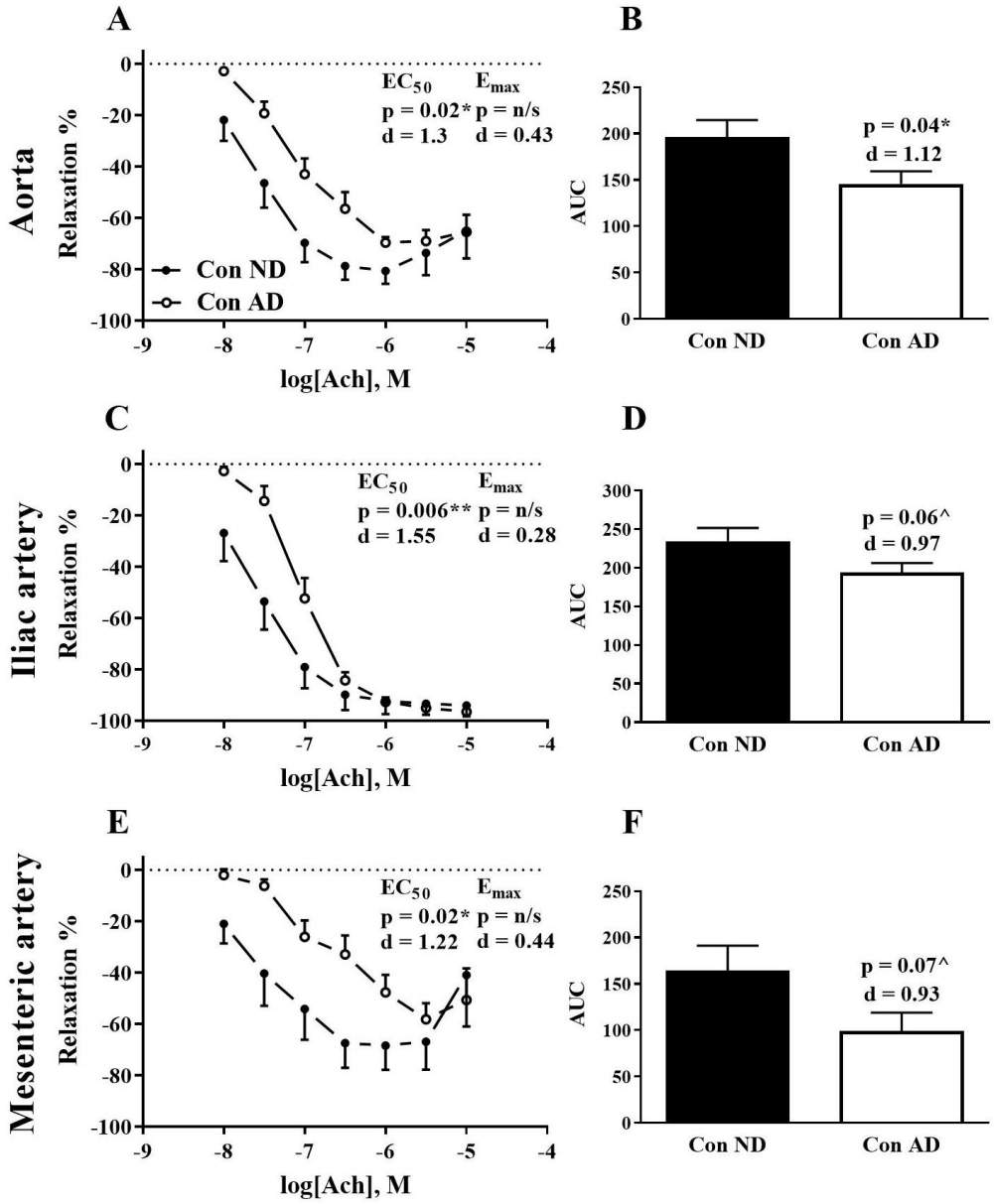

Figure 3.1 ACh-induced dose response curves in (A) abdominal aorta, (C) iliac artery, and (E) mesenteric artery incubated *ex vivo* for 2 h. Comparison between normal diet (closed circles) and atherogenic diet (open circles). Inset: Log EC_{50} and E_{max} statistical significance (p) and effect size (d) between diets. AUC in (B) abdominal aorta, (D) iliac artery, and (F) mesenteric artery presented as arbitrary values; numbers above columns represent the statistical significance (p) and effect size (d) between diets. n = 7 – 12 per group. All data mean ± SEM. * $p < 0.05$ ND vs AD, ** $p < 0.01$ ND vs AD, ^ p 0.05 – 0.099 ND vs AD.

Abbreviations - ACh: acetylcholine, AD: atherogenic diet, AUC: area under the curve, Con: normal Krebs, d: Cohen's d, ND: normal diet.

3.3.2 Endothelial function following the high glucose vs normal glucose incubation

Incubation of the aorta in 20 mM glucose from rabbits fed a normal diet produced a strong trend towards a reduction in AUC (18%, $p = 0.08$) and E_{max} was reduced by 10%, but this was not significant ($p > 0.1$) (**Figure 3.2 A + C**, **Table 3.1**). Incubation of the aorta in 20 mM glucose from the atherogenic diet-fed rabbits caused a shift to the right of the dose response curve reducing EC_{50} ($p < 0.05$) (**Figure 3.2 B** and **Table 3.1**). No dysfunction was caused in the iliac artery following the normal diet, irrespective of glucose incubation (**Figure 3.2 D + F**). Whereas, relaxation of the iliac artery from the atherogenic diet-fed animals altered EC_{50} in the 40 mM ($p < 0.05$) incubated group (**Figure 3.2 E** and **Table 3.1**). Endothelial dependent relaxation of the mesenteric artery was not negatively affected by the high glucose incubations following either the normal or atherogenic diet (**Figure 3.2 G - I** and **Table 3.1**).

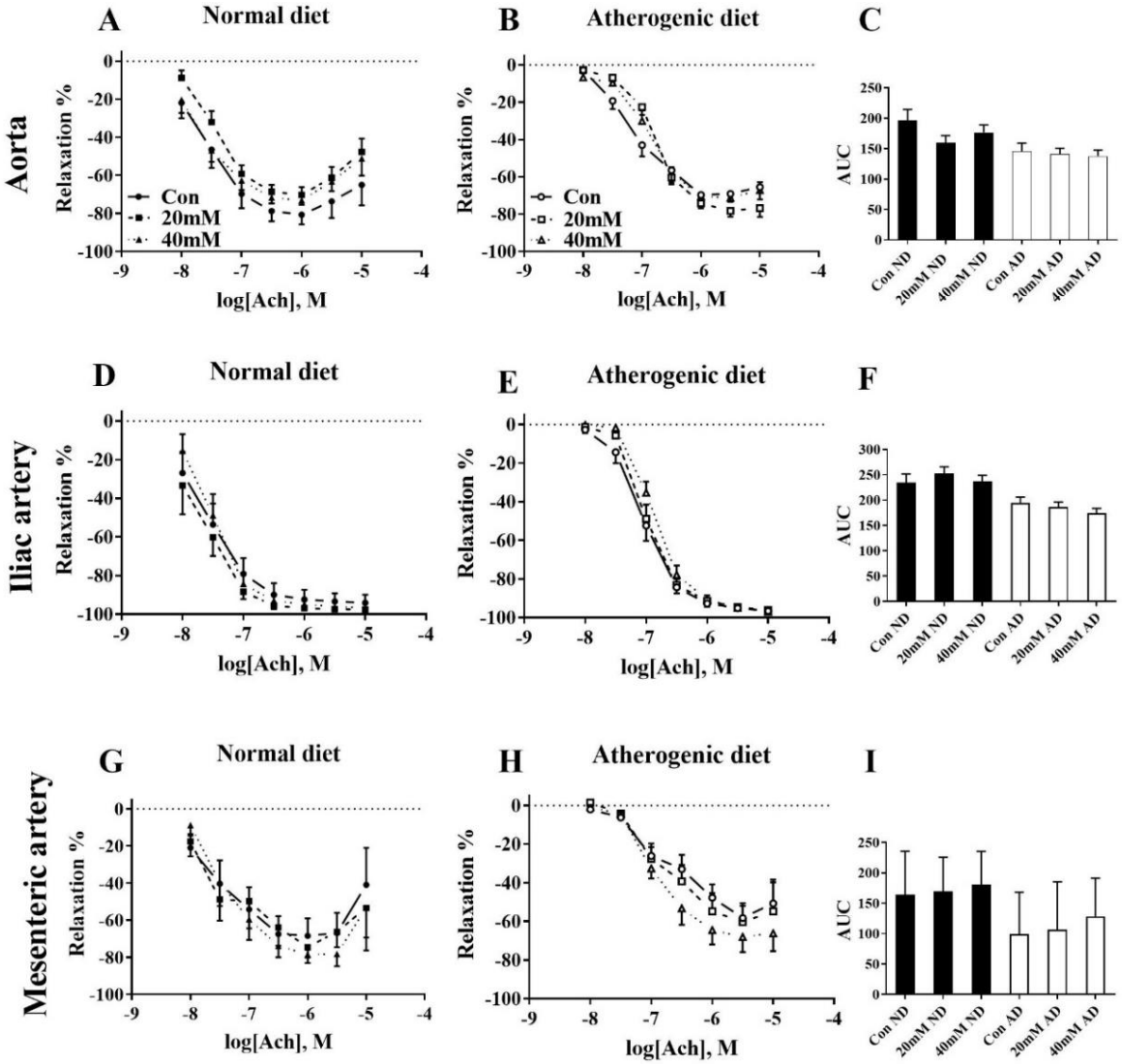

Figure 3.2 ACh-induced endothelium-dependent dose response curves in (A, B) abdominal aorta, (D, E) iliac artery, and (G, H) mesenteric artery incubated *ex vivo* for 2 h in normal, 20 mM glucose or 40 mM glucose solution. Comparison between Con (circles + line), 20 mM (squares + dashes), and 40 mM (triangles + dots). AUC (C, F, I) presented as arbitrary values. n = 6 – 12 per group. All data mean ± SEM.

Abbreviations - 20mM: 20 mM glucose Krebs, 40 mM: 40mM glucose Krebs, ACh: acetylcholine, AD: atherogenic diet, AUC: area under the curve, Con: normal Krebs, ND: normal diet.

Table 3.1 Log EC_{50}, E_{max} and AUC for ND and AD fed rabbits incubated *ex vivo* for 2 h in control, 20 mM or 40 mM glucose solutions.

Abdominal aorta	**n**	**Log EC_{50} ± SEM**	**p vs con**	**d vs Con**	**E_{max} ± SEM**	**p vs con**	**d vs Con**	**AUC ± SEM**	**p vs con**	**d vs con**
ND Con	7	-7.59 ± 0.12			-81 ±13			196 ± 18		
ND 20 mM	7	-7.43 ± 0.09	n/s	0.54	-70 ± 11	n/s	0.32	160 ± 11	**0.08^**	0.92
ND 40 mM	7	-7.65 ± 0.1	n/s	0.18	-73 ± 2	n/s	0.29	177 ± 12	n/s	0.48
AD Con	10	-7.10 ± 0.13			-70 ± 2			146 ± 13		
AD 20 mM	11	-6.81 ± 0.06	**0.03***	0.88	-78 ± 3	**0.04***	0.97	141 ± 9	n/s	0.13
AD 40 mM	11	-6.93 ± 0.07	n/s	0.52	-71 ± 3	n/s	0.18	138 ± 10	n/s	0.21
Iliac artery		**Log EC_{50} ± SEM**	**p vs con**	**d vs Con**	**E_{max} ± SEM**	**p vs con**	**d vs Con**	**AUC ± SEM**	**p vs con**	**d vs con**
ND Con	7	-7.52 ± 0.12			-94 ± 4			235 ± 17		
ND 20 mM	7	-7.64 ± 0.11	n/s	0.41	-98 ± 1	n/s	0.43	252 ± 14	n/s	0.42
ND 40 mM	6	-7.55 ± 0.13	n/s	0.1	-97 ± 2	n/s	0.31	237 ± 12	n/s	0.05
AD Con	10	-7.07 ± 0.08			-97 ± 1			194 ± 12		
AD 20 mM	11	-7.00 ± 0.06	n/s	0.32	-96 ± 1	n/s	0.11	186 ± 10	n/s	0.23
AD 40 mM	10	-6.85 ± 0.06	**0.03***	0.98	-97 ± 1	n/s	0.09	175 ± 9	n/s	0.59
Mesenteric artery		**Log EC_{50} ± SEM**	**p vs con**	**d vs Con**	**E_{max} ± SEM**	**p vs con**	**d vs Con**	**AUC ± SEM**	**p vs con**	**d vs con**
ND Con	7	-7.44 ± 0.19			-68 ± 9			164 ± 27		
ND 20 mM	6	-7.46 ± 0.23	n/s	0.04	-75 ± 6	n/s	0.3	170 ± 23	n/s	0.08
ND 40 mM	6	-7.35 ± 0.18	n/s	0.2	-79 ± 5	n/s	0.52	181 ± 22	n/s	0.26
AD Con	12	-6.74 ± 0.18			-58 ± 6			99 ± 20		
AD 20 mM	11	-6.50 ± 0.18	n/s	0.4	-60 ± 10	n/s	0.08	107 ± 24	n/s	0.11
AD 40 mM	11	-6.81 ± 0.12	n/s	0.12	-68 ± 8	n/s	0.41	128 ± 19	n/s	0.45

Statistical significance (p) and effect size (d) in comparison to the control group for each diet. * $p < 0.05$ vs control, ^ p 0.05 – 0.99 vs control.

Abbreviations - 2 h: 2 hour incubation, 20 mM: 20 mM glucose Krebs, 40 mM: 40 mM glucose Krebs, AD: atherogenic diet, AUC: area under the curve, Con: normal Krebs, d: Cohen's d, ND: normal diet.

3.3.3 Immunoreactivity of NT and eNOS

Representative images of IHC stained vessels are presented in **Figure 3.3**. The incubation of blood vessels in 20 mM and 40 mM glucose for 2 h did not significantly affect the immunoreactivity of eNOS and NT in any blood vessel. In the aorta NT was increased in the 40 mM glucose normal diet group by 0.9-fold compared to the control, which had a trend towards significance ($p = 0.9$) and a large effect ($d = 0.99$) (**Figure 3.4 A**). A medium to large effect (d) was present in a number of groups, but this was not associated with statistical significance (**Figure 3.4 A – F**).

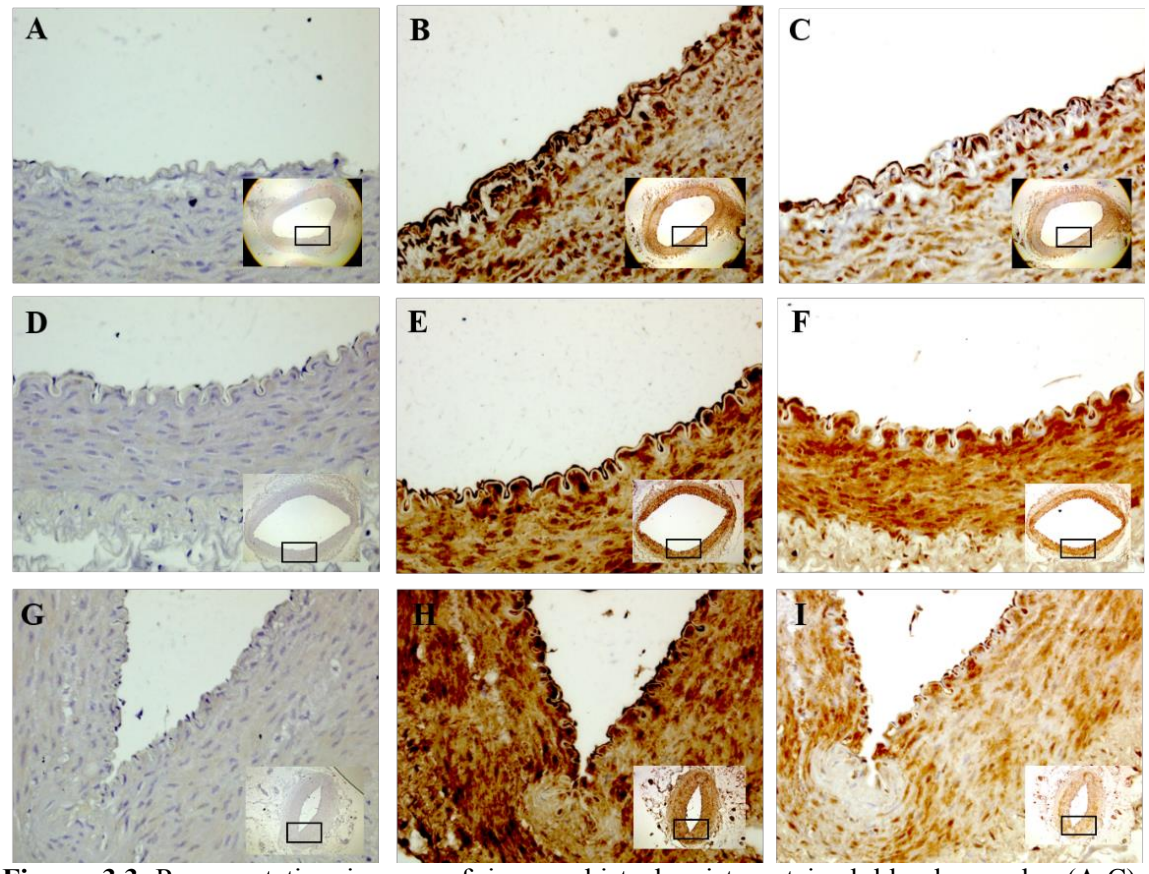

Figure 3.3 Representative images of immunohistochemistry stained blood vessels; (A-C) abdominal aorta, (D-F) iliac artery and (G-I) mesenteric artery from normal diet-fed rabbits. (A, D, G) no primary antibody control, (B, E, H) NT and (C, F, I) eNOS taken at 40× magnification. Inset - image of whole vessel taken at 4× magnification (abdominal aorta) or 10× magnification (iliac and mesentery).

Abbreviations - eNOS: endothelial nitric oxide synthase, NT: nitrotyrosine.

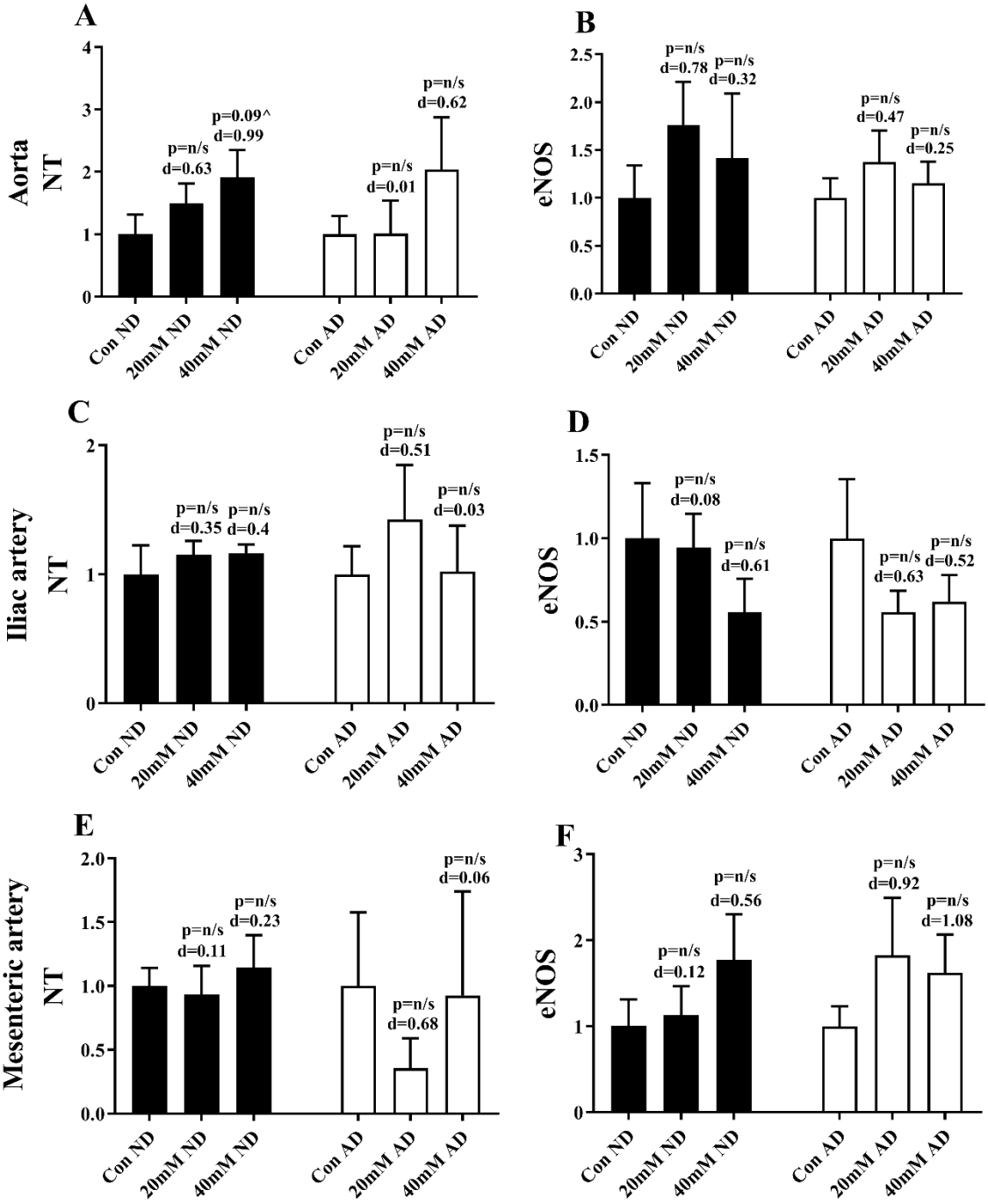

Figure 3.4 Immunoreactivity of NT and eNOS in (A, B) abdominal aorta, (C, D) iliac artery, and (E, F) mesenteric artery. Immunoreactivity is calculated based on the intensity of the staining present on the endothelium, which is an arbitrary unit and expressed as fold change from the respective control. Numbers above columns represent the statistical significance (*p*) and effect size (*d*) in comparison to the control group for each diet. ^ *p* 0.05 – 0.99 vs control. *Abbreviations - 20mM: 20 mM glucose Krebs, 40mM: 40 mM glucose Krebs, AD: atherogenic diet, Con: normal Krebs, d: Cohen's d, eNOS: endothelial nitric oxide synthase, ND: normal diet, NT: nitrotyrosine.*

3.4 Discussion

We report for the first time that high glucose-induced endothelial dysfunction is blood vessel specific. The abdominal aorta is the most susceptible to high glucose-induced dysfunction, with the iliac artery affected to a lesser degree, and the mesenteric artery exhibited no signs of dysfunction. High fat diets are commonly used to study the development of endothelial dysfunction and atherosclerosis in animals. The four week atherogenic diet used in this study has previously been shown to exhibit endothelial dysfunction in abdominal aorta of rabbits (Zulli and Hare, 2009). We confirm the findings of atherogenic diet-induced endothelial dysfunction in the aorta and demonstrate endothelial dysfunction in the peripheral iliac and mesenteric arteries. Altogether, this suggests that the atherogenic diet functions systemically to cause dysfunction.

Hyperglycaemia is a major clinical risk factor for the development of endothelial dysfunction and atherosclerosis. This is the first study to examine the effect of high glucose incubations on endothelial function of blood vessels in various locations. We demonstrate that the abdominal aorta is the artery that is most prone to developing endothelial dysfunction following both the normal and atherogenic diet. This confirms findings from several previous studies, which reported endothelial dysfunction in rat and rabbit aorta following acute high glucose incubations (Guo et al., 2000, Qian et al., 2010, Tesfamariam et al., 1991). The iliac artery exhibited minor high glucose-induced dysfunction following the atherogenic diet, but not following the normal diet. As such, the iliac artery appears to be more susceptible to developing high glucose-induced dysfunction in a disease state and not in a healthy environment. Alternatively, the mesenteric artery did not develop any signs of endothelial dysfunction. Susceptibility to atherosclerosis can depend on haemodynamic factors such as shear stress and oscillating

flow, which can vary between vascular sites depending on the location of arterial branches or bifurcations (VanderLaan et al., 2004). The exposure of the endothelium to low shear stress is one of the most important factors in atherosclerosis development and is an important consideration when examining endothelial dysfunction *in vivo* (Traub and Berk, 1998). Furthermore, whilst endothelial dysfunction a systemic condition it can present locally in some blood vessels more than others as certain blood vessels can resist the development of dysfunction (Motwani and Topol, 1998). For example, vascular beds such as the internal mammary artery and other conduit arteries have increased NO production, decreased vasoconstriction, and have higher shear stress than other vessels (Motwani and Topol, 1998, Ozkor et al., 2011). Overall, there is variance in the effect of the high glucose incubations on endothelial function in different blood vessels, which may be explained, at least in part, by variations in the structure, physiological effects, and disease susceptibility of each vessel.

In this study, the development of endothelial dysfunction to high glucose incubation was not dose-dependent. The 20 mM glucose incubation caused the largest reduction in endothelium dependent vasodilation in the aorta from both the normal diet-fed and atherogenic diet-fed rabbits. This finding is in contrast with a previous study, which reported that incubation of rabbit aorta in 44 mM glucose aggravated dysfunction compared to the 20 mM incubation (Tesfamariam et al., 1991). Similarly, the relaxation of the third order branches of the mesenteric artery from female Wistar rats following incubations in 20 mM and 45 mM glucose solution for 2 h elicited a dose-dependent reduction in endothelial-dependent vasodilation (Taylor and Poston, 1994). The conflicting results in this study possibly occurred as a result of species or methodological differences. Taken together, this study demonstrates endothelial dysfunction in the aorta

following 2 h high glucose incubations in rabbits fed normal and atherogenic diets. The dysfunction caused by the 2 h 20 mM glucose incubation provides a model for studying high glucose-induced blood vessel dysfunction that mimics an acute post-prandial response.

In a normal physiological environment, eNOS synthesises NO, which has a number of anti-atherogenic functions including vasodilation (Jamwal and Sharma, 2018). An acute state of hyperglycaemia can reduce eNOS expression and subsequently NO bioavailability, resulting in endothelial dysfunction (Sena et al., 2013). Hyperglycaemia also promotes electron transport chain overproduction of superoxide anion and via signalling pathways, produces peroxynitrite, a potent ROS (Ceriello, 2005). Mechanistically, NT is used as a marker of peroxynitrite production, indicating the presence of nitrative stress (Ceriello, 2005). Although not significant, the increase in NT observed in the aorta following high glucose incubations suggests the presence of nitrative stress in the current study, an effect that has previously been reported in rabbit aorta in a disease state (Rai et al., 2009). Several recent studies, in both human and animal models, have identified that increased fasting glucose levels as a result of a high fat diet cause reductions in eNOS and plasma nitrate (Alarcon et al., 2018, Parry et al., 2019). Overall, the evidence suggests that an increase in oxidative/nitrative stress and a reduction in eNOS are characteristic of hyperglycaemia-induced dysfunction. In this study, we did not find any significant alterations in NT or eNOS, but moderate to large changes in the effect size suggests that future research should examine this in more detail.

A potential limitation of the current study is that superoxide anion or other ROS forms were not directly measured to determine the exact mechanistic effect of the high glucose incubations. Furthermore, NT alone may not provide the most accurate

representation of hyperglycaemia-induced oxidative stress as it may be influenced by other factors including the atherogenic diet (Choi et al., 2008). We examined total eNOS expression in combination with NT as an indirect measure of superoxide overproduction and peroxynitrite-induced oxidative stress.

In conclusion, the effect of acute high glucose incubations on blood vessel function is blood vessel specific and in some cases, is aggravated by an atherogenic diet. The abdominal aorta may be the optimal artery to study potential therapeutic treatments of hyperglycaemia-induced endothelial dysfunction and atherosclerosis in rabbit models.

Chapter 4. Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells

In **Chapter 3** (**Study 1**) we reported that the development of endothelial dysfunction in rabbit arteries is blood vessel specific. The abdominal aorta was the artery most prone to endothelial dysfunction following incubation in acute hyperglycaemic conditions and this was enhanced by an atherogenic diet. As such, in **Chapter 4** (**Study 2**) we utilised the same model of dysfunction to determine whether the administration of ucOC *ex vivo* would improve or worsen endothelial function. This chapter also examines whether ucOC treatment alters markers of endothelial function in human endothelial cells incubated in high glucose media.

This chapter was published as a study in *The Journal of Cellular Physiology* as follows:

Tacey, **A**., Millar, S., Qaradakhi, T., Smith, C., Hayes, A., Anderson, S., Zulli, A., O'Sullivan, S., Levinger, I. (2020). Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells. *J. Cell. Physiol*. 236(4), 2840-2849. (Scimago rank - Q1)

The published study is included in **Appendix C**.

Declaration of co-authorship and co-contribution

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS

Title of Paper/Journal/Book:	Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells

Surname:	Tacey	First name:	Alexander
Institute:	Institute for Health and Sport	Candidate's Contribution (%):	50

Status:

Accepted and in press:	☐	Date:	
Published:	☑	Date:	31/08/2020

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 09:46:03+11'00'	16/12/2020
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Victoria University

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
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Tawar Qaradakhi	10	Data collection and editing of manuscript		15/12/20
Cassandra Smith	5	Editing of manuscript		15/12/20
Alan Hayes	5	Editing of manuscript		15/12/20
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Anthony Zulli	5	Editing of manuscript		15/12/20
Saoirse O'Sullivan	5	Editing of manuscript		15/12/20
Itamar Levinger	5	Editing of manuscript		15/12/20

Chapter 5. Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo*

In **Chapter 4** (**Study 2**) we report that ucOC does not directly influence endothelial function in the abdominal aorta of rabbits or in human endothelial cells incubated in high glucose conditions. In **Chapter 5** (**Study 3**) we further examine the effect of ucOC treatment on endothelial function using perfusion myography, a technique that closely mimics a physiological environment, while allowing the examination of isolated vascular tissue. In this chapter we also examine the association between ucOC and vascular function in older adults, to provide a translational aspect of the thesis in humans.

This chapter has been accepted for publication in *PLoS One* as follows:

Tacey, **A**., Smith, C., Woessner, M. N., Chubb, P., Neil, C., Duque, G., Hayes, A., Zulli, A., Levinger, I. (2020). Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery ex vivo. *PLoS One*, 15(11). (Scimago rank - Q1).

The published study is included in **Appendix D**.

VICTORIA UNIVERSITY

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Declaration of co-authorship and co-contribution

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS

Title of Paper/Journal/Book:	Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery ex vivo

Surname:	Tacey	First name:	Alexander
Institute:	Institute for Health and Sport	Candidate's Contribution (%):	55

Status:

Accepted and in press:	☐	Date:	
Published:	✔	Date:	25/11/2020

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Signature	Date
Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 09:46:03+11'00'	16/12/2020

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

6. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
7. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

8. There are no other authors of the publication according to these criteria;
9. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
10. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Victoria University

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
Cassandra Smith	10	Data collection and editing of manuscript	[redacted]	16/12/20
Mary Woessner	5	Data analysis and editing of manuscript		16/12/20
Paul Chubb	5	Data analysis and editing of manuscript		16/12/20
Christopher Neil	5	Editing of manuscript		16/12/20
Gustavo Duque	5	Editing of manuscript		16/12/20
Alan Hayes	5	Editing of manuscript		16/12/20
Anthony Zulli	5	Editing of manuscript		16/12/20
Itamar Levinger	5	Editing of manuscript		16/12/20

5.1 Introduction

The bone-derived hormone osteocalcin (OC) is a vitamin K-dependent protein that exists in several biological forms. The post-translational γ-carboxylation of less than three glutamic acid residues produces undercarboxylated osteocalcin (ucOC), which has a low affinity for hydroxyapatite and is predominantly found in circulating blood (Li et al., 2016). In recent years ucOC has been suggested as a mediator of a cross-talk between bone and metabolic outcomes (Lin et al., 2018a). In humans, higher levels of ucOC are associated with a reduced risk of metabolic syndrome and T2DM (Yeap et al., 2015b, Riquelme-Gallego et al., 2020, Urano et al., 2018). Similarly, ucOC has been reported to improve glucose regulation, adiposity and insulin sensitivity in animal models (Lin et al., 2017, Lee et al., 2007, Ferron et al., 2012). However, not all studies are in agreement (Diegel et al., 2020, Moriishi et al., 2020). Given these findings, it is of interest to investigate whether ucOC is involved in other biological functions within the body (Levinger et al., 2017, Rossi et al., 2019). As metabolic and CVD share common pathological links (Rask-Madsen and King, 2013), it is of particular interest to examine the interaction of ucOC with endothelial function and atherosclerosis progression. This is important, not only in the context of CVD, but also because ucOC could be targeted as a future therapy for metabolic diseases.

The association between OC and its isoforms with CVD in humans remains unknown (Millar et al., 2017, Tacey et al., 2018). A number of cross-sectional studies have reported that higher circulating total OC (tOC) is associated with improved vascular health and function (Confavreux et al., 2013, Gu et al., 2014, Yang et al., 2013). Yet, others have reported that higher tOC has adverse (Reyes-Garcia et al., 2012, Kanazawa et al., 2011a, Okura et al., 2010), or even no association (Ling et al., 2018), with vascular

health (**Table 2.2**). However, only a limited number of studies have investigated the role of ucOC in the vasculature. As ucOC is suggested to be the active circulating form of OC, it is particularly important to investigate whether ucOC is associated with vascular function, and if so, whether these effects are beneficial or detrimental.

In animal models, administration of tOC and ucOC *in vivo* improve blood vessel function. For example, daily tOC (30 ng/gram) injections for 12 weeks significantly improved pulse wave velocity (PWV), a measure of arterial stiffness, in rats induced with diabetes mellitus (Huang et al., 2017). Daily tOC also enhanced vasodilation *ex vivo* in the aorta of apolipoprotein E -/- mice (Dou et al., 2014). In another study, 30 ng/gram of ucOC administered for 10 weeks in female C57BL/6 mice produced an increase in nitric oxide availability, a key vasoactive molecule (Kondo et al., 2016). While these *in vivo* studies indicate potential links between OC administration and improvements in vascular health, they also reported concurrent improvements in metabolic outcomes, such as improved glycaemic control and lower adiposity. Therefore, it is unclear whether the improvement in blood vessel function resulted from a direct effect of OC, or an indirect effect from improved metabolic outcomes.

The aims of this study were to a) investigate the association of circulating ucOC levels with endothelial function, arterial stiffness and BP in older adults via a cross-sectional analysis, and b) examine the direct effect of ucOC on endothelial function in rabbit arteries.

5.2 Methods

5.2.1 Human participants

Twenty six healthy, community dwelling older women (mean age of 73 years) and 12 older men (mean age of 74 years) participated in this study. Inclusion criteria

included adults over 60 years old and women > 12 months post-menopause. Exclusion criteria included a current diagnosis of diabetes, a BMI over 40 kg/m^2, a fracture within the last three months or participation in resistance exercise > 2 days per week. Participants were on a range of medications to control for hypertension, cholesterol and CVD, however all were stable and controlled for at least three months as per their medical records. Each participant received written and verbal explanations about the nature of the study before signing an informed consent document. This study was approved by Melbourne Health and Victoria University Human Research Ethics Committees. The data were collected as part of a larger clinical trial (ACTRN12618001756213).

5.2.2 BP and vascular function

Participants were asked to complete an overnight fast and to abstain from their regular medication until after the testing had been completed. They were also asked to refrain from intense exercise for 24 hours prior to the testing. Brachial artery systolic BP, diastolic BP and mean arterial pressure measurements were recorded using the non-invasive SphygomoCor-XCEL® (AtCor Medical, Sydney, NSW, Australia) diagnostic system. Two measurements were captured, with the lower of the two readings recorded. If the two BP readings were > 6 mmHg apart, a third measure was recorded to ensure a true resting value and the average of the two lowest BP measurements were recorded. Participants were split into groups based on hypertension guidelines; normal (< 130 mmHg and < 80 mmHg) n = 7, stage 1 hypertension (130 – 139 mmHG and 80 – 89 mmHg) n = 14 or stage 2 hypertension (> 140 mmHG and > 90mmHG) n = 17 (Carey et al., 2018). None of the participants with normal BP were taking antihypertensive medication, five of the participants with stage 1 hypertension and 10 of the participants with stage 2 hypertension were taking antihypertensive medication. Arterial stiffness was

measured by PWV using the subtraction method, with the thigh cuff placed on the thigh and a tonometer used to measure the carotid artery waveform (SphygomoCor-XCEL®) (Butlin and Qasem, 2017).

Endothelial function was assessed via brachial artery flow mediated dilation (BAFMD) using a high-resolution ultrasound (Terason, LifeHealthcare, New South Wales, Australia) with R wave trigger. Brachial artery diameter was assessed for approximately 10 s at baseline (in duplicate and averaged) and during forearm occlusion. Brachial artery diameter was continuously captured after the occlusion cuff release for about 2 min (reactive hyperaemia). Peak change was calculated as the peak percentage change in brachial artery diameter from baseline to immediately following peak hyperaemia (Harris et al., 2010).

5.2.3 Circulating osteocalcin

Serum samples were taken in the morning following an overnight fast and stored at -80°C until analysis. Serum tOC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics) (Smith et al., 2020). Serum ucOC was measured using the hydroxyapatite binding method, a commonly used, well established method (Gundberg et al., 1998). Each sample was measured once and the inter-assay coefficients of variation were 5.4% and 9.2% for tOC and ucOC, respectively.

5.2.4 Animals

Male New Zealand White Rabbits at 12 weeks of age were randomised into either a normal chow diet (n = 7) (Guinea pig and rabbit pellets, Specialty Feeds, Australia) or an atherogenic diet (n = 10) (a normal diet combined with 1% methionine, 0.5% cholesterol and 5% peanut oil (#SF00-218, Specialty Feeds, Australia)) for four weeks (Zulli and Hare, 2009). This atherogenic diet has previously been reported to cause

endothelial dysfunction in rabbits (Zulli and Hare, 2009, Tacey et al., 2020b). The rabbits were housed in individual cages on a 12 h light/dark cycle at 21°C, with access to water and their assigned chow diet *ad libitum.* At the completion of the four week diet, the rabbits were sedated (0.25 mg/kg medetomidine) and anaesthetised (4% isoflurane) before exsanguination via severing of the inferior vena cava. The arterial system was immediately flushed with ice cold Krebs solution (118 mM NaCl; 4.7 mM KCl; 1.2 mM $MgSO_4.7H_2O$; 1.2 mM KH_2PO_4; 25 mM $NaHCO_3$; 1.25 mM CaCl and 11.7 mM glucose) and the carotid arteries were carefully dissected and placed in Krebs solution. The animal experiments were approved by the Victoria University Animal Ethics Committee (#14/005) and complied with the Australian National Health and Medical Research Council code for the care and use of animals for scientific purposes (8^{th} edition).

5.2.5 Perfusion myography

The carotid arteries were cleaned of connective tissue and fat, with care taken to avoid damaging the arterial wall and the endothelium. Arterial branches were identified, and the carotid arteries were cut to a length of 15 – 20mm, ensuring no branches were present. The arteries were placed in individual chambers within a perfusion myography system (Zultek Engineering, Melbourne, Australia). Each artery was immersed in either normal Krebs solution (11 mM glucose) or a high glucose Krebs solution (20 mM glucose) as previously described in **Chapter 3** (**Study 1**) and (Tacey et al., 2020b). The organ baths were warmed to 37ºC and bubbled with 95% oxygen and 5% carbon dioxide and were refreshed every 30 min over a 2 h period with the respective Krebs solution. Subsequently, the arteries were cannulated, and the respective Krebs solution pumped through the artery while pressure transducers monitored the intraluminal pressure of the vessel.

The carotid arteries were constricted with phenylephrine (3×10^{-7} M), which was added intraluminally and extraluminally. Once a stable constriction was achieved, a dose response curve was completed to ucOC (0.3, 3, 30 and 45 ng/ml) (Glu13, 17, 20, osteocalcin (1 – 46) (mouse) trifluoroacetate salt (Auspep, Australia, H-6552.0500)) or to Krebs (control), each concentration was administered internally via the endothelium and separated by 2 min intervals. The same mouse ucOC has previously been shown to improve relaxation in rabbit arteries (Qaradakhi et al., 2019). Following the dose response curve a bolus of acetylcholine (ACh) (10^{-5} M) was added internally and 2 min later a bolus of sodium nitroprusside (SNP) (10^{-5} M) externally, to determine the maximal endothelium-dependent and endothelium-independent relaxation, respectively. The vasoactive response of the vessels were analysed using the MEDIDAQ software program (MEDIDAQ, Melbourne, Australia). The vasoactivity of the carotid artery was measured as percentage change from the phenylephrine peak pressure and compared to the baseline pressure. Area under the curve (AUC) was calculated as the total relaxation below the phenylephrine plateau caused by the dose response curve, ACh and SNP bolus doses. The endothelium-dependent E_{max} was considered as the relaxation produced by ACh, and the endothelium-independent E_{max} was considered as the relaxation produced by SNP.

5.2.6 Statistical analysis

Human data were analysed using Statistical Package for the Social Sciences (SPSS, Inc. Chicago, IL, USA, version 22). A one-way analysis of variance (ANOVA) was used to examine the difference in ucOC concentration when participants were split into groups based on BP levels. Spearman rho correlations were used to examine the correlation between ucOC and measures of vascular function (BP, BAFMD and PWV) in all participants. Spearman partial correlations were used for the additional adjustments

of age, BMI or age and BMI, as they are strong influencers of ucOC levels (Li et al., 2016, Smith et al., 2020).

Animal data were analysed using Graphpad prism (version 7.1, Graphpad software Inc, USA). A one-way ANOVA was used to examine the effect of the ucOC dose response curves in rabbit carotid artery segments. AUC was calculated as the total area of relaxation below the maximum phenylephrine pressure and a one-way ANOVA was used to determine the difference in AUC between the ucOC dose response curves. All data is reported as mean ± SEM and statistical analysis was conducted at the 95% confidence level of significance ($p < 0.05$). Trends were reported when $p = 0.05 – 0.099$.

5.3 Results

5.3.1 Human data

Participant characteristics are presented in **Table 5.1**. In older adults with stage 2 hypertension ucOC was reduced by 34% compared to normotensive individuals ($p < 0.05$, **Figure 5.1 A**). When split by sex, ucOC was reduced by 43% ($p < 0.01$, **Figure 5.1 B**) and tOC was reduced by 30% ($p < 0.05$, **Figure 5.1 E**) in women with stage 2 hypertension compared to normotensive women. There was no difference between groups in older men ($p > 0.05$, **Figure 5.1 C + F**).

Table 5.1 Participant characteristics.

Variable	n	mean ± SEM
Participant number (n) [Female/Male]		38 [26/12]
Age (years)	38	73 ± 1
BMI (kg/m^2)	38	28 ± 1
Waist circumference (cm)	36	91 ± 1.5
Currently smoking (n) [%]	38	2 [5]
Cholesterol medication (n) [%]	38	13 [34]
Hypertensive medication (n) [%]	38	15 [39]
Heart disease medication (n) [%]	38	10 [26]
tOC (ng/ml)	37	21 ± 1.31
ucOC (ng/ml)	37	8 ± 0.61
ucOC/tOC ratio	37	0.39 ± 0.01
Systolic BP (mmHg)	38	139 ± 2
Diastolic BP (mmHg)	38	79 ± 1
MAP (mmHg)	38	98 ± 1
PWV (m/s)	34	8 ± 0.28
BAFMD – peak dilation (%)	29	4.62 ± 0.44
BAFMD – time to peak dilation (s)	29	58 ± 2.56

Abbreviations - BAFMD: brachial artery flow mediated dilatation, BMI: Body mass index, BP: blood pressure, MAP: mean arterial pressure, PWV: pulse wave velocity, s: second, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin.

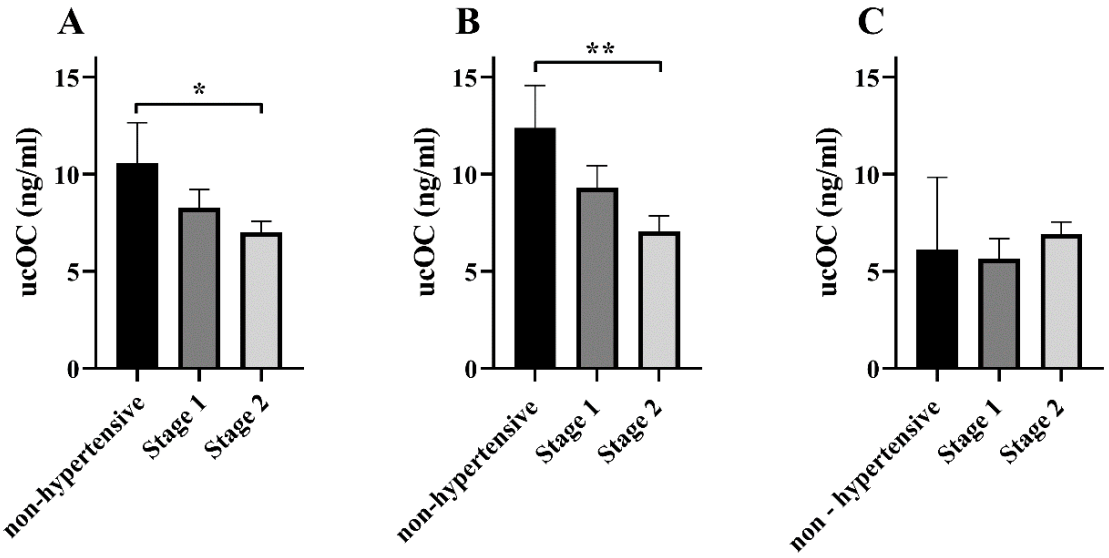

Figure 5.1 Concentration of ucOC based on hypertension category. (A) all participants, (B) women and (C) men split into groups based hypertension category; non-hypertensive (< 130 mmHg and < 80 mmHg) (women n = 5, men n = 2), stage 1 hypertension (130 – 139 mmHg and 80 – 89 mmHg) (women n = 10, men n = 4) and stage 2 hypertension (> 140 mmHg and > 90 mmHg) (women n = 11, men n = 6). All data mean ± SEM. * $p < 0.05$, ** $p < 0.01$ between groups. *Abbreviations - ucOC: undercarboxylated osteocalcin.*

5.3.2 Correlation between ucOC and vascular function

In the unadjusted model, high circulating ucOC was associated with lower systolic BP and PWV with all participants combined ($p < 0.05$ for both, **Table 5.2**). In women only, higher levels of circulating ucOC and tOC was associated with lower systolic BP ($p < 0.01$). There were trends for associations between lower MAP and PWV with higher levels of ucOC in women ($p = 0.05 – 0.099$ for both, **Table 5.2**). When adjusted for age, higher ucOC was associated with lower diastolic BP in all participants and with lower systolic BP in women ($p < 0.05$ for both, **Table 5.2**). Increased ucOC levels tended to correlate with both lower DBP and MAP in women after adjusting for age ($p = 0.05 – 0.099$ for both, **Table 5.2**). Adjusting for BMI, and BMI and age together, removed all associations of ucOC and tOC with BP and PWV

outcomes ($p > 0.05$). There were no significant correlations between ucOC and tOC with BAFMD peak % dilation in any model, and ucOC or tOC was not associated with any vascular function outcome in men ($p > 0.05$).

Table 5.2 Correlation between ucOC and vascular function outcomes.

Vascular function outcome	All (n = 38)	Women (n = 26)	Men (n = 12)
SBP			
Model 1	**-0.39***	**-0.58****	0.25
Model 2	-0.28	**-0.48***	0.01
Model 3	0.15	-0.07	0.39
Model 4	0.25	0.05	0.26
DBP			
Model 1	-0.21	-0.3	0.12
Model 2	**-0.41***	**-0.44^**	-0.51
Model 3	-0.04	-0.14	-0.05
Model 4	-0.13	-0.14	-0.75
MAP			
Model 1	-0.2	**-0.35^**	0.18
Model 2	-0.32	**-0.44^**	-0.06
Model 3	0.12	-0.05	0.05
Model 4	0.09	-0.01	-0.42
PWV			
Model 1	**-0.41***	**-0.41^**	-0.32
Model 2	-0.18	-0.26	0.25
Model 3	-0.03	0.02	-0.23
Model 4	0.19	0.13	0.54
BAFMD peak %			
Model 1	0.14	0.00	0.39
Model 2	0.22	0.13	0.32
Model 3	0.02	-0.18	0.51
Model 4	0.05	-1.14	0.29

Model 1 - unadjusted, Model 2 - adjusted for age, Model 3 - adjusted for BMI, Model 4 - adjusted for age and BMI. * $p < 0.05$, ** $p < 0.01$, ^ p 0.05 - 0.099 ucOC vs vascular function outcome. *Abbreviations - BAFMD: brachial artery pulse wave velocity, BMI: body mass index, DBP: diastolic blood pressure, MAP: mean arterial pressure, PWV: pulse wave velocity, SBP: systolic blood pressure, ucOC: undercarboxylated osteocalcin.*

5.3.3 Perfusion myography

The carotid artery segments from the animals fed the atherogenic diet did not exhibit a reduction in endothelium dependent relaxation in comparison to the arteries from the normal diet-fed animals ($p > 0.05$). The carotid artery vasoactive response from

rabbits fed a normal or atherogenic diet and treated *ex vivo* with ucOC was unaltered in both normal and high glucose environments ($p > 0.05$, **Figure 5.2 A + C**). The endothelium-dependent (ACh) and endothelium-independent (SNP) E_{max} were also unaltered following ucOC treatment, in comparison to the control, suggesting ucOC did not enhance the maximal relaxation of the vessel ($p > 0.05$, **Figure 5.2 A + C**). The AUC was unaltered by ucOC treatment following both the normal and atherogenic diet and incubation in normal and high glucose conditions ($p > 0.05$, **Figure 5.2 B + D**).

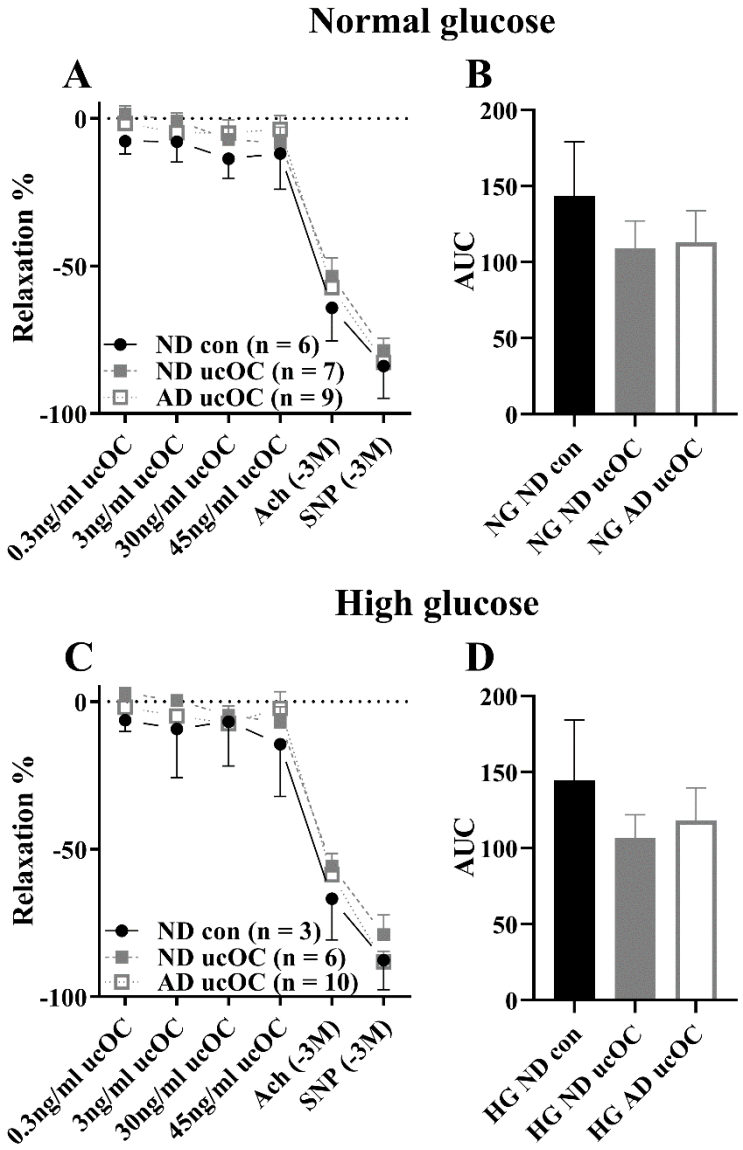

Figure 5.2 ucOC administration to carotid artery following 2 h incubation in NG or HG solution. (A) ucOC dose response curve in carotid artery incubated in NG solution and (B) AUC of dose response curve. (C) ucOC dose response curve in carotid artery incubated in HG solution and (D) AUC of dose response curve. All data mean ± SEM. No significant differences were detected. *Abbreviations - ACh: acetylcholine, AD: atherogenic diet, AUC: area under the curve, con: control treatment, HG: high glucose, ND: normal diet, NG: normal glucose, SNP: sodium nitroprusside, ucOC: undercarboxylated osteocalcin.*

5.4 Discussion

The major findings of the current study are a) in humans, higher circulating ucOC is associated with lower BP and increased arterial stiffness, which is particularly evident

in older women and b) ucOC treatment has no beneficial but also no adverse effect on carotid artery function from rabbits fed a normal or atherogenic diet or exposed acutely to normal and high glucose environments.

A number of studies have examined the correlation of tOC with vascular health and function outcomes. It was reported that tOC was lower in men but not women with hypertension (aged 24 – 78 years) (Xu et al., 2018b). Further, in 3,604 middle to older aged men and women higher levels of tOC were associated with lower PWV in men but higher PWV in women. However, when controlled for age and menopause status there was no longer an association between tOC and PWV in women (Yun et al., 2016). In middle and older aged men, but not post-menopausal women, higher tOC was associated with lower brachial artery PWV and intima media thickness (IMT), even after adjustment for confounding variables including age and BMI (Kanazawa et al., 2009). Yet, not all studies are in agreement, as higher tOC levels were associated with increased IMT, carotid plaque and aortic calcification in middle to older-aged women but not men (Reyes-Garcia et al., 2012). Overall, the findings are conflicting, and this appears to be largely driven by the differences between men and women. A major limitation of these studies is that they do not report the concentration of the individual forms of OC, in particular ucOC, which is important as ucOC is the putative bioactive form of the hormone.

Evidence examining the association of ucOC with vascular function is lacking, but crucial, if we are to understand the role of ucOC in CVD, specifically hypertension and atherosclerosis. In the current study we report that higher levels of ucOC are associated with lower BP in older post-menopausal women, but not in older men. However, the relatively small sample size of men in the current study means that

definitive conclusions cannot be established. However, similar to the current study, a previous study in older men and women (mean age 64 years old), reported that those with a higher cardiovascular risk score had increased MAP and lower circulating ucOC levels (Riquelme-Gallego et al., 2020). Conflictingly, in 162 community dwelling men (mean age 48 years old) and women (mean age 55 years old), ucOC was not correlated with systolic BP or diastolic BP (Choi et al., 2015). The conflicting outcomes may be related to the age difference between the study cohorts, as age is an important factor in determining ucOC levels (Smith et al., 2020). Furthermore, hormone variations between sexes and between pre- and post-menopausal women may also explain some of the diverse findings reported. Overall, whether ucOC is a mediator or a marker of CVD processes requires further investigation. In addition, taking into account several factors including sex, age and hormonal status will be important considerations for future studies.

As the association of ucOC with vascular function in humans is unclear and given the exact biological functions of ucOC are yet to be fully elucidated, examining its bioactive effect on the vasculature in animal models is important. The most commonly used method of examining the vasoactivity of blood vessels *ex vivo* is via isometric tension analysis. However, in this study we have utilised a novel perfusion myography system. This technique utilises haemodynamic forces such as shear stress, pressure and pulsatile flow which are mechanical factors important in the regulation of normal endothelial function, thus creating a more physiological environment (Cahill and Redmond, 2016). We found that ucOC did not directly influence the vasoactivity of isolated rabbit carotid arteries in either normal or high glucose solutions following an atherogenic or normal diet. A potential limitation of this study is that the atherogenic diet did not cause endothelial dysfunction. This suggests that the carotid artery may be

resistant to the development of endothelial dysfunction, as previous studies have reported that the same atherogenic diet caused endothelial dysfunction after four weeks in rabbit aorta, iliac and mesenteric arteries (Tacey et al., 2020b, Zulli and Hare, 2009). Carotid arteries were used in this study as they lack arterial branches, allowing effective cannulation and perfusion, which would not have been possible in other vessels due to the presence of branches. Notwithstanding, ucOC did not influence vasoactivity when vessels were exposed to a high glucose solution. In support of this, a previous *ex vivo* study, utilising the isometric tension analysis technique, reported similar findings to the current study. The administration of ucOC (10 ng/ml and 30 ng/ml) to rabbit aorta following an atherogenic diet or normal diet, with incubation in normal or high glucose solution, did not influence endothelium-dependent or endothelium-independent vasodilation (Tacey et al., 2020a). While another study reported that ucOC caused a slight enhancement in ACh-induced endothelium-dependant relaxation in dysfunctional rabbit aorta following an atherogenic diet, this did not occur after a normal diet, suggesting that ucOC may only function to enhance endothelium-dependent vasodilation in a dysfunctional state (Qaradakhi et al., 2019). However, this requires further investigation. Overall, while we report a correlation between ucOC and BP in older post-menopausal women, the *ex vivo* data indicates that ucOC has minimal direct biological influence on vascular function. There are several potential reasons for these findings. Firstly, ucOC may not act directly on the vasculature, and the associations observed in some studies may be through indirect pathways, such as via improvements in glycaemic control. Secondly, given recent reports, ucOC may not be as biologically active outside of the skeleton as initially suggested (Diegel et al., 2020, Moriishi et al., 2020).

This study has several limitations. Firstly, the relatively small sample size of older adult men means that definitive conclusions on the association of ucOC with vascular function in males cannot be made. Further research should examine in detail the potential association of ucOC with vascular function in females and males, taking into account confounding variables such as age and BMI. Secondly, a number of human participants were on hypertensive medication which may have influenced their BP measurement, highlighting the important role animal models can play in determining any direct effects of ucOC. Finally, due to only male rabbits being studied, the direct effect of ucOC on endothelial function in arteries from female rabbits is unclear.

In conclusion, increased ucOC is associated with lower BP and increased arterial stiffness in older post-menopausal women, but has no direct effect on endothelial function in rabbit carotid arteries. Future studies should explore whether treatment with ucOC *in vivo* has direct or indirect effects on blood vessel function.

Chapter 6. Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet

In **Chapter 5** (**Study 3**) we reported that circulating ucOC levels were lower in older females with hypertension, but not in older males with hypertension, however, when adjusted for confounding factors no association was present. In **Chapter 6** (**Study 4**) we examine whether a reduction in circulating ucOC by dietary intervention is associated with a change in blood pressure and vascular stiffness in middle aged and older men and women.

This chapter has been accepted for publication in *The Annals of Nutrition and Metabolism* as follows:

Tacey, **A**., Sim, M., Smith, C., Woessner, M. N., Byrnes, E., Lewis, J. R., Brennan-Speranza, T., Hodgson, J. M., Blekkenhorst, L. C., Levinger, I. (2020). Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet. *Ann. Nutr. Metab.,* 76(5), 361-367. (Scimago rank - Q1).

The published study is included in **Appendix E**.

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Declaration of co-authorship and co-contribution

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS

Title of Paper/Journal/Book:	Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet

Surname:	Tacey	First name:	Alexander
Institute:	Institute for Health and Sport	Candidate's Contribution (%):	45

Status:

Accepted and in press:	☐	Date:	
Published:	✔	Date:	24/11/2020

2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

Alexander Tacey Digitally signed by Alexander Tacey Date: 2020.12.16 09:46:03+11'00'	16/12/2020
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and
5. The original data will be held for at least five years from the date indicated below and is stored at the following **location(s)**:

Victoria University and Edith Cowan University

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
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Itamar Levinger	5	Editing of manuscript		16/12/20

This is the accepted manuscript version of an article published by S. Karger AG in Annals of nutrition & metabolism 2020;76:361-367. doi: 10.1159/000511660 and available on https://www.karger.com/Article/Abstract/511660

6.1 Introduction

CVD is the leading cause of death worldwide (Organization, 2019). A diet rich in fruit and vegetables is an important, non-therapeutic approach to reduce CVD development and progression (Benjamin et al., 2017, Struijk et al., 2014). Evidence suggests that diets rich in green leafy vegetables increase NO bioavailability and can improve vascular health (Blekkenhorst et al., 2017, Bondonno et al., 2018). However, we have previously shown that a four week dietary intervention involving an increased intake of leafy green vegetables, did not reduce BP or arterial stiffness (Blekkenhorst et al., 2018b). One potential explanation for the absence of a beneficial effect on BP and arterial stiffness may be related to other bioactive components found in leafy green vegetables that concomitantly influence vascular health. For example, vit K1 is abundant in leafy green vegetables and regulates several coagulation factors including vit K-dependent proteins (VKDP) (Kidd, 2010).

One such protein is OC, a VKDP-derived from osteoblasts that exists in two forms: cOC and ucOC (Booth et al., 2013, Booth and Al Rajabi, 2008, Gundberg et al., 2012). The carboxylated form of OC has a high affinity to hydroxyapatite within the bone matrix and is therefore thought to reflect bone mineralisation (Hauschka et al., 1989, Price et al., 1981), whereas ucOC is proposed as the bioactive form of OC in several target tissues (Lee et al., 2007). Growing evidence suggests an association between OC, in particular tOC and ucOC with hypertension, vascular calcification, atherosclerosis and CVD mortality (Magni et al., 2016, Levinger et al., 2017, Polgreen et al., 2012). However, the literature is conflicting and it is unclear whether tOC or its isoforms are associated with positive or negative effects on cardiometabolic health (Millar et al., 2017, Tacey et

al., 2018). We have previously shown that a diet rich in leafy green vegetables, and thus vit K1, reduces circulating ucOC levels (Sim et al., 2020).

The current study was a sub-analysis examining the cardiometabolic implications of ucOC suppression following an increased intake of predominantly leafy green vegetables. It was of interest to investigate whether a reduction in ucOC levels was correlated with changes in cardiometabolic risk factors, and whether this could explain, at least in part, the lack of a beneficial effect on blood pressure following an increase in dietary nitrate. Participants from the high vit K1 intervention were divided into high and low responders based on the suppression of ucOC following the intervention. The aim was to determine if a large reduction in ucOC (high responders) would be associated with alterations in cardiometabolic risk factors including blood pressure, arterial stiffness and blood glucose and lipid concentrations.

6.2 Methods

6.2.1 Human participants

The data for this paper was collected for the Vegetable Intake and Blood Pressure (VIABP) study (ACTRN12615000194561). The study was approved by The University of Western Australia Human Research Ethics Committee and was completed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was a randomised, controlled crossover trial and methodology has been described in full elsewhere (Blekkenhorst et al., 2018b). In brief, middle and older aged (40 – 74 years of age) community dwelling men and women with pre-hypertension or untreated grade one hypertension were recruited to participate. Each participant received three 4 week dietary interventions, each interspersed with a four week washout period. The VIABP study was originally designed with the following dietary

interventions: (1) increased intake of nitrate-rich leafy green vegetables (high nitrate); (2) increased intake of nitrate-poor vegetables (low nitrate); and (3) no increase in vegetables (control). As vit K1 is also found predominately in leafy green vegetables, these three dietary interventions have been equated to: (1) high vit K1 intake (HK); (2) low vit K1 intake (LK); and (3) control diet (CON) (Sim et al., 2020). Considering the primary aim of this study is to examine the association between the suppression of ucOC and cardiometabolic risk factors (and given the LK diet did not suppress ucOC), we predominantly considered data from the HK intervention.

6.2.2 Vascular analysis

Participants were asked to complete an overnight fast and to abstain from their regular medication until after the testing had been completed. They were also asked to refrain from intense exercise for 24 hours prior to the testing. Resting BP and PWV (SphygmoCor XCEL 2012, AtCor Medical Pty. Ltd.) were measured pre and post the four week dietary intervention, as previously described (Blekkenhorst et al., 2018b). Ambulatory BP was recorded over a 24 h period, every 20 min during the day and every 30 min during the night, mean BP was determined for the 24 h period (Blekkenhorst et al., 2018b).

6.2.3 Biochemical analysis

Plasma concentrations of glucose, triglycerides, total cholesterol, HDL cholesterol and calculated LDL cholesterol were analysed by PathWest laboratories (Fiona Stanley Hospital, Perth, Australia). Serum tOC was measured by sandwich electrochemiluminescence immunoassay using the Roche Cobas N-Mid OC assay (Roche Diagnostics, Mannheim). The inter-assay coefficients of variation were 2.3% and 4.8% at levels of 18 and 90 ng/mL, respectively. Serum ucOC was determined using the

hydroxyapatite binding method (Calbiochem) (Gundberg et al., 1983, Gundberg et al., 1998). The inter-assay imprecision for percentage binding of cOC was 8% and 12% at OC of 100 and 15 ng/mL, respectively. Plasma creatinine was measured at baseline and glomerular filtration rate was estimated using plasma creatinine levels based on the known equation (Levey et al., 2009). Vit K intake was estimated as previously described (Sim et al., 2020).

6.2.4 Statistical analysis

All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL, USA, version 22). Independent samples t-tests were conducted to examine OC concentrations between males and females and if characteristics known to influence ucOC (BMI, age, vit K intake and glomerular filtration rate) were different between the high responders and low responders at baseline. Spearman rho correlations were used to assess the relationship between pre-intervention OC concentrations and pre-intervention outcome measures. Spearman partial correlations were used for the additional adjustments of age and BMI as they are strong influencers of ucOC levels (Smith et al., 2020, Li et al., 2016).

When considering post intervention data from the HK diet intervention, participants were divided into high responders (suppression of ucOC ≥ median [≥ 30%]) and low responders (suppression of ucOC < median [< 30%]), based on the percent change in ucOC. The between groups (high versus low responders) effect of the HK diet on changes in OC, vascular and metabolic outcomes were assessed using one-way ANOVA. Within groups effects for pre- and post-intervention were assessed using paired samples t-tests, as previously reported (Sim et al., 2020). All data reported as mean ±

SEM and statistical analysis was conducted at the 95% confidence level of significance ($p < 0.05$).

6.3 Results

6.3.1 Baseline characteristics

Baseline characteristics are presented in **Table 6.1**. Serum tOC, cOC and ucOC levels at pre-intervention data points were not different between women (n = 10) or men (n = 20) ($p > 0.05$ for all, **Table 6.1**). With pre-intervention data points combined together, a higher ucOC/tOC ratio was associated with lower PWV when adjusted for BMI and age ($r = -0.493$, $p < 0.05$). A higher concentration of cOC was associated with a higher PWV when adjusted for BMI and age ($r = .638$, $p < 0.01$). All other pre-intervention correlations were not significant ($p > 0.05$ for all, **Table 6.2**).

Table 6.1 Participant characteristics.

Variable	mean ± SEM
Participant number [male/female]	30 [20/10]
tOC (male/female) (ng/ml)	21.82 ± 1.53 / 22.23 ± 1.79
cOC (male/female) (ng/ml)	14.05 ± 1.17 / 13.41 ± 2.01
ucOC (male/female) (ng/ml)	7.77 ± 0.88 / 8.82 ± 0.77
Age (years)	61.80 ± 9.90
BMI (kg/m^2)	26.99 ± 3.87
Waist circumference (cm)	89.48 ± 2.18
Waist to hip ratio	0.87 ± 0.02
Systolic BP (mmHg)	133.56 ± 1.53
Diastolic BP (mmHg)	77.67 ± 1.45
Heart rate (bpm)	61.59 ± 1.46
Glucose (mmol/L)	5.29 ± 0.08
Total Cholesterol (mmol/L)	5.54 ± 0.26
HDL (mmol/L)	1.35 ± 0.06
LDL (mmol/L)	3.61 ± 0.22
Triglycerides (mmol/L)	1.28 ± 0.11
eGFR (ml/min/1.73m)	92.57 ± 2.17
Vit K intake (ug/d)	120.84 ± 11.14

Abbreviations - BMI: body mass index, BP: blood pressure, cOC: carboxylated osteocalcin, eGFR: estimated glomerular filtration rate, HDL: high density lipoprotein, LDL: low density lipoprotein, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin, vit K: vitamin K.

Table 6.2 Correlation between OC variables and cardiovascular health outcomes at baseline.

CV health outcomes	ucOC	ucOC/tOC ratio	cOC
Amb SBP			
Model 1	.078	.013	.014
Model 2	.093	-.068	.302
Amb DBP			
Model 1	.199	.160	-.137
Model 2	.197	.120	.077
Resting SBP			
Model 1	.063	.017	.061
Model 2	.141	.063	.121
Resting DBP			
Model 1	.193	.196	-.164
Model 2	.191	.141	.109
PWV			
Model 1	-.076	-.237	.191
Model 2	-.245	**-.493***	**.638****
Glucose			
Model 1	-.027	.057	-.210
Model 2	.254	.281	-.106
Total Chol			
Model 1	.003	-.092	.183
Model 2	.124	-.012	.139
LDL			
Model 1	-.051	-.095	.156
Model 2	.051	-.080	.163
HDL			
Model 1	.168	.057	.141
Model 2	.223	.218	-.129
Triglycerides			
Model 1	-.096	-.001	-.150
Model 2	.032	-.034	.163

Model 1 - unadjusted, Model 2 - adjusted for BMI and age. * $p < 0.05$, ** $p < 0.01$ OC variable vs cardiovascular health outcome.

Abbreviations - Amb: ambulatory, BMI: body mass index, Chol: cholesterol, cOC: carboxylated osteocalcin, CV: cardiovascular, DBP: diastolic blood pressure, HDL: high density lipoprotein, LDL: low density lipoprotein, OC: osteocalcin, PWV: pulse wave velocity, SBP: systolic blood pressure, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin.

6.3.2 Correlation between OC and vascular function

We have previously shown that the HK intervention, but not the LK or CON intervention suppressed tOC, ucOC and the ucOC/tOC ratio (Sim et al., 2020). In the high

responder's tOC, ucOC and ucOC/tOC were reduced post-intervention compared to pre-intervention, following the four week HK diet ($p < 0.001$ for all, **Table 6.3**). While in the low responders, ucOC ($p < 0.001$) and ucOC/tOC ($p < 0.01$), as well as resting systolic BP (2%, $p < 0.05$) were reduced post intervention. As expected, the change in ucOC and ucOC/tOC ratio was significantly greater in the high responders versus the low responders ($p < 0.05$ for both, **Table 6.3**). The change in tOC, cOC, markers of vascular (ambulatory systolic BP, ambulatory diastolic BP, resting systolic BP, resting diastolic BP or PWV) and metabolic (glucose, total cholesterol, LDL, HDL or triglycerides) health were not significantly different between the low and high responders (**Table 6.3**). There was no difference in BMI, vit K intake, age or estimated glomerular filtration rate (eGFR) between the high or low responders at baseline ($p > 0.05$ for all, **Table 6.4**).

Table 6.3 OC, vascular and metabolic outcomes pre and post treatment by high and low responders. Delta (Δ) change of OC, vascular and metabolic outcomes following the high vit K1 diet (pre to post).

	Low responders			**High responders**		
	Pre mean ± SEM	Post mean ± SEM	Δchange	Pre mean ± SEM	Post mean ± SEM	Δchange
Sample (n) male/female	11/4	11/4		9/6	9/6	
tOC (µg/L)	21.61 ± 1.39	20.61 ± 1.52	-.99 ± .86	22.31 ± 1.92	**18.38 ± 1.42*****	-3.93 ± .77
ucOC (µg/L)	8.86 ± .88	**7.76 ± .93*****	-1.10 ± .24	7.39 ± .92	**4.33 ± .44*****	**-3.06 ± .51**^##^**
cOC (µg/L)	12.75 ± 1.44	12.85 ± 1.25	.10 ± .74	14.92 ± 1.42	14.05 ± 1.19	-0.87 ± .68
ucOC/tOC	0.42 ± .04	**0.38 ± .04****	-0.04 ± .01	0.34 ± .03	**0.24 ± .02*****	**-0.09 ± .01**^##^**
Amb SBP (mmHg)	125.40 ± 1.86	126.20 ± 1.73	.81 ± 1.24	125.79 ± 1.85	126.83 ± 1.60	1.04 ± 1.13
Amb DBP (mmHg)	76.15 ± 2.14	76.26 ± 2.23	.12 ± 1.17	74.41 ± 2.10	74.34 ± 2.06	-0.07 ± .76
Resting SBP (mmHg)	130.13 ±1.46	**127.33 ± 2.18***	-2.8 ± 1.26	130.37 ± 2.52	129.53 ± 2.45	-0.83 ± 1.97
Resting DBP (mmHg)	77.9 ±1.57	75.53 ± 1.64	-2.37 ± 1.25	75.30 ± 2.00	75.07 ± 2.12	-0.23 ± 1.20
PWV (m/s)	8.34 ± .36	8.38 ± .35	.04 ± -.21	8.31 ± .26	8.17 ± .24	-.13 ± .16
Glucose	5.17 ±.15	5.06 ± .13	-0.11 ± .14	4.79 ±.16	4.88 ± .13	0.09 ± .12
Total Chol	5.64 ±.28	5.59 ± .23	-0.05 ± .17	5.32 ±.36	4.96 ± .33	-0.36 ± .22
LDL	3.68 ±.27	3.68 ± .22	0.01 ± .14	3.26 ±.30	3.04 ± .28	-0.23 ± .15
HDL	1.38 ±.07	1.35 ± .09	-0.03 ± .03	1.44 ±.09	1.39 ± .10	-0.06 ± .05
Triglycerides	1.26 ±.16	1.21 ± .10	-0.05 ± .11	1.34 ±.25	1.17 ± .16	-0.17 ± .14

High and low responders based on median split in percent change of ucOC from pre to post high vit K1 diet. Data reported as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ pre vs post high vit K1 diet, ## $p < 0.01$ Δhigh responders vs Δlow responders.

Abbreviations - Amb: ambulatory, Chol: cholesterol, cOC: carboxylated osteocalcin, DBP: diastolic blood pressure, HDL: high density lipoprotein, LDL: low density lipoprotein, OC: osteocalcin, PWV: pulse wave velocity, SBP: systolic blood pressure, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin.

Table 6.4 Differences between HR and LR in baseline variables known to regulate ucOC.

	Mean ± SEM
BMI (kg/m^2)	
HR	26.87 ± 0.93
LR	27.12 ± 1.09
Vit K intake (ug/d)	
HR	108.60 ±13.66
LR	133.07 ± 17.50
Age (years)	
HR	63.1 ± 2.44
LR	60.47 ± 2.71
eGFR (ml/min/1.73m)	
HR	92.40 ± 3.26
LR	92.73 ± 2.99

Abbreviations - BMI: body mass index, eGFR: estimated glomerular filtration rate, HR: high responders, LR: low responders, ucOC: undercarboxylated osteocalcin, vit K: vitamin K.

Using unadjusted Spearman rho correlation and Spearman partial correlation there was no association between the change in ucOC or the ucOC/tOC ratio with the change in any cardiometabolic risk factor in the high responders ($p > 0.05$ for all, **Table 6.5**). Using unadjusted spearman rho correlation, a positive association was present between the change in ucOC and the change in LDL when all participants were combined (i.e. high and low responders combined) ($p < 0.05$, **Table 6.5**). When adjusted for age and BMI using Spearman partial correlations, a positive correlation was present between the change in ucOC/tOC ratio and change in ambulatory diastolic BP when all participants were combined ($r = .435$, $p < 0.05$). In low responders only, there was a strong positive correlation between the change in ucOC/tOC ratio and change in glucose levels ($r = .793$, $p < 0.05$). All other correlations were not significant ($p > 0.05$ for all, **Table 6.5**).

Table 6.5 Correlation between ΔucOC and ΔucOC/tOC ratio with Δvascular and metabolic outcomes following the high vit K1 diet.

	ΔucOC			ΔucOC/tOC ratio		
	All participants	High responders	Low responders	All participants	High responders	Low responders
ΔAmb SBP						
Model 1	.197	.396	.041	-.014	.175	-.033
Model 2	.400	.512	.152	.040	.197	.224
ΔAmb DBP						
Model 1	.099	.489	-.267	.210	.136	.319
Model 2	.284	.551	-.051	**.435***	.249	.611
ΔResting SBP						
Model 1	.014	-.052	.334	-.240	-.275	-.014
Model 2	-.226	-.251	.498	-.355	-.480	-.625
ΔResting DBP						
Model 1	-.090	.073	.052	-.170	.141	-.066
Model 2	-.296	-.408	.030	-.224	-.264	-.343
ΔPWV						
Model 1	.238	.071	.041	.164	.011	.264
Model 2	-.048	-.123	.021	-.022	-.315	-.136
ΔGlucose						
Model 1	-.300	-.074	-.120	-.182	-.261	.290
Model 2	-.285	-.046	-.583	.145	-.367	**.793***
ΔTotal Chol						
Model 1	.314	.296	.234	.070	.071	-.107
Model 2	.257	.369	.186	.025	-.024	-.487
ΔLDL						
Model 1	**.375***	.336	.388	.156	.139	.064
Model 2	.276	.547	.205	.141	.205	-.398
ΔHDL						
Model 1	.154	.093	.008	-.107	-.264	-.043
Model 2	.006	.011	-.155	-.175	-.329	-.383
ΔTriglycerides						
Model 1	.018	-.064	-.018	-.202	-.200	-.389
Model 2	.171	.073	.252	-.255	-.167	-.566

Model 1 - unadjusted; Model 2 - adjusted for BMI and age. * $p < 0.05$ ΔucOC/tOC vs vascular/metabolic outcome.

Abbreviations - Amb: ambulatory, Chol: cholesterol, DBP: diastolic blood pressure, HDL: high density lipoprotein, LDL: low density lipoprotein, PWV: pulse wave velocity, SBP: systolic blood pressure, tOC: total osteocalcin, ucOC: undercarboxylated osteocalcin.

6.4 Discussion

The major finding of this study is that the suppression of ucOC was not associated with increased cardiometabolic risk factors, even in individuals who responded the most to the intervention (high responders). As such, it appears that the suppression of ucOC following a leafy green-rich diet does not impact, either negatively or positively, on cardiometabolic risk factors.

Currently, there are conflicting reports regarding the relationship between OC and blood pressure. Some have reported that lower tOC levels are associated with a higher prevalence of hypertension in adult men and women (Oosterwerff et al., 2013, Tan et al., 2011). Others however, have described no association between tOC and systolic or diastolic BP in adult men and women (Lerchbaum et al., 2014, Lerchbaum et al., 2013). As cOC and ucOC may have diverse biological functions, the examination of tOC alone, as often reported in these studies, limits our understanding of the exact function of each form of OC (Li et al., 2016, Ling et al., 2018). In the current study, we have examined each form of OC and report that a reduction in ucOC and ucOC/tOC ratio via dietary modification is not correlated with changes in BP. This is interesting and suggests several possibilities. Firstly, ucOC may simply not have a regulatory role in the maintenance of blood vessel function and BP. Secondly, the HK (leafy green rich) diet may regulate other bioactive factors that influence vascular health. For example, we have previously shown that the four week leafy green-rich diet increased plasma nitrate levels (Blekkenhorst et al., 2018b). An increase in plasma nitrate enhances the bioavailability of NO, an anti-atherogenic molecule that regulates blood vessel function and BP (Blekkenhorst et al., 2017, Blekkenhorst et al., 2018a). Interestingly, ucOC has also been implicated as a regulatory factor responsible for the maintenance of blood vessel function and BP (Tacey

et al., 2018). Therefore, it is possible that the reduction in ucOC was offset by an increase in NO bioavailability. Consequently, cross-talk mechanisms may exist, which may explain the lack of changes in BP. This hypothesis should be explored in further mechanistic studies.

Circulating ucOC has been established as a regulator of energy homeostasis, at least in animal models (Brennan-Speranza and Conigrave, 2015, Rossi et al., 2019). A large number of cross-sectional studies in humans show that ucOC is associated with metabolic responses and diseases. For example, a reduction in circulating ucOC is associated with an increased risk or presence of metabolic disorders, such as metabolic syndrome and T2DM (Levinger et al., 2017). Lower circulating tOC and ucOC has been associated with increased concentrations of blood glucose and triglycerides and decreased levels of HDL (Alfadda et al., 2013, Kanazawa et al., 2011b). However, few interventional studies have modified ucOC and examined the effect on metabolic outcomes. One study administered a single dose of prednisolone, a glucocorticoid, which suppressed circulating tOC and ucOC and also caused a reduction in insulin sensitivity and fasting blood glucose (Parker et al., 2019, Tacey et al., 2019). In the current study, despite a 41% reduction in ucOC and 29% reduction in ucOC/tOC after the HK diet, there were no changes in fasting glucose or lipid levels in the high responders. Potential mechanisms for the lack of change are not clear, but it may be related to other bioactive components present in green leafy vegetables that can caused a compensatory effect and prevented any change in metabolic variables.

The development of vascular calcification is a process comparable to the development of bone within the skeleton. As OC is involved in bone mineralisation within the skeleton, it has also been implicated in the development of mineralisation within the

vasculature (Kapustin and Shanahan, 2011, Li et al., 2016). The form of OC predominantly involved with bone development in the skeleton is cOC, as such, it is possible that cOC is the form of OC involved in the development of calcification within the vasculature. However, research in this area is lacking. We have shown that baseline cOC is associated with baseline PWV, a measure of arterial stiffness which suggests the presence of vascular calcification (Cheng et al., 2018). However, we saw no correlation of cOC with PWV following the HK diet in the high or low responders. While, it is possible that OC is involved in vascular calcification, future large scale studies are needed to assess the effect of each form of OC, in particular cOC, on arterial stiffness and the development of vascular calcification.

A limitation of the current study is that the four week intervention period may not have been long enough or the dose of vit K1 large enough to observe a change in measures of cardiometabolic risk. Previous studies administering vit K1 supplementation (500 – 1000 μg per day) for three years found improvements in vascular compliance and reductions in coronary artery calcification (Braam et al., 2004, Shea et al., 2009). In the current study, it was estimated that participants increased their vit K1 intake by 150 μg per day over the four weeks (Sim et al., 2020). As such, a prolonged intervention may be needed to demonstrate changes in cardiometabolic risk factors. Another potential limitation was the inclusion of people who are relatively healthy. It is possible that those with diabetes or cardiovascular disease will respond differently to the intervention and that the correlation between ucOC and cardiovascular risk factors may be apparent in these populations. Finally, the generalisation of the results are somewhat limited due to the relatively small sample size. As such, further large scale studies, in particular randomised controlled trials, are needed to confirm our findings.

In conclusion, this study demonstrated that the suppression of ucOC following increased daily intake of leafy green vit K1-rich vegetables over four weeks was not associated with unfavourable changes in cardiometabolic risk factors. This may be due to the presence of compensatory mechanisms, or the fact that ucOC has a limited regulatory role over cardiometabolic risk factors in apparently healthy individuals. Such hypothesis should be explored by future mechanistic studies.

Chapter 7. General Discussion

7.1 Major findings

The undercarboxylated form of osteocalcin (ucOC) is considered as a potential therapeutic target to manage and treat metabolic diseases such as insulin resistance and T2DM. Prior to this research the biological role of ucOC within the vasculature was unclear as some research suggested ucOC negatively influenced blood vessel function and CVD development. Thus, it was important to assess the effect of ucOC within the vasculature to exclude negative off-target effects before further translational research could be completed. This thesis investigated whether ucOC directly regulates endothelial function in experimental studies and explored the association of ucOC with blood vessel function in humans. The major findings of this thesis are summarised in **Figure 7.1**.

The major novel findings of this thesis are:

1. The development of endothelial dysfunction in rabbit arteries following acute hyperglycaemia and an atherogenic diet is blood vessel specific (**Chapter 3**, **Study 1**).
2. The direct administration of ucOC to rabbit arteries *ex vivo* or human vascular cells *in vitro* does not influence endothelial function in either normoglycaemic or hyperglycaemic conditions (**Chapter 4** and **5**, **Studies 2** and **3**).
3. Higher levels of ucOC may be associated with vascular outcomes in older women (**Chapter 5**, **Study 3**). However, dietary-induced reductions in circulating ucOC do not alter blood vessel function (**Chapter 6**, **Study 4**).

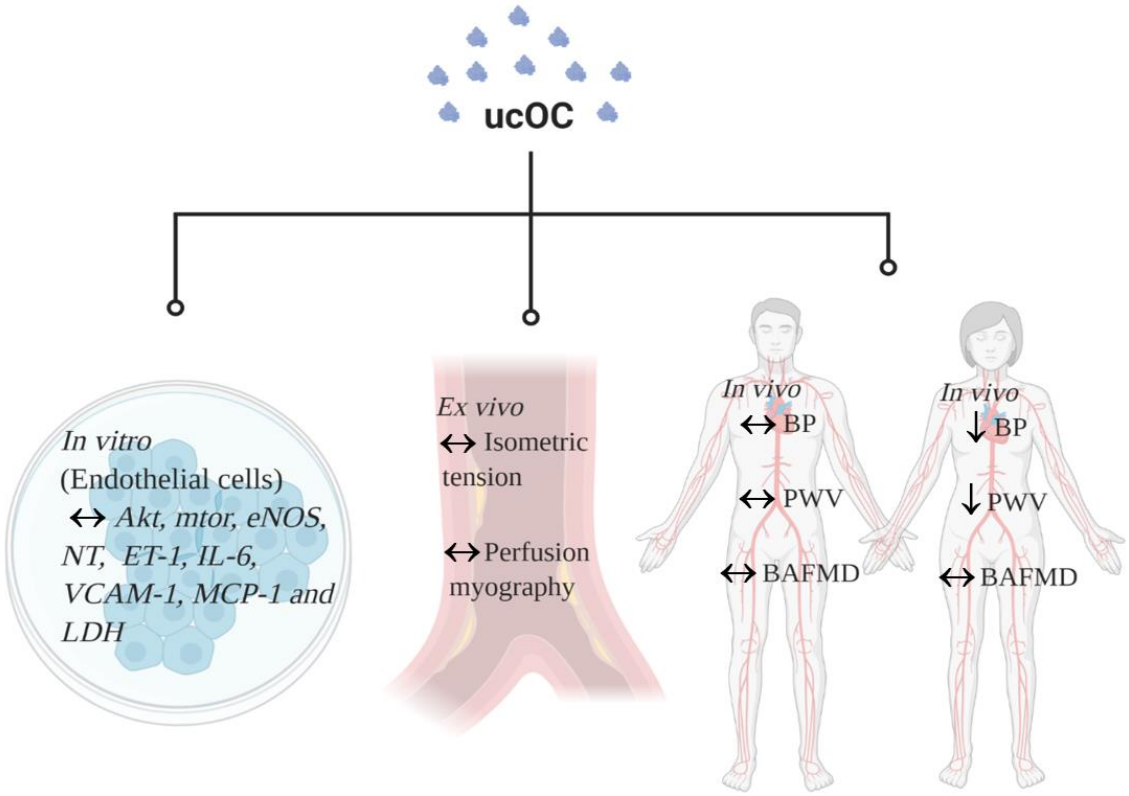

Figure 7.1 Summary of the major findings of this thesis. *In vitro* ucOC had no direct effect, positive or negative, on any examined marker of endothelial cell function. *Ex vivo* ucOC did not influence endothelial or smooth muscle cell function either positively or negatively. *In vivo*, ucOC shared some association with BP and PWV, but this not after adjustment for confounding variables, while the suppression of ucOC did not alter vascular function. ↔ no correlation between OC and vascular function, ↓ Lower OC associated with adverse function. Created with BioRender.com

Abbreviations - Akt: protein kinase b, BAFMD: brachial artery flow mediated dilation, BP: blood pressure, eNOS: endothelial nitric oxide synthase, ET-1: endothelin 1, IL-6: interleukin 6, LDH: lactate dehydrogenase, MCP-1: monocyte chemoattractant protein 1, mTOR: mammalian target of rapamycin, NT: nitrotyrosine, PWV: pulse wave velocity, ucOC: undercarboxylated osteocalcin, VCAM-1: vascular cell adhesion molecule 1.

7.1.1 The development of a model of endothelial dysfunction in rabbit arteries

Endothelial dysfunction is the initiating stage in the development of atherosclerosis and CVD. Hyperglycaemia and poor dietary choices are major risk factors in the development of endothelial dysfunction, but whether different arteries respond to

these pathological factors in a similar or diverse fashion was unclear. This thesis examined the development of endothelial dysfunction in rabbit arteries following a short-term atherogenic diet and exposure to acute hyperglycaemia. The abdominal aorta was the artery most susceptible to develop endothelial dysfunction following a four week atherogenic diet (**Chapter 3**, **Section 3.3.1**). Similarly, the acute incubation of rabbit arteries in hyperglycaemic conditions (20 mM) revealed that the aorta was more prone to develop dysfunction than the iliac or mesenteric arteries (**Chapter 3**, **Section 3.3.2**). This data suggests that the aorta is more vulnerable to the development of endothelial dysfunction than the iliac and mesenteric arteries. Given the role of the aorta as the main distributor of blood to the abdomen and lower extremities, this is concerning. Further work to establish reasons for discrepancies may open new avenues for treatment, but this was outside the scope of this thesis. As the development of endothelial dysfunction to acute hyperglycaemia and an atherogenic diet were most pronounced in the abdominal aorta, this is the vessel that was used to assess the vasoactivity of ucOC in **Chapter 4**, **Study 2**.

7.1.2 Direct administration of ucOC does not influence endothelial function in normal or hyperglycaemic conditions

Strong evidence suggests that ucOC positively influences energy regulation and glycaemic control (Lin et al., 2020b). However, before ucOC treatment can be translated to clinical trials, it was important to identify whether it has a negative off-target effects that may lead to adverse health outcomes. This thesis focused on endothelial function as there is conflicting data regarding the effect of ucOC on the blood vessels. Some research has suggested that ucOC may negatively influence atherosclerosis and vascular calcification (Kanazawa et al., 2011a, Liu et al., 2019). Whereas others demonstrated that

in vivo administration of OC improves BP and blood vessel function in animal models (Dou et al., 2014, Huang et al., 2017). However, it was unknown whether the improvement in vascular function in these studies occurred directly or whether there was an indirect effect via the improvement in glucose homeostasis and adiposity. In this thesis two *ex vivo* techniques were used to examine whether ucOC has a direct biological effect on endothelial function in the abdominal aorta (**Chapter 4**, **Study 2**) and carotid artery of rabbits (**Chapter 5**, **Study 3**). The findings from this thesis demonstrate for the first time that ucOC has no direct effect, either positively or negatively, on endothelial function in physiological conditions or in pathological hyperglycaemic conditions.

Using isometric tension analysis techniques the vasoactive function of recombinant ucOC was assessed in the aorta of New Zealand White Rabbits following four weeks on a normal or atherogenic diet and incubated in normal or high glucose solution (20 mM) for two hours. Administration of recombinant ucOC at physiological concentrations did not alter the vasoactive response to endothelium-dependent or endothelium-independent vasodilators (**Chapter 4**, **Section 4.3.2**). This suggests that if ucOC was used therapeutically, it is unlikely to cause any direct alterations in endothelial function. However, given that the experiments were completed in isolated vascular rings, a novel perfusion myography method was utilised to more closely mimic a physiological environment. This method allowed for the assessment of whole vessel vasoactivity simulating endogenous flow and haemodynamics (**Chapter 5**, **Section 5.3.3**). The vasoactivity of the carotid artery was unaltered by the administration of a ucOC dose response curve following acute incubations in normal or high glucose conditions, providing further confidence that ucOC has no deleterious effects on endothelial function. The results of these experiments suggest that any alteration in blood vessel function that

is reported following ucOC administration *in vivo* probably occurs indirectly. Likely through changes in metabolic outcomes in which ucOC improves glucose regulation and the resulting reduction in glucose concentrations leads to the improvement in blood vessel function. These experiments were limited by the fact that the high glucose incubation did not cause endothelial dysfunction, despite following the same methodology that was established in **Chapter 3**, and by others (Tesfamariam et al., 1991, Zulli and Hare, 2009). Thus, it could be that ucOC does not have a vasoactive effect unless administered in a dysfunctional state, as was reported in one previous study (Qaradakhi et al., 2019). This hypothesis should be explored in further studies.

In support of the *ex vivo* findings, *in vitro* experiments in HAECs revealed that co-treatment of ucOC does not attenuate markers of endothelial dysfunction (IL-6, VCAM-1, ET-1, MCP-1 and LDH) which were increased due to culture in high glucose media (**Chapter 4**, **Section 4.3.4**). This data expand on previous studies which reported that human vascular cell function and common vascular signalling pathways were minimally altered in HAECs and HASMCs following ucOC treatment (Millar et al., 2019a). Further, markers of dysfunction and inflammation, such as VCAM-1 and IL-6, were unaltered by ucOC treatment in vascular cells cultured in inflammatory media (Millar et al., 2019c). Overall, it appears that ucOC has minimal direct influence on endothelial and smooth muscle cell function in the conduit arteries of rabbits and human endothelial cells in normoglycaemic or hyperglycaemic conditions.

7.1.3 The association of ucOC with vascular function in humans

Findings in animal models often do not translate to humans. Accordingly, this thesis explored the association of ucOC with vascular function in humans to support the results of the preclinical studies. To date, it is unclear whether OC is related to CVD

outcomes such as the presence of plaque, calcification or adverse vascular events (Millar et al., 2017). Even less is known about the association of OC with blood vessel function and BP. In **Chapter 5** (**Study 3**) lower levels of ucOC were associated with hypertension and arterial stiffness but not endothelial function in older women. However, the association was dependent on confounding variables such as age and BMI, which are known to influence OC levels. The data from **Chapter 5** (**Study 3**) supports this, identifying a correlation between ucOC and vascular function, particularly in post-menopausal women, but the lack of association after adjustment for confounding factors suggests other factors likely mediate this association.

A known limitation of cross-sectional studies is that they are correlational only and thereby do not describe cause and effect. Currently, the administration of ucOC to humans is not possible given that the extent of its biological effects is still unknown. However, it is possible to manipulate the circulating levels of ucOC using dietary modifications, pharmaceuticals or exercise to explore whether changes are observed in metabolic and cardiovascular parameters. Vit K is a major influencer of ucOC bioavailability (Shea et al., 2017, Tanaka et al., 2020). This thesis demonstrates for the first time that the suppression of circulating ucOC following a four week leafy green diet, which is high in vit K, does not influence markers of vascular function in middle aged and older adults (**Chapter 6**, **Section 6.3.2**). It is important to acknowledge that leafy green vegetables also increase circulating nitrate (Blekkenhorst et al., 2018b), which is likely beneficial to vascular health as it increases the bioavailability of NO (Blekkenhorst et al., 2017, Bondonno et al., 2018). As such, the reduction in ucOC and the increase in nitrate that occurred in this study may have compensated for each other to maintain vascular homeostasis. Overall these results support the findings from the experimental

models and suggest that ucOC does not directly influence vascular function. However, the number of studies are still small and further interventional studies are needed to confirm these findings. The results from this thesis provide strong evidence to support the theory that ucOC has no direct regulatory function in the vasculature, suggesting ucOC may be targeted as a therapeutic treatment for metabolic disease without the risk of adverse effects on vascular function.

7.2 Limitations of thesis

Specific limitations of each study are described in the relevant sections (**Chapters 3 – 6**). General limitations of this thesis are described below.

1. It is possible that the acute administration of ucOC *ex vivo* in **Chapters 4** and **5** (**Studies 2** and **3**) was not long enough to induce a physiological effect. Treatment for a longer duration may be necessary to induce an effect on vascular function and this should be explored in future studies.
2. The lack of dysfunction resulting from the high glucose incubation and the atherogenic diet in **Chapter 4** (**Study 2**) despite observing dysfunction in **Chapter 3** (**Study 1**) is a limitation. Examining the influence of ucOC on endothelial function in both normal and dysfunctional conditions would strengthen the findings.
3. As the primary aim of was to examine function outcomes, this thesis examined only several mechanistic pathways by which ucOC may interact with endothelial cells. Future research should examine potential pathways that link the metabolic processes to vascular function.

7.3 General conclusion and suggestions for future research

This thesis adds significant knowledge on the bioactive role of ucOC within the vasculature. In particular, it provides some of the first evidence on the biological effect of ucOC directly on vascular tissue free from the influence from other systems. Overall, the results suggest that ucOC has no, or minimal, direct biological regulation of endothelial function in rabbit arteries and human endothelial cells. The majority of evidence provided by this thesis suggests that ucOC does not directly regulate blood vessel function. As such, ucOC may be targeted as a therapeutic treatment for metabolic disease without the risk of any adverse effects in the vasculature.

Future investigations into the potential regulatory role of OC are warranted. Several suggestions for future research are described below.

1. Firstly, it will be important to investigate whether changes in circulating OC influence vascular function in humans following a more aggressive treatment to suppress or increase ucOC. Studies may use a longer duration of vit K supplementation, glucocorticoid treatment, exercise or some other treatment that may modify the circulating levels of ucOC. It will be important for studies to investigate this in both healthy and clinical populations, particularly those with cardiovascular and metabolic conditions.
2. Secondly, using *ex vivo* and *in vitro* techniques that focus on pathological disease models of dysfunction and assess ucOC administration for longer durations will be important.
3. Finally, vascular calcification is a critical later stage of atherosclerosis development and is characterised by the differentiation of VSMCs into an osteoblast like phenotype which expresses bone-derived factors including OC. As

such, investigating the role of OC, in particular cOC, the isoform of OC most abundant in mineralised tissue is warranted.

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Appendices

Appendix A: Potential role for osteocalcin in the development of atherosclerosis and blood vessel disease

nutrients

MDPI

Review

Potential Role for Osteocalcin in the Development of Atherosclerosis and Blood Vessel Disease

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Received: 1 September 2018; Accepted: 29 September 2018; Published: 4 October 2018

Abstract: There is increasing evidence for the involvement of the skeleton in the regulation of atherosclerotic vascular disease. Osteocalcin, an osteoblast derived protein, exists in two forms, carboxylated and undercarboxylated osteocalcin. Undercarboxylated osteocalcin has been linked to the regulation of metabolic functions, including glucose and lipid metabolism. Features of atherosclerosis have been associated with circulating osteocalcin; however, this association is often conflicting and unclear. Therefore, the aim of this review is to examine the evidence for a role of osteocalcin in atherosclerosis development and progression, and in particular endothelial dysfunction and vascular calcification. The current literature suggests that undercarboxylated osteocalcin stimulates the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway to upregulate nitric oxide and nuclear factor kappa β (NF-κβ) in vascular cells, possibly protecting endothelial function and preventing atherogenesis. However, this effect may be mediated by metabolic factors, such as improvements in insulin signaling, rather than through a direct effect on the vasculature. Total osteocalcin is frequently associated with vascular calcification, an association that may occur as a result of vascular cells eliciting an osteogenic phenotype. Whether osteocalcin acts as a mediator or a marker of vascular calcification is currently unclear. As such, further studies that examine each form of osteocalcin are required to elucidate if it is a mediator of atherogenesis, and whether it functions independently of metabolic factors.

Keywords: undercarboxylated osteocalcin; carboxylated osteocalcin; endothelial dysfunction; vascular calcification; atherosclerosis; humans; animal models

1. Introduction

Cardiovascular disease describes a group of disorders that affect the myocardium and vasculature and is the leading cause of death worldwide [1,2]. Ischemic heart disease, or atherosclerosis, is the most common cause of cardiovascular related deaths [3,4]. The development of atherosclerosis is characterized by distinct phases, including endothelial dysfunction, intimal thickening, plaque development, and, in the chronic stage, vascular calcification [5]. Numerous risk factors are responsible for the development of atherosclerosis, including diabetes, ageing, smoking, low physical activity levels, poor diet, and family history [6,7]. Diabetes, which is characterized by hyperglycemia and insulin resistance, is a leading risk factor for the development of atherosclerosis [2]. Abnormal insulin

signaling, advanced glycation end-products, and oxidative stress caused by hyperglycemia and insulin resistance may promote the pathological interaction between diabetes and atherosclerosis [8–11].

Recently, the skeleton has been established as an endocrine organ participating in several metabolic processes, including the maintenance of circulating blood glucose and lipid levels. This bone/endocrine "cross talk" is thought to be regulated, at least in part, via osteocalcin, a vitamin K-dependent, osteoblast-derived protein [12–15]. Total osteocalcin includes both undercarboxylated osteocalcin (ucOC) and carboxylated osteocalcin (cOC). UcOC is characterized by the presence of 0–2 gamma-carboxyl groups on glutamic acid residues and is predominantly released into circulation [16]. The presence of 3 gamma-carboxyglutamic acid residues produces carboxylated osteocalcin (cOC), which has a high affinity for hydroxyapatite and is located predominantly in the bone matrix [17,18]. Vitamin K is essential for carboxylation and, as such, circulating levels of osteocalcin are used as a marker of vitamin K status [19]. Generally, ucOC makes up between 40 and 60% of total circulating osteocalcin [20–22]. The role of osteocalcin in the regulation of endocrine outcomes has been discussed in a number of recent review studies [23–27] and is thus not examined in this review.

The discovery of osteocalcin as a regulator of metabolic processes has led to investigations considering associations with cardiovascular disease. Several cross-sectional studies have demonstrated that circulating levels of total osteocalcin and ucOC are associated with both metabolic and cardiovascular disorders [28–32]. However, it is unclear whether osteocalcin has a direct role in the vasculature, independent of metabolic outcomes, or whether the association is mediated indirectly, via the metabolic effects of ucOC. Furthermore, advanced atherosclerotic plaques are characterized by the development of calcification, a process that involves a shift in vascular cells to an osteogenic phenotype [33]. Whether total osteocalcin, or one of the forms of osteocalcin, is a mediator or a marker of this process, is of interest. Osteocalcin has the potential to be a target in future therapeutic interventions for metabolic diseases such as diabetes and obesity [34]. Therefore, elucidating whether total osteocalcin and each of its forms have a biological function in the vasculature, independent from endocrine or metabolic outcomes, is of importance.

2. Atherosclerosis

Atherosclerosis typically presents in the coronary, cerebral, and peripheral arteries. The clinical manifestations are myocardial infarction, stroke, and peripheral arterial disease [35]. Each layer of the blood vessel contributes to the pathogenesis of atherosclerosis in a distinct way. For instance, the tunica intima, which is comprised primarily of a layer of endothelial cells, forms a semi-permeable barrier between the blood and the blood vessel wall [36]. Endothelial cell dysfunction is the initiating factor in atherogenesis and involves a reduction in the bioavailability of nitric oxide (NO), a major vasodilator and anti-atherogenic molecule. The reduction in NO results in an inflammatory response that is characterized by the migration of smooth muscle cells from the tunica media. The adherence of circulating molecules such as low-density lipoprotein (LDL) and cells, including leukocytes, contributes to the formation of a fatty streak [37]. Subsequently, a lipid rich plaque develops and collagen content increases. Finally, the advanced stages of atherosclerosis development are characterized by calcification and fibrosis [38]. Evidently, the phenotype of an atherosclerotic plaque alters dramatically over the life cycle of the disease. As such, it is of importance to distinguish between the stages when reporting atherosclerotic outcomes [39]. This review will focus on two distinct features of atherosclerosis, namely, the development of endothelial dysfunction and vascular calcification.

Endothelial dysfunction (vascular dysfunction) occurs in the initial stages of atherogenesis and is present throughout the life cycle of the disease. It is a predictor of future adverse cardiovascular events [40]. Normal endothelial function is regulated by a balance between anti-atherogenic (NO, endothelial derived hyperpolarizing factor, and prostacyclin) and pro-atherogenic (endothelin-1, thromboxane A2, and angiotensin II) factors [41,42]. The presence of a diseased state upsets the balance, suppressing atheroprotective factors and promoting atherogenic factors. This imbalance results in

several pathological effects, principally the inability of the endothelium to regulate vasomotor tone (vasodilation and vasoconstriction) [36,43,44]. Abnormal vasomotor tone results in damage to the endothelial cells lining the vascular wall and is associated with an inflammatory and coagulatory response [43].

Advanced atherosclerosis, characterized by vascular calcification, involves the accumulation of scattered calcium-like deposits [38,45]. The presence of calcification reduces blood vessel compliance, causes stiffening of the vasculature, and is a predictor of future adverse health outcomes [46,47]. The development process of vascular calcification is similar to the process of bone formation in the skeleton [48]. This process involves the accumulation of hydroxyapatite, the differentiation of smooth muscle cells into osteoblast-like cells, the downregulation of calcification inhibitors, and the presence of osteogenic proteins, such as Runx-2 and osteocalcin [49–51].

3. Association between Osteocalcin and Atherosclerosis Outcomes

3.1. Measurement of Osteocalcin in Humans

To date, it is not clear whether osteocalcin is biologically active during atherosclerosis development in humans [16,27]. A recent meta-analysis included 46 studies examining the association between osteocalcin and atherosclerosis outcomes. No clear relationship was reported between osteocalcin and markers of atherosclerosis and calcification [52]. The presence of atherosclerosis was examined via several methods, including aortic calcification score (ACS), coronary artery calcification score, pulse wave velocity (PWV), and carotid intima-media thickness (C-IMT). Conflicting results were reported across all the outcomes. For example, in the studies reporting the association between osteocalcin and C-IMT, four reported that higher osteocalcin levels were associated with a higher C-IMT, four reported that higher osteocalcin levels were associated with a lower C-IMT, and three reported no correlation. Interestingly, all studies that examined osteocalcin-positive mononuclear cells or completed histological staining for osteocalcin reported that higher osteocalcin levels were associated with increased markers of atherosclerosis and calcification [52]. This suggests that osteocalcin may be present in atherosclerosis, although, whether it has a regulatory function is not clear.

The conflicting outcomes presented in the meta-analysis may be due to a number of limitations. First, total osteocalcin was reported in 43 of the studies, whereas some also included ucOC and cOC, reported in 7 studies and 1 study, respectively. This is a major limitation as each form has a distinct function [53,54]. Furthermore, different techniques were used to analyze osteocalcin. For example, total osteocalcin has been measured with enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), as well as flow cytometry and immunostaining, which may explain the different findings.

Taken together, the meta-analysis did not provide a clear association between osteocalcin and atherosclerosis. Consequently, the remainder of this review will focus on experimental studies that report the effect or expression of osteocalcin, specifically examining if each form of osteocalcin has distinct functions.

3.2. In Vivo Osteocalcin Treatment and Cardiovascular Function in Animal Models

The biological effects of osteocalcin are not fully known. A small number of studies have examined the effect of total osteocalcin administration in vivo on the cardiovascular system in animal models of disease. Tail cuff blood pressure (BP) was measured in apolipoprotein E-deficient ($ApoE^{-/-}$) mice, and the animals received daily injections of total osteocalcin (30 ng/g) for 12 weeks [55]. Total osteocalcin treatment had a protective effect on high-fat-diet-induced hypertension by reducing mean and diastolic BP by ~5 and 7 mmHg, respectively. Similar trends occurred for systolic BP, which was decreased by ~3 mmHg. Although an improvement in blood pressure occurred, there was also a concomitant improvement in body weight, fasting blood glucose levels, glucose tolerance, circulating lipids, and markers of inflammation in animals receiving osteocalcin treatment [55]. As such,

it is not clear whether the observed improvements were due to a direct effect of osteocalcin on the cardiovascular system, or indirectly via the improvement in the animals' metabolic profile.

In another study, the administration of total osteocalcin repaired the alteration to PWV, concurrently with improvements in fasting blood glucose and circulating lipids [56]. Specifically, PWV, which is a measure of arterial stiffness, was increased (by 6%) in a rat model fed a high-fat diet and induced with diabetes via streptozotocin injection. Following 12 weeks of a high-fat diet, daily intraperitoneal injections of total osteocalcin (30 ng/g) reversed the alteration in PWV, albeit by a small magnitude. Blood pressure, heart rate, and mean arterial pressure were not altered by the high-fat diet or the osteocalcin injections [56]. This study suggests that a daily injection of total osteocalcin can repair abnormal cardiovascular function (Table 1). However, whether the protective effect occurred directly, or as a result of improved metabolic outcomes, is still not clear.

Table 1. Summary of studies examining the effects of in vivo osteocalcin treatment on vascular function outcomes in animals.

First Author, Year [Ref.]	Experimental Overview	Measurement of Vascular Function	Results
Dou, 2014 [55]	ApoE$^{-/-}$ mice received ND or HFD and treatment daily for 12 weeks with vehicle or total osteocalcin (30 ng/g)	BP, heart rate, and isometric myography	In vivo: mean and diastolic BP normalized by osteocalcin treatment in HFD group, no change in systolic BP or heart rate. Ex vivo: 20% improvement in relaxation in osteocalcin-treated mice on HFD
Huang, 2017 [56]	Sprague Dawley rats induced with diabetes via STZ injection and received ND or HFD, daily treatment of vehicle or total osteocalcin (30 ng/g) for 12 weeks	BP, PWV, heart rate, pulse pressure, and mean arterial pressure	PWV normalized in osteocalcin-treated rats with diabetes compared to diabetic rats treated with vehicle, no change in BP, heart rate, mean arterial pressure, and pulse pressure
Kondo, 2016 [57]	Wild type C57BL/6 mice received HFD and treated 5 times a week for 10 weeks with vehicle or ucOC (30 ng/g)	Nitric oxide production	Increased nitric oxide concentration in ucOC-treated mice compared to vehicle-treated mice
Zhou, 2013 [58]	C57BL/6J mice received ND or HFD for 8 weeks with daily injections of vehicle or ucOC (30 ng/g)	Autophagy and ER stress	Autophagy and ER stress attenuated in mice receiving ucOC

ApoE = apolipoprotein E, HFD = high -fat diet, ND = normal chow diet, STZ = streptozotocin, ucOC = undercarboxylated osteocalcin, BP = blood pressure, PWV = pulse wave velocity, ER = endoplasmic reticulum.

3.3. In Vivo Osteocalcin Treatment and Markers of Atherosclerosis Risk in Animal Models

Isometric/isotonic myography is an ex vivo technique used to examine endothelial function directly, independent of factors such as sheer stress and circulation hormones. This technique was used to examine the function of the thoracic aorta in ApoE$^{-/-}$ mice following 12 weeks on a high-fat diet, receiving simultaneous daily injections of total osteocalcin (30 ng/g) or vehicle [55]. Vehicle-treated mice had a reduction in endothelial function by 20%, a pathological effect that was attenuated in the mice receiving osteocalcin treatment. An examination of the mechanisms revealed that co-incubation with N^{G}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, blocked the relaxation of all groups. However, co-incubation with sodium nitroprusside (SNP), a nitric oxide donor that is endothelium-independent, resulted in a similar relaxation between total osteocalcin-treated and non-treated tissue. The relaxation of all vessels to SNP demonstrates that the high-fat diet or total osteocalcin does not affect the ability of vascular smooth muscle cells to respond to nitric oxide [55]. Thus, total osteocalcin appears to have a protective effect on endothelial function that may assist in the prevention of atherosclerosis.

Whether one or both forms of osteocalcin were responsible for this effect is unclear. As a result, each form of osteocalcin was administered to female wild type C57BL/6 mice, to determine the effect on nitric oxide availability. Treatment with ucOC (30 ng/g), but not an equivalent dose of cOC, increased serum nitric oxide, providing further evidence that it is the bioactive form of the protein, at least in mice [57]. Subsequently, ucOC (30 ng/g) was administered via intraperitoneal injection 5 times per week for 10 weeks into mice fed an atherogenic (F2HFD1) diet. The diet did not induce the development

of atherosclerotic plaques, but it did increase total cholesterol, LDL, and LDL/high density lipo-protein (HDL) ratio, all of which are associated with an increased risk of atherosclerosis. UcOC administration significantly lowered all the lipid markers and produced a 1.7-fold increase in serum nitric oxide bioavailability compared to saline-treated mice [57]. The improvement in lipid markers and serum nitric oxide availability would likely assist in the prevention of atherosclerosis development.

Furthermore, eight weeks of daily ucOC (30 ng/g) treatment following a high-fat diet produced an improvement in insulin signaling and a reduction in autophagy and ER stress in the aorta of C57BL/6J mice [58]. Several markers of autophagy (Atg7, p62, and light chain 3 II (LC3-II)) and ER stress (protein kinase-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2α (eIF2α)) were increased in the high-fat diet-fed mice. However, the administration of ucOC following the high-fat diet attenuated the pathologic autophagy and ER stress marker response. Moreover, insulin resistance was detected in the high-fat-diet-fed mice, as measured by a reduction in the phosphorylation of insulin receptor β (IRβ) subunit tyrosine 1162/1163 and protein kinase B (Akt) Ser-473. An improvement in insulin signaling in ucOC-treated mice, as seen by an increase in the IRβ subunit and Akt Ser-473 phosphorylation, demonstrates that ucOC rescues high-fat-diet-induced insulin resistance in mouse aorta [58].

In summary, in vivo ucOC treatment protects vascular function and pathological disease markers that often contribute to or are involved in the development of atherosclerosis (Table 1). However, the protective effects of ucOC on the vasculature are often associated with improved metabolic outcomes, such as improvements in insulin signaling or lipid markers. As such, in vitro studies are needed to confirm (1) whether osteocalcin and its forms are acting directly on vascular tissue, and (2) that ucOC is the active form of osteocalcin mediating these effects.

4. Osteocalcin and Endothelial Function

4.1. In Vitro Osteocalcin Treatment in Human Cells

The endothelium has an important role in maintaining vascular homeostasis because it mediates the release of a number of regulatory factors [41]. Molecular signaling mechanisms that regulate vascular function have been examined in several studies to determine if there is a direct link between osteocalcin and atherosclerosis development (Table 2).

Human umbilical vein endothelial cells (HUVECs) cultured with total osteocalcin (10–150 ng/mL) displayed a dose-dependent upregulation of Akt and endothelial nitric oxide synthase (eNOS) phosphorylation—up to 100 ng/mL of total osteocalcin [55]. Akt is a common protein kinase involved in numerous cellular signaling pathways, including the phosphorylation of eNOS via serine1177; eNOS synthesizes NO [59]. When treated with 100 ng/mL of total osteocalcin, Akt and eNOS phosphorylation increased, peaking at 1 h and 2 h following treatment, respectively [55]. Of note, the properties of HUVECs are not ubiquitous to all endothelial cells, therefore the findings cannot be directly associated with adult endothelial function [60]. Despite this, similar results have been reported in human aortic endothelial cells (HAECs). HAECs incubated with ucOC or cOC for 30 min resulted in an increase in eNOS phosphorylation in cells treated with 25 and 100 ng/mL of ucOC by ~1-fold and ~2.5-fold, respectively. However, equivalent doses of cOC had no effect [57]. Similarly, eNOS phosphorylation and nitric oxide secretion were increased in a dose-dependent manner between 0.3–30 ng/mL of ucOC treatment in HAECs [61]. Mechanistic investigation demonstrated that the phosphorylation of Akt/eNOS by ucOC was inhibited by the addition of wortmannin, an inhibitor of phosphoinositide 3-kinase (PI3K), which is the protein kinase responsible for phosphorylating Akt [61].

Taken together, these results indicate that ucOC may upregulate nitric oxide synthesis via the activation of the PI3K/Akt/eNOS signaling pathway in human endothelial cells, which may have a protective effect against endothelial dysfunction. Again, these findings support previous research suggesting that ucOC is the biologically active form of the protein. UcOC may have a protective

function in the vasculature, independent from its influence on metabolic outcomes, however further studies are required to confirm this (Figure 1).

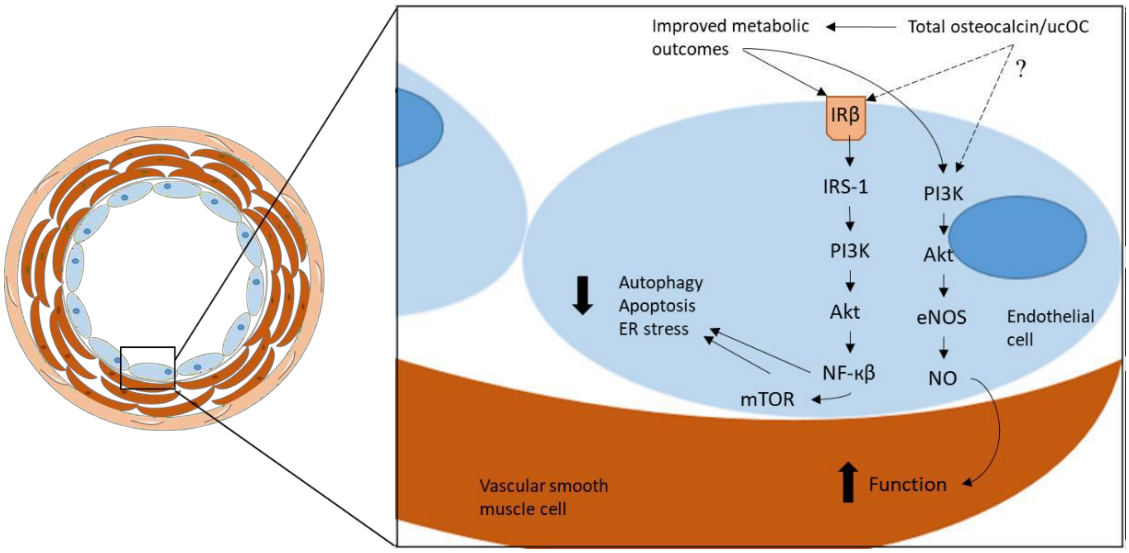

Figure 1. The proposed mechanism through which total osteocalcin/ucOC has been reported to elicit atheroprotective functions in vascular cells. By improving metabolic outcomes, total osteocalcin/ucOC reduces pathological mechanisms, including autophagy, apoptosis, and ER stress, through the β-subunit of the insulin receptor (IRβ) and via the IRS-1/PI3K/Akt/NF-κβ/mTOR signaling pathway. Vascular function is improved via the PI3K/Akt/eNOS signaling pathway which stimulates NO in the smooth muscle cells. ucOC = undercarboxylated osteocalcin, PI3K = phosphoinositide 3-kinase, Akt = protein kinase B, eNOS = endothelial nitric oxide synthase, NO = nitric oxide, IRβ = insulin receptor β, IRS-1 = insulin receptor substrate 1, NF-κβ = nuclear factor kappa β, mTOR = mammalian target of rapamycin, ER = endoplasmic reticulum.

Several pathological mechanisms promote the development of endothelial dysfunction and atherosclerosis, including elevated apoptosis and endoplasmic reticulum (ER) stress. UcOC (30 ng/mL) treatment prior to the administration of linoleic acid, which acts as a free fatty acid, inhibited the induction of apoptosis in HAECs via the PI3K/Akt pathway [61]. Additionally, HUVECs exhibited ER stress and insulin resistance when incubated with tunicamycin; however, co-incubation with ucOC (5 ng/mL) for 4 h reduced the ER stress and increased the phosphorylation of insulin receptor substrate 1 (IRS-1), a molecule involved in insulin signal transduction [62]. The co-incubation of wortmannin and Akti−1/2 (an Akt inhibitor) blocked the insulin sensitizing effect of ucOC. However, U0126 (a mitogen-activated protein kinase (MAPK) inhibitor) did not block the effect of ucOC. Additionally, nuclear factor kappa β (NF-κβ), a key cellular signaling molecule, which was suppressed by tunicamycin, exhibited a normalization when co-incubated with ucOC. Furthermore, the inhibition of NF-κβ signaling and the silencing of the NF-κβ p65 gene confirmed that NF-κβ was involved in the regulation of ER stress and insulin signaling by ucOC [62]. The results from this study suggest that ucOC suppresses ER stress via the PI3K/Akt/NF-κβ signaling pathway and that improved insulin sensitivity initiates this response.

Collectively, it appears that ucOC may elicit an atheroprotective effect in human endothelial cells. The protective effects of ucOC often occurred through improved insulin signaling or in the presence of high lipid content. However, ucOC also produced a protective effect in endothelial cells without the presence of any metabolic mediators, suggesting that osteocalcin may have a direct bioactive influence in human endothelial cells.

Table 2. Summary of cell culture studies examining the effects of in vitro osteocalcin treatment in human and animal vascular cells.

First Author, Year [Ref.]	Experimental Overview	Outcomes	Results
Dou, 2014 [55]	HUVECs incubated with total osteocalcin (10–150 ng/mL) for 15 min–2 h. Descending aorta of ApoE$^{-/-}$ mice, previously treated with osteocalcin, incubated with LY294002 (10 μmol/L) and Akt inhibitor V (5 μmol/L)	eNOS, Akt, and PI3K phosphorylation and expression	Max phosphorylation of eNOS and Akt with 100 ng/mL of osteocalcin. Max phosphorylation of eNOS and Akt occurred after 1 h and 2 h, respectively. In aorta, PI3K, Akt, and eNOS phosphorylation and expression increased, inhibited with LY294002 and Akt inhibitor V
Kondo, 2016 [57]	HAECs incubated with ucOC (5, 25, and 100 ng/mL) and cOC (25 and 100 ng/mL) for 30 min	eNOS phosphorylation	Incubation of ucOC increased eNOS phosphorylation in a dose-dependent manner, cOC had no effect
Jung, 2013 [61]	HAECs incubated with ucOC (0.3–30 ng/mL), linoleic acid (100 μmol/L for 16 h), and wortmannin (100 nmol/L for 15 min)	Nitric oxide concentration, eNOS and Akt phosphorylation and apoptosis	UcOC increased eNOS and Akt phosphorylation and nitric oxide concentration, which was inhibited by wortmannin. UcOC attenuated linoleic acid-induced apoptosis
Guo, 2017 [62]	HUVECs incubated with ucOC (5 ng/mL for 4 h), tunicamycin (5 μg/mL for 4 h), insulin (10 nM for 10 min), wortmannin, and Akti-1/2 (10 μM for 4 h)	Insulin resistance, ER stress	UcOC blocked ER stress and insulin resistance, which was inhibited by wortmannin and Akti-1/2
Zhou, 2013 [58]	Mouse VECs and VSMCs incubated with tunicamycin (5 μg/mL for 4 h), ucOC (5 ng/mL for 0, 2, 4, and 8 h), Akti-1/2 (10 μM for 4 h) and rapamycin (10 nM for 4 h)	Autophagy and ER stress	UcOC attenuated autophagy and ER stress in mouse VECs and VSMCs, which was inhibited by Akti-1/2 and rapamycin

HUVECs = human umbilical vein endothelial cells, ApoE = apolipoprotein E, HAECs = human aortic endothelial cells, ucOC = undercarboxylated osteocalcin, VECs = vascular endothelial cells, VSMCs = vascular smooth muscle cells, eNOS = endothelial nitric oxide synthase, Akt = protein kinase B, PI3K = phosphoinositide 3-kinase, ER = endoplasmic reticulum.

4.2. In Vitro Osteocalcin Treatment and Markers of Atherosclerosis Risk in Animal Cells

Animal studies have also been used to examine the effect of osteocalcin in vascular tissue and cells. Experiments using cultured aortic strips obtained from ApoE$^{-/-}$ mice revealed that total osteocalcin treatment increased the phosphorylation and expression of PI3K, Akt, and eNOS. Furthermore, the phosphorylation of Akt and eNOS was blocked by the co-incubation of LY294002 and Akt inhibitor V, which inhibit the signaling of PI3K and Akt, respectively [55]. UcOC (5 ng/mL) incubations in mouse vascular endothelial cells (VECs) and vascular smooth muscle cells (VSMCs) protected against tunicamycin-induced autophagy and ER stress. The protective effect was mediated through the Akt/mammalian target of rapamycin (mTOR) signaling pathway as a result of NF-κβ activation [58]. Similar results in cells from other organs revealed that these effects may be systemic. For example, ER stress and insulin resistance were both alleviated in L6 muscle cells when treated with 5 ng/mL of ucOC for 4 h [63].

Overall, total osteocalcin and ucOC appear to protect against the development of atherosclerosis through the activation of several signaling pathways (Table 2). NO is likely increased via the activation of the PI3K/Akt/eNOS signaling pathway, which would result in an improvement in endothelial function. However, increased eNOS expression can also enhance endothelial dysfunction by increasing eNOS uncoupling and oxidative stress [64,65].

Further studies are needed to determine if the upregulation of eNOS prevents or enhances endothelial dysfunction. Additionally, pathological abnormalities such as increased endothelial cell apoptosis and ER stress, which contribute to the development of atherosclerosis, are abrogated by ucOC treatment, likely acting through PI3K/Akt/NF-κβ/mTOR signaling. Whether these effects occur exclusively through metabolic signaling pathways, or if ucOC has a direct biological effect in the vasculature requires further investigation.

5. Osteocalcin and Vascular Calcification

5.1. Osteocalcin and Calcified Human Vascular Tissue

Several osteogenic factors, including the osteoblastic "master regulator" transcription factor Runx2, have been shown to be expressed by smooth muscle cells located at the site of vascular calcified plaques [66]. The expression of such factors may play a role in the osteogenic potential of these smooth muscle cells. In addition, osteocalcin, which has been used clinically as a marker of bone formation, has also been found at these sites [67]. It has thus been posited that osteocalcin may play a role in the calcification of plaques, although whether this is true, or whether the association is coincidental, requires further investigation.

Advanced atherosclerotic plaques are characterized by arterial stiffening, resulting in a reduction in vessel compliance; this occurs as a consequence of vascular calcification [68]. Vascular calcification increases the risk of adverse cardiovascular events, including aortic stenosis, reduced vasomotor tone, and plaque instability [46]. Since the early 1980s, osteocalcin has been detected to a larger degree in calcified plaques and aortic valves than in non-calcified and healthy vessels [69,70]. In fact, in vessels obtained from men and women during autopsy, the concentration of total osteocalcin in calcified vascular plaques was considerably higher (50.9 ng/mL) than the concentration present in fatty streaks and fibrous plaques (1.1 ng/mL) and normal aortic tissue (0.33 ng/mL) [69]. Furthermore, a study a decade later reported that the level of osteocalcin mRNA was increased between 8- and 14-fold in calcified plaques and aorta compared to healthy aorta [71]. These findings predate the hypothesis that osteocalcin may have a role in vascular function, yet demonstrate that the concentration of total osteocalcin is positively correlated to the stage of atherosclerotic plaque progression. It is possible that the increase in total osteocalcin occurs as a result of atherosclerotic plaques developing an osteogenic phenotype [33], yet this requires further validation.

Vascular smooth muscle cells (VSMCs) are responsible for the development of atherosclerotic calcification by differentiating into osteoblast-like cells [50]. Cultured VSMCs in the initial stages of atherosclerosis formation undergo a downregulation of proteins that inhibit mineralization, leading to a shift in the VSMCs to an osteo/chondrocytic phenotype. This change is characterized by an increase in transcription factors regulating the expression of osteogenic proteins, including total osteocalcin [67]. Furthermore, total osteocalcin was shown to be minimally expressed in human aortic VSMCs cultured to mimic the early stages of atherosclerosis [72]. In situ hybridization analysis demonstrated that osteocalcin expression was significantly increased in lipid-rich, calcified VSMCs obtained from the media and intima of explanted human aorta, compared to VSMCs without plaque development [73]. Modification of lipid content by treating VSMCs for 28 days with acylated low-density lipoprotein did not alter osteocalcin expression, despite a 3-fold increase in calcification. Interestingly, incubation of lipoprotein-deficient serum for 28 days in VSMCs was associated with an inhibition of calcium formation, but an upregulation of osteocalcin expression [73]. This study demonstrates that VSMCs developing an osteogenic phenotype modify the expression of total osteocalcin and are influenced by the presence of lipids.

Overall, osteocalcin expression is increased in calcified lesions throughout the intimal and medial layers of the vascular wall (Table 3). Whilst the increase in osteocalcin is often associated with the development of an osteogenic phenotype, it is also associated with lipids. Of note, no studies have differentiated between the forms of osteocalcin when discussing vascular calcification. Consequently, an examination of ucOC and cOC and an investigation into the mechanisms that are associated with the increase in osteocalcin in calcified tissue are required.

Table 3. Summary of studies examining the interaction between osteocalcin and calcification in human and animal tissue and cells.

First Author, Year [Ref.]	Experimental Overview	Outcomes	Results
Levy, 1983 [69]	Human aortic and valve tissue	Osteocalcin and Gla levels	Osteocalcin and Gla levels higher in calcified tissue than in non-calcified tissue
Levy, 1980 [70]	Human aortic and valve tissue	Gla levels	Higher Gla levels in calcified aorta and valves than non-calcified tissue
Fleet, 1994 [71]	Human aortic tissue	Osteocalcin mRNA levels	Osteocalcin mRNA increased in calcified aorta and plaque compared to non-calcified aorta
Tyson, 2003 [67]	Human aortic and carotid tissue	Osteocalcin expression	Calcified vessels had an increase in the expression of osteocalcin
Severson, 1995 [72]	Cultured human aortic VSMCs	Immunostaining for osteocalcin	Minimal immunostaining of human VSMCs
Proudfoot, 2002 [73]	Cultured human aortic VSMCs with lipid content modification	Osteocalcin expression	Osteocalcin expression increased in calcified cells compared to non-calcified cells, which was altered with the modification of lipid content
Murshed, 2004 [74]	$MGP^{-/-}$ mice inter-crossed with pSM22α-Osteocalcin	Mineralization of aorta	Osteocalcin gain of function model did not inhibit the mineralization of mouse aorta
Pal, 2010 [75]	$OPG^{+/+}$ and $OPG^{-/-}$ mice	Calcification and mononuclear cells expressing osteocalcin	Increased calcification in $OPG^{-/-}$ mice, which was associated with an increased percentage of osteocalcin positive mononuclear cells
Morony, 2008 [76]	$Ldlr^{-/-}$ mice fed HFD for 5 months and treated with OPG	Calcification, osteocalcin mRNA and circulating levels	Osteocalcin mRNA levels were unchanged, circulating osteocalcin increased over the 5 months, which was associated with calcification
Akiyoshi, 2016 [77]	Thoracic aorta of C57BL/6 mice cultured to induced calcification	Osteocalcin expression	Osteocalcin expression increased in calcified thoracic aortas
Idelevich, 2011 [78]	Cultured MOVAS cells induced with calcification and overexpressed with osteocalcin. Sprague Dawley rats induced with calcification	Mineralization, osteocalcin mRNA, metabolic signaling pathways	In vitro: overexpression of osteocalcin in MOVAS cells associated with mineralization and upregulation of insulin signaling In vitro: osteocalcin mRNA is increased in calcified vasculature and associated with activation of metabolic signaling pathways

VSMCs = vascular smooth muscle cells, MGP = matrix Gla protein, OPG = osteoprotegerin, ldlr = low-density lipoprotein receptor, HFD = high-fat diet, MOVAS = mouse vascular smooth muscle cells.

5.2. *Vascular Calcification and Osteocalcin in Animal Models*

Vascular homeostasis is regulated by a tight balance between pro- and anti-mineralizing osteogenic factors. Several of these regulatory factors include matrix Gla protein (MGP), osteoprotegerin (OPG), fetuin A, and inorganic phosphate [79]. MGP and osteocalcin belong to the same family of mineral-binding Gla proteins, both characterized by vitamin K-dependent post translational y-carboxylation, and MGP is a known inhibitor of vascular calcification [80]. MGP-deficient mice are characterized by the development of calcified aortas and early death due to vascular thrombosis and hemorrhage [74,81]. MGP knockout mice were inter-crossed with pSM22α-Osteocalcin to create an osteocalcin gain-of-function model. Four-week-old pSM22α-Osteocalcin mice exhibited no difference in mineralization from the MGP knockout model, despite a 6- to 8-fold increase in serum osteocalcin concentration [74]. The results from this study demonstrate that total osteocalcin has no anti-mineralization effect.

Osteoprotegerin (OPG) is a circulating bone marker that functions to inhibit osteoclast activity [82]. OPG-deficient mice are characterized by osteoporosis [83]. Interestingly, OPG-deficient mice are associated with an increase in vascular calcification, suggesting that OPG may have a protective role in vascular calcification [75,83]. In a low-density lipoprotein receptor (ldlr) knockout mouse model treated with OPG and fed a high-fat diet, circulating total osteocalcin was reduced compared to vehicle-treated mice after two and five months. This was associated with the development of significantly less calcified lesions, suggesting that lower circulating total osteocalcin is correlated with a reduction in mouse aortic calcification [76]. Furthermore, cultured thoracic aorta of C57BL/6 mice revealed that increased calcification was positively correlated with an increase in osteocalcin expression [77]. As such, it appears that total osteocalcin is increased in animal tissue undergoing calcification, which may result from the differentiation of VSMCs to an osteogenic phenotype. Whether

this association results in a biological effect, either protective or negative, cannot be determined from these studies.

A recent study reported that total osteocalcin stimulates glucose metabolism in vascular cells via hypoxia-inducible factor 1α (HIF-1α), a process that also results in the increase of vascular calcification [78]. Specifically, osteocalcin overexpression and treatment upregulated insulin signaling and the expression of glucose transporters through the increase in HIF-1α in mouse vascular smooth muscle cells (MOVAS). Furthermore, MOVAS cultured in DMEM medium for 21 days developed mineralized nodules and shifted to an osteogenic phenotype, a process that increases the expression of osteocalcin [78]. In an in vivo rat model of vascular calcification, total osteocalcin mRNA was positively associated with the presence of calcification. Immunohistochemistry analysis demonstrated similar expression patterns between total osteocalcin and the hypoxia-inducible factor 1α (HIF-1α) protein. Moreover, the silencing of osteocalcin RNA prevented HIF-1α stabilization and inhibited HIF-1α expression, which resulted in a reduction of calcification and a suppression of osteochondrogenic differentiation [78]. Altogether, this study demonstrates that total osteocalcin activates HIF-1α to upregulate glucose transport and utilization. The alteration to glucose metabolism stimulates osteogenic differentiation and promotes vascular calcification.

To date, there are a limited number of studies that examine the biological effect of osteocalcin on vascular calcification. A number of different methodologies and models of calcification have been used, and importantly, no studies distinguished between each form of osteocalcin. Yet, most studies demonstrate that osteocalcin expression and concentration increase with the degree of calcification. However, the increase in total osteocalcin expression may be due to the fact that VSMCs differentiate into an osteogenic phenotype and produce osteocalcin. Further investigation is needed to determine if osteocalcin is a mediator or a marker of vascular calcification.

6. The Putative Osteocalcin Receptor: GPRC6A

Osteocalcin's receptor in the vasculature is yet to be identified. The G protein-coupled receptor family C, group 6, subtype A (GPRC6A) has been suggested as the putative receptor for osteocalcin in several tissues [84–86]. Previous studies have identified GPRC6A as the osteocalcin receptor in the testes [87], β-cells [88], and skeletal muscle [89,90] of mice. In humans, GPRC6A has been identified as the osteocalcin receptor in the testes [91,92]. However, several studies report that ucOC does not activate this G-protein [93,94].

There is minimal evidence regarding the role of GPRC6A as the receptor for osteocalcin in the vasculature. GPRC6A is present in the vasculature of rats [95], but whether osteocalcin interacts with this receptor is presently unknown. Overall, future studies should aim to elucidate whether GPRC6A is the receptor for osteocalcin in the vasculature, and also examine whether ucOC affects the MAPK pathway in human vascular cells.

7. Future Directions

The current review highlights that to date, most of the evidence for a link between osteocalcin and atherosclerosis and blood vessel function focused on total osteocalcin and, to a lesser extent, its forms (cOC and ucOC). Therefore, future studies should focus on the effect of each form of ucOC on atherosclerosis, blood vessels, and vascular calcification. Furthermore, it will be important to identify if there is a direct effect of ucOC on the vasculature or whether the interaction is indirect via an improvement in glucose regulation and glycemic control. As such, it is important to identify off-target effects.

8. Summary and Conclusions

The aim of this review was to examine the biological functions of osteocalcin and its forms throughout the atherosclerosis disease process and to determine whether it functions independently of metabolic outcomes. It appears that total osteocalcin and ucOC have the potential to improve

endothelial function and reduce the pathological mechanisms that promote the development of atherosclerosis. This effect is often a result of improved metabolic outcomes; however, whether there is a direct osteocalcin/vascular interaction is yet to be fully elucidated. Further, the effect of osteocalcin on vascular calcification is unclear. In most cases, calcification was associated with the presence of total osteocalcin, which increased relative to the degree of calcification. However, whether it is a mediator or a marker of vascular calcification requires further investigation.

In conclusion, there is evidence to suggest that a cross-talk exists between the skeleton and the vascular system, which is associated with aspects of atherogenesis. Further research is required to determine whether total osteocalcin or each of its forms has a direct effect on the vasculature, independent from other systems.

Author Contributions: Conceptualization, A.T. and I.L.; Investigation, A.T.; Writing-Original Draft Preparation, A.T.; Writing-Review and Editing, A.T., T.Q., T.B-S., A.H., A.Z. and I.L.; Funding Acquisition, I.L.

Funding: This research received no external funding. The APC was funded by I.L. research budget.

Acknowledgments: Itamar Levinger was supported by a Future Leader Fellowship (ID: 100040) from the National Heart Foundation of Australia.

Conflicts of Interest: The authors declare no conflict of interest.

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Appendix B: The effect of an atherogenic diet and acute hyperglycaemia on endothelial function in rabbits is artery specific

nutrients

MDPI

Article

The Effect of an Atherogenic Diet and Acute Hyperglycaemia on Endothelial Function in Rabbits Is Artery Specific

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Received: 29 May 2020; Accepted: 15 July 2020; Published: 16 July 2020

Abstract: Hyperglycaemia has a toxic effect on blood vessels and promotes coronary artery disease. It is unclear whether the dysfunction caused by hyperglycaemia is blood vessel specific and whether the dysfunction is exacerbated following an atherogenic diet. Abdominal aorta, iliac, and mesenteric arteries were dissected from New Zealand White rabbits following either a 4-week normal or atherogenic diet (n = 6–12 per group). The arteries were incubated ex vivo in control or high glucose solution (20 mM or 40 mM) for 2 h. Isometric tension myography was used to determine endothelial-dependent vasodilation. The atherogenic diet reduced relaxation as measured by area under the curve (AUC) by 25% ($p < 0.05$), 17% ($p = 0.06$) and 40% ($p = 0.07$) in the aorta, iliac, and mesenteric arteries, respectively. In the aorta from the atherogenic diet fed rabbits, the 20 mM glucose altered EC_{50} ($p < 0.05$). Incubation of the iliac artery from atherogenic diet fed rabbits in 40 mM glucose altered EC_{50} ($p < 0.05$). No dysfunction occurred in the mesentery with high glucose incubation following either the normal or atherogenic diet. High glucose induced endothelial dysfunction appears to be blood vessel specific and the aorta may be the optimal artery to study potential therapeutic treatments of hyperglycaemia induced endothelial dysfunction.

Keywords: atherosclerosis; nitric oxide; nitrative stress; diabetes; immunohistochemistry

1. Introduction

Type 2 diabetes is a major risk factor for cardiovascular complications, including atherosclerosis and subsequently coronary artery disease (CAD) [1,2]. Whilst diabetes and CAD can occur independently, diabetes often accelerates atherosclerosis development, increasing the risk of adverse cardiovascular events such as myocardial infarction [1]. The devastating effect of diabetes on the vascular system is caused, in part, by hyperglycaemia, which is characterised by toxic levels of circulating blood glucose [3,4].

Endothelial dysfunction is the first detectable sign of atherogenesis [5] and is a significant predictor of future cardiovascular events [6]. The impairment of nitric oxide (NO) mediated endothelial dependant vasodilation is a hallmark and one of the earliest indications of endothelial dysfunction [5]. Hyperglycaemia promotes endothelial dysfunction via a number of pathways, each of which are associated with a common link, the generation of reactive oxygen species (ROS), and oxidative/nitrative stress [7]. Specifically, hyperglycaemia induced mitochondrial electron transport system overproduction

of superoxide binds with NO to produce peroxynitrite, reducing the bioavailability of NO and promoting endothelial dysfunction [8,9].

Acute elevations in circulating blood glucose, such as that which occurs in the post-prandial state, are a major risk factor for diabetes-induced endothelial dysfunction [10,11], perhaps more so than fasting blood glucose and haemoglobin A1c (HbA1c) [12]. A number of studies have reported that acute (2 to 6 h) ex vivo high glucose incubations can reduce endothelial-dependent vasodilation in arteries of rabbits [13–15] and rats [16–19]. However, no previous studies have completed high glucose incubations following a diet that mimics an atherosclerotic milieu, which is important to understand the effects of acute hyperglycaemia in a disease state. Furthermore, a study from our laboratory has shown that different vascular beds (thoracic aorta, renal, carotid, and iliac arteries) respond differently to hormonal stimulus, indicating that vascular beds are not homogeneous in their responses [20].

As such, the aim of this study was to determine if acute ex vivo high glucose incubations would impair endothelial function in aorta, iliac, and mesenteric arteries and whether the impairment would be exacerbated by an atherogenic diet. We hypothesised that high glucose incubations would reduce endothelium-dependant relaxation and that the impairment would be aggravated following an atherogenic diet.

2. Materials and Methods

2.1. Ethical Approval

This study was approved by the Victoria University Animal Ethics Committee (#14/005) and complied with the Australian National Health and Medical Research Council code for the care and use of animals for scientific purposes (8th edition).

2.2. Animal Model

Male New Zealand White rabbits (n = 6–12) at 3 months of age were randomly allocated into two groups and were fed a normal chow diet (Specialty Feeds, Glen Forrest, WA, Australia) or an atherogenic diet (a normal diet combined with 1% methionine, 0.5% cholesterol, and 5% peanut oil; SF00-218, Specialty Feeds, Glen Forrest, WA, Australia) for 4 weeks [21]. The animals were housed in separate cages on a 12 h light/dark cycle at a constant temperature of 21 °C. Food and water were supplied *ad libidum*.

2.3. Isometric Tension Myography

Following the 4-week diet, the rabbits were sedated with medetomidine (0.25 mL/kg), anaesthetised with 4% isoflurane, and exsanguinated via severing the inferior vena cava. The arterial system was immediately flushed with ice cold Krebs ((mM) 118 NaCl; 4.7 KCl; 1.2 $MgSO_4$▪$7H_2O$; 1.2 KH_2PO_4; 25 $NaHCO_3$; 1.25 CaCl and 11.7 glucose). The abdominal aorta (2 to 3 cm below the diaphragm), external iliac artery (immediately after the aortic bifurcation), and main mesenteric artery were excised, cleaned of connective tissue and fat, and cut into 3 mm rings. Blood vessel reactivity was measured via an isometric tension organ bath system (Zultek Engineering, Melbourne, Australia), as previously described [22,23]. Briefly, each vessel was incubated in physiological Krebs solution warmed to 37 °C and bubbled with 95% oxygen and 5% carbon dioxide. Following 30 min acclimatisation, the rings were strung up between 2 metal hooks attached to a force transducer to measure the tension of the vessel. Each vessel was passively stretched to a tension comparative to its size—the abdominal aorta to 2 g, the iliac artery to 1 g, and the mesenteric artery to 0.5 g. After 30 min, the vessels were again stretched to their respective tension for a further 30 min. Subsequently, the vessels were incubated in Krebs (11 mM glucose) or high glucose Krebs (20 mM or 40 mM glucose). Vasodilation of blood vessels in 11 mM glucose has previously been shown to cause relaxation equivalent to incubation in 5 mM glucose [14]. The respective Krebs solutions were refreshed every 30 min and incubated for a total of 2 h. Following the incubation, blood vessels were pre-contracted with 3×10^{-7} M phenylephrine

(aorta and iliac artery) or 3×10^{-7} M cirazoline (mesenteric artery). Once the contraction reached a plateau, endothelium-dependant vasodilation was determined via a cumulative dose response curve to acetylcholine (ACh) in half-log increments (10^{-8} M to 10^{-5} M). Maximal relaxation (E_{max}) was determined as the maximal dilation below the phenylephrine/cirazoline plateau. The log dose of ACh that produced half the maximal relaxation was reported as EC_{50}. The area under the curve (AUC) was determined as the total area of relaxation below the phenylephrine/cirazoline plateau. Endothelial dysfunction was considered when there was an alteration to one or a combination of E_{max}, EC_{50}, and AUC that represented a reduction in the vasodilation of the blood vessels. All chemicals and reagents were supplied by Sigma Aldrich, St. Louis, MO, USA unless otherwise specified.

2.4. Immunohistochemistry (IHC)

The blood vessel rings were placed into 4% paraformaldehyde, left overnight, and then transferred into 1× phosphate buffered saline (PBS) at 4 °C. This was followed by paraffin processing (Microm STP120, Thermo Scientific, Waldorff, Germany) and embedding in paraffin blocks. Sections were cut at 5 µm, deparaffinised in xylene, rehydrated, and blocked with 1% goat serum in 10 mm TrisCl (pH 7.4) for 20 min. Primary mouse monoclonal anti-bodies Anti-3-Nitrotyrosine [39B6] (Abcam 61392) and eNOS type III (BD Biosciences 610296) at 1:100 dilution were applied overnight. A no primary antibody control was completed to detect non-specific protein binding. Samples were subsequently incubated with anti-mouse IgG for 1 h (Immpress HRP reagent kit, MP-7452 Vector laboratories). Diaminobenzidine (DAB) (BD Biosciences 550880) was applied as a chromogen before counterstaining with hematoxylin, dehydration, and mounting in Dibutylphthalate Polystyrene Xylene (DPX) [24].

2.5. IHC Semiquantification

Images of each vessel were taken at 40× magnification (Leica DFC 450F, Leica Microsystems, Wetzlar, Germany). The endothelium was traced and the degree of staining (brown from DAB) was quantified using the MCID programme (MCID 7.0, Interfocus, Linton, UK). Researchers were blinded to the samples for quantification, using methods previously established [25–30]. The proportional intensity (arbitrary unit) of staining was calculated as a ratio of colour intensity to proportional area, normalised to the no primary antibody control. Finally, the immunoreactivity of each protein was calculated based on a fold change from the respective control vessel (the control ring from the normal diet or atherogenic diet groups).

2.6. Statistical Analysis

All results were expressed as mean ± standard error of the mean (SEM). Unpaired Student's t test was used for comparison between the diets. A one-way analysis of variance (ANOVA) was used to analyse the comparison between glucose incubations and *Post-hoc* analysis was completed using Fisher's least significance difference (LSD) test to identify the differences between groups. Data was analysed in Graphpad prism (version 7.1, Graphpad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant, trends were reported when $p = 0.05$–0.099, and >0.099 was considered not significant (n/s). Effect sizes are commonly used to study the clinical relevance of an intervention and show the magnitude of the effect that it is producing [31–33]. The Cohen's d (d) equation was used to examine the magnitude of the effect of the high glucose incubations on blood vessel relaxation and immunohistochemistry results. A large effect is considered when d is **>**0.8, a medium effect between 0.5 and 0.79, and a small effect between 0.2 and 0.49 [34].

3. Results

The atherogenic diet significantly reduced the relaxation of the abdominal aorta as measured by AUC (25%, $p < 0.05$) and EC_{50} ($p < 0.05$) compared to the normal diet (Figure 1A,B). In the iliac artery, the atherogenic diet reduced EC_{50} ($p < 0.05$) and there was a strong trend for a reduction in AUC (17%, $p = 0.06$) compared to the normal diet (Figure 1C,D). Similarly, in the mesenteric artery, the

atherogenic diet shifted EC_{50} to the right ($p < 0.05$) and there was a strong trend for a reduction in AUC (40%, $p = 0.07$) (Figure 1E,F).

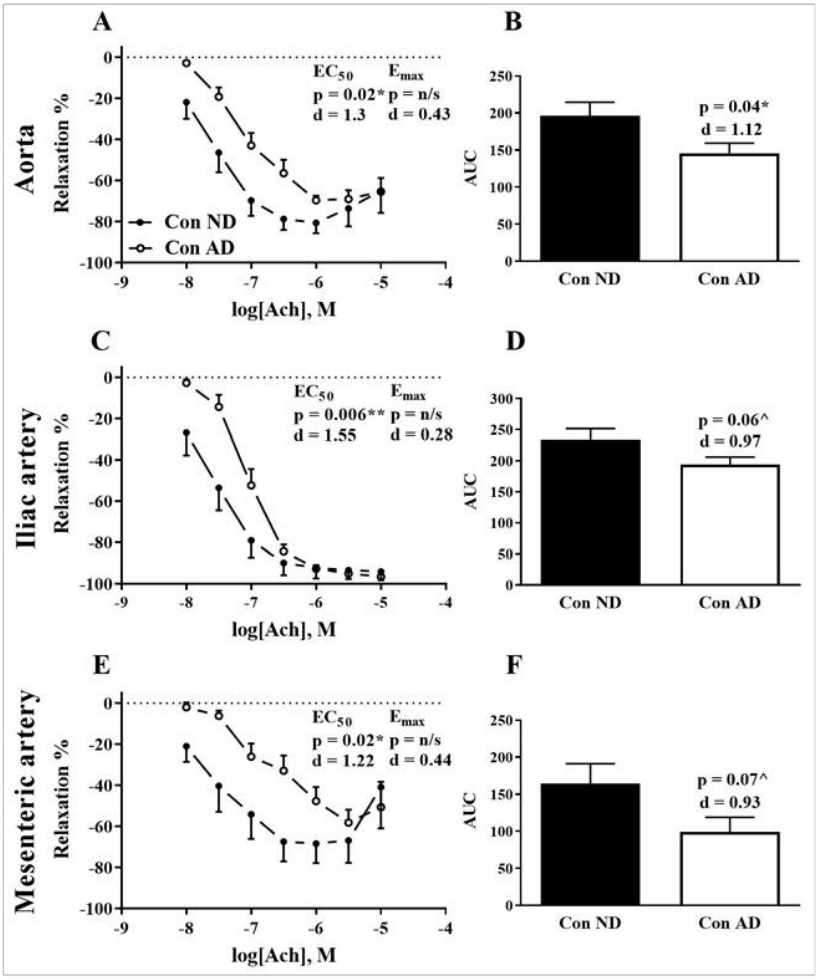

Figure 1. Ach-induced dose response curves in abdominal aorta (**A**), iliac artery (**C**), and mesenteric artery (**E**) incubated ex vivo for 2 h. Comparison between normal diet (closed circles) and atherogenic diet (open circles). Inset: EC_{50} and E_{max} statistical significance (p) and effect size (d) between diets. AUC in abdominal aorta (**B**), iliac artery (**D**), and mesenteric artery (**F**) presented as arbitrary values; numbers above columns represent the statistical significance (p) and effect size (d) between diets. n = 7–12 per group. All data mean ± SEM. * $p < 0.05$ ND vs. AD, ** $p < 0.01$ ND vs AD, ^ p 0.05–0.09 ND vs. AD. ND: normal diet; AD: atherogenic diet; Con: normal Krebs, AUC: area under the curve, d: Cohen's d.

For the rabbits who were fed a normal diet, incubation of the aorta in 20 mM glucose produced a strong trend towards a reduction in AUC (18%, $p = 0.08$) and E_{max} was reduced by 10%, but this was not significant ($p > 0.1$) (Figure 2A,C, Table 1). Incubation of the aorta in 20 mM glucose for the atherogenic diet fed rabbits caused a shift to the right of the dose response curve reducing EC_{50} ($p < 0.05$) (Figure 2B and Table 1). No dysfunction was caused in the iliac artery following the normal diet, irrespective of glucose incubation (Figure 2D,F). Whereas, relaxation of the iliac artery from the atherogenic diet fed animals altered EC_{50} in the 40 mM ($p < 0.05$) incubated group (Figure 2E and Supplementary Table S1). Endothelial dependent relaxation of the mesenteric artery was not negatively affected by the high glucose incubations following either the normal or atherogenic diet (Figure 2G–I and Supplementary Table S1).

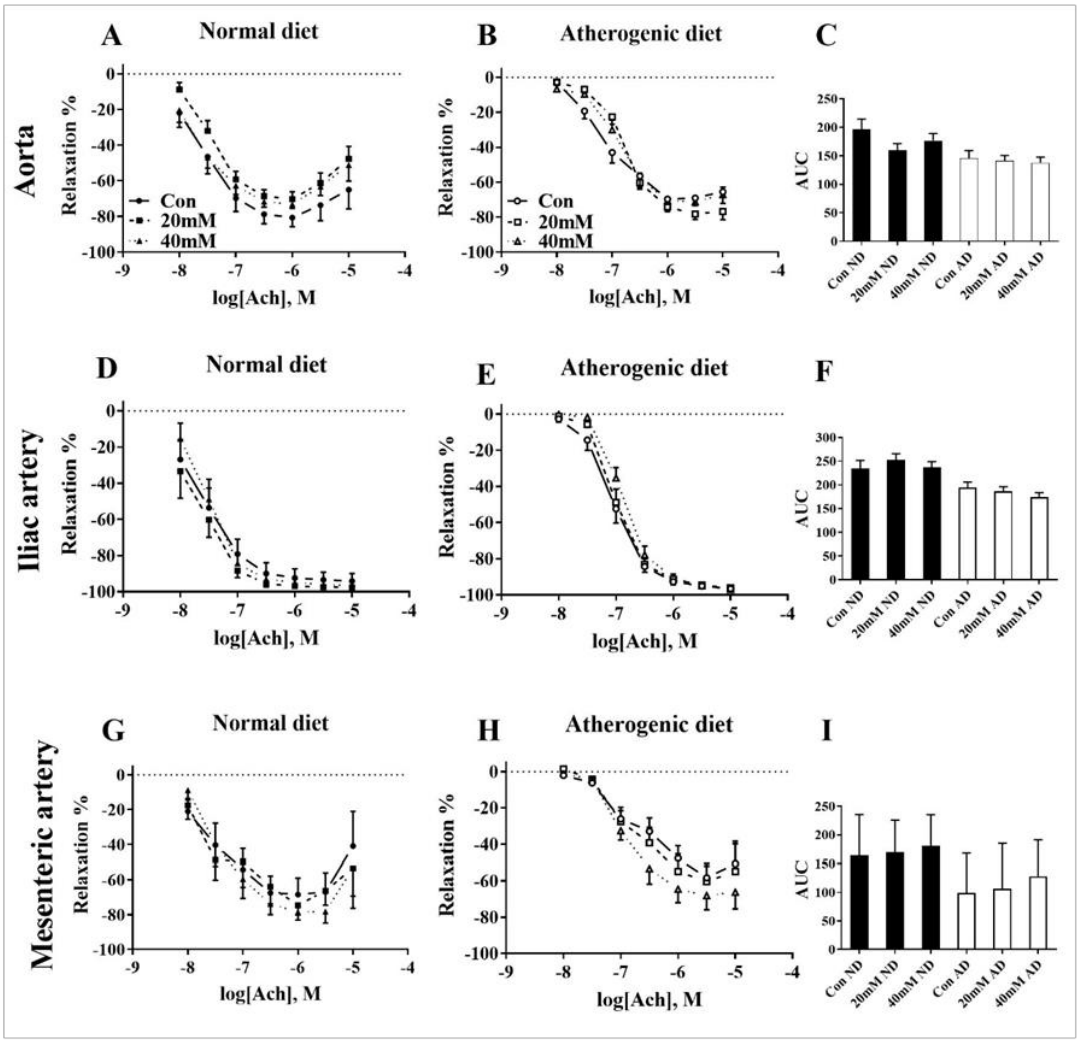

Figure 2. Ach-induced endothelium-dependent dose response curves in abdominal aorta (**A**,**B**), iliac artery (**D**,**E**), and mesenteric artery (**G**,**H**) incubated ex vivo for 2 h in respective solution. Comparison between Con (circles + line), 20 mM (squares + dashes), and 40 mM (triangles + dots). AUC (**C**,**F**,**I**) presented as arbitrary values. n = 6–12 per group. All data mean ± SEM. Con: normal Krebs; 20 mM: 20 mM glucose Krebs; 40 mM: 40 mM glucose Krebs; ND: normal diet; AD: atherogenic diet; AUC: area under the curve; Ach: acetylcholine.

Table 1. Log EC_{50}, E_{max} and AUC results from ND and AD fed rabbits incubated ex vivo for 2 h in control, 20 mM, or 40 mM glucose solution.

Abdominal Aorta	± *n*	Log EC50 SEM	*p* vs. Con	*p* vs. Con	Emax ± SEM	*p* vs. Con	*d* vs. Con	AUC ± SEM	*p* vs. Con	*d* vs. Con
ND Con	7	**−7.59 ± 0.12**			**−81 ±13**			196 **±8**		
ND 20 mM	7	**−7.43 ± 0.09**	n/s	0.54	**−70 ± 11**	n/s	0.32	160 **± 11**	**0.08** ^	0.92
ND 40 mM	7	**−7.65 ± 0.1**	n/s	0.18	**−73 ± 2**	n/s	0.29	177 **± 12**	n/s	0.48
AD Con	10	−7.10 ± 0.13			−70 ± 2			146 ± 13		
AD 20 mM	11	−6.81 ± 0.06	**0.03** *	0.88	−78 ± 3	**0.04** *	0.97	141 ± 9	n/s	0.13
AD 40 mM	11	−6.93 ± 0.07	n/s	0.52	−71 ± 3	n/s	0.18	138 **± 10**	n/s	0.21

ND: normal diet; AD: atherogenic diet; Con: normal Krebs; 20 mM: 20 mM glucose Krebs; 40 mM: 40 mM glucose Krebs; 2 h: 2 h incubation; AUC: area under the curve; *d*: Cohen's d; *n* = number of rabbits. Statistical significance (*p*) and effect size (Cohen's d) in comparison to the control group for each diet. * $p <$ 0.05 vs. control, ^ p 0.05–0.99 vs. control.

Representative images of IHC stained vessels are presented in Figure 3. The incubation of blood vessels in 20 mM and 40 mM glucose for 2 h did not significantly affect the immunoreactivity of eNOS and NT in any group. NT was increased in the 40 mM glucose normal diet group by 0.9 fold compared to the control, which had a trend towards significance ($p = 0.9$) and a large effect ($d = 0.99$) (Figure 4A). A medium to large effect (d) was present in a number of groups, but this was not associated with statistical significance (Figure 4A–F).

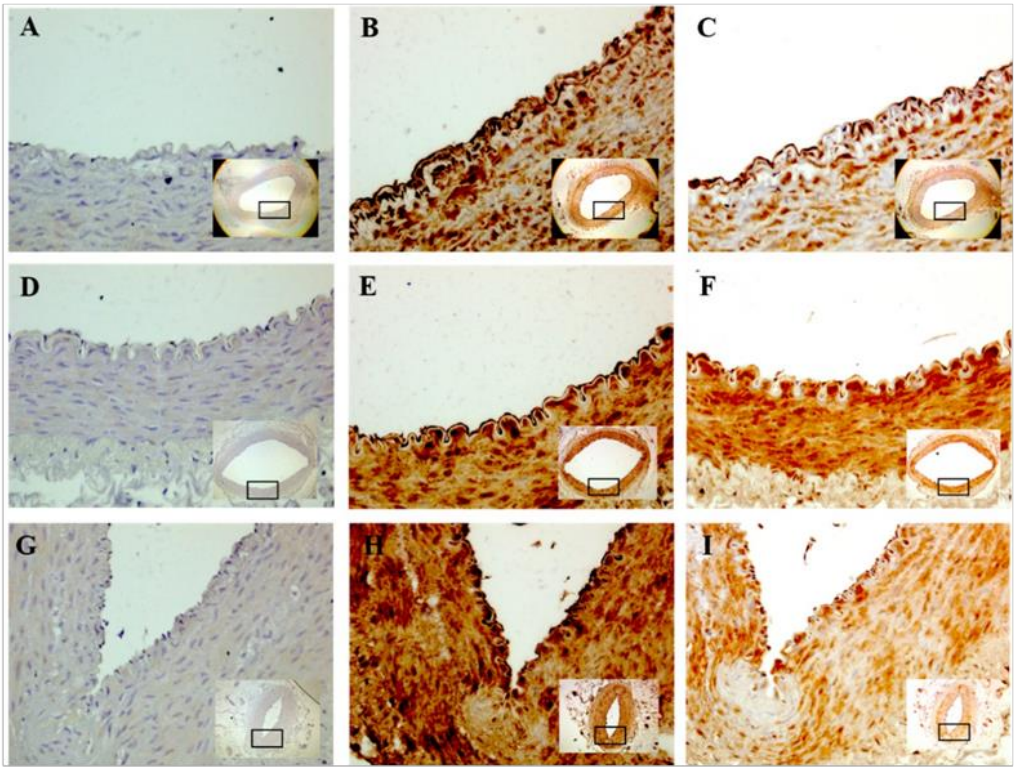

Figure 3. Representative images of immunohistochemistry stained blood vessels; abdominal aorta (**A**–**C**), iliac (**D**–**F**), and mesentery (**G**–**I**) from normal diet fed rabbits. No primary antibody control (**A**,**D**,**G**), nitrotyrosine (NT) (**B**,**E**,**H**), and endothelial nitric oxide synthase (eNOS) (**C**,**F**,**I**) taken at 40× magnification. Inset—image of whole vessel taken at 4× magnification (abdominal aorta) or 10× magnification (iliac and mesentery).

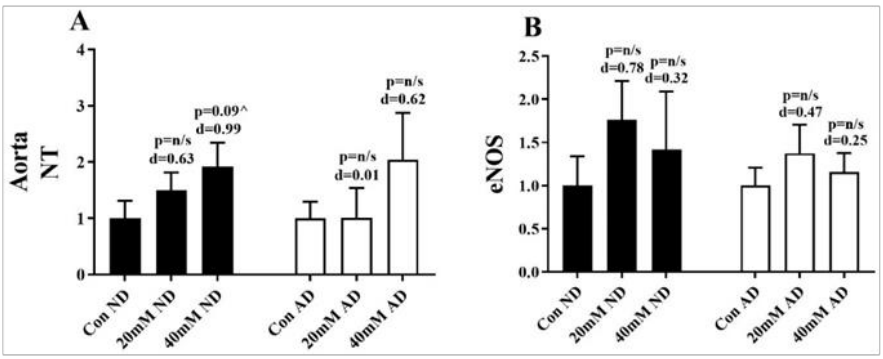

Figure 4. *Cont.*

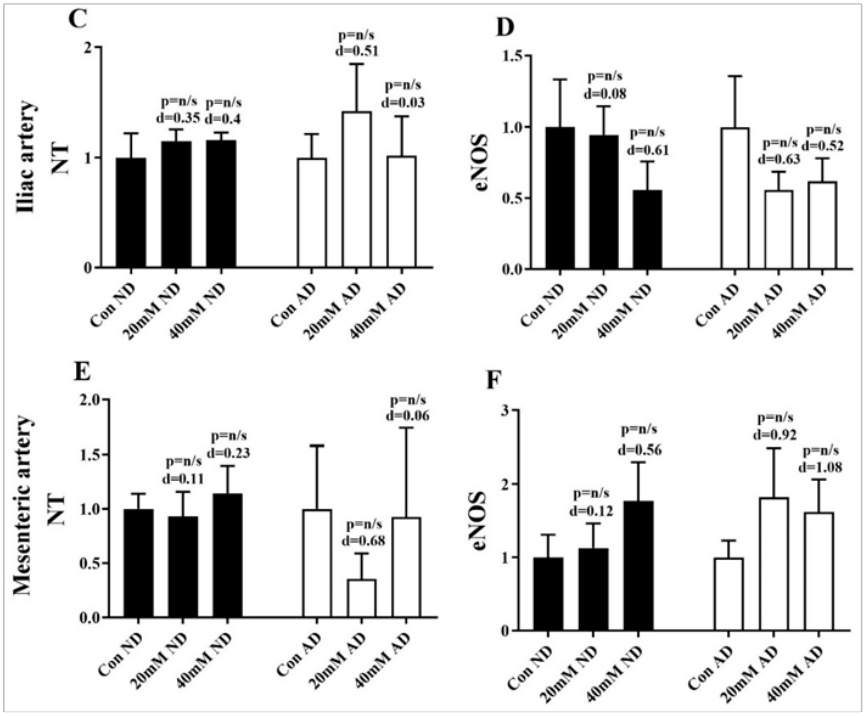

Figure 4. Immunoreactivity of NT and eNOS in abdominal aorta (**A**,**B**), iliac artery (**C**,**D**), and mesenteric artery (**E**,**F**). Immunoreactivity is calculated based on the intensity of the staining present on the endothelium, which is an arbitrary unit and expressed as fold change from the respective control. Numbers above columns represent the statistical significance (p) and effect size (d) in comparison to the control group for each diet. ^ p 0.05–0.99 vs. control. Con: normal Krebs; 20 mM: 20 mM glucose Krebs; 40 mM: 40 mM glucose Krebs; ND: normal diet; AD: atherogenic diet; NT: nitrotyrosine; eNOS: endothelial nitric oxide synthase.

4. Discussion

We report for the first time that high glucose-induced endothelial dysfunction is blood vessel specific. The abdominal aorta is the most susceptible to high glucose induced dysfunction, with the iliac artery affected to a lesser degree, and the mesenteric artery exhibited no signs of dysfunction.

High fat diets are commonly used to study the development of endothelial dysfunction and atherosclerosis in animals. The 4-week atherogenic diet used in this study has previously been shown to exhibit endothelial dysfunction in abdominal aorta of rabbits [21]. We confirm the findings of atherogenic diet induced endothelial dysfunction in the aorta and demonstrate endothelial dysfunction in the peripheral iliac and mesenteric arteries. Altogether, this suggests that the atherogenic diet functions systemically to cause dysfunction.

Hyperglycaemia is a major clinical risk factor for the development of endothelial dysfunction, atherosclerosis, and CAD. This is the first study to examine the effect of high glucose incubations on endothelial function of blood vessels in various locations. We demonstrate that the abdominal aorta is the artery that is most prone to developing endothelial dysfunction following both the normal and atherogenic diet. This confirms findings from several previous studies, which reported endothelial dysfunction in rat and rabbit aorta following acute high glucose incubations [13,16,35]. The iliac artery exhibited minor high glucose-induced dysfunction following the atherogenic diet, but not following the normal diet. As such, the iliac artery appears to be more susceptible to developing high glucose-induced dysfunction in a disease state and not in a healthy environment. Alternatively, the mesenteric artery did not develop any signs of endothelial dysfunction. Susceptibility to atherosclerosis can depend on haemodynamic factors such as shear stress and oscillating flow, which

can vary between vascular sites depending on the location of arterial branches or bifurcations [36]. The exposure of the endothelium to low shear stress is one of the most important factors in atherosclerosis development and is an important consideration when examining endothelial dysfunction in vivo [37]. Furthermore, endothelial dysfunction is not a systemic condition and some blood vessels can often resist the development of dysfunction more than others [38]. For example, vascular beds such as the internal mammary artery and other conduit arteries have increased NO production, decreased vasoconstriction, and have higher shear stress than other vessels [38,39]. Overall, there is variance in the effect of the high glucose incubations on endothelial function in different blood vessels, which may be explained, at least in part, by variations in the structure, physiological effects, and disease susceptibility of each vessel.

In this study, the development of endothelial dysfunction to high glucose incubation was not dose-dependent. The 20 mM glucose incubation caused the largest reduction in endothelium dependent vasodilation in the aorta from both the normal diet fed and atherogenic diet fed rabbits. This finding is in contrast with a previous study, which reported that incubation of rabbit aorta in 44 mM glucose aggravated dysfunction compared to the 20 mM incubation [13]. Similarly, the relaxation of the third order branches of the mesenteric artery from female Wistar rats following incubations in 20 mM and 45 mM glucose solution for 2 h elicited a dose-dependent reduction in endothelial-dependent vasodilation [19]. The conflicting results in this study possibly occurred as a result of species or methodological differences. Taken together, this study demonstrates endothelial dysfunction in the aorta following 2 h high glucose incubations in the normal and atherogenic diets. The dysfunction caused by the 2 h 20 mM glucose incubation provides a model for studying high glucose-induced blood vessel dysfunction that mimics an acute post-prandial response.

In a normal physiological environment, eNOS synthesises NO, which has a number of anti-atherogenic functions including vasodilation [40]. An acute state of hyperglycaemia can reduce eNOS expression and subsequently NO bioavailability, resulting in endothelial dysfunction [41]. Hyperglycaemia also promotes electron transport system overproduction of superoxide anion and via signalling pathways, produces peroxynitrite, a potent ROS [42]. Mechanistically, NT is used as a marker of peroxynitrite production, indicating the presence of nitrative stress [42]. Although not significant, the increase in NT observed in the aorta following high glucose incubations suggests the presence of nitrative stress in the current study—an effect that has previously been reported in rabbit aorta in a disease state [43]. Several recent studies, in both human and animal models, have identified that increased fasting glucose levels as a result of a high fat diet cause reductions in eNOS and plasma nitrate [44,45]. Overall, the evidence suggests that an increase in oxidative/nitrative stress and a reduction in eNOS are characteristic of hyperglycaemia-induced dysfunction. In this study, we did not find any significant alterations in NT or eNOS, but moderate to large changes in the effect size suggests that future research should examine this in more detail.

A potential limitation of the current study is that superoxide anion or other ROS forms were not directly measured to determine the exact mechanistic effect of the high glucose incubations. Furthermore, NT alone may not provide the most accurate representation of hyperglycaemia induced oxidative stress as it may be influenced by other factors including the atherogenic diet [46]. We examined total eNOS expression in combination with NT as an indirect measure of superoxide overproduction and peroxynitrite induced oxidative stress.

In conclusion, the effect of acute high glucose incubations on blood vessel function is blood vessel specific and in some cases, is aggravated by an atherogenic diet. The abdominal aorta may be the optimal artery to study potential therapeutic treatments of hyperglycaemia-induced endothelial dysfunction and CAD in rabbit models.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/7/2108/s1. Table S1: Log EC50, Emax and AUC results from ND and AD fed rabbits incubated ex vivo for 2 hr in control, 20 mM or 40 mM glucose solution.

Author Contributions: The study conception and design were completed by A.T., A.Z., and I.L. Material preparation, data collection, and analysis were performed by A.T., T.Q., C.S., and C.P. The first draft of the manuscript was written by A.T., A.T., T.Q., C.S., C.P., A.H., A.Z., and I.L. reviewed and edited the manuscript and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Rebecca L. Cooper Medical Research Foundation.

Acknowledgments: We wish to acknowledge Kristen McSweeney, Laura Gadanec, and Jemma Abraham for their assistance with data collection.

Conflicts of Interest: The authors declare no conflict of interest.

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Appendix C: Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells

Received: 14 July 2020 | Accepted: 31 August 2020
DOI: 10.1002/jcp.30048
ORIGINAL RESEARCH ARTICLE
Journal of Cellular Physiology WILEY

Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells

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Funding information
Rebecca L. Cooper Medical Research Foundation

Abstract

Undercarboxylated osteocalcin (ucOC) improves glucose metabolism; however, its effects on endothelial cell function are unclear. We examined the biological effect of ucOC on endothelial function in animal models ex vivo and human cells in vitro. Isometric tension and immunohistochemistry techniques were used on the aorta of male New Zealand white rabbits and cell culture techniques were used on human aortic endothelial cells (HAECs) to assess the effect of ucOC in normal and high-glucose environments. Overall, ucOC, both 10 and 30 ng/ml, did not significantly alter acetylcholine-induced blood vessel relaxation in rabbits ($p > .05$). UcOC treatment did not cause any significant changes in the immunoreactivity of cellular signalling markers ($p > .05$). In HAEC, ucOC did not change any of the assessed outcomes ($p > .05$). UcOC has no negative effects on endothelial function which is important to reduce the risks of off target adverse effects if it will be used as a therapeutic option for metabolic disease in the future.

KEYWORDS
cardiovascular disease, cell culture techniques, hyperglycaemia, immunohistochemistry, osteocalcin

1 | INTRODUCTION

The link between diabetes-associated hyperglycaemia and the development of cardiovascular disease is well established (Ebong et al., 2013). Hyperglycaemia is a major independent risk factor for the development of atherosclerosis and vascular disease (Bornfeldt & Tabas, 2011; Rask-Madsen & King, 2013). Exposure of endothelial cells to high glucose levels perturbs cell homeostasis, and an imbalance of biochemical pathways contributes ultimately to endothelial dysfunction (Bakker, Eringa, Sipkema, & van Hinsbergh, 2009). This imbalance causes several pathological effects, principally, the inability of the endothelium to regulate vasodilation and vasoconstriction (Bonetti, Lerman, & Lerman, 2003; Cahill & Redmond, 2016; Lerman & Zeiher, 2005).

Recent advances in the understanding of bone physiology have established bone as an active endocrine organ. Osteocalcin (OC) in its undercarboxylated form (ucOC) plays a role in glucose regulation and energy metabolism (Levinger et al., 2017; Li, Zhang, Yang, Li, & Dai, 2016). ucOC has been linked to enhanced secretion of insulin from pancreatic beta cells, improvements in insulin sensitivity, and regulation of glucose homeostasis (Ferron, Hinoi, Karsenty, & Ducy, 2008; Lin et al., 2017; Oury et al., 2011). Given

these bioactive effects, it has been suggested that ucOC be tar- geted as a therapeutic treatment for metabolic diseases, including diabetes (Villafan-Bernal, Sanchez-Enriquez, & Munoz-Valle, 2011). However, some studies report that OC may be associated with endothelial dysfunction and atherosclerosis. For example, in men and women with diabetes, lower levels of circulating total OC (tOC) have been associated with increased pulse wave velocity, intima–media thickness (IMT), and vascular complications (Q. Guo et al., 2017; Kanazawa et al., 2009). On the other hand, higher levels of tOC have also been associated with increased plaque de- velopment and IMT men and women with diabetes (Kanazawa et al., 2011; Reyes-Garcia et al., 2012). Overall, the evidence is conflicting and the exact role of tOC and ucOC in the vasculature is unclear (Millar, Patel, Anderson, England, & O'Sullivan, 2017; Tacey et al., 2018). One major limitation of previous studies is that they only examined the serum levels of circulating tOC, and not its individual forms, in particular ucOC. Given the bioactivity of ucOC, its direct effect on blood vessels must be explored before any use as a therapeutic option for hyperglycaemia.

The aim of the current study was to determine (a) whether ucOC has an effect on endothelium-dependent and endothelium-independent vasodilation in rabbit aorta following incubations in normal and high glucose solutions, and (b) whether the treatment of human aortic endothelial cells (HAECs) with ucOC alters en- dothelial cell homeostasis following incubation in high glucose media.

2 | METHODOLOGY

2.1 | Animals

Male New Zealand White rabbits were housed in individual cages on a 12-h light/dark cycle at 21°C, with access to water and standard chow diet ad libidum. At 12 weeks of age, the rabbits were randomised onto a normal chow diet (Guinea pig and rabbit pellets) or an atherogenic diet (a normal diet combined with 1% methionine, 0.5% cholesterol and 5% peanut oil (#SF00-218) for 4 weeks (Zulli & Hare, 2009). At the completion of the 4-week diet, the rabbits were weighed and then sedated (0.25 mg/kg medetomidine) and anaesthetised (4% isoflurane) before ex- sanguination via severing of the inferior vena cava. A random blood glucose sample was obtained from the inferior vena cava immediately upon exsanguination and was recorded with a blood glucose monitor (Freestyle Optimum Neo). A serum sample was obtained from the inferior vena cava to determine insulin con- centration. Insulin was measured using an enzyme-linked im- munosorbent assay (ELISA) Kit and was completed according to the manufacturer's instructions (#90186; Australian Biosearch). This study was approved by the Victoria University Animal Ethics Committee (#14/005) and complied with the Australian National Health and Medical Research Council code for the care and use of animals for scientific purposes (8th edition).

2.2 | Isometric tension myography

The abdominal aorta (immediately before the iliac bifurcation) was dissected and placed in ice-cold Krebs solution ([mM] 118 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 $KH \cdot 2PO_4$, 25 $NaHCO_3$, 11.7 glucose, and 1.25 CaCl). The aorta was cleaned of connective tissues, cut into rings (2–3 mm) and placed in individual organ baths containing Krebs warmed to 37°C and bubbled with 95% O_2/5% CO_2. This was fol- lowed by 30-min acclimatisation. Blood vessel reactivity was mea- sured via an isometric tension organ bath system (Zultek Engineering), as previously described (El-Hawli et al., 2017; R. M. Smith, Rai, Kruzliak, Hayes, & Zulli, 2019). In brief, the aortic rings were carefully mounted on parallel hooks (one of which was con- nected to a force transducer) and stretched to a basal tension twice over a 1-h period. Thereafter, the vessels were incubated for 2 h in normal Krebs solution (11.7 mM glucose) or high-glucose Krebs so- lution (20 mM glucose) which has previously shown to cause a re- duction in endothelium-dependent vasodilation (X. Guo, Liu, Chen, & Guo, 2000; Taylor & Poston, 1994). Each organ bath was refreshed every 30 min with its respective Krebs solution. Aortic rings were constricted with phenylephrine (3×10^{-7} M) until a plateau occurred followed by a 5-min incubation with either 10 or 30 ng/ml ucOC (Glu13, 17, 20, osteocalcin [1–46] [mouse] trifluoroacetate salt [H- 6552.0500; Auspep]) or control solution [Krebs]. The concentration of ucOC administered to each aortic ring was chosen based on physiological ranges (Hiam et al., 2019). Blood vessel reactivity was determined via cumulative dose-response curves to the endothelium- dependent vasodilator acetylcholine (ACh) or with the endothelium- independent vasodilator sodium nitroprusside (SNP) in half-log increments (10^{-8} to 10^{-5} M). The response of the vessels was measured on a software program (MEDIDAQ) which displays the tension of the vessel in grams. The log dose of ACh/SNP that pro- duced the maximal relaxation was indicated by the E_{max} and the EC_{50} as the log dose that produced 50% of the E_{max}. The area under the curve (AUC) was determined as the total area of relaxation below the phenylephrine plateau.

2.3 | Immunohistochemistry

Following the isometric testing, aortic rings were immediately placed into 4% paraformaldehyde, left overnight, and then transferred into 1X phosphate-buffered saline at 4°C. This was followed by paraffin processing (Microm STP120) and embedding in paraffin blocks. Sections were cut at 5 µm, deparaffinised in xylene, rehydrated and blocked with 1% goat serum in 10 mm Tris-Cl (pH 7.4) for 20 min. Primary mouse monoclonal antibodies anti-3-nitrotyrosine [39B6] (#61392; Abcam) and endothelial nitric oxide synthase (eNOS) type III (#610296; BD Biosciences), p-protein kinase B (Akt) 1/2 [Ser473] (NOVNB10056749; Novus Biologicals) and p-mammalian target of rapamycin (mTOR) [59. Ser 2448] (SANTSC-293133; Santa Cruz Biotechnology) at 1:100 dilution were applied overnight. Samples were also prepared where the primary antibody was omitted from

the solution as a negative control. Samples were subsequently incubated with antimouse immunoglobulin G for 1 h (Immpress HRP Reagent Kit MP-7452; Vector Laboratories). Diaminobenzidine (#550880; BD Biosciences) was applied as a chromogen before counterstaining with hematoxylin, dehydration, and mounting in dibutylphthalate polystyrene xylene (Arora, Hare, & Zulli, 2012). All chemicals and reagents were supplied by Sigma-Aldrich unless otherwise specified.

2.4 | Immunohistochemistry quantification

Images of each aortic ring were taken at ×40 magnification (Leica DFC 450F; Leica Microsystems). The endothelium was traced and the degree of brown immunoprecipitate (indicative of positive antigenic sites) was quantified using the MCID programme (MCID 7.0; Interfocus), as previously described (Qaradakhi et al., 2017; Zulli et al., 2006). Researchers were blinded to the samples for quantification. The proportional intensity (arbitrary unit) of brown immunoprecipitate was calculated as a ratio of colour intensity to proportional area, normalised to the negative control. Finally, the immunoreactivity of each protein was calculated based on a fold change from the respective control vessel (the control ring from the normal diet or atherogenic diet groups).

2.5 | Cell culture

HAECs were purchased from PromoCell and maintained in commercial endothelial cell media with supplements (PromoCell) containing 1% penicillin–streptomycin (Sigma-Aldrich) in a humidified incubator (5% CO_2, 37°C), as previously established (Millar, Zala, Anderson, & O'Sullivan, 2019). Cells were used for experiments at passages 4 and 5. Cells were treated with either 5.6 mM normal glucose media (NG) or 16 mM high glucose media (HG) for 7 days with or without ucOC (10 ng/ml) to induce endothelial dysfunction. Media and cell lysates were collected at the end of the experiments. Each experiment was repeated independently three times. Human uncarboxylated osteocalcin (ucOC; amino acids 1–49, [Glu17, 21, 24]) was purchased from US Biological (O8060-09C-USB). D-(+)-glucose was purchased from Sigma-Aldrich (#I9278 and #G7021). Cell lysis buffer was purchased from Cell Signalling Technology (#9803) and was supplemented with protease and phosphatase inhibitors (A32959; Thermo Fisher Scientific).

2.6 | ELISAs, lactate dehydrogenase activity assay, and total protein content

Secreted interleukin-6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1), endothelin (ET), and monocyte chemoattractant protein-1 (MCP-1) were measured in cell culture media by ELISA as per manufacturers' instructions (catalogue numbers DY206, DT809, DY1160, and DY279; R&D Systems). A lactate dehydrogenase (LDH) (Colorimetric) Assay Kit (category number ab102526; Abcam) was performed on cell media as per the manufacturer's instructions. A bicinchoninic acid protein assay was performed to quantify the total protein content in the cell lysates collected at the end of the experiments (P. K. Smith et al., 1985). A total osteocalcin ELISA which does not differentiate between uncarboxylated and carboxylated osteocalcin was performed to assess predicted and actual concentrations of ucOC added to experimental wells and to validate the purchased protein (DY1419; R&D Systems).

2.7 | Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.0 Graphpad Software Inc). Unpaired Student's *t* test was used to compare between the normal and atherogenic diet and between the NG and HG incubations in the ex vivo rabbit model. A one-way analysis of variance (ANOVA) was used to analyse the effect of the ucOC treatment on blood vessel relaxation and immunohistochemistry staining. One-way ANOVA was also used to detect differences between groups following the in vitro cell culture experiments. Post hoc analysis was completed using Fisher's least significance difference test. All data are reported as mean ± *SEM* and statistical analysis was conducted at the 95% level of significance ($p < .05$). Trends were reported if p was between 0.05 and 0.09. Effect sizes are commonly used to study the clinical relevance of intervention and show the magnitude of the effect that it is producing (Maylor, Zakrzewski-Fruer, Stensel, Orton, & Bailey, 2019; Rodevand et al., 2019; Silva, Lacerda, & da Mota, 2019). The Cohen's d (d) equation was used to examine the magnitude of effect. A large effect is considered when $d > 0.8$, a medium effect between 0.5 and 0.79 and a small effect between 0.2 and 0.49 (Cohen, 2013).

3 | RESULTS

3.1 | Atherogenic diet versus normal diet in rabbits

No difference in body mass occurred between the rabbits fed a normal diet and those fed the atherogenic diet ($p > .05$, $d = 0.16$; Figure 1a). Circulating blood glucose levels was increased by 25% following the atherogenic diet compared to the normal diet ($p < .01$, $d = 1.48$; Figure 1b). Circulating insulin concentration did not change following both diets, suggesting the presence of insulin resistance in the animals fed with atherogenic diet ($p > .05$, $d = 0.44$; Figure 1c). Endothelial function was not altered by the atherogenic diet as shown by EC_{50}, E_{max}, and AUC ($p > .05$ for all, $d = 0.03$, 0.08 and 0.07, respectively; Figure 1d) compared to the normal diet.

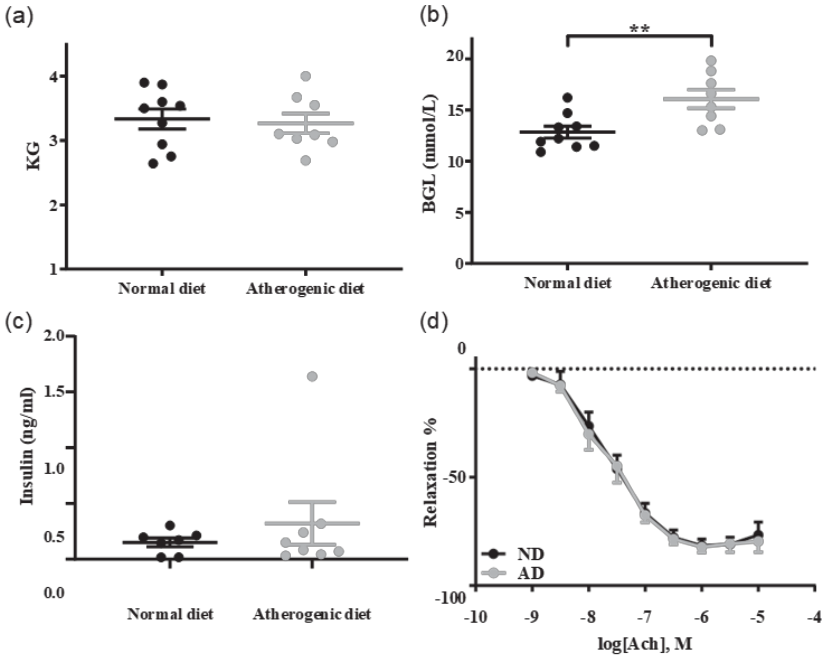

FIGURE 1 Comparison between the 4-week normal and atherogenic diets for (a) Body mass, (b) random blood glucose concentration, (c) random insulin concentration, and (d) blood vessel relaxation. All data mean ± *SEM*. AD, atherogenic diet; BGL, blood glucose level; ND, normal diet. $^{**}p < .01$ between diets

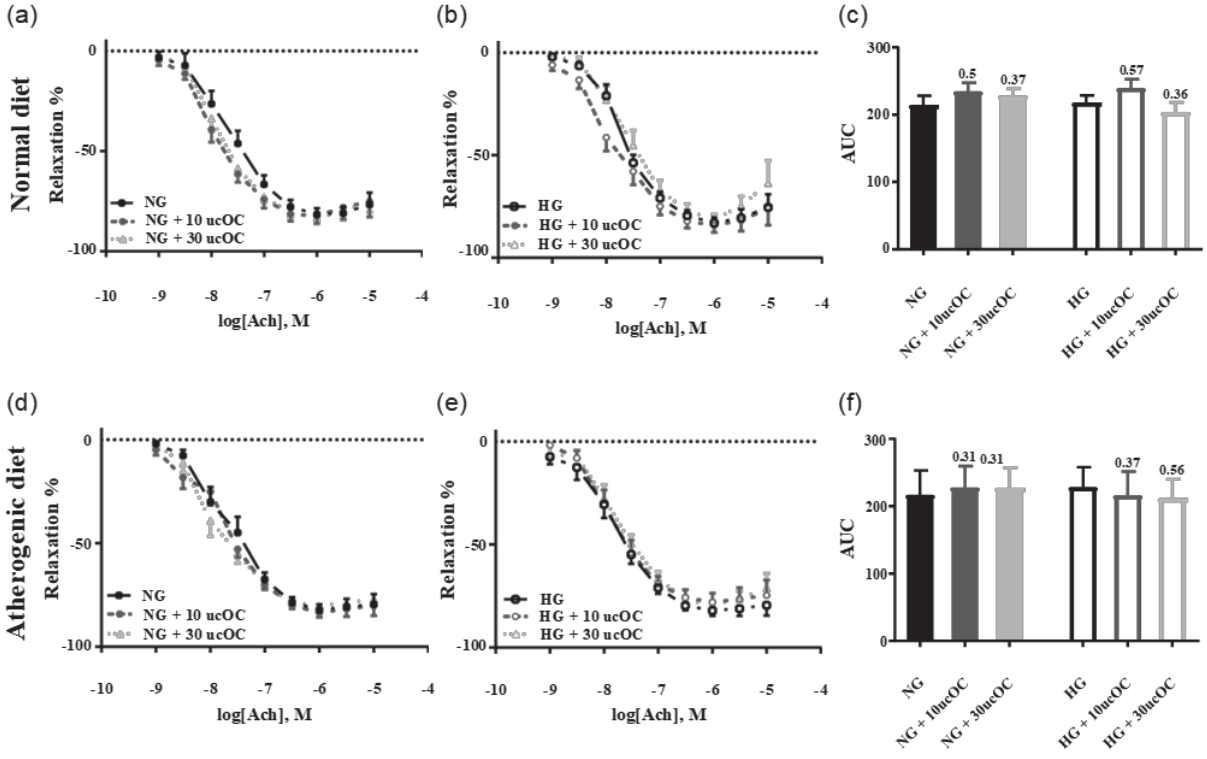

FIGURE 2 ACh-induced endothelium-dependent dose-response curves in abdominal aorta following a 5-min ucOC preincubation. ND (a–c) and AD (d–f) fed rabbits. All data mean ± *SEM*. Numbers above columns represent the effect size (Cohen's *d*) in comparison to the respective NG/HG control group. 10 ucOC, 10 ng/ml ucOC treatment; 30 ucOC, 30 ng/ml ucOC treatment; ACh, acetylcholine; AD, atherogenic diet; AUC, area under the curve; HG, high glucose media; ND, normal diet; NG, normal glucose media; ucOC, undercarboxylated osteocalcin

3.2 | Blood vessel reactivity

The 20 mM high-glucose incubation did not alter endothelium-dependent vasodilation following either the normal diet (d = EC_{50}: 0.17; E_{max}: 0.18) or atherogenic diet (d = EC_{50}: 0.52; E_{max}: 0.01; p > .05 for all; Figure S1). UcOC (10 and 30 ng/ml) did not alter ACh-induced blood vessel relaxation in rabbits fed a normal or atherogenic diet or in aortic rings incubated in NG or HG solution (p > .05; Figure 2a–f; Table 1). The 10 ng/ml ucOC treatment produced a trend in Log EC_{50} (p = .05–.09; Table 1) and moderate improvements in AUC (~10%; Figure 2c) as indicated by Cohen's d in NG and HG from normal diet-fed rabbits. Administration of ucOC (10 and 30 ng/ml) before SNP induced endothelium-independent relaxation did not significantly alter any measure of blood vessel relaxation (Figure S2 and Table S1).

3.3 | Immunohistochemistry

The ucOC (10 and 30 ng/ml) treatment did not cause any significant changes in the immunoreactivity of NT, eNOS, p-Akt, or p-mTOR following either the normal or atherogenic diet (p > .05; Figure 3a–h). Analysis of Cohen's d revealed moderate to large increases in the reactivity of eNOS in HG incubated aorta following the normal diet (10 ng/ml ucOC = 2.5-fold and 30 ng/ml ucOC 0.9-fold) following ucOC administration. This was also found in the NG incubated aorta following the atherogenic diet (30 ng/ml ucOC = 1.2-fold; Figure 3c,d). ucOC administration also increased the phosphorylation of mTOR at ser2448 in the NG condition following both normal (10 ng/ml ucOC = 1.3-fold and 30 ng/ml ucOC = 2.2-fold) and atherogenic diets (10 ng/ml ucOC = 1-fold and 30 ng/ml = 0.9-fold), while less effect occurred in the HG incubated vessels (Figure 3g,h).

3.4 | Cell culture

Total protein content was unaltered between the three experimental conditions (Figure 4a). After 7 days cultured in HG media (16 mM), the secretion of IL-6, VCAM-1, ET-1, MCP-1 and LDH were increased compared to NG controls (53%, 64%, 29%, 108% and 30%, respectively; p < .01 for all; Figure 4b–f). The addition of ucOC to HG media did not attenuate the increases in IL-6, VCAM-1, ET-1, MCP-1 or LDH activity (p > .05 for all). The HG + ucOC was also significantly increased compared to the NG controls for IL-6, VCAM-1, MCP-1 and LDH (p < .01 for all). There was a trend for HG + ucOC to be elevated above the NG controls for ET-1 (p = .07).

4 | DISCUSSION

We report that acute ucOC treatment has no negative, or positive, effect on endothelial function or endothelial cell homeostasis in rabbit aorta ex vivo or human vascular cells in vitro in the presence or absence of high glucose.

Functionally, an impairment of endothelium-dependent vasodilation is one of the first signs of endothelial dysfunction, and a marker of early atherosclerosis development (Bonetti et al., 2003; Esper et al., 2006). Previous research has examined endothelium-dependent vasodilation in the thoracic aorta of apolipoprotein E (ApoE)−/− mice ex vivo following tOC administration. Following a 12-week high-fat diet with daily injections of vehicle or tOC (30 ng/g), the dilation of the thoracic aorta was improved by 20% in the tOC-treated mice compared to the vehicle-treated mice (Dou et al., 2014). However, the tOC-treated mice also had improvements in body weight, blood glucose concentration, lipids and inflammatory

TABLE 1 Log EC_{50} and E_{max} results from ACh-induced endothelium-dependent dose-response curves in abdominal aorta

	n	Log EC_{50} ± SEM	p vs. NG/HG	d vs. NG/HG	E_{max} ± SEM	p vs. NG/HG	d vs. NG/HG
ND + NG	9	−7.67 ± 0.15			−81.54 ± 3.05		
ND + NG + 10 ng/ml ucOC	8	−7.96 ± 0.08	.07^	0.82	−82.46 ± 3.72	NS	0.09
ND + NG + 30 ng/ml ucOC	8	−7.88 ± 0.09	NS	0.58	−83.16 ± 2.4	NS	0.2
ND + HG	9	−7.73 ± 0.07			−83.1 ± 2.77		
ND + HG + 10 ng/ml ucOC	8	−7.96 ± 0.08	.09^	1.06	−84.21 ± 3.38	NS	0.12
ND + HG + 30 ng/ml ucOC	8	−7.59 ± 0.13	NS	0.46	−81.43 ± 2.61	NS	0.21
AD + NG	8	−7.68 ± 0.17			−82.18 ± 2.59		
AD + NG + 10 ng/ml ucOC	8	−7.76 ± 0.12	NS	0.18	−83.62 ± 2.15	NS	0.21
AD + NG + 30 ng/ml ucOC	8	−7.96 ± 0.12	NS	0.68	−80.3 ± 1.54	NS	0.31
AD + HG	7	−7.93 ± 0.18			−82.09 ± 2.56		
AD + HG + 10 ng/ml ucOC	7	−7.89 ± 0.12	NS	0.1	−78.08 ± 4.47	NS	0.42
AD + HG + 30 ng/ml ucOC	7	−7.74 ± 0.1	NS	0.49	−79.3 ± 1.94	NS	0.46

Note: 10 ucOC, 10 ng/ml ucOC treatment; 30 ucOC, 30 ng/ml ucOC treatment. ^p = .05–.099.

Abbreviations: ACh, acetylcholine; AD, atherogenic diet; d, Cohen's d; HG, high glucose media; n, total number of animals; ND, normal diet; NG, normal glucose media; NS, not significant; ucOC, undercarboxylated osteocalcin.

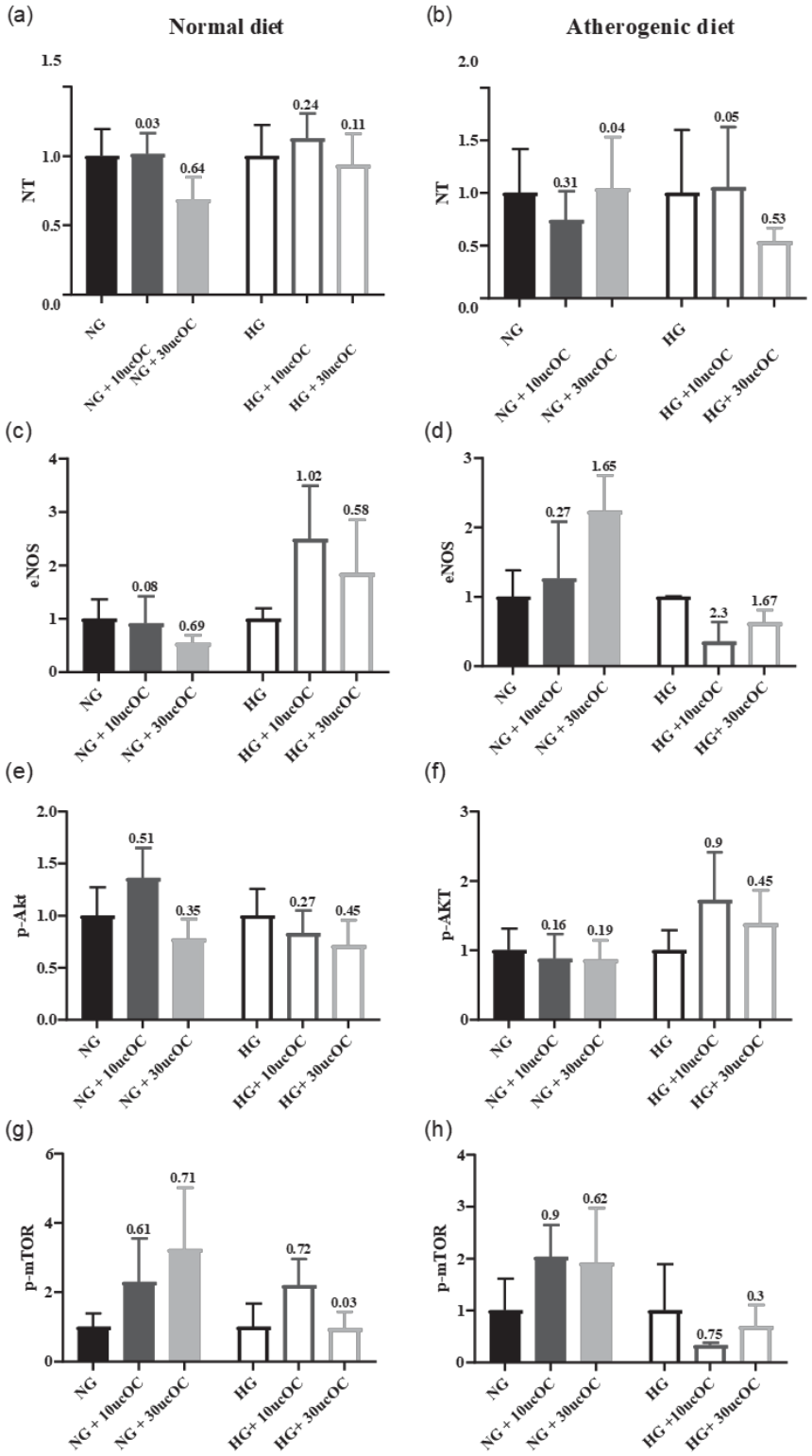

FIGURE 3 Immunoreactivity of (a and b) NT, (c and d) eNOS, (e and f) p-AKT, and (g and h) p-mTOR in aorta following normal diet (a,c,e,h) and atherogenic diet (b,d,g,i). Reactivity is calculated based on intensity of staining present on the endothelium which is an arbitrary unit and expressed as fold change from the respective NG/HG control group. Numbers above columns represent the effect size (Cohen's *d*) in comparison to the respective NG/HG control group. All data mean ± *SEM*. eNOS, endothelial nitric oxide synthase; HG, high glucose; NG, normal glucose; NT, nitrotyrosine; p-Akt, phosphorylated protein kinase B; p-mTOR, phosphorylated mammalian target of rapamycin; ucOC, undercarboxylated osteocalcin

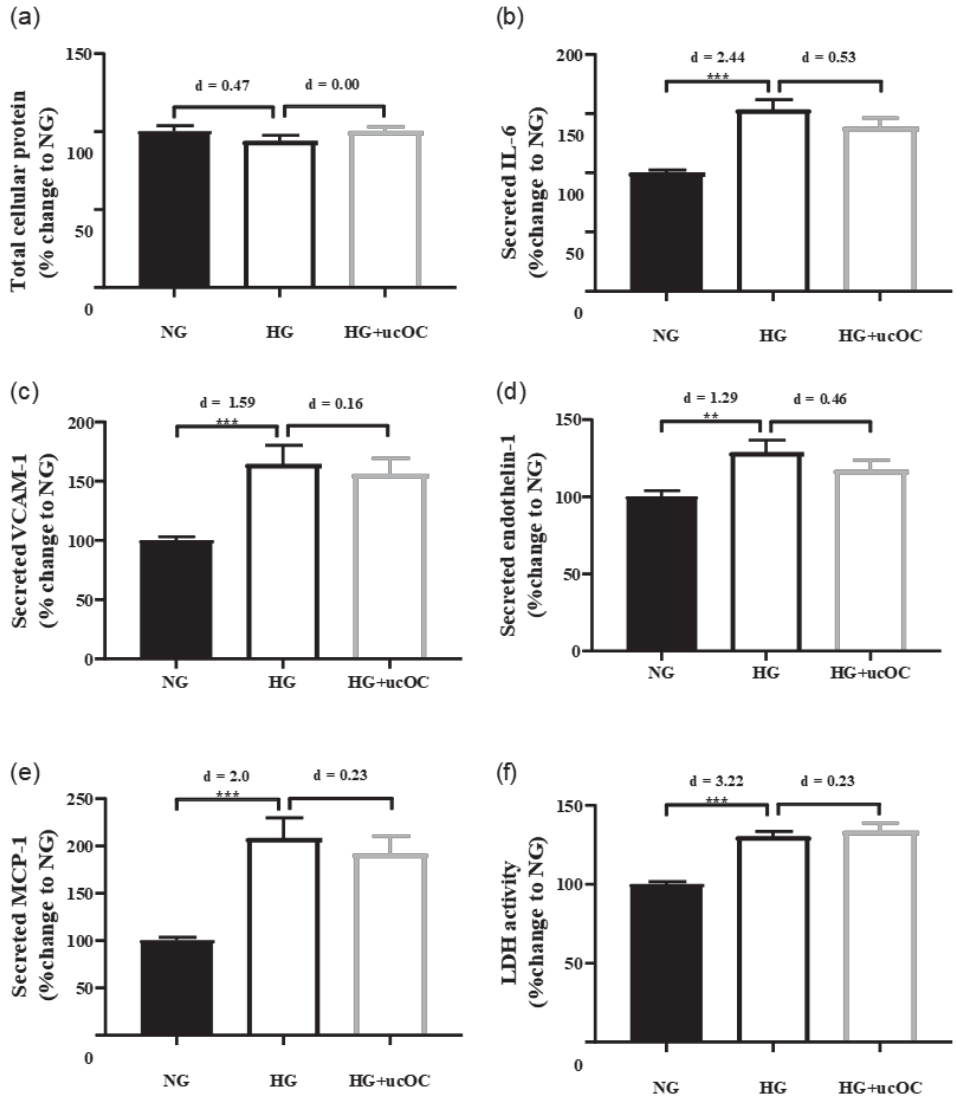

FIGURE 4 Human aortic endothelial cells (HAECs) cultured in NG media (NG; 5.5 mM) or HG media (HG; 16 mM) with or without ucOC (10 ng/ml). (a) Total protein content, (b) IL-6, (c) VCAM-1, (d) endothelin-1, (e) MCP-1 and (f) LDH activity were measured after 7 days. Numbers above columns represent the effect size (Cohen's *d*) in comparison to NG column. $^{**}p < 0.01$, $^{***}p < .001$ compared to NG. $n = 12$ for each condition from three experimental repeats. All data mean ± *SEM*. HG, high glucose; IL-6, interleukin 6; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; NG, normal glucose; ucOC, undercarboxylated osteocalcin; VCAM-1, vascular adhesion molecule 1

markers (Dou et al., 2014). Therefore, whether improved blood vessel relaxation occurred as a direct response to OC or as a result of improved metabolic parameters cannot be determined from this study. Recently, ucOC was detectable within the endothelium of rabbit arteries, and administration of ucOC (10 ng/ml) improved endothelium-dependent relaxation of the rabbit aorta (Qaradakhi et al., 2019). Suggesting that ucOC is present in the vasculature and can direct regulate blood vessel function.

As ucOC is known to exert a metabolic function (Ferron et al., 2008), we sought to determine if the effect of ucOC on blood vessel function remains under high glucose conditions. In the current study, the aortic sections from rabbits incubated for 2 h in 20 mM high glucose solution did not have altered endothelium-dependent vasodilation, which is in contrast to previous studies (X. Guo et al., 2000; Taylor & Poston, 1994). Similarly, the atherogenic diet, which has previously been shown to cause endothelial dysfunction in rabbit aorta (Qaradakhi et al., 2019; Zulli & Hare, 2009), did not cause dysfunction in this study. It is unclear why in the endothelial dysfunction did not occur, but we were able to examine the effect of ucOC following acute high glucose incubations and in an insulin-resistant state following the atherogenic diet. Whilst the administration of 10 ng/ml ucOC produced a trend towards an improvement in endothelium-dependent relaxation, overall ucOC did not alter relaxation under any condition. This finding is in contrast to previous studies, but suggests that ucOC may not have a biological role in the regulation of endothelial function. Importantly, there was no indication of an adverse effect of ucOC on blood vessel function.

To examine the effect of ucOC on smooth muscle cells, independent of the endothelium, we administered ucOC to rabbit aorta before completing dose-response curves with SNP, a nitric oxide donor. The acute ucOC treatment had no effect on SNP- induced, endothelium-independent relaxation of the rabbit aorta and suggests that any effect of ucOC in the vasculature is likely occurring via endothelium-dependent mechanisms.

Previous in vivo experiments in mice demonstrated an improvement in endothelial relaxation following treatment with tOC (30 ng/ml) for 12 weeks. The authors suggested that the activation of the phosphatidylinositol 3-kinase (PI3k)/Akt/eNOS signalling path- way was necessary to induce the enhancement in relaxation (Dou et al., 2014). In the current study, Akt and eNOS immunoreactivity was unaltered by treatment with ucOC. This is the same for the presence of NT and mTOR and suggests that the duration of ucOC treatment may not have been long enough to induce changes in the cellular signalling mechanisms necessary to alter endothelial func- tion. Overall, in the current study, ucOC did not have a regulatory effect on the vasoactivity of rabbit aorta. Whilst the focus of this study was predominantly on vasodilation, the examination of the proposed signalling pathways that ucOC activates in endothelial cells did not reveal any changes following ucOC treatment. It is possible that a different dose of ucOC or a longer incubation is needed before ACh-induced relaxation to cause a change in the signalling pathways or functional outcomes. This should be explored in future studies.

Endothelial cells have an important role in the maintenance of vascular homeostasis and the protection against the development of vascular disease (Brown, Shantsila, Varma, & Lip, 2017). Recent data suggest that ucOC may be involved in endothelial function. Administration of ucOC (25 and 100 ng/ml), but not the carboxylated form (cOC), increases eNOS phosphorylation in HAECs in a dose-dependent manner (Kondo et al., 2016). Similarly, in HAEC incubation of ucOC for 1 h caused a dose-dependent increase in eNOS phosphorylation at serine 1177. This was associated with dose-dependent increases of Akt, an upstream activator of eNOS and of nitric oxide (Jung et al., 2013). Furthermore, increased eNOS, Akt and PI3k phosphorylation was reported in human umbilical cord vein endothelial cells following tOC (100 ng/ml) and ucOC (5 ng/ml) treatment (Dou et al., 2014; Q. Guo et al., 2017). Altogether, these studies support the hypothesis that OC, via ucOC, has an active role in endothelial cells, protecting against pathological processes and improving endothelial function via the PI3K/Akt signalling pathway (Tacey et al., 2018). However, we have recently reported some conflicting findings, ucOC treatment (10 ng/ml) did not alter the phosphorylation of Akt, mTOR, nuclear factor-κB and several other markers of intracellular signalling in HAECs (Millar, Anderson, & O'Sullivan, 2019). In addition, ucOC did not alter markers of angiogenesis in HAEC, such as migration and matrix degradation and inflammatory markers that are commonly involved in endothelial dysfunction (Millar, Anderson et al., 2019). Furthermore, under acute and chronic inflammatory conditions that mimic an atherogenic environment, ucOC (10 ng/ml) had no anti-inflammatory effect in human HAECs (Millar, Zala, et al., 2019). These results are supported by the findings of the current study. Here we show that ucOC administration did not attenuate inflammatory or dysfunction markers altered by high glucose treatment. One potential explanation for our finding is due to the dose of ucOC used. We used 10 ng/ml of ucOC, which is lower than what was used in some previous studies; however, it is in the physiological range (Hiam et al., 2019). Future research may complete a dose-response curve to determine if there is an optimal dose of ucOC. Overall, our findings suggest that ucOC does not regulate endothelial cell signalling or function in physiological or pathophysiological conditions.

Although it was not examined in this study, there is evidence to suggest a link between OC and the advanced stages of atherosclerotic cardiovascular disease development, in particular the development of vascular calcification (Levinger et al., 2017). The mineralisation of plaque during atherosclerosis development is similar to the formation of bone within the skeleton (Zhu, Mackenzie, Farquharson, & Macrae, 2012). The form of OC present within calcified plaques is yet to be identified, given the role of cOC in the mineralisation of bone it is possible that cOC is responsible for the association with vascular calcification. The exact mechanisms by which OC mediates the interaction with vascular calcification is still to be fully identified, but should be investigated in future studies.

A limitation of this study is that the atherogenic diet and the 20 mM glucose incubation which have previously been shown to cause endothelial dysfunction, did not alter endothelial function, in- dicating heterogeneity of blood vessel dysfunction. However, we were still able to assess the effect of ucOC on endothelial function in normal and high glucose environments.

In conclusion, acute ucOC treatment does not have a negative, or positive, effect on endothelial function or endothelial cell homeostasis in rabbit aorta or human vascular cells in the presence or absence of a high glucose environment. As no adverse effects occurred, it is proposed that ucOC may be considered as a therapeutic option for metabolic disease.

ACKNOWLEDGEMENT

This study was supported by the Rebecca L. Cooper Medical Research Foundation.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

The research was designed by Alexander Tacey, Sophie Millar, Susan Anderson, Anthony Zulli, Saoirse O'Sullivan and Itamar Levinger. The experiments were completed by Alexander Tacey, Sophie Millar and Tawar Qaradakhi. The original draft was written by Alexander Tacey. The manuscript was reviewed and edited by Sophie Millar, Tawar Qaradakhi, Cassandra Smith, Alan Hayes, Susan Anderson, Anthony Zulli, Saoirse O'Sullivan and Itamar Levinger. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data from this study are available upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Tacey A, Millar S, Qaradakhi T, et al. Undercarboxylated osteocalcin has no adverse effect on endothelial function in rabbit aorta or human vascular cells. *J Cell Physiol*. 2020;1–10. https://doi.org/10.1002/jcp.30048

Appendix D: Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo*

PLOS ONE

RESEARCH ARTICLE

Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo*

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Citation: Tacey A, Smith C, Woessner MN, Chubb P, Neil C, Duque G, et al. (2020) Undercarboxylated osteocalcin is associated with vascular function in female older adults but does not influence vascular function in male rabbit carotid artery *ex vivo*. PLoS ONE 15(11): e0242774. https://doi.org/10.1371/journal.pone.0242774

Editor: Michael Bader, Max Delbruck Centrum fur Molekulare Medizin Berlin Buch, GERMANY

Received: August 19, 2020

Accepted: November 9, 2020

Published: November 25, 2020

Data Availability Statement: The Human Ethics approval obtained for this study states that only the investigators will have access to the data. Sharing the data with a wide audience will be a breach of the participants' confidentiality and will not be approved by the Ethics Committee. As such, interested researchers should contact the corresponding author and Ethics Committee if they wish to have access to the data. The corresponding author will then need to receive special approval from the Human Ethics Committee that approved

Abstract

Background

There are conflicting reports on the association of undercarboxylated osteocalcin (ucOC) in cardiovascular disease development, including endothelial function and hypertension. We tested whether ucOC is related to blood pressure and endothelial function in older adults, and if ucOC directly affects endothelial-mediated vasodilation in the carotid artery of rabbits.

Methods

In older adults, ucOC, blood pressure, pulse wave velocity (PWV) and brachial artery flow-mediated dilation (BAFMD) were measured (n = 38, 26 post-menopausal women and 12 men, mean age 73 ± 0.96). The vasoactivity of the carotid artery was assessed in male New Zealand White rabbits following a four-week normal or atherogenic diet using perfusion myography. An ucOC dose response curve (0.3–45 ng/ml) was generated following incubation of the arteries for 2-hours in either normal or high glucose conditions.

Results

ucOC levels were higher in normotensive older adults compared to those with stage 2 hypertension ($p < 0.05$), particularly in women ($p < 0.01$). In all participants, higher ucOC was associated with lower PWV ($p < 0.05$), but not BAFMD ($p > 0.05$). In rabbits, ucOC at any dose did not alter vasoactivity of the carotid artery, either following a normal or an atherogenic diet ($p > 0.05$).

the study to disclose this information. The contact information for the Victoria University Human Ethics Committee is as follows - A/Prof Deborah Zion, Chair of Victoria University Ethics Committee (deborah.zion@vu.edu.au) and VU Research Ethics Committee Secretary (researchethics@vu.edu.au).

Funding: This study was supported by funding from the Rebecca Cooper Medical Research Foundation (IL) (https://www.cooperfoundation.org.au/) and the Tom Penrose Community Service Grant (IL) (https://www.essa.org.au/) from Exercise and Sports Science Australia (ESSA). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusion

Increased ucOC is associated with lower blood pressure and increased arterial stiffness, particularly in post-menopausal women. However, ucOC administration has no direct short-term effect on endothelial function in rabbit arteries. Future studies should explore whether treatment with ucOC, *in vivo*, has direct or indirect effects on blood vessel function.

Introduction

The bone derived hormone osteocalcin (OC) is a vitamin K-dependent protein that exists in several biological forms. The post-translational γ-carboxylation of less than three glutamic acid residues produces undercarboxylated osteocalcin (ucOC), which has a low affinity for hydroxyapatite and is predominantly found in circulating blood [1]. In recent years ucOC has been suggested as a mediator of a cross-talk between bone and metabolic outcomes [2]. In humans, higher levels of ucOC are associated with a reduced risk of metabolic syndrome and type II diabetes [3–5]. Similarly, ucOC has been reported to improve glucose regulation, adiposity and insulin sensitivity in animal models [6–8]. However, not all studies are in agreement [9, 10]. Given these findings, it is of interest to investigate whether ucOC is involved in other biological functions within the body [11, 12]. As metabolic and cardiovascular diseases (CVD) share common pathological links [13], it is of particular interest to examine the interaction of ucOC with endothelial function and atherosclerosis progression. This is important, not only in the context of CVD, but also because ucOC could be targeted as a future therapy for metabolic diseases.

The association between OC and its isoforms with CVD in humans remains unknown [14, 15]. A number of cross-sectional studies have reported that higher circulating total OC (tOC) is associated with improved vascular health and function [16–18]. Yet, others have reported that higher tOC has adverse [19–21], or even no association [22], with vascular health. However, only a limited number of studies have investigated the role of ucOC in the vasculature. As ucOC is suggested to be the active circulating form of OC, it is particularly important to investigate whether ucOC is associated with vascular function, and if so, whether these effects are beneficial or detrimental.

In animal models, administration of tOC and ucOC *in vivo* improve blood vessel function. For example, daily tOC (30ng/gram) injections for 12 weeks significantly improved pulse wave velocity (PWV), a measure of arterial stiffness, in rats with induced diabetes mellitus [23]. Daily tOC also enhanced vasodilation *ex vivo* in the aorta of apolipoprotein $E^{-/-}$ mice [24]. In another study, 30ng/gram of ucOC administered for 10 weeks in female C57BL/6 mice produced an increase in nitric oxide availability, a key vasoactive molecule [25]. While these *in vivo* studies indicate potential links between OC administration and improvements in vascular health, they also reported concurrent improvements in metabolic outcomes, such as improved glycaemic control and lower adiposity. Therefore, it is unclear whether the improvement in blood vessel function resulted from a direct effect of OC, or an indirect effect from improved metabolic outcomes.

The aims of this study were to a) investigate the association of circulating ucOC levels with endothelial function, arterial stiffness and blood pressure (BP) in older adults via a cross-sectional analysis, and b) examine the direct effect of ucOC on endothelial function in rabbit arteries.

Methods

Human participants

Twenty six healthy, community dwelling post-menopausal women (mean age of 73 years) and 12 older men (mean age of 74 years) participated in this study. Inclusion criteria included

adults over 60 years old and women >12 months post-menopause. Exclusion criteria included a current diagnosis of diabetes, a body mass index (BMI) over 40kg/m^2, a fracture within the last 3 months or participation in resistance exercise >2 days per week. Participants were on a range of medications to control for hypertension, cholesterol and CVD, however all were stable and controlled for at least 3 months as per their medical records. Each participant received written and verbal explanations about the nature of the study before signing an informed consent document. This study was approved by Melbourne Health and Victoria University Human Research Ethics Committees. The data were collected as part of a larger clinical trial (ACTRN12618001756213).

Blood pressure (BP) and vascular function

Brachial artery systolic BP, diastolic BP and mean arterial pressure measurements were recorded using the non-invasive SphygomoCor-XCEL1 (AtCor Medical, Sydney, NSW, Australia) diagnostic system. Two measurements were captured, with the lower of the two readings recorded. If the two BP readings were >6 mmHg apart, a third measure was recorded to ensure a true resting value and the average of the two lowest BP measurements were recorded. Participants were split into groups based on hypertension guidelines; normal (<130mmHg/<80mmHg) n = 7, stage 1 hypertension (130–139mmHg/80-89mmHg) n = 14 or stage 2 hypertension (>140mmHG/>90mmHG) n = 17 [26]. None of the participants with normal BP were taking antihypertensive medication, five of the participants with stage 1 hypertension and 10 of the participants with stage 2 hypertension were taking antihypertensive medication. Arterial stiffness was measured by PWV using the subtraction method, with the thigh cuff placed on the thigh and a tonometer used to measure the carotid artery waveform (SphygomoCor-XCEL1) [27].

Endothelial function was assessed via brachial artery flow mediated dilation (BAFMD) using a high-resolution ultrasound (Terason, LifeHealthcare, New South Wales, Australia) with R wave trigger. Brachial artery diameter was assessed for ~10 seconds at baseline (in duplicate and averaged) and during forearm occlusion. Brachial artery diameter was continuously captured after the occlusion cuff release for ~2 minutes (reactive hyperaemia). Peak change was calculated as the peak percentage change in brachial artery diameter from baseline to immediately following peak hyperaemia [28].

Circulating osteocalcin measurements

Serum samples were taken in the morning following an overnight fast and stored at -80˚C until analysis. Serum tOC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics) [29]. Serum ucOC was measured using the hydroxyapatite binding method, a commonly used, well established method [30]. Each sample was measured once and the inter-assay coefficients of variation were 5.4% and 9.2% for tOC and ucOC, respectively.

Animals

Male New Zealand White rabbits at 12 weeks of age were randomised into either a normal chow diet (n = 7) (Guinea pig and rabbit pellets, Specialty Feeds, Australia) or an atherogenic diet (n = 10) (a normal diet combined with 1% methionine, 0.5% cholesterol and 5% peanut oil (#SF00-218, Specialty Feeds, Australia)) for 4 weeks [31]. This atherogenic diet has previously been reported to cause endothelial dysfunction in rabbits [31, 32]. The rabbits were housed in individual cages on a 12-hour light/dark cycle at 21˚C, with access to water and their assigned chow diet *ad libitum*. At the completion of the 4-week diet, the rabbits were sedated (0.25mg/kg medetomidine) and anaesthetised (4% isoflurane) before exsanguination

via severing of the inferior vena cava. The arterial system was immediately flushed with ice cold Krebs solution ((mM) 118 NaCl; 4.7 KCl; 1.2 $MgSO_4$|$7H_2O$; 1.2 KH_2PO_4; 25 $NaHCO_3$; 1.25 CaCl and 11.7 glucose) and the carotid arteries were carefully dissected and placed in Krebs solution. The animal experiments were approved by the Victoria University Animal Ethics Committee (#14/005) and complied with the Australian National Health and Medical Research Council code for the care and use of animals for scientific purposes (8^{th} edition).

Perfusion myography

The carotid arteries were cleaned of connective tissue and fat, with care taken to avoid damaging the arterial wall and the endothelium. Arterial branches were identified, and the carotid arteries were cut to a length of 15–20mm, ensuring no branches were present. The arteries were placed in individual chambers within a perfusion myography system (Zultek Engineering, Melbourne, Australia). Each artery was immersed in either normal Krebs solution (11mM glucose) or a high glucose Krebs solution (20mM glucose) as previously described [32]. The organ baths were warmed to 37˚C and bubbled with 95% oxygen and 5% carbon dioxide and were refreshed every 30 minutes over a 2-hour period with the respective Krebs solution. Subsequently, the arteries were cannulated, and the respective Krebs solution pumped through the artery while pressure transducers monitored the intraluminal pressure of the vessel.

The carotid arteries were constricted with phenylephrine ($3x10^{-7}$M), which was added intraluminally and extraluminally. Once a stable constriction was achieved, a dose response curve was completed to ucOC (0.3, 3, 30 and 45ng/ml) (Glu13, 17, 20, osteocalcin (1–46) (mouse) trifluoroacetate salt (Auspep, Australia, H-6552.0500)) or to Krebs (control), each concentration was administered internally via the endothelium and separated by 2 minute intervals. The same mouse ucOC has previously been shown to improve relaxation in rabbit arteries [33]. Following the dose response curve a bolus of acetylcholine (ACh) (10^{-5}M) was added internally and two minutes later a bolus of sodium nitroprusside (SNP) (10^{-5}M) externally, to determine the maximal endothelium-dependent and endothelium-independent relaxation, respectively. The vasoactive response of the vessels were analysed using the MEDIDAQ software program (MEDIDAQ, Melbourne, Australia). The vasoactivity of the carotid artery was measured as percentage change from the phenylephrine peak pressure and compared to the baseline pressure. Area under the curve (AUC) was calculated as the total relaxation below the phenylephrine plateau caused by the dose response curve, ACh and SNP bolus doses. The endothelium-dependent E_{max} was considered as the relaxation produced by ACh, and the endothelium-independent E_{max} was considered as the relaxation produced by SNP.

Statistical analysis

Human data were analysed using Statistical Package for the Social Sciences (SPSS, Inc. Chicago, IL, USA, version 22). A one-way analysis of variance (ANOVA) was used to examine the difference in ucOC concentration when participants were split into groups based on BP levels. Spearman rho correlations were used to examine the correlation between ucOC and measures of vascular function (BP, BAFMD and PWV) in all participants. Spearman partial correlations were used for the additional adjustments of age, BMI or age and BMI, as they are strong influencers of ucOC levels [1,29].

Animal data were analysed using Graphpad prism (version 7.1, Graphpad software Inc, USA). A one-way ANOVA was used to examine the effect of the ucOC dose response curves in rabbit carotid artery segments. AUC was calculated as the total area of relaxation below the maximum phenylephrine pressure and a one-way ANOVA was used to determine the difference in AUC between the ucOC dose response curves. All data is reported as mean ± SEM and

statistical analysis was conducted at the 95% confidence level of significance ($p < 0.05$). Trends were reported when $p = 0.05–0.099$.

Results

Human data

Participant characteristics are presented in Table 1. In older adults with stage 2 hypertension ucOC was reduced by 34% compared to normotensive individuals ($p < 0.05$, Fig 1A). When split by sex, ucOC was reduced by 43% ($p < 0.01$, Fig 1B) and tOC was reduced by 30% ($p < 0.05$, Fig 1E) in women with stage 2 hypertension compared to normotensive women. There was no difference between groups in older men ($p > 0.05$, Fig 1C and 1F).

Correlation between ucOC and vascular function outcomes

In the unadjusted model, high circulating ucOC was associated with lower systolic BP and PWV with all participants combined ($p < 0.05$ for both, Table 2). In women only, higher levels of circulating ucOC and tOC was associated with lower systolic BP ($p < 0.01$). There were trends for associations between lower MAP and PWV with higher levels of ucOC in women ($p = 0.05–0.09$ for both, Table 2). When adjusted for age, higher ucOC was associated with lower diastolic BP in all participants and with lower systolic BP in women ($p < 0.05$ for both, Table 2). Increased ucOC levels tended to correlate with both lower DBP and MAP in women after adjusting for age ($p = 0.05–0.09$ for both, Table 2). Adjusting for BMI, and BMI and age together, removed all associations of ucOC and tOC with BP and PWV outcomes ($p > 0.05$). There were no significant correlations between ucOC and tOC with BAFMD peak % dilation in any model, and ucOC or tOC was not associated with any vascular function outcome in men ($p > 0.05$).

Table 1. Baseline characteristics.

Variable	n	Mean ± SEM
Participant number (n) [F/M]		38 [26/12]
Age (years)	38	73 ± 0.96
BMI (kg/m^2)	38	28 ± 0.59
Waist circumference (cm)	36	91 ± 1.58
Currently smoking (n) [%]	38	2 [5]
Caucasian ethnicity (n) [%]	38	38 [100]
Cholesterol medication (n) [%]	38	13 [34]
Antihypertensive medication (n) [%]	38	15 [39]
Heart disease medication (n) [%]	38	10 [26]
tOC (ng/ml)	37	21 ± 1.31
ucOC (ng/ml)	37	8 ± 0.61
ucOC/tOC ratio	37	0.39 ± 0.01
Systolic BP (mmHg)	38	139 ± 2.53
Diastolic BP (mmHg)	38	79 ± 1.43
MAP (mmHg)	38	98 ± 1.74
PWV (m/s)	34	8 ± 0.28
BAFMD–peak dilation (%)	29	4.62 ± 0.44
BAFMD–time to peak dilation (s)	29	58 ± 2.56

Abbreviations: BMI; Body mass index, tOC; total osteocalcin, ucOC; undercarboxylated osteocalcin, BP; blood pressure, MAP; mean arterial pressure, PWV; pulse wave velocity, BAFMD; brachial artery flow mediated dilatation.

https://doi.org/10.1371/journal.pone.0242774.t001

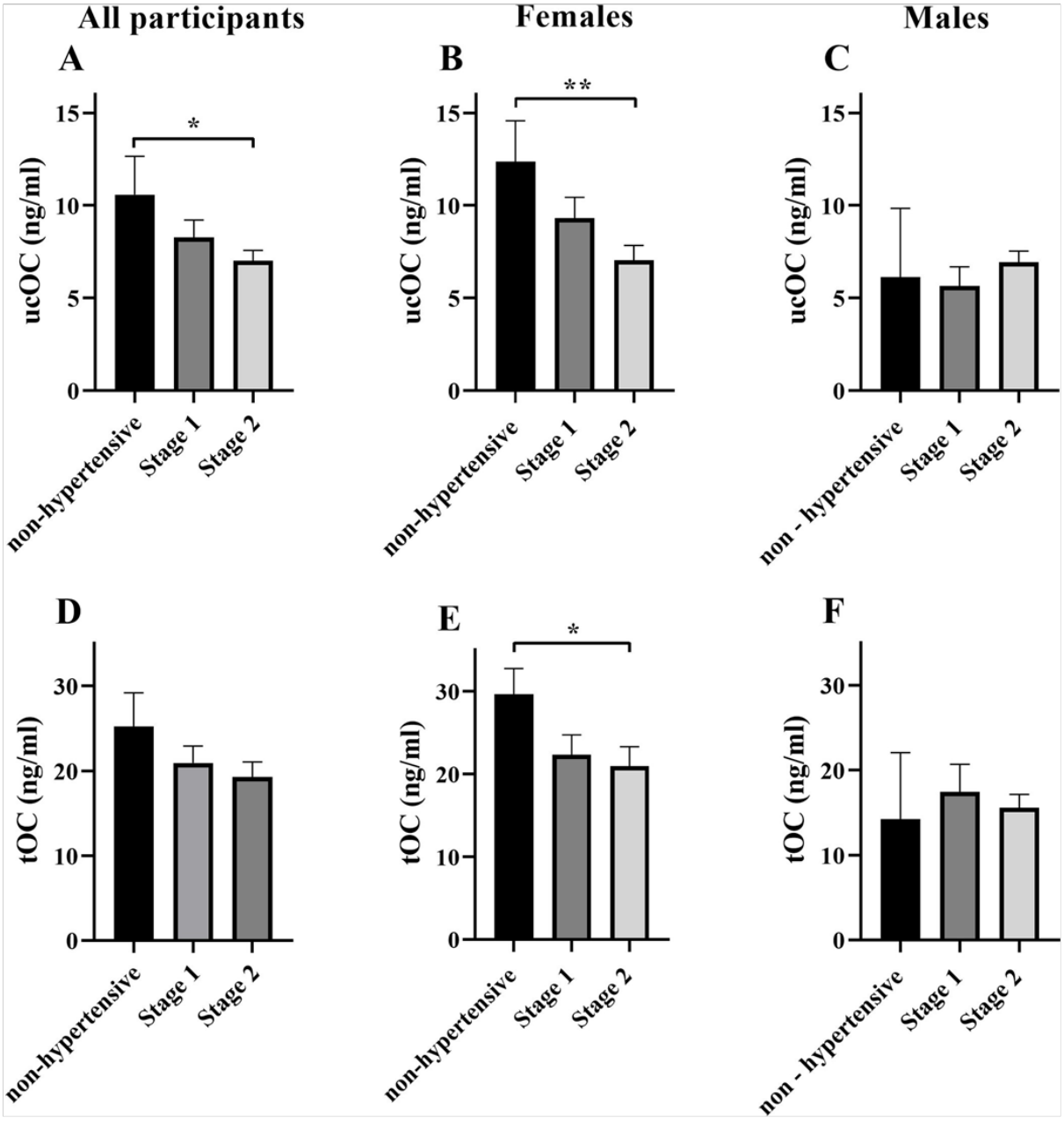

Fig 1. Concentration of ucOC and tOC based on hypertension category. ucOC concentration in all participants (A), women (B) and men (C) and tOC concentration in all participants (D), women (E) and men (F) split into groups based hypertension category; non-hypertensive (<130/<80mm/Hg) (women n = 5, men n = 2), stage 1 hypertension (130-139/80-89mm/Hg) (women n = 10, men n = 4) and stage 2 hypertension (>140/>90mm/Hg) (women n = 11, men n = 6). Given the small sample size in each group, particularly for males, the data are not conclusive and further examination of sex specific effects should be explored. All data mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ between groups. *Abbreviations: ucOC; undercarboxylated osteocalcin, tOC; total osteocalcin.*

https://doi.org/10.1371/journal.pone.0242774.g001

Table 2. Correlation of ucOC and tOC with vascular function outcomes.

	ucOC			tOC		
	All (n = 38)	Women (n = 26)	Men (n = 12)	All (n = 38)	Women (n = 26)	Men (n = 12)
SBP						
Model 1	**-0.39**{	**-0.58**{{	0.25	**-0.31**^	**-0.4**{	0.18
Model 2	-0.28	**-0.48**{	0.01	-0.23	**-0.39**^	0.07
Model 3	0.15	-0.07	0.39	0.18	-0.004	0.37
Model 4	0.25	0.05	0.26	0.2	0.04	0.41
DBP						
Model 1	-0.21	-0.3	0.12	-0.23	-0.22	-0.07
Model 2	**-0.41**{	**-0.44**^	-0.51	-0.34	-0.3	-0.42
Model 3	-0.04	-0.14	-0.05	-0.08	-0.02	-0.4
Model 4	-0.13	-0.14	-0.75	-0.09	-0.02	-0.58
MAP						
Model 1	-0.2	**-0.35**^	0.18	-0.19	-0.24	-0.06
Model 2	-0.32	**-0.44**^	-0.06	-0.25	-0.33	-0.24
Model 3	0.12	-0.05	0.05	0.1	0.03	-0.19
Model 4	0.09	-0.01	-0.42	0.1	0.04	-0.22
PWV						
Model 1	**-0.41**{	**-0.41**^	-0.32	-0.25	-0.32	-0.01
Model 2	-0.18	-0.26	0.25	-0.14	-0.26	0.4
Model 3	-0.03	0.02	-0.23	0.11	0.003	0.37
Model 4	0.19	0.13	0.54	0.16	0.03	0.76
BAFMD peak %						
Model 1	0.14	0.00	0.39	0.23	0.09	0.29
Model 2	0.22	0.13	0.32	0.26	0.16	0.63
Model 3	0.02	-0.18	0.51	0.13	-0.06	0.46
Model 4	0.05	-1.14	0.29	0.14	-0.04	0.64

Model 1—unadjusted; Model 2—adjusted for age; Model 3—adjusted for BMI; Model 4—adjusted for age and BMI. Given the small sample size in each group, particularly for males, the data are not conclusive and further examination of sex specific effects should be explored.

{ $p < 0.05$

{{ $p < 0.01$

^p 0.05–0.09 ucOC and tOC vs vascular function outcome.

Abbreviations: ucOC; undercarboxylated osteocalcin, tOC; total osteocalcin SBP; systolic blood pressure, DBP; diastolic blood pressure, PWV; pulse wave velocity, BAFMD; brachial artery pulse wave velocity.

https://doi.org/10.1371/journal.pone.0242774.t002

Perfusion myography

The carotid artery segments from the animals fed the atherogenic diet did not exhibit a reduction in endothelium dependent relaxation in comparison to the arteries from the normal diet fed animals ($p > 0.05$). The carotid artery vasoactive response from rabbits fed a normal or atherogenic diet and treated *ex vivo* with ucOC was unaltered in both normal and high glucose environments ($p > 0.05$, Fig 2A and 2C). The endothelium-dependent (ACh) and endothelium-independent (SNP) E_{max} were also unaltered following ucOC treatment, in comparison to the control, suggesting ucOC did not enhance the maximal relaxation of the vessel ($p > 0.05$, Fig 2A and 2C). The AUC was unaltered by ucOC treatment following both the normal and atherogenic diet and incubation in normal and high glucose conditions ($p > 0.05$, Fig 2B and 2D).

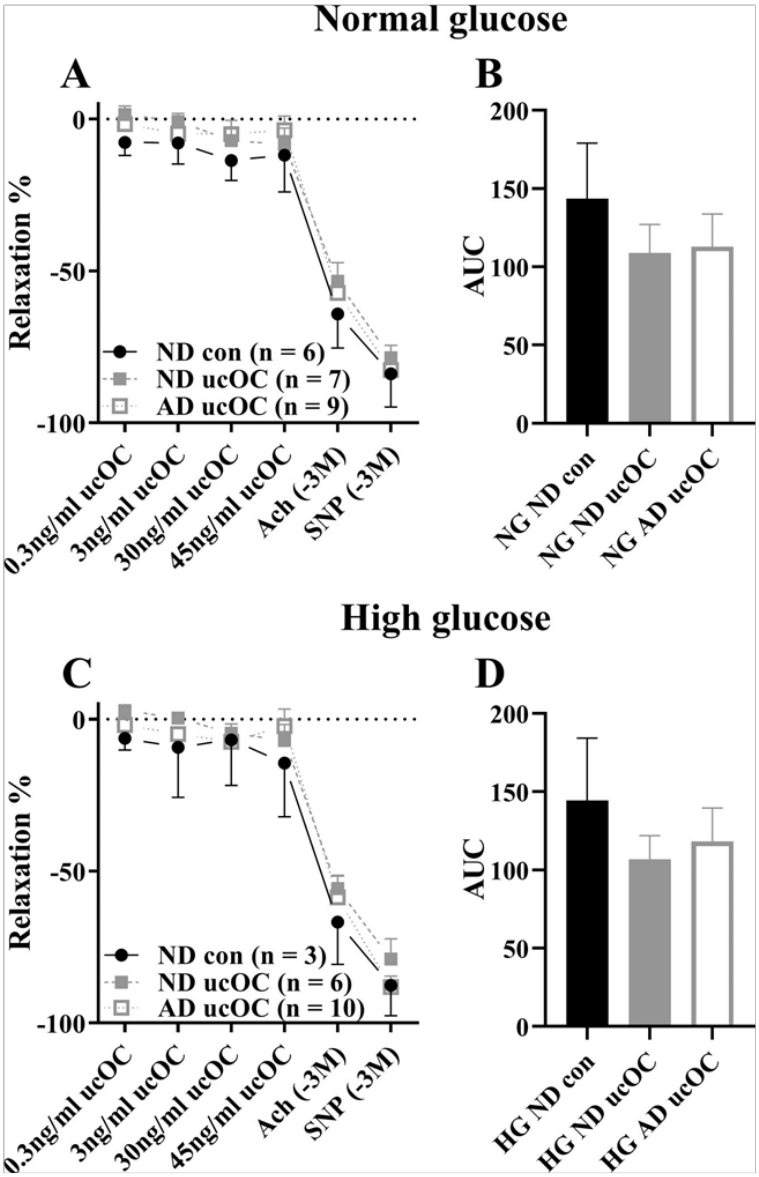

Fig 2. ucOC administration to carotid artery following 2-hour incubation in NG or HG solution. (A) ucOC dose response curve in carotid artery incubated in NG solution and (B) AUC of dose response curve. (C) ucOC dose response curve in carotid artery incubated in HG solution and (D) AUC of dose response curve. All data mean ± SEM. No significant differences were detected. *Abbreviations*: *ucOC, undercarboxylated osteocalcin; NG, normal glucose media; HG, high glucose media; ND, normal diet; AD, atherogenic diet; Ach, acetylcholine; SNP, sodium nitroprusside.*

https://doi.org/10.1371/journal.pone.0242774.g002

Discussion

The major findings of the current study are a) in humans, higher circulating ucOC is associated with lower BP and increased arterial stiffness, which is particularly evident in post-menopausal women, and b) ucOC treatment has no beneficial, but also no adverse, effect on carotid

artery function from rabbits fed a normal or atherogenic diet, or exposed acutely to normal and high glucose environments.

A number of studies have examined the correlation of tOC with vascular health and function outcomes. It was reported that tOC was lower in men, but not women with hypertension (aged 24–78 years) [34]. Further, in 3,604 middle to older aged men and women, higher levels of tOC were associated with lower PWV in men, but higher PWV in women. However, when controlled for age and menopause status there was no longer an association between tOC and PWV in women [35]. In middle and older aged men, but not post-menopausal women, higher tOC was associated with lower brachial artery PWV and intima media thickness (IMT), even after adjustment for confounding variables including age and BMI [36]. Yet, not all studies are in agreement, as higher tOC levels were associated with increased IMT, carotid plaque and aortic calcification in middle to older-aged women, but not men [19]. Overall, the findings are conflicting, and this appears to be largely driven by the differences between men and women. A major limitation of these studies is that they do not report the concentration of the individual forms of OC, in particular ucOC, which is important as ucOC is the putative bioactive form of the hormone.

Evidence examining the association of ucOC with vascular function is lacking, but crucial, if we are to understand the role of ucOC in CVD, specifically hypertension and atherosclerosis. In the current study we report that higher levels of ucOC are associated with lower BP in post-menopausal women, but not in older men. However, the relatively small sample size of men in the current study means that definitive conclusions cannot be established. However, similar to the current study, a previous study in older men and women (mean age 64 years old), reported that those with a higher cardiovascular risk score had increased MAP and lower circulating ucOC levels [4]. Conflictingly, in 162 community dwelling men (mean age 48 years old) and women (mean age 55 years old), ucOC was not correlated with systolic BP or diastolic BP [37]. The conflicting outcomes may be related to the age difference between the study cohorts, as age is an important factor in determining ucOC levels [29]. Furthermore, hormone variations between sexes and between pre- and post-menopausal women may also explain some of the diverse findings reported. Overall, whether ucOC is a mediator or a marker of CVD processes requires further investigation. In addition, taking into account several factors including sex, age and hormonal status will be important considerations for future studies.

As the association of ucOC with vascular function in humans is unclear, and given the exact biological functions of ucOC are yet to be fully elucidated, examining its bioactive effect on the vasculature in animal models is important. The most commonly used method of examining the vasoactivity of blood vessels *ex vivo* is via isometric tension analysis. However, in this study, we have utilised a novel perfusion myography system. This technique utilises haemodynamic forces such as shear stress, pressure and pulsatile flow, which are mechanical factors important in the regulation of normal endothelial function, thus creating a more physiological environment [38]. We found that ucOC did not directly influence the vasoactivity of isolated rabbit carotid arteries in either normal or high glucose solutions following an atherogenic or normal diet. A potential limitation of this study is that the atherogenic diet did not cause endothelial dysfunction. This suggests that the carotid artery may be resistant to the development of endothelial dysfunction, as previous studies have reported that the same atherogenic diet caused endothelial dysfunction after 4-weeks in rabbit aorta, iliac and mesenteric arteries [31, 32]. Carotid arteries were used in this study as they lack arterial branches, allowing effective cannulation and perfusion, which would not have been possible in other vessels due to the presence of branches. Notwithstanding, ucOC did not influence vasoactivity when vessels were exposed to a high glucose solution. In support of this, a previous *ex vivo* study, utilising the isometric tension analysis technique, reported similar findings to the current study. The

administration of ucOC (10ng/ml and 30ng/ml) to rabbit aorta following an atherogenic diet or normal diet, with incubation in normal or high glucose solution, did not influence endothelium-dependent or endothelium-independent vasodilation [39]. Whilst another study reported that ucOC caused a slight enhancement in ACh-induced endothelium-dependant relaxation in dysfunctional rabbit aorta following an atherogenic diet, this did not occur after a normal diet, suggesting that ucOC may only function to enhance endothelium-dependent vasodilation in a dysfunctional state [33]. However, this requires further investigation. Overall, whilst we report a correlation between ucOC and BP in post-menopausal women, the *ex vivo* data indicates that ucOC has minimal direct biological influence on vascular function. There are several potential reasons for these findings. Firstly, ucOC may not act directly on the vasculature, and the associations observed in some studies may be through indirect pathways, such as via improvements in glycaemic control. Secondly, given recent reports, ucOC may not be as biologically active outside of the skeleton as initially suggested [9, 10].

This study has several limitations. Firstly, the relatively small sample size of older adult men means that definitive conclusions on the association of ucOC with vascular function in males cannot be made. Further research should examine in detail the potential association of ucOC with vascular function in females and males, taking into account confounding variables such as age and BMI. Secondly, a number of human participants were on hypertensive medication which may have influenced their BP measurement, highlighting the important role animal models can play in determining any direct effects of ucOC. Finally, due to only male rabbits being studied, the direct effect of ucOC on endothelial function in arteries from female rabbits is unclear.

In conclusion, increased ucOC is associated with lower BP and arterial stiffness in post-menopausal women, but has no direct effect on endothelial function in rabbit carotid arteries. Future studies should explore whether treatment with ucOC *in vivo* has direct or indirect effects on blood vessel function.

Acknowledgments

We acknowledge the participants who completed the study for their time and effort.

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Appendix E: Association between circulating osteocalcin and cardiometabolic risk factors following a 4-week leafy green vitamin K-rich diet

Annals of Nutrition and Metabolism

Clinical Study: Research Article

Ann Nutr Metab
DOI: 10.1159/000511660

Received: April 24, 2020
Accepted: September 17, 2020
Published online: November 24, 2020

Association between Circulating Osteocalcin and Cardiometabolic Risk Factors following a 4-Week Leafy Green Vitamin K-Rich Diet

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Keywords
Vitamin K · Undercarboxylated osteocalcin · Carboxylated osteocalcin · Arterial stiffness · Blood glucose · Blood lipids

Abstract
Background: Evidence suggests that lower serum undercarboxylated osteocalcin (ucOC) may be negatively associated with cardiometabolic health. We investigated whether individuals with a suppression of ucOC following an increase in dietary vitamin K1 exhibit a relative worsening of cardiometabolic risk factors. ***Materials and Methods:*** Men ($n = 20$) and women ($n = 10$) aged 62 ± 10 years participated in a randomized, controlled, crossover study. The primary analysis involved using data obtained from participants following a high vitamin K1 diet (HK; 4-week intervention of increased leafy green vegetable intake). High and low responders were defined based on the median percent reduction (30%) in ucOC following the HK diet. Blood pressure (resting and 24 h), arterial stiffness, plasma glucose, lipid concentrations, and serum OC forms were assessed. ***Results:*** Following the HK diet, ucOC and ucOC/tOC were suppressed more ($p < 0.01$) in high responders (41 and 29%) versus low responders (12 and 10%). The reduction in ucOC and ucOC/tOC was not associated with changes in blood pressure, arterial stiffness, plasma glucose, or lipid concentrations in the high responders ($p > 0.05$). ***Discussion/Conclusion:*** Suppression of ucOC via consumption of leafy green vegetables has no negative effects on cardiometabolic health, perhaps, in part, because of cross-talk mechanisms.

Alexander Tacey and Marc Sim shared first authorship; Lauren C. Blekkenhorst and Itamar Levinger shared last authorship.

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Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. A diet rich in fruit and vegetables is an important, nontherapeutic approach to reduce CVD development and progression [2, 3]. Evidence suggests that diets rich in green leafy vegetables increase nitric oxide bioavailability and can improve vascular health [4, 5]. However, we have previously shown that a 4-week dietary intervention involving an increased intake of leafy green vegetables did not reduce blood pressure (BP) or arterial stiffness [6]. One potential explanation for the absence of a beneficial effect on BP and arterial stiffness may be related to other bioactive components found in leafy green vegetables that concomitantly influence vascular health. For example, vitamin K1 is abundant in leafy green vegetables and regulates several coagulation factors including vitamin K-dependent proteins [7].

One such protein is osteocalcin (OC), a vitaminK-dependent protein derived from osteoblasts that exists in 2 forms: carboxylated OC (cOC) and undercarboxylated OC (ucOC) [8–10]. cOC has a high affinity to hydroxyapatite within the bone matrix and is therefore thought to reflect bone mineralization [11, 12], whereas ucOC is proposed as the bioactive form of OC in several target tissues [13]. Growing evidence suggests an association between OC, in particular total OC (tOC) and ucOC with hypertension, vascular calcification, atherosclerosis, and CVD mortality [14–17]. However, the literature is conflicting, and it is unclear whether tOC or its isoforms are associated with positive or negative effects on cardiometabolic health [18, 19]. We have previously shown that a diet rich in leafy green vegetables, and thus vitamin K1, reduces circulating ucOC levels [20].

The current study was a subanalysis examining the cardiometabolic implications of ucOC suppression following an increased intake of predominantly leafy green vegetables. It was of interest to investigate whether a reduction in ucOC levels was correlated with changes in cardiometabolic risk factors, and whether this could explain, at least in part, the lack of a beneficial effect on BP following an increase in dietary nitrate. Participants from the high vitamin K1 intervention were divided into high/low responders based on the suppression of ucOC following the intervention. The aim was to determine if a large reduction in ucOC (high responders) would be associated with alterations in cardiometabolic risk factors including BP, arterial stiffness, blood glucose, and lipidconcentrations.

Table 1. Participant characteristics (mean±SEM)

Variable	Mean±SEM
Participant, *n* (M/F)	30 [20/10]
tOC (M/F), ng/mL	21.82±1.53/22.23±1.79
cOC (M/F), ng/mL	14.05±1.17/13.41±2.01
ucOC (M/F), ng/mL	7.77±0.88/8.82±0.77
Age, years	62±9.90
BMI, kg/m^2	27±3.87
Waist circumference, cm	89±2.18
Waist-to-hip ratio	0.87±0.02
Systolic BP, mm Hg	134±1.53
Diastolic BP, mm Hg	78±1.45
Heart rate, bpm	62±1.46
Glucose, mmol/L	5.29±0.08
Total cholesterol, mmol/L	5.54±0.26
HDL, mmol/L	1.35±0.06
LDL, mmol/L	3.61±0.22
Triglycerides, mmol/L	1.28±0.11
eGFR, mL/min/1.73 m	92.57±2.17
Vitamin K intake, µg/day	121±11

tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; cOC, carboxylated osteocalcin; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate.

Methods

The data for this paper were collected from the Vegetable IntakeandBloodPressure(VIABP)study(ACTRN12615000194561). The study was approved by the University of Western Australia Human Research Ethics Committee and was completed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. The study was a randomized, controlled crossover trial, and methodology has been described in full elsewhere [6]. In brief, middle- and older-aged (40–74 years of age) community-dwelling men and women with prehypertension or untreated grade 1 hypertension were recruited to participate. Each participant received three 4-week dietary interventions, each interspersed with a 4-week washout period. The VIABP study was originally designed with the following dietary interventions: (1) increased intake of nitrate-rich leafy green vegetables (high nitrate); (2) increased intake of nitrate-poor vegetables (low nitrate); and (3) no increase in vegetables (control). As vitamin K1 is also found predominately in leafy green vegetables, these 3 dietary interventions have been equated to (1) high vitamin K1 intake (HK); (2) low vitamin K1 intake (LK); and (3) control diet (CON) [20]. Considering the primary aim of this study is to examine the association between the suppression of ucOC and cardiometabolic risk factors (and given the LK diet did not suppress ucOC), we predominantly considered data from the HK intervention.

Resting BP and pulse wave velocity (PWV) (SphygmoCor XCEL 2012; AtCor Medical Pty., Ltd.) were measured before and after the 4-week dietary intervention, as previously described [6]. Ambulatory BP was recorded over a 24-h period, every 20 min

Table 2. OC, vascular, and metabolic outcomes before and after high vitamin K diet, separated into high and low responders

	Low responders			High responders		
	before, mean±SEM	after, mean±SEM	Δchange	before, mean±SEM	after, mean±SEM	Δchange
Sample, *n* (F/M)	4/11	4/11		6/9	6/9	
tOC, µg/L	21.61±1.39	20.61±1.52	−1±0.86	22.31±1.92	18.38±1.42***	−3.93±0.77
ucOC, µg/L	8.86±0.88	7.76±0.93***	−1.10±0.24	7.39±0.92	4.33±0.44***	**−3.06±0.51**##
cOC, µg/L	12.75±1.44	12.85±1.25	0.1±0.74	14.92±1.42	14.05±1.19	−0.87±0.68
ucOC/tOC	0.42±0.04	0.38±0.04**	−0.04±0.01	0.34±0.03	0.24±0.02***	**−0.1±0.01**##
Amb SBP, mm Hg	125.40±1.86	126.20±1.73	0.8±1.24	125.79±1.85	126.83±1.60	1.04±1.13
Amb DBP, mm Hg	76.15±2.14	76.26±2.23	0.12±1.17	74.41±2.10	74.34±2.06	−0.07±0.76
Resting SBP, mm Hg	130.13±1.46	127.33±2.18*	−2.8±1.26	130.37±2.52	129.53±2.45	−0.83±1.97
Resting DBP, mm Hg	77.9±1.57	75.53±1.64	−2.37±1.25	75.30±2.00	75.07±2.12	−0.23±1.20
PWV, m/s	8.34±0.36	8.38±0.35	0.04±−0.21	8.31±0.26	8.17±0.24	−0.13±0.16
Glucose	5.17±0.15	5.06±0.13	−0.11±0.14	4.79±0.16	4.88±0.13	0.09±0.12
Total chol	5.64±0.28	5.59±0.23	−0.05±0.17	5.32±0.36	4.96±0.33	−0.36±0.22
LDL	3.68±0.27	3.68±0.22	0.01±0.14	3.26±0.30	3.04±0.28	−0.22±0.15
HDL	1.38±0.07	1.35±0.09	−0.03±0.03	1.44±0.09	1.39±0.10	−0.05±0.05
Triglycerides	1.26±0.16	1.21±0.10	−0.05±0.11	1.34±0.25	1.17±0.16	−0.17±0.14

Delta (Δ) change of OC, vascular, and metabolic outcomes following the high vitamin K1 diet (before to after). High and low responders based on median split in percent change of ucOC from before to after high vitamin K1 diet. Data reported as mean±SEM. OC, osteocalcin; tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; cOC, carboxylated osteocalcin; amb, ambulatory; SBP, systolic blood pressure; DBP, diastolic blood pressure; PWV, pulse wave velocity; chol, cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein. * $p<0.05$. ** $p<0.01$. *** $p<0.001$ before versus after high vitamin K1 diet. ## $p<0.01$ Δhigh responders versus Δlow responders.

during the day and every 30 min during the night, and mean BP was determined for the 24-h period [6]. Plasma concentrations of glucose, triglycerides, total cholesterol, HDL cholesterol, and calculated LDL cholesterol were analyzed by PathWest laboratories (Fiona Stanley Hospital, Perth, Australia). Serum tOC was measured by the sandwich electrochemiluminescence immunoassay using the Roche Cobas N-Mid OC assay (Roche Diagnostics, Mannheim). The interassay coefficients of variation were 2.3 and 4.8% at levels of 18 and 90 ng/mL, respectively. Serum ucOC was determined using the hydroxyapatite binding method (Calbiochem) [21]. The interassay imprecision for percentage binding of cOC was 8 and 12% at an OC concentration of 100 and 15 ng/mL, respectively. Plasma creatinine was measured at baseline, and glomerular filtration rate (GFR) was estimated using plasma creatinine levels based on the known equation [22]. Vitamin K intake was estimated as previously described [20].

Statistical Analysis

All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA, version 22). Independent samples *t* tests were conducted to examine OC concentrations between males and females and if characteristics known to influence ucOC (BMI, age, vitamin K intake, and GFR) were different between the high responders and low responders at baseline. Spearman rho correlations were used to assess the relationship between preintervention OC concentrations and preintervention outcome measures. Spearman partial correlations were used for the additional adjustments of age and BMI as they are strong influencers of ucOC levels [23, 24].

When considering postintervention data from the HK diet intervention, participants were divided into high responders (suppression of ucOC ≥median [≥30%]) and low responders (suppression of ucOC <median [<30%]), based on the percent change in ucOC. The between-groups (high vs. low responders) effect of the HK diet on changes in OC, vascular, and metabolic outcomes was assessed using one-way ANOVA. Within-groups effects for pre- and postintervention were assessed using paired samples *t* tests, as previously reported [20]. All data were reported as mean ± SEM, and statistical analysis was conducted at the 95% confidence level of significance ($p<0.05$).

Results

Baseline characteristics are presented in Table 1. Serum tOC, cOC, and ucOC levels at preintervention data points were not different between women ($n=10$) and men ($n=20$) ($p>0.05$ for all, Table 1). With preintervention data points combined together, a higher ucOC/tOC ratio was associated with lower PWV when adjusted for BMI and age ($r=-0.493$, $p<0.05$). A higher concentra-

Table 3. Correlation between ΔucOC and ΔucOC/tOC ratio and Δvascular and metabolic outcomes following the high vitamin K1 diet

	ΔucOC			ΔucOC/tOC ratio		
	all participants	high responders	low responders	all participants	high responders	low responders
ΔAmb SBP						
Model 1	0.197	0.396	0.041	−0.014	0.175	−0.033
Model 2	0.400	0.512	0.152	0.040	0.197	0.224
ΔAmb DBP						
Model 1	0.099	0.489	−0.267	0.210	0.136	0.319
Model 2	0.284	0.551	−0.051	**0.435***	0.249	0.611
ΔResting SBP						
Model 1	0.014	−0.052	0.334	−0.240	−0.275	−0.014
Model 2	−0.226	−0.251	0.498	−0.355	−0.480	−0.625
ΔResting DBP						
Model 1	−0.090	0.073	0.052	−0.170	0.141	−0.066
Model 2	−0.296	−0.408	0.030	−0.224	−0.264	−0.343
ΔPWV						
Model 1	0.238	0.071	0.041	0.164	0.011	0.264
Model 2	−0.048	−0.123	0.021	−0.022	−0.315	−0.136
ΔGlucose						
Model 1	−0.300	−0.074	−0.120	−0.182	−0.261	0.290
Model 2	−0.285	−0.046	−0.583	0.145	−0.367	**0.793***
ΔTotal chol						
Model 1	0.314	0.296	0.234	0.070	0.071	−0.107
Model 2	0.257	0.369	0.186	0.025	−0.024	−0.487
ΔLDL						
Model 1	**0.375***	0.336	0.388	0.156	0.139	0.064
Model 2	0.276	0.547	0.205	0.141	0.205	−0.398
ΔHDL						
Model 1	0.154	0.093	0.008	−0.107	−0.264	−0.043
Model 2	0.006	0.011	−0.155	−0.175	−0.329	−0.383
ΔTriglycerides						
Model 1	0.018	−0.064	−0.018	−0.202	−0.200	−0.389
Model 2	0.171	0.073	0.252	−0.255	−0.167	−0.566

Model 1, unadjusted; Model 2, adjusted for BMI and age. tOC, total osteocalcin; ucOC, undercarboxylated osteocalcin; amb, ambulatory; SBP, systolic blood pressure; DBP, diastolic blood pressure; PWV, pulse wave velocity; chol, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein. * $p < 0.05$ ΔucOC/tOC versus vascular/metabolic outcome.

tion of cOC was associated with a higher PWV when adjusted for BMI and age ($r = 0.638$, $p < 0.01$). All other preintervention correlations were not significant ($p > 0.05$ for all, see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000511660).

We have previously shown that the HK intervention, but not the LK or CON intervention, suppressed tOC, ucOC, and the ucOC/tOC ratio [20]. In the high responders, tOC, ucOC, and ucOC/tOC were reduced postintervention compared to preintervention, following the 4-week HK diet ($p < 0.001$ for all, Table 2). Whilst in the low responders, ucOC ($p < 0.001$) and ucOC/tOC ($p < 0.01$), as well as resting systolic BP (2%, $p < 0.05$), were reduced postintervention. As expected, the change in ucOC and the ucOC/tOC ratio was significantly greater in the high responders versus low responders ($p < 0.05$ for both, Table 2). The change in tOC, cOC, and markers of vascular (ambulatory systolic BP, ambulatory diastolic BP, resting systolic BP, resting diastolic BP, or PWV) and metabolic (glucose, total cholesterol, LDL, HDL, or triglycerides) health was not significantly different between the low and high responders (Table 2). There was no difference in BMI, vitamin K intake, age, or estimated GFR (eGFR) between the high and low responders at baseline ($p > 0.05$ for all, online suppl. Table 2).

Using unadjusted Spearman rho correlation and Spearman partial correlation, there was no association between the change in ucOC or the ucOC/tOC ratio and the change

in any cardiometabolic risk factor in the high responders ($p > 0.05$ for all, Table 3). Using unadjusted spearman rho correlation, a positive association was present between the change in ucOC and the change in LDL when all participants were combined (i.e., high and low responders combined) ($p < 0.05$, Table 3). When adjusted for age and BMI using Spearman partial correlations, a positive correlation was present between the change in the ucOC/tOC ratio and change in ambulatory diastolic BP when all participants were combined ($r = 0.435$, $p < 0.05$). In low responders only, there was a strong positive correlation between the change in the ucOC/tOC ratio and change in glucose levels ($r = 0.793$, $p < 0.05$). All other correlations were not significant ($p > 0.05$ for all, Table 3).

Discussion

The major finding of this study is that the suppression of ucOC was not associated with increased cardiometabolic risk factors, even in individuals who responded the most to the intervention (high responders). As such, it appears that the suppression of ucOC following a leafy green-rich diet does not impact, either negatively or positively, on cardiometabolic risk factors.

Currently, there are conflicting reports regarding the relationship between OC and BP. Some have reported that lower tOC levels are associated with a higher prevalence of hypertension in adult men and women [25, 26]. Others however have described no association between tOC and systolic or diastolic BP in adult men and women [27, 28]. As cOC and ucOC may have diverse biological functions, the examination of tOC alone, as often reported in these studies, limits our understanding of the exact function of each form of OC [23, 29]. In the current study, we have examined each form of OC and report that a reduction in ucOC and the ucOC/tOC ratio via dietary modification is not correlated with changes in BP. This is interesting and suggests several possibilities. Firstly, ucOC may simply not have a regulatory role in the maintenance of blood vessel function and BP. Secondly, the HK (leafy green rich) diet may regulate other bioactive factors that influence vascular health. For example, we have previously shown that the 4-week leafy green-rich diet increased plasma nitrate levels [6]. An increase in plasma nitrate level enhances the bioavailability of nitric oxide, an antiatherogenic molecule that regulates blood vessel function and BP [4, 30]. ucOC has also been implicated as a regulatory factor responsible for the maintenance of blood vessel function and BP [19]. Therefore, it is possible that the reduction in ucOC was offset by an increase in NO bioavailability. Consequently, cross-talk mechanisms may exist, which may explain the lack of changes in BP. This hypothesis should be explored in further mechanistic studies.

ucOC has been established as a regulator of energy homeostasis, at least in animal models [31, 32]. A large number of cross-sectional studies in humans show that ucOC is associated with metabolic responses and diseases. For example, a reduction in circulating ucOC is associated with an increased risk or presence of metabolic disorders, such as metabolic syndrome and type 2 diabetes [17]. Lower circulating tOC and ucOC has been associated with increased concentrations of blood glucose and triglycerides and decreased levels of HDL [33, 34]. However, few interventional studies have modified ucOC and examined the effect on metabolic outcomes. One study administered a single dose of prednisolone, a glucocorticoid, which suppressed circulating tOC and ucOC and also caused a reduction in insulin sensitivity and fasting blood glucose [35, 36]. In the current study, despite a 41% reduction in ucOC and 29% reduction in ucOC/tOC after the HK diet, there were no changes in fasting glucose or lipid levels in the high responders. Potential mechanisms for the lack of change are not clear, but it may be related to other bioactive components present in green leafy vegetables that can cause a compensatory effect and prevent any change in metabolic variables.

The development of vascular calcification is a process comparable to the development of bone within the skeleton. As OC is involved in bone mineralization within the skeleton, it has also been implicated in the development of mineralization within the vasculature [23, 37]. cOC is the form of OC predominantly involved with bone development in the skeleton; as such, it is possible that cOC is the form of OC involved in the development of calcification within the vasculature. However, research in this area is lacking. We have shown that baseline cOC is associated with baseline PWV, a measure of arterial stiffness which suggests the presence of vascular calcification [38]. However, we saw no correlation of cOC with PWV following the HK diet in the high or low responders. Whilst it is possible that OC is involved in vascular calcification, future large-scale studies are needed to assess the effect of each form of OC, in particular cOC, on arterial stiffness and the development of vascular calcification.

A limitation of the current study is that the 4-week intervention period may not have been long enough or the dose of vitamin K1 not large enough to observe a change in measures of cardiometabolic risk. Previous studies ad-

ministering vitamin K1 supplementation (500–1,000 μg p/day) for 3 years found improvements in vascular compliance and reductions in coronary artery calcification [39, 40]. In the current study, it was estimated that participants increased their vitamin K1 intake by ~150 μg p/day over the 4 weeks [20]. As such, a prolonged intervention may be needed to demonstrate changes in cardiometabolic risk factors. Another potential limitation was the inclusion of people who are relatively healthy. It is possible that those with diabetes or CVD will respond differently to the intervention and that the correlation between ucOC and cardiovascular risk factors may be apparent in these populations. Finally, the generalization of the results is somewhat limited due to the relatively small sample size. As such, further large-scale studies, in particular RCTs, are needed to confirm our findings.

In conclusion, this study demonstrated that the suppression of ucOC following increased daily intake of leafy green vitamin K1-rich vegetables over 4 weeks was not associated with unfavorable changes in cardiometabolic risk factors. This may be due to the presence of compensatory mechanisms or the fact that ucOC has a limited regulatory role over cardiometabolic risk factors in apparently healthy individuals. Such hypothesis should be explored by future mechanistic studies.

Acknowledgements

The authors wish to thank all of the participants for their involvement in this study. They would also like to thank Nasima Shirzad for conducting the total osteocalcin and undercarboxylated osteocalcin analysis.

Statement of Ethics

The Vegetable Intake and Blood Pressure (VIABP) Study (registered at www.anzctr.org.au as ACTRN12615000194561) was approved by the University of Western Australia Human Research Ethics Committee and was carried out in accordance with the Declaration of Helsinki.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was funded by the National Health and Medical Research Council of Australia (NHMRC), grant 1084922. The salary of JMH is supported by an NHMRC of Australia Senior Research Fellowship (ID: 1116973). The salary of JRL is supported by a National Heart Foundation of Australia Future Leader Fellowship (ID: 102817). The salary of LCB is supported by an NHMRC of Australia Emerging Leadership Investigator Grant (ID: 1172987) and a National Heart Foundation of Australia Post-Doctoral Research Fellowship (ID: 102498). The funders had no role in study design; collection, management, analysis, and interpretation of data; writing of the manuscript; and the decision to submit the manuscript for publication.

Author Contributions

M.S., J.R.L., J.M.H., and L.C.B. designed the research; E.B. and L.C.B. conducted the research; A.T., C.S., M.W., and I.L. analyzed the data; A.T. wrote the first draft manuscript; all authors revised the manuscript and approved the final version.

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