

# **Investigating the effects of inorganic nitrate on vascular function, inflammation and platelet reactivity in healthy volunteers and patients with stable angina**

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## **Declaration**

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text. All sources of information have been properly referenced and all help has been acknowledged. Finally, this work has not been submitted for any other degree or professional qualification except as specified.

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## **Abstract**

This thesis is studying nitric oxide (NO) as a fundamental basis for health and disease and investigating as well as modifying its properties for therapeutic purposes. In the UK, nearly 3.5 million people have angina symptoms or have had a myocardial infarction (MI), or both. The mainstay treatment for reducing the symptoms of angina and long-term risk of MI in patients with heart disease is stent implantation in the diseased coronary artery. While this procedure has revolutionised treatment, the incidence of secondary events remains a concern. These repeat events are thought to be due, in part, to an increased inflammatory state that predisposes to continued enhanced platelet reactivity, endothelial dysfunction, and ultimately restenosis of the stented artery. In addition, CVD in general including atherosclerotic disease occurs at a lower incidence in premenopausal females compared with age-matched males. There has been considerable interest in why this might be the case but whether sex differences in inflammation underlie these differences is uncertain. Greater understanding of the role of inflammation and strategies that might selectively reduce inflammation within the CVD setting may offer novel approaches to therapeutics.

Thus, I prospectively investigated whether inflammation and components of the inflammatory response are altered in females compared with males using two healthy volunteer studies. In the first study, I assessed systemic inflammatory markers and vascular function using brachial artery flow-mediated dilation (FMD). Typhoid vaccine induced mild systemic inflammation at 8 hours, reflected by increased white cell count in both sexes. Systemic inflammation in turn caused

a decrease in FMD in males, but an increase in females, at 8 hours. To investigate the differences in inflammatory responses between the sexes further in a separate study, I measured inflammatory exudate mediators and cellular recruitment in cantharidin-induced skin blisters. At 24 hours, cantharidin formed blisters of similar volume in both sexes; however, at 72 hours, blisters had only resolved in females. The activation state of all major leukocytes was lower in blisters of females, which was associated with enhanced levels of the resolving lipids. Together these findings suggest that in females, resolution of inflammation is accelerated compared with males, and that within the cardiovascular system this is likely to result in improved vascular function.

The second part of my thesis studies was focussed on using the techniques established in the healthy volunteer studies to investigate whether a once a day inorganic nitrate ( $\text{NO}_3^-$ ) administration might, through modification of inflammatory pathways, favourably modulate platelet reactivity and endothelial function leading to a decrease in restenosis. This was conducted in the form of the NITRATE-OCT study, which is a phase II trial enrolling 246 patients with stable angina due to have elective stent implantation. Patients have been randomised to receive 6 months of a once a day dose of either  $\text{NO}_3^-$ -rich beetroot juice or  $\text{NO}_3^-$ -deplete beetroot juice (placebo) before their procedure. The primary outcome is reduction of in-stent late loss assessed by quantitative coronary angiography (QCA) and optical coherence tomography (OCT) at 6 months. Thus far, we have baseline demographic data for 200 patients in total and the study is still ongoing to ensure the investigators remain blinded. At baseline there were no significant differences in demographic or procedural characteristics between the two groups.

## Publications

**The following publications have resulted from this thesis:**

### Manuscripts

Randomised, double-blind, placebo-controlled study investigating the effects of inorganic nitrate on vascular function, platelet reactivity and restenosis in stable angina: protocol of the NITRATE-OCT study. **Rathod KS**, Jones DA, Van-Eijl TJ, Tsang H, Warren H, Hamshere SM, Kapil V, Jain AK, Deaner A, Poulter N, Caulfield MJ, Mathur A, Ahluwalia A. *BMJ Open*. 2016 Dec 20;6(12):e012728. doi: 10.1136/bmjopen-2016-012728. PMID: 27998900.

Accelerated resolution of inflammation underlies sex differences in inflammatory responses in humans. **Rathod KS**, Kapil V, Velmurugan S, Khambata RS, Siddique U, Khan S, Van Eijl S, Gee LC, Bansal J, Pitrola K, Shaw C, D'Acquisto F, Colas RA, Marelli-Berg F, Dalli J, Ahluwalia A. *J Clin Invest*. 2017 Jan 3;127(1):169-182. doi: 10.1172/JCI89429. Epub 2016 Nov 28. PMID: 27893465.

A 'green' diet-based approach to cardiovascular health? Is inorganic nitrate the answer? **Rathod KS**, Velmurugan S, Ahluwalia A. *Mol Nutr Food Res*. 2016 Jan;60(1):185-202. doi: 10.1002/mnfr.201500313. Epub 2015 Sep 13. Review.



Intravascular Ultrasound Versus Optical Coherence Tomography for Coronary Artery Imaging - Apples and Oranges? **Rathod KS**, Hamshere SM, Jones DA, Mathur A. *Interv Cardiol.* 2015 Mar;10(1):8-15. doi: 10.15420/icr.2015.10.1.8. PMID: 26256112.

### **Abstracts**

Accelerated resolution of inflammation underlies sex differences in inflammatory responses in humans. **KS Rathod**, V Kapil, S Velmurugan, R Khambata, U Siddique, S Khan, L Gee, J Bansal, K Pitrola, C Shaw, F D'Acquisto, R Colas, F Marelli-Berg, J Dalli, A Ahluwalia. *pA2 Online* May 2017.

Sex differences in the inflammatory response and inflammation induced vascular dysfunction. **KS Rathod**, V Kapil, S Velmurugan, R Khambata, U Siddique, S Khan, L Gee, J Bansal, K Pitrola, C Shaw, F D'Acquisto, R Colas, F Marelli-Berg, J Dalli, A Ahluwalia. *The Lancet* 2017;389,S20.doi.org/10.1016/S0140-6736(17)30416-6 .

Investigation of the influence of sex on cantharidin-induced inflammation in healthy volunteers. **Rathod K.**, U Siddiqui, A Hartley, RS Khambata, A Ahluwalia. *The FASEB Journal* (2016) 30 (1 supplement): 716.9-716.9.

**The following publications during studentship not contained within this thesis:**

### **Manuscripts**

Sodium Nitrite-Mediated Cardioprotection in Primary Percutaneous Coronary Intervention for ST-Segment Elevation Myocardial Infarction: A Cost-Effectiveness Analysis. Jones DA, Whittaker P, **Rathod KS**, Richards AJ, Andiapen M, Antoniou S, Mathur A, Ahluwalia A. *J Cardiovasc Pharmacol Ther.* 2018 Aug. PMID: 30081658.

Sex differences in the nitrate-nitrite-NO $\bullet$  pathway: role of oral nitrate-reducing bacteria. Kapil V, **Rathod KS**, Khambata RS, Bahra M, Velmurugan S, Purba A, Watson D, Barnes MR, Wade WG, Ahluwalia A. *Free Radic Biol Med.* 2018 Jul 19. pii: S0891-5849(18)31248-6. doi: 10.1016/j.freeradbiomed.2018.07.010. [Epub ahead of print]. PMID: 30031863.

The effect of intracoronary sodium nitrite on the burden of ventricular arrhythmias following primary percutaneous coronary intervention for acute myocardial infarction. Jones DA, **Rathod KS**, Williamson A, Harrington D, Andiapen M, van Eijl S, Westwood M, Antoniou S, Schilling RJ, Ahluwalia A, Mathur A. *Int J Cardiol.* 2018 Sep 1;266:1-6. doi: 10.1016/j.ijcard.2018.01.029. PMID: 29887423.

Antiinflammatory actions of inorganic nitrate stabilize the atherosclerotic plaque. Khambata RS, Ghosh SM, **Rathod KS**, Thevathasan T, Filomena F, Xiao Q, Ahluwalia A. *Proc Natl Acad Sci U S A*. 2017 Jan 24;114(4):E550-E559. doi: 10.1073/pnas.1613063114. Epub 2017 Jan 5. PMID: 28057862.

Intracoronary nitrite suppresses the inflammatory response following primary percutaneous coronary intervention. Jones DA, Khambata RS, Andiapen M, **Rathod KS**, Mathur A, Ahluwalia A. *Heart*. 2017 Apr;103(7):508-516. doi: 10.1136/heartjnl-2016-309748. Epub 2016 Sep 28. PMID: 27683405.

Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. Velmurugan S, Gan JM, **Rathod KS**, Khambata RS, Ghosh SM, Hartley A, Van Eijl S, Sagi-Kiss V, Chowdhury TA, Curtis M, Kuhnle GG, Wade WG, Ahluwalia A. *Am J Clin Nutr*. 2016 Jan;103(1):25-38. doi: 10.3945/ajcn.115.116244. Epub 2015 Nov 25.

Randomized phase 2 trial of intracoronary nitrite during acute myocardial infarction. Jones DA, Pellaton C, Velmurugan S, **Rathod KS**, Andiapen M, Antoniou S, van Eijl S, Webb AJ, Westwood MA, Parmar MK, Mathur A, Ahluwalia A. *Circ Res*. 2015 Jan 30;116(3):437-47. doi: 10.1161/CIRCRESAHA.116.305082. Epub 2014 Dec 15. Erratum in: *Circ Res*. 2015 Mar 27;116(7):e55. PMID: 25512434.

## Abstracts

Dietary Nitrate Suppresses Leukocyte Recruitment and Activation in Apolipoprotein E Knockout Mice. RS Khambata, SM Ghosh, **Rathod K.**, T Thevathasan, A Ahluwalia. *The FASEB Journal* (2016) 30 (1 supplement): 165.2-165.2.

Nitrite reductase activity is elevated in atherosclerotic mice: role for xanthine oxidoreductase. **Krishnaraj Rathod**, Suborno M Ghosh, Rayomand S Khambata and Amrita Ahluwalia. *pA2 Online* Dec 2014.

Nitrite reductase activity is elevated in atherosclerotic mice: role for xanthine oxidoreductase. **Krishnaraj Rathod**, Suborno M Ghosh, Rayomand S Khambata and Amrita Ahluwalia. *Nitric Oxide* 2014. Nov 15;42C:139. doi:10.1016/j.niox.2014.09.119. Epub 2014 Nov 24.

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## Abbreviations

$\alpha_2\beta_1$	Alpha 2 beta1
$\alpha_{IIb}\beta_3$	Alpha IIb beta 3
AA	Arachodonic acid
AAR	Area at risk
ACh	Acetylcholine
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AE	Adverse events
ALDH-2	Aldehyde dehydrogenase-2
AMI	Acute myocardial infarction
ANT	Adenine nucleotide translocase
ANOVA	Analysis of variance
APC	Allophycocyanin
ApoE	Apolipoprotein E
aPWV	Aortic pulse wave velocity
ATP	Adenosine Triphosphate
AU	Arbitrary aggregation units
AUC	Area under the curve
AI	Augmentation index
B/Bo	% bound
$\beta_3$	Beta-3
bFGF	Basic fibroblast growth factor
BH <sub>4</sub>	Tetrahydrobiopterin
BMI	Body mass index

BMS	Bare-metal stent
BP	Blood pressure
BR	Binary restenosis
CABG	Coronary artery bypass grafting
Ca <sup>2+</sup>	Calcium
cAMP	Cyclic adenosine monophosphate
CAD	Coronary artery disease
CADCTs	Collagen adenosine diphosphate closure times
CCL-2	Chemokine (C-C motif) ligand 2
CD40L	CD40 ligand
cGMP	Cyclic guanylate monophosphate
CHD	Coronary heart disease
CI	Confidence Interval
CMR	Cardiac magnetic resonance
CNG	Cyclic nucleotide-gated
CRP	C-reactive protein
CRF	Case report form
CK	Creatine Kinase
CTU	Clinical trials unit
Cu	Copper
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
Cyp-D	Cyclophilin D
CVD	Cardiovascular disease
CX3CL-1	Chemokine (C-X3-C motif) ligand 1

CXCL-1	Chemokine (C-X-C motif) ligand 1
CXCL-2	Chemokine (C-X-C motif) ligand 2
CXCL-5	Chemokine (C-X-C motif) ligand 5
CXCL-8	Chemokine (C-X-C motif) ligand 8
CXCL-12	Chemokine (C-X-C motif) ligand 12
DASH	Dietary approaches to stop hypertension
DBP	Diastolic blood pressure
DES	Drug-eluting stent
deoxyHb	Deoxyhaemoglobin
deoxyMb	Deoxymyoglobin
DHA	Docosahexaenoic acid
DOH	Department of health
DS	Diameter restenosis
DSMB	Data Safety Monitoring Board
ecto-ADPase	Ectoadenosine diphosphatase
e <sup>-</sup>	Electron
EC	Endothelial cell
ECG	Electrocardiogram
ECM	Extra-cellular matrix
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPA	Eicosapentaenoic
eNOS	Endothelial nitric oxide synthase

FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide
Fe	Iron
FD-OCT	Frequency domain optical coherence tomography
FITC	Fluorescein isothiocyanate
FMN	Flavin mononucleotide
FMD	Flow-mediated dilatation
FSC	Forward scatter
GDN	Glyceryl dinitrate
GI	Gastrointestinal
GTN	Glyceryl trinitrate
GPIb/IX/V	Glycoprotein Ib/IX/V
GPVI	Glycoprotein VI
GP1b $\alpha$	Glycoprotein 1b alpha
GPIIb/IIIa	Glycoprotein IIb/IIIa
GTP	Guanosine triphosphate
GC	Guanylate cyclase
Hb	Haemoglobin
HDL	High-density lipoprotein
hr	Hour(s)
HCAEC	Human Coronary artery endothelial cell
HIF	Hypoxia inducible factor
HmCASMIC	Human Coronary artery smooth muscle cell
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HNO <sub>2</sub>	Nitrous acid

HR	Heart rate
hsCRP	High sensitive c-reactive protein
HUVEC	Human Umbilical vein endothelial cell
IARC	International Agency for Research on Cancer
IBMX	3-isobutyl-1-methylxanthine
IHD	Ischaemic heart disease
IS	Infarct size
ICAM-2	Intercellular adhesion molecule-2
IL-1	Interleukin-1
IL-1 $\beta$	Interleukin-1-beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
i.m.	Intramuscular
IM	Inflammatory monocytes
JAM-3	Junctional adhesion molecule-3
I/R	Ischaemia-Reperfusion
ISR	In-stent restenosis
ISRCTN	International standard randomized controlled trials number
IVUS	Intravascular ultrasound
K <sup>+</sup>	Potassium
KNO <sub>3</sub>	Potassium nitrate
KNO <sub>2</sub>	Potassium nitrite

KO	Knock out
LCM	Laser-capture microdissection
LDL	Low-density lipoprotein
LGE	Late Gadolinium enhancement
LL	Late loss
L-NMMA	L-nitro mono methyl arginine
LT	Leukotriene
LV	Left ventricular
LVEDV	Left ventricular end-diastolic volume
LVESV	Left ventricular end-systolic volume
Mac-1	Macrophage 1 antigen
MACE	Major adverse cardiac events
MAPK	Mitogen-activated protein kinase
Mb	Myoglobin
MCP-1	Monocyte chemoattractant protein 1
metHb	Methaemoglobin
MFI	Median frequency intensity
Mo	Molybdenum cofactor
MI	Myocardial infarction
Min	Minute(s)
MIP-1 $\alpha$	Macrophage inflammatory protein-1-alpha
MLD	Minimal luminal diameter
MMP-2	Matrix metalloproteinase -2
mPTP	Mitochondrial permeability transition pore
MRM	Multiple reaction monitoring

MSI	Myocardial salvage index
MVO	Microvascular obstruction
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NaF	Sodium fluoride
NaNO <sub>2</sub>	Sodium nitrite
Na <sub>3</sub> VO <sub>4</sub>	Sodium orthovanadate
NG	Nitroglycerin
NHS	National health service
NICE	National institute of clinical excellence
NK	Nuclear factor
NMDA	N-methyl-D-aspartate
nNOS	Neuronal NOS
NO	Nitric oxide
NO <sub>3</sub> <sup>-</sup>	Nitrate
NO <sub>2</sub> <sup>-</sup>	Nitrite
NOA	Nitric oxide analysor
NOS	Nitric oxide synthase
NSB	Non-specific binding
O <sub>2</sub>	Oxygen
O <sub>2</sub> <sup>·-</sup>	Superoxide
O <sub>3</sub>	Ozone
OCT	Optical Coherence Tomography
OPLS-DA	Orthogonal-partial least squares discriminant analysis
OTW	Over-the-wire



oxyHb	Oxyhaemoglobin
ONOO <sup>-</sup>	Peroxynitrite
PAI-1	Plasminogen activator inhibitor-1
PAF	Platelet-activating factor
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate buffer solution
PPAR	Proliferator-activator receptor
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PDE	Phosphodiesterase
PE	Phycoerythrin
PEG-SOD	Polyethylene-glycolated SOD
PF <sub>4</sub>	Platelet factor 4
PMA	Platelet monocyte aggregate
PRP	Platelet-rich plasma
PGI <sub>2</sub>	Prostaglandin I <sub>2</sub>
PKG	Protein kinase G
PSGL-1	P-selectin glycoprotein ligand-1
PE	Phycoerythrin
PKG	Protein kinase G
PMT	Photomultiplier tubes
PRP	Platelet-rich plasma
PWA	Pulse wave analysis
PWV	Pulse wave velocity
QCA	Quantitative coronary angiography

REC	Research ethics committee
RIC	Remote ischaemic conditioning
RISK	Reperfusion injury salvage kinases
RM	Resident monocytes
ROS	Reactive oxygen species
RT	Retention time
RVD	Reference vessel diameter
SAE	Serious adverse events
SAFE	Survivor Activating Factor Enhancement
SBP	Systolic blood pressure
sCD40L	soluble CD40 ligand
SD	Standard deviation
SEM	Standard error of the mean
SES	Sirolimus-eluting stent
sGC	Soluble guanylate cyclase
sICAM-1	Soluble intercellular Adhesion Molecule 1
SMC	Smooth muscle cell
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SPM	Specialised pro-resolving mediators
SSC	Side scatter
STEMI	ST-segment elevation myocardial infarction
TCFA	Thin cap fibroatheroma
TD-OCT	Time domain optical coherence tomography
TGF- $\beta$	Transforming growth factor beta

TIMI	Thrombolysis in myocardial infarction
TLR	Target lesion revascularisation
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TSC	Trial steering committee
Tx	Thromboxane
US	United States
UK	United Kingdom
UKCRN	United Kingdom Clinical Research Network
VSMC	Vascular smooth muscle cell
VDAC	Voltage dependent anion channel
vWF	Von Willebrand factor
WBC	White blood cell count
WHO	World Health Organisation
XOR	Xanthine Oxidoreductase

## Materials

### Materials

### Manufacturer/City/Country

#### Antibodies

Anti human CD42b	Biolegend, San Diego, USA
Anti-human CD62E/CD62P	Serotec, Oxford, UK
Anti-human CD14-FITC	Becton, Dickinson & company, New Jersey, USA
Anti-human CD16-PE	Becton, Dickinson & company, New Jersey, USA
Anti-human CD16b-PE	Becton, Dickinson & company, New Jersey, USA
Anti-human CD11b-PB	Becton, Dickinson & company, New Jersey, USA
Anti-human CD3-FITC	eBioscience Inc, San Diego, USA
Anti-human CD4-PE-Cy7	eBioscience Inc, San Diego, USA
Anti-human CD8-PerCp-cy5.5	eBioscience Inc, San Diego, USA
Anti-human CD162-APC	eBioscience Inc, San Diego, USA
Anti-human CD62L-APC efluor 780	eBioscience Inc, San Diego, USA

#### Isotype controls

IsoCD11b-PB Mouse IgG1k	Becton, Dickinson & company, New Jersey, USA
IsoCD162-APC Mouse IgG2ak	eBioscience Inc, San Diego, USA
Iso-CD62L-APC efluor 780	eBioscience Inc, San Diego, USA
Iso-CD42b-Mouse IgG1 x	Biolegend, San Diego, USA
Iso-CD62E/CD62P-Mouse IgG1	Serotec, Oxford, UK

## **Solutions/Drugs**

Allopurinol	Sigma-Aldrich Company Ltd, Dorset, UK
CaCl <sub>2</sub> Tablets	Sigma-Aldrich Company Ltd, Dorset, UK
Glacial acetic acid	Sigma-Aldrich Company Ltd., Dorset, UK
HEPES Powder	Sigma-Aldrich Company Ltd, Dorset, UK
Hydrochloric acid	Prolabo VWR, Lutterworth, UK
Immunolyse Solution	Beckman Coulter, California, USA
3-isobutyl-1-methylxanthine	Sigma-Aldrich Company Ltd, Dorset, UK
Isotonic Glucose	Nycomed, Zurich, Switzerland
L-NMMA	Sigma-Aldrich Company Ltd, Dorset, UK
MilliQ NO <sub>x</sub> -free water	Millipore Corporation, Billerica, USA
ODQ	Sigma-Aldrich Company Ltd, Dorset, UK
Paraformaldehyde Powder	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphate Buffered Saline tablets	Sigma-Aldrich Company Ltd, Dorset, UK
Potassium iodide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium hydroxide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium nitrate	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium nitrite	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium nitrite for injection (4350M in 10mls)	Ipswich Hospital Manufacturing Unit, UK
Vanadium (III) chloride	Sigma-Aldrich Company Ltd, Dorset, UK

## **Platelet Agonists**

ADP	Chrono-log Corp, Havertown, USA
Collagen	Nycomed Ltd, Zurich, Switzerland

## **Instruments/Equipment**

Haemocytometer	Hawksley, Sussex, UK
Multiplate Aggregometer	Dynabyte Medical, Munich, Germany
Sigma 4KIS Centrifuge	Sigma-Aldrich Company Ltd, Dorset, UK
Multiplate Test Cells	Verum Diagnostica GmbH
Optical Coherence Tomography (OCT)	
Catheters	Abbott Laboratories, Illinois, USA
OCT Console	Abbott Laboratories, Illinois, USA

## **Cell culture reagents**

Annexin V	Trevigen, Gaithersburg, USA
Annexin binding buffer	Biolegend, San Diego, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphate buffered solution (PBS)	Sigma-Aldrich Company Ltd, Dorset, UK
Propidium Iodide	Trevigen, Gaithersburg, USA
Trypan blue	Gibco (Paisley, UK)
Trypsin/EDTA	Sigma-Aldrich Company Ltd, Dorset, UK

## **ELISA**

cGMP	GE Healthcare, Little Chalfont, UK
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## **Chapter 1.0**

# **Introduction**

## 1.0 Introduction

Some cardiovascular disorders are characterised by an underlying vascular inflammation that is considered to play a major causative role in pathogenesis (Willerson and Ridker, 2004).

This thesis is separated into two distinct sections. In the first part, we decided to use the typhoid-induced model of systemic inflammation to further interrogate the changes in endothelial regulation of vascular tone in the arterial circulation. In addition, to study the inflammatory response in humans more closely, we explored whether a suitable model of innate inflammation might be available. Since the cantharidin model allows us to explore localised inflammation, we decided to use this model to characterise the leukocytes, chemokines, cytokines and resolution factors generated during an inflammatory response.

To test both of these methodologies and to establish both models of inflammation in our laboratory, we investigated the responses to typhoid and cantharidin in both healthy male and female volunteers in two healthy volunteer studies.

In the second section, we decided to conduct a clinical trial to assess the effects of inorganic nitrate ( $\text{NO}_3^-$ ) on restenosis and inflammation by conducting the NITRATE-OCT study. Inorganic  $\text{NO}_3^-$ , when converted to nitrite ( $\text{NO}_2^-$ ) via the enterosalivary circuit, has been shown to reduce smooth muscle cell proliferation and promote endothelial cell growth in our laboratory. This thesis contains a sub-analysis of our large Phase II double blinded randomised controlled trial (NITRATE-OCT) that is investigating whether there is a reduction in the growth of



smooth muscle cells (preventing restenosis within the stent), following ingestion of inorganic  $\text{NO}_3^-$  in patients undergoing percutaneous coronary intervention (PCI) with stent implantation for stable angina.

The aim of the second part of the thesis is to explore the possibility that the beneficial effects of inorganic  $\text{NO}_3^-$  with regards to reducing restenosis in stable angina patients might relate to effects on inflammation, by assessing the levels of specific pro-inflammatory components and circulating cell subtypes as exploratory endpoints in the NITRATE-OCT cohort.

### **1.1 Coronary Heart Disease**

In the last 20 years, the management of cardiovascular disease (CVD) has greatly improved, however despite this it remains the top cause of death worldwide (Lim et al., 2012). This level of mortality coupled with increasing levels of morbidity over the past 10 years have had huge economic implications both to developing countries (as western lifestyles continue to be adopted) and within developed countries including Europe. For instance, this health burden is estimated to cost the UK economy £9 billion in 2016. The UK remains one of the most affected countries in Europe in terms of CVD with 152,465 deaths out of a total mortality of 525,048 due to CVD in 2016, with the majority (66,076) a consequence of coronary heart disease (CHD), the latter remaining the single biggest cause of death in the UK (BHF, 2016).

That said, overall figures for mortality in the UK continue to improve each year. Since the early 1970's death rates from CVD have been on the decrease. Indeed,

for the first time since CVD statistics have been compiled by the British Heart Foundation, mortality due to CVD (26%) has been surpassed by cancer (29%). In those under 75 years, death rates have fallen by 34% in the last ten years (<http://www.bhf.org.uk/research/statistics/mortality.aspx>). This has been attributed largely to improvement in lifestyle factors and to highly efficacious treatment advances following an acute event. Presently, timely PCI with stent implantation coupled with prolonged (1 year) anti-platelet therapy following a heart attack remains the most effective treatment strategy for limiting secondary events and improving clinical outcomes (Kastrati et al., 2005; Stone et al., 2013). However, despite these advances, substantial morbidity persists. Thus, there is an urgent imperative to identify and develop efficacious and sustainable approaches for primary and secondary prevention.

CHD can manifest in a number of different ways, the main ones being angina (either stable or unstable) and myocardial infarction (MI). In particular, my PhD research has focused upon trying to identify therapeutic strategies for one of these i.e. stable angina.

## **1.2 Stable angina and its management**

In the UK, nearly 3.5 million people have angina symptoms or have had a myocardial infarction, or both ([www.bhf.org.uk](http://www.bhf.org.uk)). Angina is classed as a symptom and not a disease (the underlying disease is CHD). It is often defined as pain or discomfort in the chest that is a result of myocardial hypoxia, secondary to a restricted coronary blood flow caused by atheromatous plaque in the coronary arteries (National et al., 2011). Patients often complain of symptoms, including

chest pain, that can radiate to the surrounding area and these symptoms are known as angina pectoris. Stable angina symptoms often occur when the demands of the heart are high (i.e. during exercise) and the supply of blood and oxygen to the heart is unable to meet the demand due to CHD. In patients suffering from angina, one or more coronary arteries usually has a stenosis of more than 70%. Therefore, even though sufficient blood can be supplied to the myocardium during rest, the myocardium is not able to compensate when there is an increased demand (Lilly, 2007).

The management of stable angina aims to relieve patient's symptoms, improving quality of life and reducing morbidity and mortality. As a first line therapy, the current NICE guidelines recommend that patients with stable angina are prescribed anti-anginal medicines. However, when these are ineffective, consideration is given to elective coronary revascularisation, either coronary artery bypass grafting surgery (CABG) or PCI (National et al., 2011), to help relieve angina symptoms by restoring the blood supply to the heart.

### **1.3 Percutaneous Coronary Intervention for stable angina**

PCI (also known as balloon angioplasty, coronary angioplasty and percutaneous transluminal coronary angioplasty) was introduced by Andreas Gruntzig in 1977 and is a procedure that is used to 're-open' patients' narrowed coronary arteries. Since this procedure is performed under local anaesthesia, it is considered a safe and less invasive option compared to CABG surgery which requires general anaesthesia (Lauck et al., 2009). In addition, CABG is generally restricted to patients with severe multi-vessel coronary artery disease while the

revascularisation modality of choice for more focal coronary disease is PCI (Patel et al., 2009). Although following significant advances in percutaneous coronary intervention in terms of both skills set as well as technology, more complex coronary disease has guideline approval for PCI as an alternate to CABG (Windecker et al., 2018).

Currently in the UK, patients undergoing elective PCI procedures are often in hospital for less than 24 hours and advances in post-procedure care mean that the majority of patients are in hospital for just a few hours as a 'day-case' patient (Brayton et al., 2013; British Heart Foundation, 2014). Furthermore, patients are often expected to return to normal activities and work, if appropriate, within two weeks (British Heart Foundation, 2009a; b). During the PCI, patients are taken to a cardiac catheterisation laboratory in the hospital, the cardiologist makes a small incision (into the radial or femoral artery) and introduces a balloon catheter into the patient's artery, which is then advanced to the site of the narrowing in the coronary artery (Reynolds et al., 2001). The balloon is then inflated several times which pushes the plaque into the inner wall of the artery, opening the blood vessel (Brar and Stone, 2009). A stent device (a small stainless steel mesh) is inserted to hold the artery open and improve the blood flow. Once the procedure is complete the balloon catheter is removed, the artery is 'sealed' and the patient transferred to a day case unit/ward area for a few hours prior to discharge (British Heart Foundation, 2014).

The aim of PCI is to reduce or completely relieve the patients' angina symptoms. However, despite this, the underlying CHD remains. Findings from the large BARI

longitudinal study (n = 1829) demonstrated that more than 75% (n = 915) of PCI patients require further revascularisation for symptom relief within ten years (Alderman et al., 2004; BARI Investigators, 2007). Such findings demonstrate that the underlying CHD continues to progress even after PCI and therefore, it is essential that this is managed as a chronic condition with long-term pharmacological treatment and risk factor modification to slow the progression of the disease.

#### **1.4 Bare metal stents and drug eluting stents**

Over the last 20 years, PCI has evolved to include the placement of intra-coronary bare metal stents (BMS) which act as scaffolding to prevent arterial recoil. Despite its short-term efficacy, long-term results with BMS have been limited by accelerated neo-intimal proliferation at the site of the stent placement, resulting in luminal restenosis (Smith et al., 2006). The rates of restenosis with BMS have been estimated to be between 10-30% (Weintraub, 2007). Restenosis is often accompanied by the recurrence of anginal symptoms and typically requires repeat PCI, or occasionally surgical revascularisation with CABG. To address this drawback of BMS, stents coated with anti-proliferative medications were developed (Smith et al., 2006). These drug-eluting stents (DES) slow down neo-intimal proliferation by the controlled release of anti-proliferative medications into the tunica media of the coronary artery. In the year 2000, the first generation of polymer-based DES, the Sirolimus-eluting CYPHER stent (Johnson & Johnson) and the Paclitaxel-eluting TAXUS stent (Boston Scientific) were introduced into clinical practice.

Since the introduction of these two first generation DES, there have been a number of advances in the design of the stent platforms, associated polymers and anti-proliferative medications that has led to the introduction of 2<sup>nd</sup> and 3<sup>rd</sup> generation DES (Akin et al., 2011), which are now available for use. All DES markedly reduce restenosis and recurrent angina when compared to BMS (Babapulle et al., 2004). However, even with the latest generation of DES, it has been estimated that in simple lesions, restenosis rates are less than 5% at 1 year but at 5 years, repeat intervention rates are approximately 10% (Weisz et al., 2009). Furthermore, in more complex lesions, restenosis has been documented at 10% within 2 years (Steinberg et al., 2009).

In addition, a number of studies have raised more serious concerns regarding the long-term safety of DES, suggesting a potential increase in late MI from very late stent thrombosis because of incomplete endothelialisation of stent struts (Babapulle et al., 2004; Garg et al., 2008; Spaulding et al., 2007; Stone et al., 2007). Although late stent thrombosis is rare, with estimates suggesting a 0.13% increased risk per year compared to BMS, it has an extremely poor prognosis, with short-term mortality estimated at 20% (Garg et al., 2008). Compared to BMS, DES stents are marginally less deliverable and have significantly increased acquisition costs. Furthermore, extended administration of dual anti-platelet coverage (typically with a combination of aspirin and a thienopyridine such as clopidogrel) until adequate stent strut endothelialisation, is essential in reducing the risk of stent thrombosis (Windecker et al., 2005). Moreover, it must be noted that the newer thienopyridines (such as prasugrel and ticagrelor (Wallentin et al., 2009; Wiviott et al., 2007)) are expensive and dual anti-platelet coverage has a non-trivial risk

of major bleeding (Ko et al., 2010). Finally, there is also data to suggest the inflammation that accompanies PCI with stent implantation is systematic as well as focal (Brasselet et al., 2007; Kozinski et al., 2005).

### **1.5 Cellular and molecular pathogenesis of restenosis**

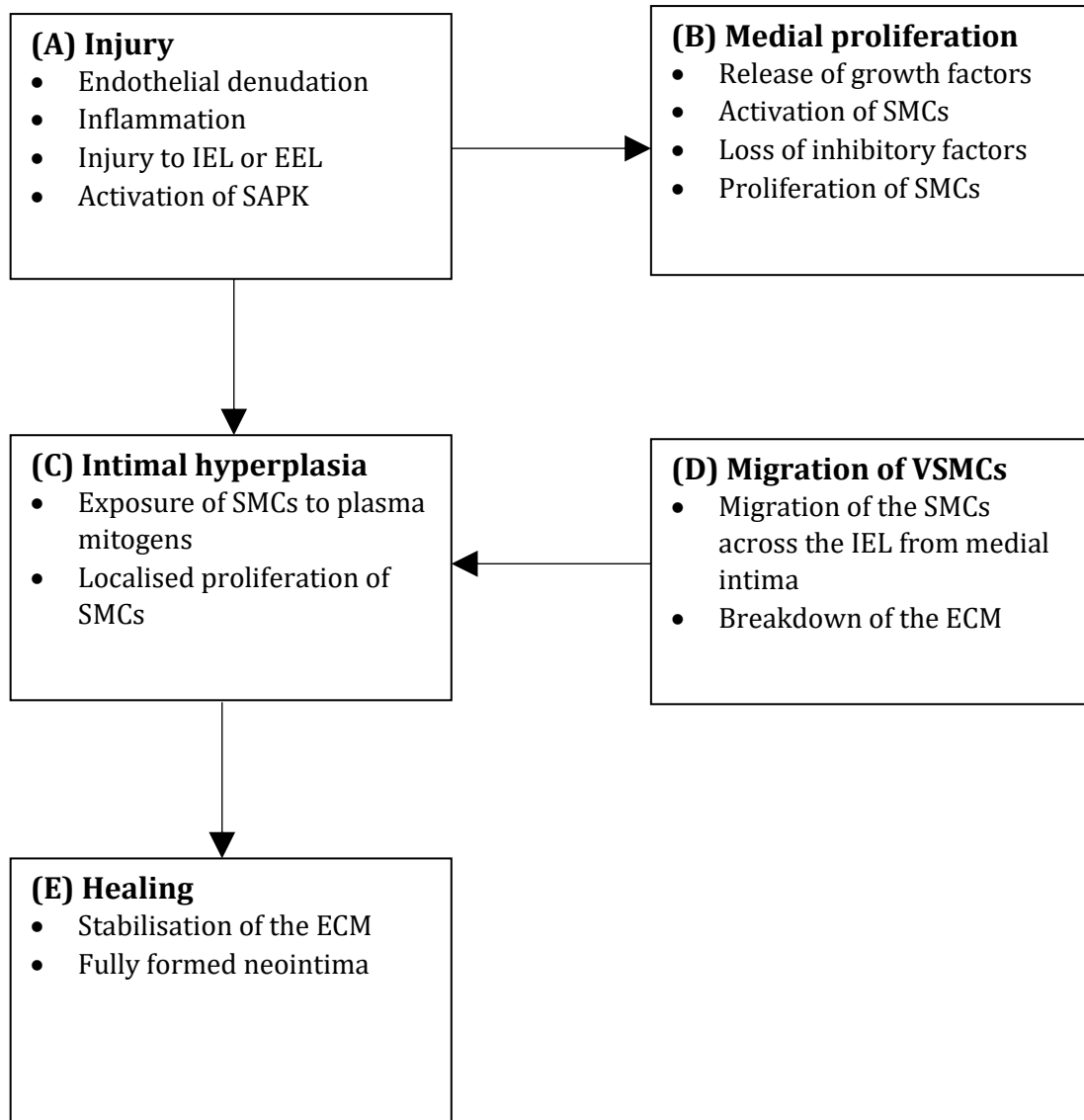
We now know that the long-term outcome of stent implantation is affected by a process called in stent restenosis (ISR). ISR is defined either clinically or on angiograms. This clinical definition includes the the presentation of recurrent angina or objective evidence of myocardial ischaemia. On the other hand, angiographic ISR is defined as the presence of stenosis (>50% in diameter) in the stented segment (Teirstein et al., 1997). Traditionally, ISR has been classified based on the length of the lesion, as diffuse (>10 mm) or focal (<10 mm).

Angiographically, ISR is classified into six different groups (I–IV) depending on the pattern and extent of restenosis with relation to the affected vessel. According to this classification, focal pattern I can be further subdivided into IA–ID and diffuse pattern can be grouped into II–IV (Mehran et al., 1999).

With the advance of intravascular imaging, particularly Intravascular ultrasound (IVUS) (described further in section 1.6) , we have a greater understanding of the fundamental differences in the process of restenosis following PCI (Mintz et al., 1996; Serruys et al., 1994; Williams et al., 2000). The pathophysiology of restenosis includes build-up of new tissue within the arterial wall (also known as neointimal hyperplasia - NIH). There is a cascade mechanism that involves platelets, polymorphonuclear leucocytes, and macrophage aggregation leading to

medial smooth muscle cell (SMC) migration and proliferation, which is the basis of NIH formation (Carter et al., 1994) (**Figure 1.1**). Furthermore, the hallmarks of the onset of restenosis are leucocyte recruitment at the site of the injury and the deposition of platelets and fibrin (Libby and Simon, 2001).

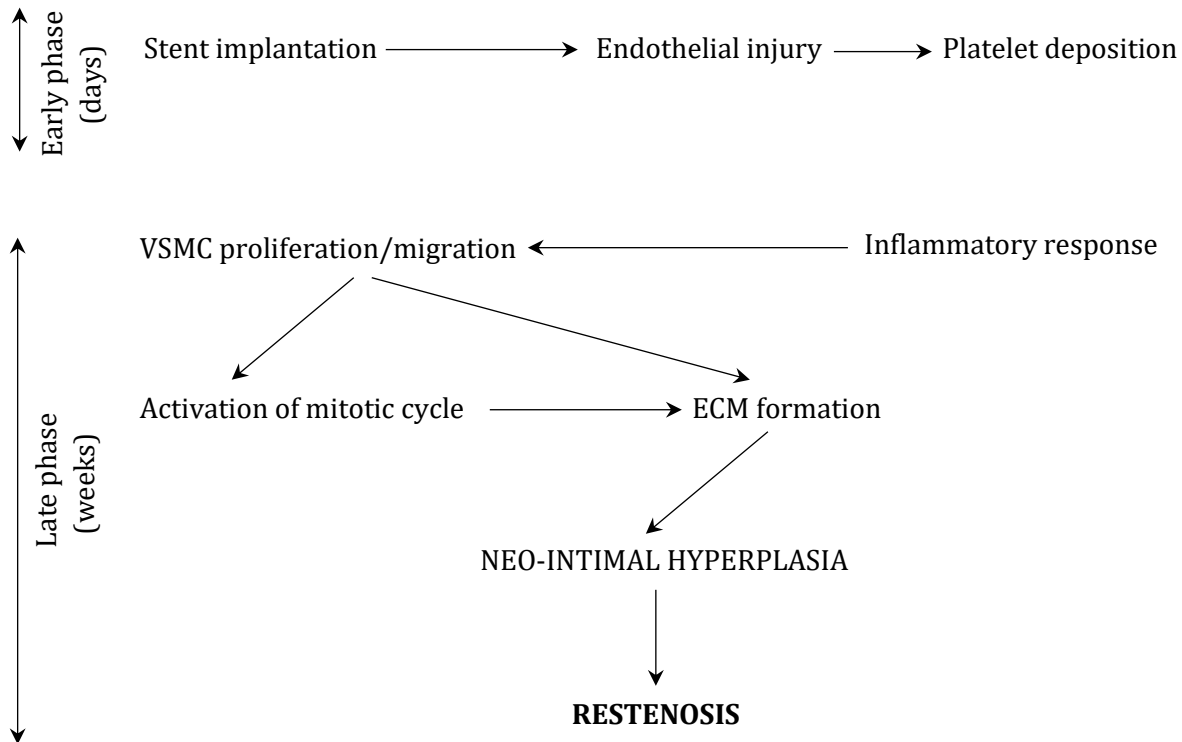




**Figure 1.1: Schematic of the “cascade” reaction of the vascular wall to stent injury involving several distinct steps.** (A) injury to the endothelium and even the elastic lamina, triggers an inflammatory reaction, which causes (B) proliferative changes in the media. (C) There is phenotypic modulation of the medial VSMCs, which causes them to migrate into the intimal layer, where (D) increased ECM production by VSMCs adds bulk to the restenotic lesion. (E) The final healing phase results in stabilization of the fully formed lesion. Legend: SMCs; smooth muscle cells, ECM; extra cellular matrix, VSMCs; vascular smooth muscle cells, IEL; internal extracellular lamina, EEL; external extracellular lamina, SAPK; stress-activated protein kinases, Adapted from (Mitra and Agrawal, 2006).

Experimental models have demonstrated that thrombocytopenia has been found to reduce restenosis (Virmani and Farb, 1999). Furthermore, glycoprotein IIb/IIIa antagonists (which are antibodies to the platelet glycoprotein receptor IIb/IIIa) have been shown clinically to have similar effects (Kereiakes et al., 1998; Topol and Serruys, 1998). The glycoprotein IIb/IIIa inhibitors have opened the field to a rapidly developing area of anti-integrin targeting as a therapeutic approach. Currently, the Fab fragment abciximab and the small molecule glycoprotein IIb/IIIa inhibitors eptifibatid and tirofiban are used in interventional cardiology practice (Ahrens et al., 2003). However, some studies challenge the benefit of these therapeutic agents in the clinical setting (Waksman et al., 2004). Transmigration of leucocytes occur across the platelet coated surface (Diacovo et al., 1996), mediated by P-selectin (Yeo et al., 1994). Neutrophil-platelet and monocyte-platelet aggregates have been found in the peripheral blood of patients with CAD and have been proposed as predictors of disease activity (Furman et al., 1998; Ott et al., 1996). A number of adhesion molecules and chemoattractant agents play a critical role in the recruitment of monocytes, platelet activation, and aggregation. These include Mac-1 (CD11b/CD18), a component of leucocyte secretory granules, which promotes adhesion of polymorphonuclear leucocytes and monocytes to endothelial cells (Inoue et al., 1998). Furthermore, Mac-1 expression increases following stent implantation (Fuster et al., 1995). Monocyte chemoattractant protein (MCP)-1 is a chemokine secreted by activated platelets (Rollins, 1997), the expression of which rises in endothelial cells and vascular smooth muscle cells (VSMCs) following stenting injury (Furukawa et al., 1999). Persistently elevated levels of MCP-1 have been demonstrated in patients who developed restenosis (Cipollone et al., 2001). Events occurring at the platelet

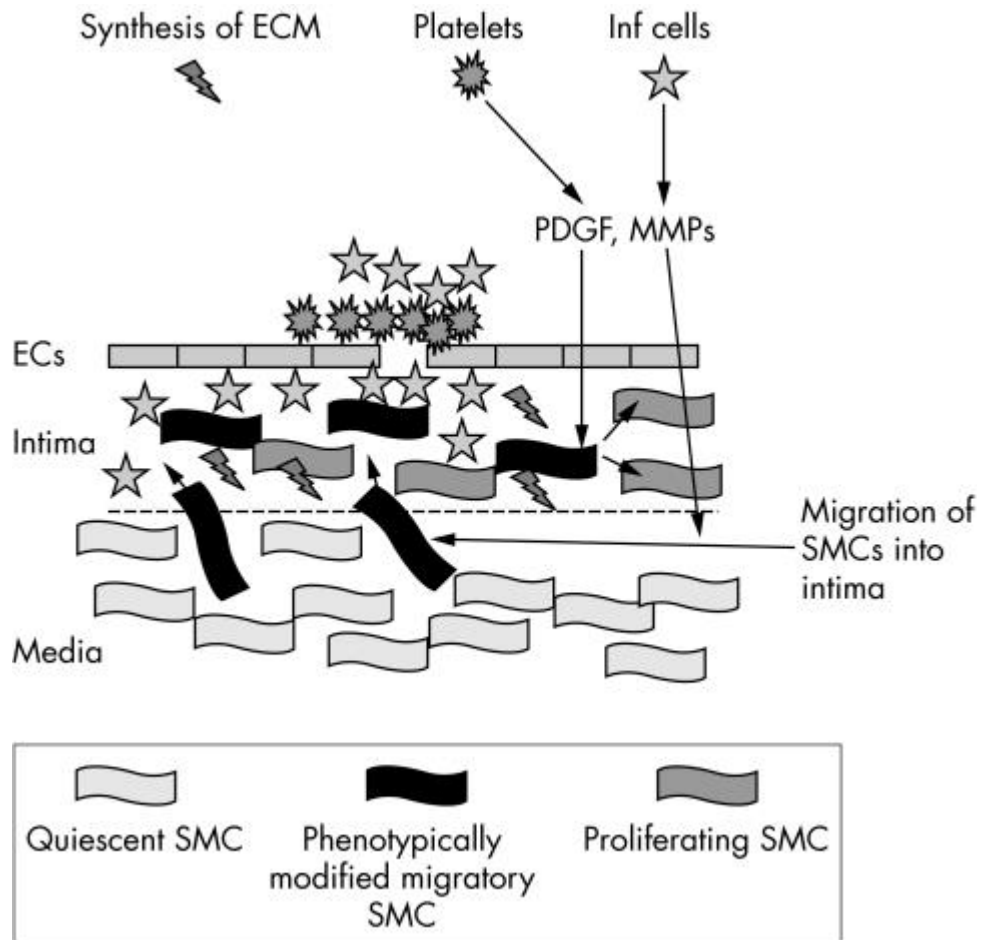
surface involve CD40L, (described in more detail in section 1.9 below). Assembly of CD40L and its soluble fragment sCD40L inhibits re-endothelialisation of the endothelial layer and predisposes to restenosis (Aukrust et al., 1999).



**Figure 1.2: Schematic of the time course reactions of restenosis.** Chronological sequence of events leading to instant restenosis can be divided into the early and the late phases. The early phase is the initial injury followed by the inflammatory reactions and the late phase essentially consists of the various mechanisms leading to increase in lesion volume (hyperplasia). Legend: ECM; extra cellular matrix, VSMCs; vascular smooth muscle cells. Adapted from (Mitra and Agrawal, 2006).

ISR development has complex mechanisms, which can be divided into an “early” (days to weeks) and a “late” (weeks to months) phase (**Figure 1.2**). Each step is regulated by unique but inter-related molecular and cellular events, the overall control of which is the result of interaction among growth factors and their

receptors, secondary messengers, cytokines and proto-oncogenes involved in transcription, translation, and post-translational events (Lincoff et al., 1994; Topol and Serruys, 1998). The early phase events start with restructuring of the thrombus, relocation of the plaque and an acute inflammatory response. In addition, studies have shown that elastic recoil is minimal in ISR compared to restenosis that occurs following PCI, due to the rigid scaffolding of the stents (Bennett, 2003). Endothelial injury produces some element of thrombus formation (Virmani et al., 2003) and fibrin and platelet deposition at the site of injury delivers the basis for the inflammatory aggregate. In addition, there is increased leucocyte trafficking (predominantly monocyte derived macrophages (Komatsu et al., 1998)) to the stent site and subsequent migration into the vessel wall (Dzavik, 2003). Un-interrupted production of adhesion molecules, chemoattractants, cytokines and growth factors by the monocytes, SMCs and platelets results in further leucocyte recruitment and infiltration. The following weeks after this injury leads the whole process into the late phase. The main outcome of the late phase is the phenotypic modification of medial SMCs followed by their migration and subsequent proliferation in the intima. Synchronised extracellular matrix (ECM) synthesis by these SMCs is responsible for the increasing volume of intimal tissue (**Figure 1.3**) and the bulk of the NIH is composed of ECM collagens and proteoglycans. Furthermore, cellular elements only make up approximately 11% of the restenosis body (Rollins, 1997). Therefore following stent implantation, there is a shift towards greater ECM synthesis rather than SMC proliferative activity over the months subsequent to stent implantation (Furman et al., 1998; Ott et al., 1996).



**Figure 1.3: Schematic diagram of the cellular mechanism of ISR.** Phenotypic modulation of the quiescent VSMCs and migration into the intimal layer. Continual SMC proliferation occurs in the intima with the deposition of de novo ECM. Legend: ECM; extra cellular matrix, ECs; endothelial cells, MMPs; Matrix metalloproteinases, PDGF; platelet-derived growth factor, SMC; smooth muscle cells, Inf cells; Inflammatory cells, Adapted from (Mitra and Agrawal, 2006)

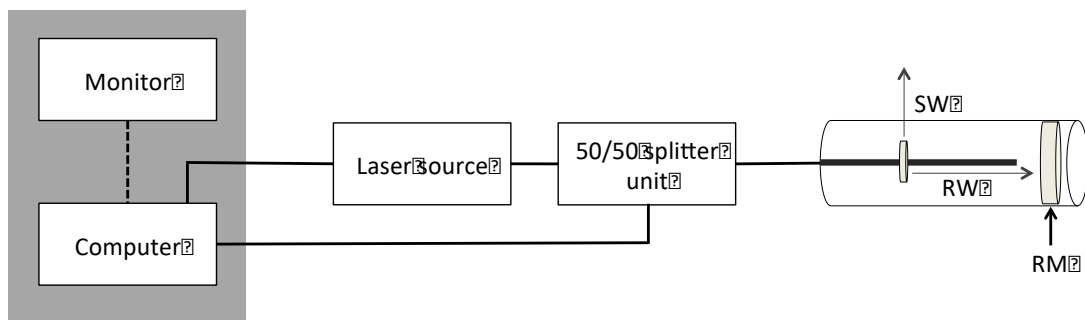
## 1.6 Optical coherence tomography (OCT)

Coronary angiography has been the gold standard technique for evaluating coronary artery disease for the past 50 years. Increasingly however, realisation of the limitations of coronary angiography, mainly the inability to supply information regarding the coronary wall, has prompted the design and development of adjunctive technologies to better evaluate not just luminal disease but also the burden and character of atherosclerotic plaque within the vessel. The

development of intracoronary imaging modalities namely IVUS and optical coherence tomography (OCT) has progressed quickly and these technologies now have established roles in the diagnosis and treatment of coronary artery disease. In general, intracoronary devices that can assess the coronary endothelium use either acoustic or optical signals that are received by a coronary catheter (IVUS uses ultrasound, OCT uses near infrared light).

OCT was first developed by two Japanese researchers at the Yamagata University (Japan) and subsequently at the Massachusetts Institute of Technology in the USA in 1991. OCT was initially performed in the retina but adopted in the coronary artery later in the same year (Mintz et al., 2001; Terashima et al., 2012). OCT uses near-infrared light, which is absorbed by water, lipids and erythrocytes (**Figure 1.4**). The high-resolution of OCT has allowed use of this technology for both clinical and research purposes (Terashima et al., 2012). OCT has widely been used in the assessment of coronary anatomy over the last decade, and has a wide range of clinical applications including coronary plaque anatomy, post PCI stent position and malapposition. Within research, OCT has improved the evaluation of stent endothelisation post implantation. Although initial OCT systems consisted of time-domain (TD-OCT) technology, this has been surpassed by frequency-domain (FD-OCT) technology. While TD-OCT uses a moving mirror as its reference arm and a broadband light source, FD-OCT uses a fixed mirror with a variable frequency light source allowing simultaneous detection of reflections from all echo time delays (Takarada et al., 2010).

Current OCT catheters are 3.2 French (The French scale or French gauge system is commonly used to measure the size of a catheter and is three times the diameter in millimeters) flexible short monorail systems with an optical emitting transducer, that emits a near-infrared wavelength of about 13,000 nm. The OCT catheter transducer lies 20mm behind the distal marker. The transducer contains optical fibres with a micro-lens transducer that is placed beyond the target lesion along a standard guide-wire. The OCT catheter does not move during image acquisition, instead the transducer moves back inside the central part of the catheter.



**Figure 1.4 Schematic of OCT imaging system:** The pulse of light from the laser source is split equally between the tissue wave (TW) and the reference wave (RW). The RW reflects off the reference mirror (RM) to calculate distance of pullback. The returning TW signal from the tissue is combined with the returning RW signal from the RM and this is converted to images within the main computer system.

The catheters have an automated pullback system at a rate of 36 mm per second with an image range of 54 to 74 mm when adequate coronary preparation has occurred. As the light source is easily absorbed by blood there is a need for coronary preparation prior to image acquisition. The use of pure contrast through a manifold to prepare the coronary artery with total blood removal is generally

recommended with most left coronary systems requiring 10-14 mls and right coronary arteries 8-10 mls. The OCT system consists of an OCT imaging catheter (ImageWite™, St Jude™) and an OCT system console, which contains the optical imaging engine and computer signal acquisition (M2/M3 CV OCT Imaging System, LightLab Imaging, Inc.).

Due to the need for complete coronary preparation, if any blood pooling remains, a high signal will remain within the image distorting the final image. In addition, as the guide wire does not run through the entire length of the OCT catheter, all images will have a silhouette of the guide wire with reduction of image quality in these areas (**Figure 1.5**).

The relatively low energy used in OCT (5.0 to 8.0 mW) does not cause functional or structural damage to the coronary tissue. The main safety concern with OCT is the use of a contrast bolus in coronary preparation, however, studies have shown that no patients suffered contrast induced nephropathy, however there is a relatively small risk of coronary spasm and ECG changes during contrast administration (Imola et al., 2010).

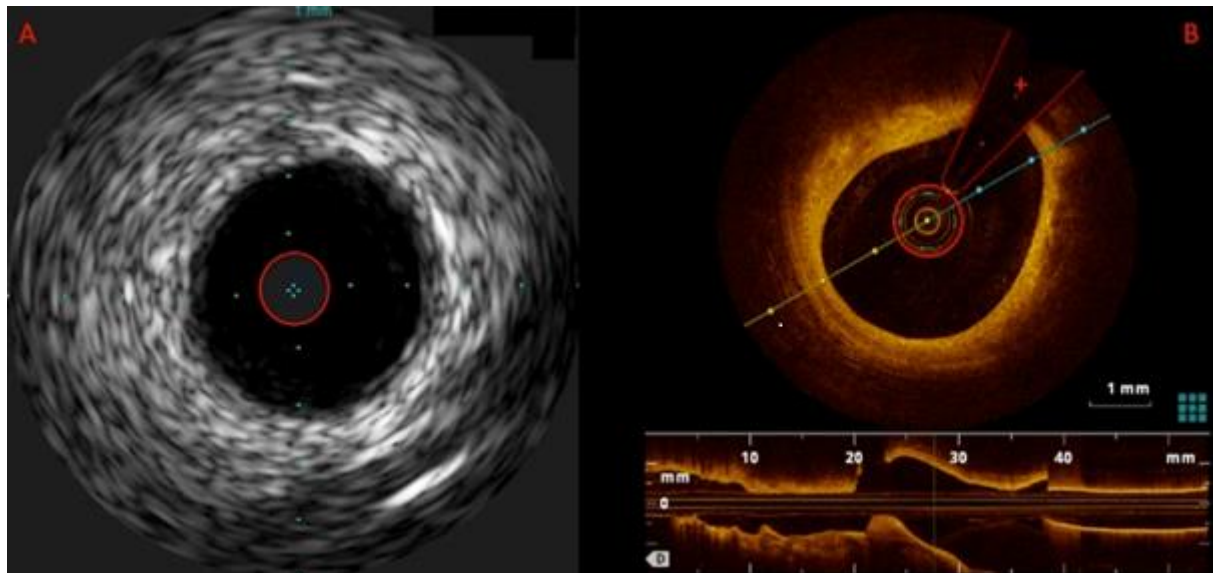
## **1.6.1 Assessment of Coronary Lesions with OCT**

### **1.6.1.1 Plaque Characterisation**

Since there is greater spatial resolution with OCT compared to IVUS (see **Figure 2**), OCT can provide more detail regarding the microstructure of the vessel wall and specifically OCT has been shown to identify thin-cap fibroatheroma (TCFA). Studies have shown a high degree of correlation between OCT imaging and fibrous



cap thickness on histologic evaluation (Calvert et al., 2011; Garcia-Garcia et al., 2011; Mintz et al., 2001). In addition, OCT can identify TCFA by measuring the thickness of the fibrous cap and the arc of the lipid-rich plaque (Jang et al., 2002; Jang et al., 2005; Tanaka et al., 2009). Lipid pools are less sharply delineated than calcification and show lower signal intensity. Lipids also exhibit more heterogeneous backscattering than fibrous plaques (Prati et al., 2010; Yabushita et al., 2002).



**Figure 1.5 Intracoronary imaging of a normal coronary artery:** (A) IVUS image (B) OCT image of normal coronary arteries. Red circle indicates position occupied by imaging catheter and + shows the 'drop-out' signal produced by the guide-wire

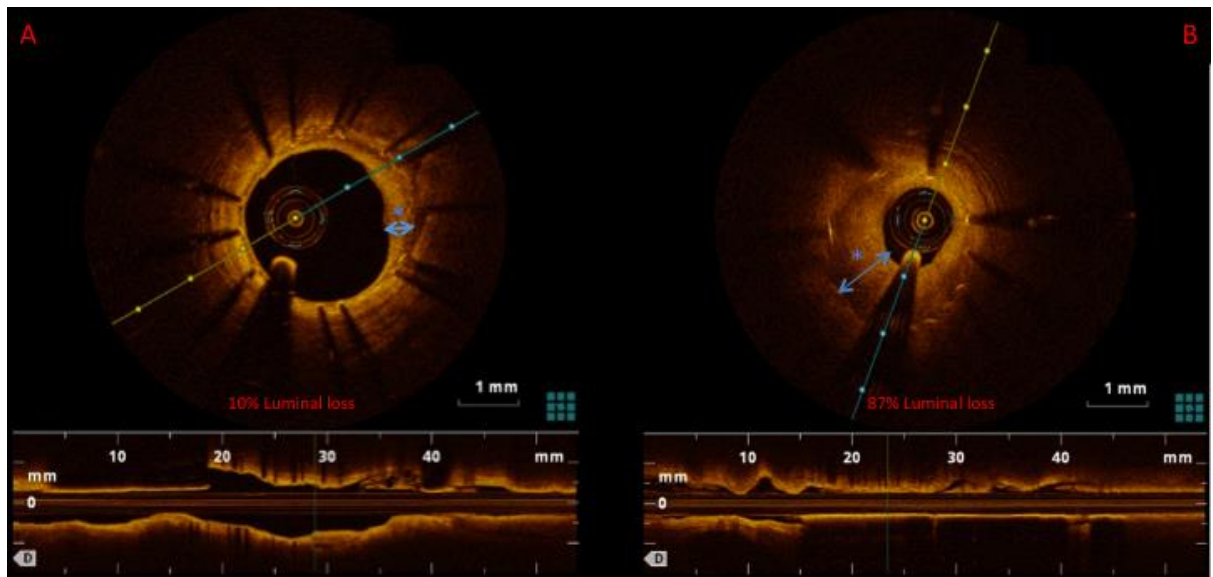
OCT has been shown to be helpful in determining prognosis by identifying vulnerable plaques. A prospective study of the characteristics of non-culprit lesions in 53 patients with coronary artery disease undergoing PCI showed that TCFA (as assessed by OCT) and the presence of micro-channels had a significant

correlation with plaque progression (defined as > 0.4 mm increase in minimal luminal diameter) at a 7-month follow-up (Uemura et al., 2012).

#### **1.6.1.2 Assessment of Neointimal Coverage with OCT**

Strut coverage is an important surrogate risk factor of stent thrombosis. Using OCT, strut coverage is clearly seen and both the coverage of individual struts and the thickness of neointimal coverage can be assessed accurately (Matsumoto et al., 2007). In one study, at 6-months follow-up, 89% of sirolimus-eluting stents (SES) lesions were covered by thin neointima, and 64% of the stent struts were covered with neointima that had a thickness of less than 100  $\mu\text{m}$  (Matsumoto et al., 2007).

Even though the introduction of DES has led to reduced rates of restenosis, this complication following PCI still occurs and our understanding of its pathophysiology is still poor. OCT has helped advance our understanding with studies demonstrating that stent restenosis is not homogenous. Furthermore, OCT imaging allows separation of restenotic tissue into homogenous, layered and heterogeneous groups. This was demonstrated in a study where paclitaxel-eluting stent restenosis could be easily classified into these three groups using OCT (Gonzalo et al., 2009). **Figure 1.6** demonstrates the sensitivity of OCT in characterising the extent of restenosis after drug-eluting stent implantation.



**Figure 1.6: Intracoronary optical coherence tomographic images of restenotic tissue following drug-eluting stent implantation. A 10% luminal loss (\* and blue arrow): B represents 87% luminal loss (\* and blue arrow)**

### **1.7 Quantitative coronary angiography (QCA)**

Coronary angiography using selective injection of radiopaque contrast agent into the coronary arteries is the gold standard for evaluating CHD. Due to significant intra-observer and inter-observer variability, subjective visual estimation of lesion severity has been shown to be inadequate (Topol and Nissen, 1995). Since the late 1980s methods and algorithms for QCA have been developed in order to objectively quantify the extent of CHD (Serruys et al., 1984). In addition, validation of specific QCA measurements associated with clinical outcomes has led to their incorporation into trials endpoint and scoring systems.

### **1.7.1 Two-dimensional QCA**

In this technique, contrast coronary angiograms allow parameters to be obtained that objectively quantify the coronary lumen and allow measurement of any significant coronary stenosis. Since coronary arteries can be highly tortuous 3D structures whereas a single coronary angiography image gives only a 2D radiographic image, at least two projections orthogonal to each other and both perpendicular to the analysed coronary segment are necessary to obtain the most complete data to avoid underestimation of stenosis severity and foreshortening.

Recent systems are based on vessel edge to edge detection algorithms to designate the arterial lumen on conventional coronary angiograms allowing the assessment of the lesion in an operator-independent way and therefore exploiting specific and dedicated software. Over the years, several generations of QCA systems have been developed based on edge detection algorithms to improve measurement accuracy. Cardiovascular X-ray imaging systems are equipped with flat-panel (FP) detectors (Spahn et al., 2003), which have replaced the combination of image intensifier and charge-couple device camera providing better image quality and enabling improved image enhancement. Novel QCA third-generation system, which took advantage of digital FP detectors instead of the conventional image intensifier systems, were better able to determine smaller diameter vessels (Van Herck et al., 2004) and analyse complex lesion morphology with irregular borders (van der Zwet and Reiber, 1994). Currently, there are different computer systems for QCA that have been validated: among them the most commonly employed are CAAS (PIE Medical, Maastricht, The Netherlands) and QAngio XA (Medis, Leiden, The Netherlands) (Garrone et al., 2009).

### **1.7.2 Clinical and research applications of QCA**

In clinical practice, QCA can be used during diagnostic coronary angiography, to provide an objective and independent parameter for the assessment of stenosis severity because the human eye does not have the resolution capability of software. Usually, visual interpretation of the severity of a coronary stenosis is expressed in intervals of percentage of stenosis while QCA produces a single specific measure for diameter stenosis, improving the accuracy and reproducibility of the severity assessment (Kalbfleisch et al., 1990). Studies have shown that the visual assessment tends to underestimate stenoses <50% and to overestimate those >50% (Fleming et al., 1991) therefore the online use of QCA assessing the lesion length and reference vessel diameter (RVD) might enable us to obtain objective parameters to decide which specific measures (mainly diameter and length) of the device (e.g., balloon or stent) should be chosen.

Within clinical research, QCA permits serial analysis of the same coronary segment over time in an independent way. In trials involving coronary devices, typically at least three QCA analyses are performed, one before treatment, another immediately after treatment, and a final one at angiographic follow-up (at a specified time). Binary restenosis (defined as a >50% diameter stenosis at follow-up (Mauri et al., 2005)) and late luminal loss (defined as the difference between the minimum luminal diameter (MLD) immediately after the procedure and the MLD at follow-up (Mauri et al., 2005)) have played an important role in several studies investigating various stents. These values, more specific and more applicable to the clinically relevant end-point of target lesion revascularisation

(TLR), allow researchers to compare different types of stents in clinical trials, with greater statistical power (and hence with fewer patients) (Tomasello et al., 2011).

Finally, another useful application of QCA is the study of plaque progression and regression that is based on at least two QCA analysis made over a pre-specified interval of time, often 6 months or 1 year. The change in MLD of the pre-determined coronary segments can then be used to determine efficacy of interventions to reduce restenosis.

### **1.8 Inflammation and restenosis**

Although factors including anatomical features (e.g. lesion length), procedural variables (e.g. residual stenosis) and clinical factors (diabetes, unstable angina, smoking, older age) (Mercado et al., 2001) are known to contribute towards restenosis, the exact mechanisms of this process are not completely understood. In addition, these factors are of only limited use in predicting those in whom restenosis will occur (Singh et al., 2004).

There is a growth of evidence suggesting that inflammation may play an important role in restenosis (Libby et al., 2003; Toutouzas et al., 2004), raising the possibility that measurement of inflammation may improve our ability to predict those at increased risk. Following PCI, balloon injury to the endothelium and vessel wall results in a 'healing' response characterised by inflammation in both the intimal and adventitial layers of the artery wall (Kornowski et al., 1998). This 'healing' response means that there is proliferation of fibrocollagenous tissues in association with the neovascularisation of the stent struts, which together

resemble granulation tissue in normal wound healing (Kornowski et al., 1998). Furthermore, as well as being prothrombotic, this inflammation has two important consequences namely, adventitial constriction and intimal hyperplasia (Kornowski et al., 1998), leading to the process of restenosis.

Constriction of the adventitia results in an overall reduction in the arterial diameter at the PCI site and is a major component of restenosis following balloon angioplasty without stenting. Although this constrictive remodelling is prevented by stent deployment, there is much more intimal hyperplasia following stenting and this accounts for virtually all in-stent restenosis (Lowe et al., 2002).

In both animal models and human pathology studies, there is a significant correlation between the extent of inflammatory reaction to angioplasty and the degree of restenosis (Kornowski et al., 1998). Experimental treatments aimed at decreasing inflammation have shown some promise in reducing restenosis (Phillips et al., 2003; Versaci et al., 2002), adding further emphasis to the theory that inflammation is a central component of the restenotic process (Farb et al., 2002; Meuwissen et al., 2002).

Hence, a better understanding of the components of the inflammatory response that contribute to restenosis may help in the development of anti-restenosis treatments. Furthermore, if measurement of circulating markers of inflammation improves our ability to predict those who will develop restenosis, they may be of use in targeting prevention strategies such as DES to those most likely to benefit.

Below, the associations between several inflammatory markers and restenosis are discussed in detail particularly those relevant to this thesis.

### **1.9 Pre-PCI inflammatory markers and restenosis**

There have been a number of studies that have investigated whether inflammatory marker levels are predictive of restenosis in patients undergoing PCI (Buffon et al., 1999; Dibra et al., 2003; Gomma et al., 2004; Rittersma et al., 2004; Segev et al., 2004; Walter et al., 2001). In particular, there has been a focus on C-Reactive Protein (CRP). CRP is an annular (ring-shaped), pentameric protein found in blood plasma, the levels of which rise in response to inflammation. It is an acute-phase protein of hepatic origin that increases following interleukin-6 secretion by macrophages and T cells (Thompson et al., 1999). Its physiological role is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system (Thompson et al., 1999). In mixed patient cohorts of unstable and stable angina, an association between raised CRP levels and subsequent angiographic restenosis has been demonstrated (Buffon et al., 1999; Walter et al., 2001). In the study by Walter *et al.* (2001) in 276 “high-risk” PCI patients (which included a number of patients with acute coronary syndrome (ACS)), pre-PCI CRP levels were predictive of angiographic restenosis, with a CRP level  $\geq 5$  mg/l associated with significantly higher restenosis rates than in those with a CRP  $< 5$  mg/l (40% vs. 19% respectively,  $p=0.002$ ) (Walter et al., 2001).

On the other hand, studies that are limited to a stable angina population alone have found no association between pre-PCI CRP levels and restenosis (Dibra et al.,



2003; Gomma et al., 2004; Rittersma et al., 2004; Segev et al., 2004). In the largest study reported to date, Dibra *et al.* investigated baseline CRP, 6 month angiographic restenosis and 1 year clinical outcome in nearly 1200 patients undergoing elective PCI for stable angina (Dibra et al., 2003). In this study, CRP appeared predictive of clinical events (death or non-fatal myocardial infarction) but not of angiographic restenosis (CRP >5 mg/l, restenosis rate 25%, CRP ≤5 mg/l, restenosis rate 24%, p=0.66). Hence, the differences in why CRP has been shown to be predictive of restenosis in some studies (Buffon et al., 1999; Walter et al., 2001) while others have not shown any difference (Dibra et al., 2003; Gomma et al., 2004; Rittersma et al., 2004; Segev et al., 2004) may be related to the differences in the population studied. As we already know, the underlying pathological processes in ACS are different from those of stable angina (Zipes et al., 2005). ACS patients have widespread coronary inflammation with evidence of unstable coronary plaques in non-culprit arteries (Buffon et al., 2002; Rioufol et al., 2002), and this is reflected in significantly higher pre-procedural CRP levels (Bogaty et al., 2001) and more marked elevation of CRP levels after PCI (Liuzzo et al., 1998) than those observed in stable angina patients (Azar et al., 2000). This may introduce confounding in studies that include a mixed population of stable and unstable angina patients, since unstable angina is associated with both raised CRP levels and increased rates of restenosis. It may also be that CRP is predictive of restenosis in ACS but not in stable angina; only study of a 'pure' ACS cohort would answer this question.

It is possible other markers directly involved in the restenotic process might be more predictive of restenosis. CD40 ligand (CD40L, also known as CD154), a

transmembrane protein, was originally identified on CD4+ T cells, but has also been found on activated platelets (Henn et al., 1998). Both membrane-bound and soluble (s) forms of this ligand may interact with CD40, which is constitutively expressed on vascular cells, resulting in various inflammatory responses (Mach et al., 1997). In addition, soluble intercellular adhesion molecule-1 (sICAM-1) represents a circulating form of ICAM-1 that is constitutively expressed or is inducible on the cell surface of different cell lines. It serves as a counter-receptor for the lymphocyte function-associated antigen (LFA-1). Interaction between ICAM-1, present on endothelial cells, and LFA-1 facilitates leukocyte adhesion and migration across the endothelium. ICAM-1 and its circulating form have been implicated in the development of any number of diseases (Witkowska and Borawska, 2004).

For example, sICAM-1 has been implicated in the pathogenesis of neointimal proliferation following vascular injury in animal models of PCI (Hwang et al., 1997) and soluble CD40 Ligand (sCD40L) has been proposed to exacerbate inflammation and prevent re-endothelialisation of the injured vessel wall thereby promoting restenosis (Andre et al., 2002). Accordingly, in a study involving 70 predominantly unstable angina patients who underwent PCI (stent deployment rate 50%) to a single non-occlusive coronary stenosis, all patients had sCD40L and sICAM-1 measured at the time of PCI and underwent 6 month angiographic follow-up. Pre-procedural levels of both sCD40L and sICAM-1 were significantly higher in patients who subsequently developed angiographic restenosis than in those who did not, suggesting that sCD40L and sICAM-1 levels may be useful in predicting restenosis in patients with unstable angina (Cipollone et al., 2003).

However, whether this might be true for stable angina patients is unknown and warrant investigation.

### **1.10 Post-PCI inflammatory markers and restenosis**

Since inflammation plays a major role in restenosis after PCI with stent implantation, measurement of post-PCI inflammatory markers could be useful in predicting rates of restenosis. There is some data suggesting a significant rise in CRP levels at 48 hours post-procedure (Azar et al., 1997; Gomma et al., 2004) and sCD40L levels between 18 and 24 hours post-procedure (Feldman et al., 2001; Quinn et al., 2004). In addition, there is some evidence to suggest that the inflammatory response can be modified and reduced by using antiplatelet agents during PCI. A sub-analysis of the EPIC trial from 2001 showed that CRP levels at 48 hours post procedure were reduced by 32% in patients receiving abciximab (glycoprotein IIb/IIIa inhibitor) compared to patients in the placebo arm of the study (Lincoff et al., 2001). Similar findings were seen in the Cleveland Clinic registry data where the periprocedural increase in CRP was reduced by 65% in patients who received clopidogrel before their PCI (Vivekananthan et al., 2004). These studies suggest that measurement of inflammatory markers post-PCI could be used to guide therapy so that patients with high levels of inflammatory markers could benefit from an increased dose or further antiplatelet therapy. Furthermore, an improved understanding of the inflammatory response to PCI in the elective setting could also lead to novel treatment strategies targeting specific aspects of the inflammatory process.

There have only been a few studies that have investigated whether the inflammatory response to PCI is predictive of future restenosis. One study involving 1800 patients with both stable and unstable angina showed an association between post-PCI inflammatory response and 6 month angiographic restenosis (Dibra et al., 2005). In this study, CRP levels were measured at 8 hours, 16 hours and then daily until discharge post-PCI. Both pre-PCI CRP levels and post-PCI rise correlated with angiographic restenosis ( $p=0.002$ ). However, this association was strongly influenced by the presence of unstable angina because after excluding unstable angina patients, the association was only borderline significant ( $p=0.05$ ) in the remaining 73% of patients who had stable angina. Three other small studies have investigated the association between post-PCI CRP and restenosis in stable angina patients (Gomma et al., 2004; Gottsauner-Wolf et al., 2000; Segev et al., 2004). Segev *et al.* found no association between post-PCI CRP levels and angiographic restenosis in 43 stable angina patients undergoing coronary stenting (Segev et al., 2004). Here CRP was measured at multiple time-points (6 hours, 24 hours, 3 days, 7 days, 1 month, 3 months and 6 months post stenting). Similarly, Gomma *et al.* measured CRP serially for up to 6 months post-PCI in 58 stable angina patients undergoing elective coronary stenting, and found no association between post-PCI CRP levels and restenosis (Gomma et al., 2004). However, Gottsauner-Wolf *et al.* studied 40 consecutive patients with stable angina, and found that CRP levels 96 hours post-procedure were significantly higher in those who subsequently went on to develop restenosis (Gottsauner-Wolf et al., 2000). Due to these discrepant results, further larger studies are needed to determine whether post-PCI levels of CRP and other inflammatory markers are predictive of restenosis in stable angina patients.

Finally, data on other inflammatory markers are very limited. One study has shown post-PCI sCD40L and sICAM-1 levels to be predictive of restenosis (Cipollone et al., 2003). This study by Cipollone *et al.*, measured levels sCD40L and sICAM-1 before, and 1, 5, 15 and 180 days after PCI. Patients who developed restenosis had higher baseline levels of these markers than patients who did not. Following PCI, there was a significant rise in levels of both markers at 24 hours, and this rise was more pronounced and more prolonged in those who developed restenosis. Although sCD40L and sICAM-1 levels returned to baseline by day five in those who did not develop restenosis, they remained elevated through to 6 months in those with restenosis. Although this is an interesting finding suggesting support for the hypothesis that these markers may be involved in the restenotic process, it is worth noting that the study cohort was mainly patients with unstable angina and therefore whether similar observations might occur in stable angina patients is not yet known.

### **1.11 Inflammatory markers and cardiovascular risk**

As mentioned above, inflammation has been implicated as playing a crucial role in the initiation, progression and even the final rupture of atherosclerotic plaques.

A number of inflammatory mediators thought to be involved in these processes can be measured and quantified in the peripheral blood. These markers have proved useful in the study of mechanisms that underlie coronary events. In addition, their measurement may also improve clinical risk prediction in addition to offering new therapeutic targets (Libby et al., 2009; Pearson et al., 2003; Szmítko et al., 2003; Vishnevetsky et al., 2004). The inflammatory markers that

have been used for risk prediction in CVD can be classified as either systemic or localised markers of inflammation.

Systemic markers, such as white blood cell count (WBC), CRP and fibrinogen have been proposed to be useful markers for risk prediction. Inflammation in local tissues stimulates the production of these makers from the liver (fibrinogen, CRP) or bone marrow (WBC) and levels reflect the overall inflammatory activity within the body. Although these markers are found in relatively high concentrations in the systemic circulation, making them easy to measure and quantify, they have low specificity since levels can be markedly elevated in response to any systemic illness and may not directly be a reflection of cardiovascular inflammation. Nevertheless, baseline levels have consistently been shown to be predictive of cardiovascular risk (Ernst et al., 1987; Ernst and Resch, 1993; Ridker and Morrow, 2003). Furthermore, CRP is now being considered for routine clinical use, in risk prediction (Pearson et al., 2003) and guidance of therapy (Ridker et al., 2008). Furthermore, a recent large randomised, double-blind controlled trial involving 10,061 patients with previous MI and a raised high-sensitivity (hs) CRP investigated the use of canakinumab (a therapeutic monoclonal antibody targeting interleukin-1 $\beta$ ) in reducing nonfatal MI, nonfatal stroke, or cardiovascular death rates. The study found that rates of cardiovascular events were significantly reduced in the treatment arm secondary to the anti-inflammatory effects of the intervention (Ridker et al., 2017).

Local mediators of inflammation such as sCD40L, sICAM-1, and pregnancy-associated plasma protein (PAPP-A) are produced locally within the

atherosclerotic plaque and are thought to contribute directly to disease progression. In addition, they are also detectable in the systemic circulation and these have been shown to be predictive of cardiovascular risk (Zakynthinos and Pappa, 2009). However, due to very low systemic concentrations measuring changes in these may not be possible or as accurate as for the systemic inflammatory markers (Pearson et al., 2003). However, since these proteins are directly involved in the atheromatous process, they may be more specific for cardiovascular inflammation than systemic markers and as such have been proposed as potential therapeutic targets (Andre et al., 2002; Bayes-Genis et al., 2001; Vishnevetsky et al., 2004). Understanding the exact role of such proteins in atherogenesis and restenosis is therefore imperative for ascertaining the therapeutic opportunity in targeting atherosclerosis and inflammation.

## **1.12 Atherosclerosis and inflammation**

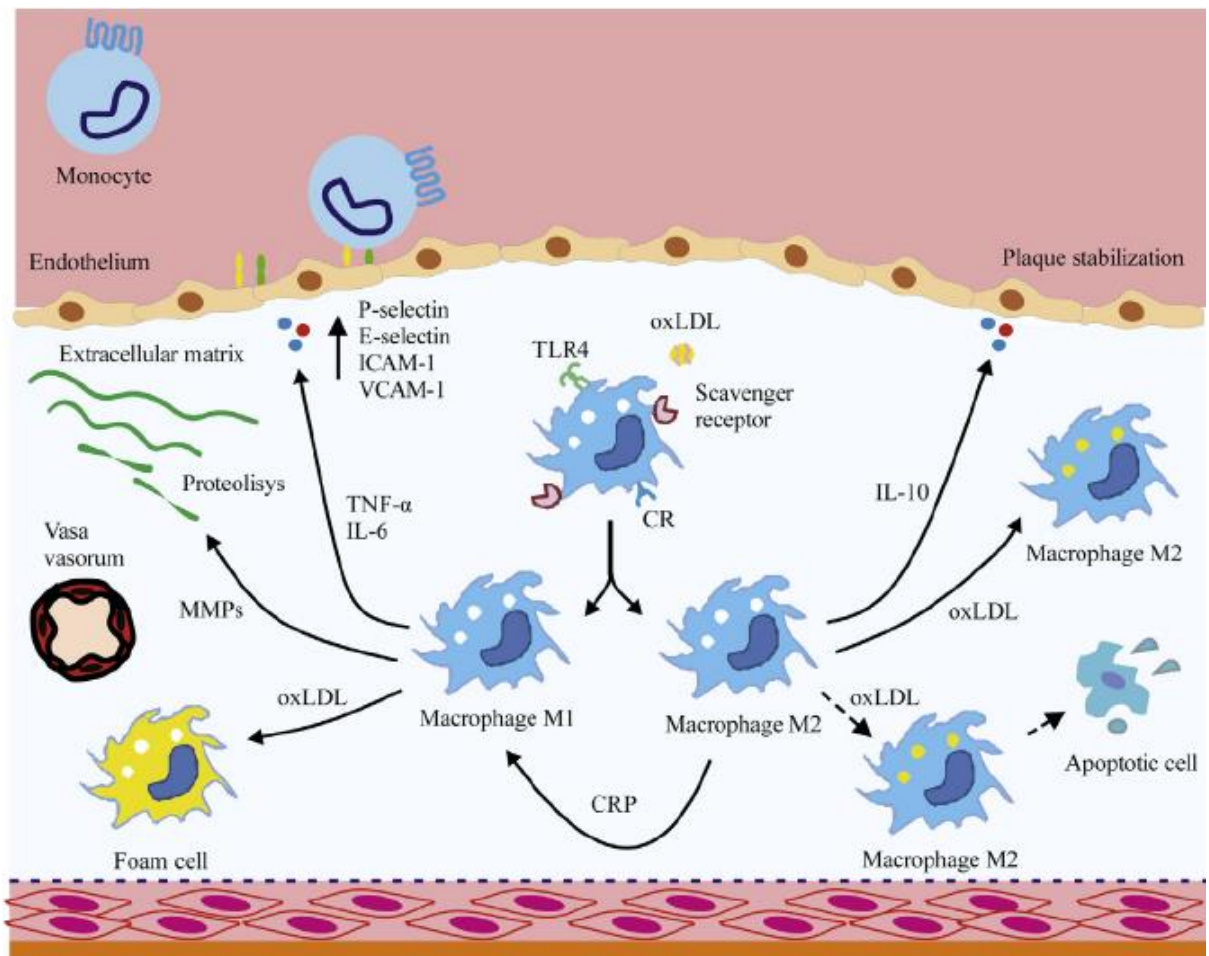
### **1.12.1 Pathogenesis**

Atherosclerosis is a chronic inflammatory process (Ross, 1999a), with immune cells acting as important mediators from the formation of the earliest fatty streaks to late-stage complex plaques. Smooth muscle cells, endothelial cells, lymphocytes, monocytes and macrophages have all been implicated in the pathogenesis of atherosclerosis (Choudhury et al., 2005) (See **Figure 1.7**).

After the initial activation of the endothelium due to disruptions in shear stress (an possibly the fatigue effect of pulse pressure on endothelial layer (Mehdizadeh and Norouzpour, 2009)), there is an increase in the permeability of the vessel to lipoproteins and upregulation of adhesion receptors (Tabas et al., 2007). This

change facilitates deposition of lipid from apolipoprotein B (apoB)-containing lipoproteins in the subendothelial space and encourages a monocytes and recruitment of circulating monocytes to atheroma vulnerable sites respectively. The adhesion of monocytes is dependent on the upregulation of a number of key cell-adhesion molecules on the luminal surface of the endothelium (Cybulsky and Gimbrone, 1991), namely, P-selectin, ICAM1 and VCAM1; followed by the expression of three major chemokine receptor families, CCR5, CCR2 and CX3C chemokine receptor 1 (CX3CR1), that facilitate the cell transmigration process (Combadiere et al., 2008; Tacke et al., 2007). After their recruitment from the spleen and bone marrow (Heidt et al., 2014) into the blood vessel wall, monocytes differentiate and locally proliferate into distinct functional phenotypes (M1 or M2) (Robbins et al., 2013), terminally differentiate into macrophages (Ley et al., 2011), or directly influence the phenotype of *in situ* cells (for example, lesional macrophages) (Khallou-Laschet et al., 2010). Via a number of processes lipids from retained apoB-containing lipoproteins are taken up, including pinocytosis of low density lipoprotein (LDL) (Barthwal et al., 2013), phagocytosis of aggregated LDL (Schissel et al., 1996), uptake by CD36 and uptake of modified apoB-lipoproteins by scavenger receptors. Ongoing lipid accumulation induces their transformation into macrophage-derived foam cells. Finally, as the foam cell population grows within lesions in the arterial wall, the rate of accumulation overcomes the rate of clearance, and eventually the foam cells amalgamate into a lipid-rich necrotic core.





**Figure 1.7 (Chavez-Sanchez et al., 2014): Schematic of the pathways involved in inflammation.** Endothelial cells, lymphocytes, smooth muscle cells, monocytes, and macrophages are all involved in the pathogenesis of atherosclerosis from earliest foam cell formation through to development of advanced plaques. Initial activation of the endothelium from disruptions to normal shear stress results in and facilitates deposition of lipid in the subendothelial space. Endothelial activation also promotes recruitment of circulating monocytes where they terminally differentiate into macrophages, or differentiate and locally proliferate into distinct functional phenotypes (M1 or M2). Activated macrophages take up lipid, which results in their transformation into macrophage-derived foam cells. As the foam cell population grows within lesions in the arterial wall, the rate of accumulation exceeds the rate of clearance, and eventually the foam cells coalesce into a lipid-rich necrotic core. CR, C-C chemokine receptor; CRP, C-reactive protein; ICAM1, intercellular adhesion molecule 1; MMP, matrix metalloproteinase; TNF, tumour necrosis factor; VCAM1, vascular cell adhesion protein 1, TLR4, toll-like receptor 4; oxLDL, oxidised-low density lipoprotein.

Secreted cytokines also play an important role in the pathogenesis of atherogenesis. Cells of both the adaptive (T and B lymphocytes, dendritic cells) and innate (macrophages) immune systems can modulate the local inflammatory setting through the secretion of cytokines. T cells can be stimulated to secrete proinflammatory TH1 cytokines such as IL-1, IL-6, and TNF; or TH2 cytokines such as IL-4, IL-10, and IL-13, which promote resolution of inflammation (Kucharzik et al., 1998; Raphael et al., 2015). IL-1 and TNF signalling is primarily mediated by p38 mitogen-activated protein kinase (MAPK) / nuclear factor (NF)- $\kappa$ B pathways (Chan et al., 2000). Conversely, activation of IL-6 signals via signal transducing protein gp130, which activates JAK1 and STAT1 and 3 (Ait-Oufella et al., 2011) results in the activation of endothelial cells and macrophages to produce adhesion molecules and chemokines (Gharavi et al., 2007).

The structure and environmental conditions within the plaque can also impact upon the propagation of the atheroma over the inflammatory response within the lesion. In human plaques, neovascularisation originating from the vasa vasorum (See **Figure 1.4**) is believed to contribute to intraplaque haemorrhage, which accelerates plaque expansion and inflammation as well as predicts future plaque rupture (Hellings et al., 2010; Michel et al., 2011). In the large lipid-rich necrotic core of advanced atheromas, the hypoxic environment induces hypoxia inducible factor (HIF) 1 $\alpha$ , which produces vascular endothelial growth factor and in turn drives angiogenesis (Sluimer and Daemen, 2009; Vink et al., 2007). *In vitro* experiments have shown that hypoxia and HIF1 $\alpha$  expression alter macrophage lipid handling and suppress cholesterol efflux via ABCA1 (the ATP-binding cassette subfamily A member 1) in both mouse (Parathath et al., 2011) and human

(Ugocsai et al., 2010) macrophages. Furthermore, *in vitro* and *ex vivo* experiments have linked hypoxia and HIF1 $\alpha$  in driving enhanced glucose uptake, metabolic activity, and polarisation of macrophages in human atheroma (Folco et al., 2011). Rong and colleagues (Rong et al., 2003) reported in 2003 that cholesterol-loaded vascular smooth muscle cells lost their differentiation markers and instead expressed macrophage markers. A number of experiments in both mice and humans (Allahverdian et al., 2014; Feil et al., 2014; Shankman et al., 2015; Vengrenyuk et al., 2015) have shown *in vivo* data consistent with these phenomena: 30–40% of cells classified as plaque macrophages were of vascular smooth muscle cell origin.

### **1.12.2 Inflammatory markers of atherosclerosis**

The association between CRP (as measured by high-sensitivity assay, which has been used as a surrogate biomarker for ‘inflammation’) and atherosclerosis has been an area of great research and interest. A number of studies and meta-analyses have demonstrated a relationship between elevated CRP levels and an increased risk of CVD (Danesh et al., 2004). Furthermore, a statin trial (the JUPITER Study (Ridker et al., 2008)) noted that a reduction in CRP level was an independent predictor of outcome (Nissen et al., 2005). In this study, which used high-sensitivity (hs) CRP to identify patients at increased risk of vascular events (without elevated LDL-cholesterol levels), patients with high basal hsCRP levels were at a significantly increased risk of future vascular events.

In addition, other circulating proteins including ICAM1 (Hwang et al., 1997), VCAM1 (Semaan et al., 2000), and P-selectin (Blann et al., 1997) have been

measured in the plasma of patients and have been demonstrated to be associated with the severity of atherosclerosis. However, as with hsCRP, exactly what these biomarkers represent 'downstream' is currently unclear.

Circulating monocytes have been the focus of research as a key cellular biomarker in the pathogenesis of atherosclerosis (Swirski et al., 2009). With the use of flow cytometry, the number of circulating CD14<sup>+</sup>CD16<sup>++</sup> monocytes has been shown to be inversely related to plasma high-density lipoprotein (HDL) levels, and CD16<sup>+</sup> monocytes are proportional to the severity of atherosclerosis (Schlitt et al., 2004). On the other hand, a reduction in the monocyte subpopulation is associated with a reduction in intima-media thickness (Poitou et al., 2011).

To explore the functional characteristics of these 'inflammatory' cells at the level of atherosclerotic plaque, a new technique using laser-capture microdissection (LCM) has been used to attain intralesional cells in a cell-type-specific and location-specific fashion. In mice, immuno-LCM coupled with downstream gene or protein expression analysis has been used to study lesional macrophages in various stages of atherogenesis, including atherosclerosis regression. The inflammatory state of plaque macrophages was shown to be dynamically regulated as the severity of disease varied (Feig et al., 2011). In humans, there have been studies that used LCM on surgical plaque explants, which has identified genes involved in lipid metabolism (such as *LEP* and *FABP4*) and activation of the adipokine / peroxisome proliferator-activator receptor (PPAR) signalling pathways (Lee et al., 2013), as well as a range of inflammatory gene 'signatures' (Puig et al., 2011), that might be important in mechanistic staging. Circulating

microvesicles derived from monocytes, platelets and erythrocytes have also been characterised specifically with regard to the miRNA that they contain, and have been shown to have roles in activation of plaque macrophages, in cell-to-cell signalling (Hulsmans and Holvoet, 2013) and VCAM1 inhibition that play a key role in the progression of atherosclerosis (Rautou et al., 2011). One of the other key phenomena critical to recruitment of cells to a site prone to atherosclerosis or restenosis is endothelial dysfunction. Indeed, not only is dysfunction a consequence of the pathology but it is also proposed as a useful biomarker. Finally, studies looking at human and murine atherosclerosis have found that the histology of lesion development in murine and humans has more similarities than differences. Unfortunately, plaque rupture and thrombosis rarely occur in mice and therefore this is a major limitation in studying atherosclerosis in murine models (Bentzon and Falk, 2010). In addition, large animal models suitable for interventional procedures and imaging (such as porcine models) are more routinely used in animal studies to develop the field of interventional cardiology (Kornowski et al., 1998; Waksman et al., 2017).

### **1.13 Differences between the sexes and cardiovascular disease**

The link between atherosclerosis and cardiovascular health has been investigated for many years. In 1933, an association between female serum cholesterol levels and dietary changes was described (Okey and Stewart, 1932). Later in 1958, during a conference on “Hormones and Atherosclerosis,” researchers found that cholesterol deposition was directly linked to blood cholesterol levels. However, other factors (such as lipid levels, sex differences, diet, hormones and stress) were

also felt to be just as important. Furthermore, it was noted that ethinyl oestradiol reduced plasma lipid levels in a group of men with previous myocardial infarction (MI), although there was no significant reduction in morbidity or mortality from coronary events as compared to the control group (Anonymous, 1958).

Apart from the traditional risk factors, researchers have also actively investigated other potential causes of CVD. In recent times, there has been a large amount of interest investigating the role of sex hormones in CVD. In a study involving a small series of men with deficient oestrogen action (due to mutations in the genes for oestrogen receptor [ER] or aromatase), found that they developed hyperinsulinemia, early-onset atherosclerosis and increased visceral adipose tissue as well as the constellation of risk factors known as metabolic syndrome. Interestingly, these same pathologies have also been identified in men with sufficient oestrogen action but low testosterone levels (Phillips, 2005). Furthermore, one hypothesis suggests that these changes in sex hormone may result in these cardiovascular abnormalities and that in fact, atherosclerotic CVD may possibly be due to an endocrinological disorder (Phillips, 2005).

There is experimental, epidemiological as well as clinical data suggesting that there are specific variations between the two sexes that affect cardiovascular risk. In fact, many medical comorbidities, including hypothyroidism, diabetes and depression are more prevalent in post-menopausal women and have been demonstrated to be associated with increased cardiovascular risk (Perez-Lopez et al., 2009). One example of this is that women are more likely than men to

experience an episode of depression and to be subsequently diagnosed with metabolic syndrome (Kinder et al., 2004). Another example is that occurrence of subclinical hypothyroidism increases with age and is higher in women and numerous studies have demonstrated an association between CAD and thyroid (Fatourechi, 2009). Finally, women with diabetes are less likely to have 'well-controlled blood sugars' and are more likely to develop and die from CVD (Evangelista and McLaughlin, 2009).

In addition to these comorbidities, the menopausal period in itself seems to be an important time of transition to increased cardiovascular risk (Mankad and Best, 2008). The incidence of CVD (that is not related to diabetes) is lower in premenopausal women compared to age-matched men (Kaseta et al., 1999). There is an association of increased cardiovascular events due to ovarian hormone insufficiency at the time of the menopause. Hence, studies have found that the development of CVD is approximately 10 years later in women compared to men (Lorenzo et al., 2007). These observations and studies have led to the hypothesis that oestrogens may in fact protect women against atherosclerotic complications.

At the cellular level, experimental data has suggested that sex differences in the regulation of physiological mechanisms are directly influenced by genetic polymorphisms. Some studies have linked CVD with variations in the nuclear hormone family of ER genes, including ER- $\alpha$  gene (ESR1) and ER- $\beta$  gene (ESR2) (Shearman et al., 2006). These receptors function as ligand-dependent

transcription factors and predominate in vascular endothelial and smooth muscle cells (Hodges et al., 2000). Oestrogen receptor  $\alpha$  gene polymorphisms may affect the response to peripheral oestradiol and, indirectly, the prevalence of different menopause-related conditions (Schuit et al., 2004). Furthermore, postmenopausal women who carry a particular ESR1 variant are at increased risk of MI and CHD, independent of known cardiovascular risk factors (Schuit et al., 2005). This association has not been found in men (Liu et al., 2005).

Oestrogen receptor  $\beta$  gene expression prevails in human VSMCs (Christian et al., 2006). Experiments in rat models have shown there was an increased expression of ESR2 following vascular injury and its expression is associated with increased coronary artery plaque surface area in both women and men (Lindner et al., 1998; Rexrode et al., 2007). Some ESR2 polymorphisms are associated with increased risk of MI that is specific to certain sex. For example, women with a particular variant allele had significantly increased risk of CVD and MI, while men with a different rare variant were at reduced risk. However, other variants confer an increased risk of MI in men only (Domingues-Montanari et al., 2008). Moreover, a near-significant association was found between some ESR2 haplotypes and obesity (which adds a significant risk to CVD) in postmenopausal women (Goulart et al., 2009).

Studies have also found that selective stimulation of a second type of ER, the intracellular G protein-coupled ER (GPER), dilates human arterial blood vessels and decreases rat blood pressure (Haas et al., 2009). In GPER knockout mice, this



effect is abolished and increased visceral obesity has been demonstrated. Female GPER knockout mice had hyperglycaemia (and subsequently impaired glucose tolerance), increased blood pressure, reduced insulin-like growth factor blood levels and reduced body growth (Martensson et al., 2009).

Endogenous sex hormones in females (especially oestrogens) are cardioprotective via a number of different mechanisms: decreased LDL, increased HDL and release of vasodilators such as nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>) from vessel walls, which results in inhibition of vascular constriction and lowering of blood pressure as well as decreased platelet aggregation (described in more detail below) (Barrett-Connor and Bush, 1991). In addition, during the menopausal transition, there is an increase in the prevalence of the metabolic syndrome, elevated body weight, hypertension dyslipidaemia and hyperinsulinemia (Ho et al., 2005; Perez Lopez F et al., 2009). Interestingly, treatment with hormone replacement therapy does not significantly reduce blood pressure in postmenopausal women. This suggests that oestrogen deficiency may not be the only factor involved in postmenopausal hypertension (Perez Lopez F et al., 2009).

In comparison to age-matched men, the lower prevalence of CVD in premenopausal women has been partly explained by differences in plasma lipoprotein levels, body fat distribution and indices of plasma glucose-insulin homeostasis (Lemieux et al., 1994; Tomaszewski et al., 2009) females appear to have higher plasma HDL levels and lower plasma insulin, triglyceride (associated with abdominal visceral adipose tissue) and apolipoprotein B levels (Mohlke et al.,

2008). Finally, oestrogen appears to contribute to glucose homeostasis via increased glucose transport into the cell and studies have suggested a positive effect of hormone replacement therapy on HbA1C levels in postmenopausal women (Okada et al., 2003).

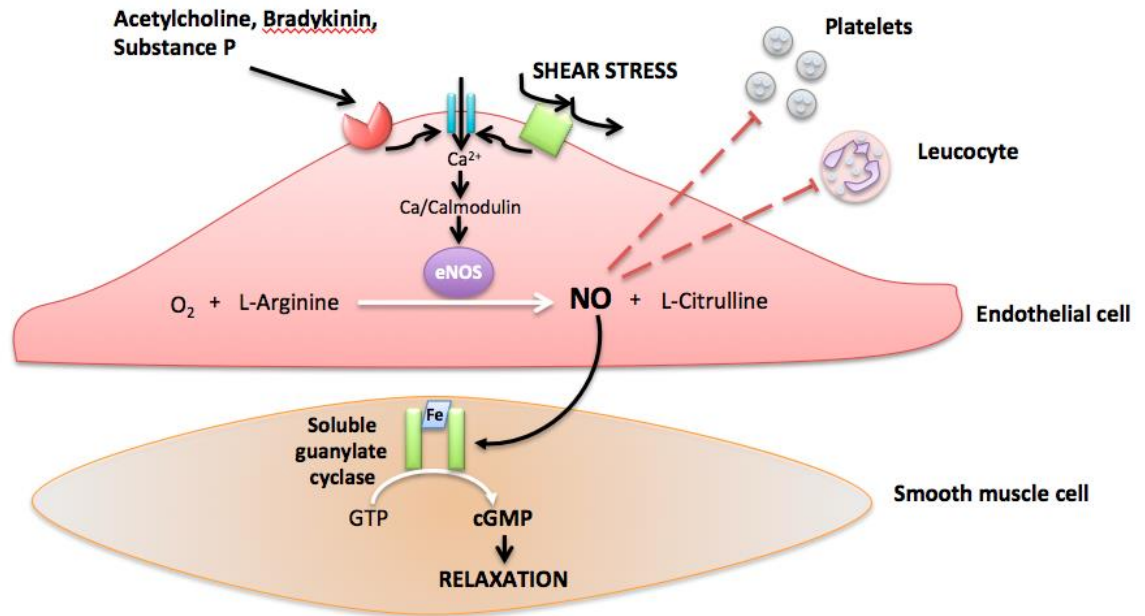
### **1.14 The healthy endothelium and nitric oxide**

In 1980, Furchgott and Zawadzki confirmed that the presence of intact endothelium is important for acetylcholine (ACh) to induce relaxation in an isolated rabbit aorta (Furchgott and Zawadzki, 1980). They showed that if the endothelium is damaged, the vessel no longer relaxes, but still responds to the NO donor glyceryl trinitrate (GTN). This highlighted the role of endothelium in ACh-induced vasorelaxation, which occurs via a messenger, initially labelled as “endothelium-derived relaxing factor” (Furchgott and Zawadzki, 1980). This sparked the interest of researchers to identify EDRF. In 1986, Ignarro and Furchgott independently proposed that NO has the same biological properties as EDRF (Furchgott, 1988; Ignarro et al., 1988). This finding was then confirmed in the following year (Ignarro et al., 1987; Palmer et al., 1987).

NO generated by the endothelium is made from the guanidine-nitrogen atoms of the amino-acid L-arginine. This reaction is catalysed by the endothelial isoform of NO synthase and generates the by-product L-citrulline (Palmer et al., 1988; Schmidt et al., 1988). NO has a short half-life in blood of approximately 6 seconds (Knowles and Moncada, 1992); as it gets oxidised to  $\text{NO}_2^-$  and then to  $\text{NO}_3^-$  by oxygenated haemoglobin and superoxide anions; and is excreted into urine

(Moncada and Higgs, 1993; Wennmalm et al., 1992). The synthesis of NO from L-arginine involves cofactors such as NADPH, tetrahydrobiopterin, oxygen and calmodulin (Bredt and Snyder, 1990; Moncada and Higgs, 1995).

Three NO synthase isoforms have been identified: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms (Forstermann et al., 1994). They all have roles in the regulation of vascular tone. eNOS is located in cell membrane invaginations called calveolae (Shaul et al., 1996). The activity of eNOS is blocked by caveolin-1 which is found bound to calmodulin (Garcia-Cardena et al., 1997). Stimuli such as shear stress, bradykinin, substance P, acetylcholine and adenosine 5'-triphosphate, trigger the release of calcium from intracellular stores (Furchgott and Vanhoutte, 1989). This promotes calcium binding to calmodulin, which releases caveolin-1 and activates eNOS, thus catalysing the production of NO **(Figure 1.8)**.



**Figure 1.8 Schematic of endothelium derived NO generation.** NO is generated in the endothelial cell via endothelial nitric oxide synthase (eNOS). NO rapidly diffuses into the underlying smooth muscle cell and leads to the generation of cGMP, resulting in relaxation of the smooth muscle cell. NO generated from the endothelium also inhibits platelet adhesion and aggregation, and leucocyte adherence. -----I means inhibition

### 1.14.1 Measurement of endothelial function in humans

Approaches to measure endothelial function in humans were first described in the 1980's. In 1986, Ludmer and colleagues infused intracoronary ACh and measured the coronary artery diameter in humans with CHD (Ludmer et al., 1986). This study included a mixture of patients ranging from normal coronary arteries, mild coronary artery disease to severe coronary artery stenosis. They found that patients with normal coronary arteries demonstrate dose-dependent dilatation following ACh infusion compared to baseline coronary artery diameter. However, in all 8 patients with severe coronary stenoses, there was a dose-dependent

constriction. A similar pattern of vasoconstriction following ACh was also seen in 5 out of 6 patients with mild CHD. Furthermore, all of the coronary arteries demonstrated dilatation following GTN. This study found that the paradoxical vasoconstriction induced by ACh occurs early as well as late in the natural history of coronary atherosclerosis and that the abnormal vascular response to ACh is likely representative of endothelial dysfunction. A further study in 1989 demonstrated dysfunction of the endothelium in patients with coronary atherosclerosis following intracoronary adenosine infusion, which resulted in an increase of blood flow through the coronary arteries (Cox et al., 1989). In patients with atherosclerosis, the increase in flow was impaired compared to individuals with no evidence of atherosclerosis. Further studies have shown that even in some apparently normal coronary arteries, there can be a degree of coronary endothelial dysfunction (Hodgson and Marshall, 1989; Werns et al., 1989).

The main disadvantage of coronary endothelial function measurement is the invasive nature of the study. Hence, this technique has been replaced in clinical trials with brachial artery flow mediated dilatation (FMD) measurement using ultrasonography. This is a cheap and non-invasive measurement that uses an ultrasound machine with computer software to accurately measure endothelial function. Although it is operator dependent, the technique is reproducible as demonstrated in a number of clinical trials (Charakida et al., 2013; Deanfield et al., 2007).

### **1.15 Endothelial Dysfunction**

The healthy vascular endothelium has vasodilator, anti-adhesive, anti-inflammatory and anti-coagulant properties, that are measurable and are induced by a number of endothelial derived mediators, with the most important being NO. Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis (Creager et al., 1990; Steinberg et al., 1997) and is characterised by reduced endothelium-dependent dilator function, increased inflammatory cell and platelet adhesion (Goldsmith et al., 2000), and increased coagulation activity (Bombeli et al., 1997). These changes are thought to tip the balance at a site of dysfunction in favour of vasospasm, thrombosis, and inflammation and may contribute to the transition between “stable” and “unstable” atheroma.

Reduced bioavailability of NO is thought to be a major contributor to the endothelial dysfunction phenomenon. This may be due to a diminished ability of the endothelium to produce NO (due to substrate or co-factor deficiency), or due to a profound imbalance in the relative amounts of oxidants that will scavenge NO (Verma and Anderson, 2002). One potential trigger for this endothelial dysfunction is inflammation (Libby et al., 1997; Mamode et al., 1995; Meier et al., 1998; Quartin et al., 1997; Ross, 1999a; Thom et al., 1992). Moreover, inflammatory cytokines have been shown to impair endothelial function in animal models (Ohkawa et al., 1995) and isolated human veins (Bhagat and Vallance, 1997).

Experimentally, endothelial dysfunction is often identified through measurement of endothelium dependant relaxation of blood vessels. The mechanisms by which

inflammation may impair endothelium-dependent relaxation are not fully understood. One possibility is that certain cytokines induce de novo expression of the inducible isoform of nitric oxide synthase (iNOS) in the vessel wall (an isoform implicated in the high-output NO production seen in inflammation and sepsis), and this high output of NO, coupled with the generation of superoxide, causes endothelial damage and scavenging of NO (Beckman and Koppenol, 1996). An alternative possibility is that the cytokines cause a decrease in the expression of the constitutive endothelial eNOS that drives endothelial NO generation. Evidence also suggests that differences in key co-factors necessary for NO generation contribute to dysfunction. Oxidation of BH<sub>4</sub> resulting in increased BH<sub>2</sub> and loss of an electron donor for NO generation has been implicated in endothelial dysfunction (Bendall et al., 2014; Maier et al., 2000). However, targeting this phenomenon has proven difficult in CVD (Channon, 2012). Separately, decreases in L-arginine due to increased expression of arginase and the competition with NOS for substrate has been implicated in CVD (Durante et al., 2007).

There has been evidence that has suggested that diet has an important role to play in improving or indeed protecting endothelial function. Nutrients, such as fish oil, antioxidants, L-arginine, folic acid and soy protein have all been shown to cause an improvement in endothelial function that can mediate, at least partially, the cardioprotective effects of these substances. Attention has been focused on dietary patterns in populations with lower prevalence of CVD. There is some evidence suggesting that Mediterranean diet characterised by high consumption of vegetables, fish, olive oil and moderate wine consumption may have a positive effect on endothelial function (Cuevas and Germain, 2004). A very recent review

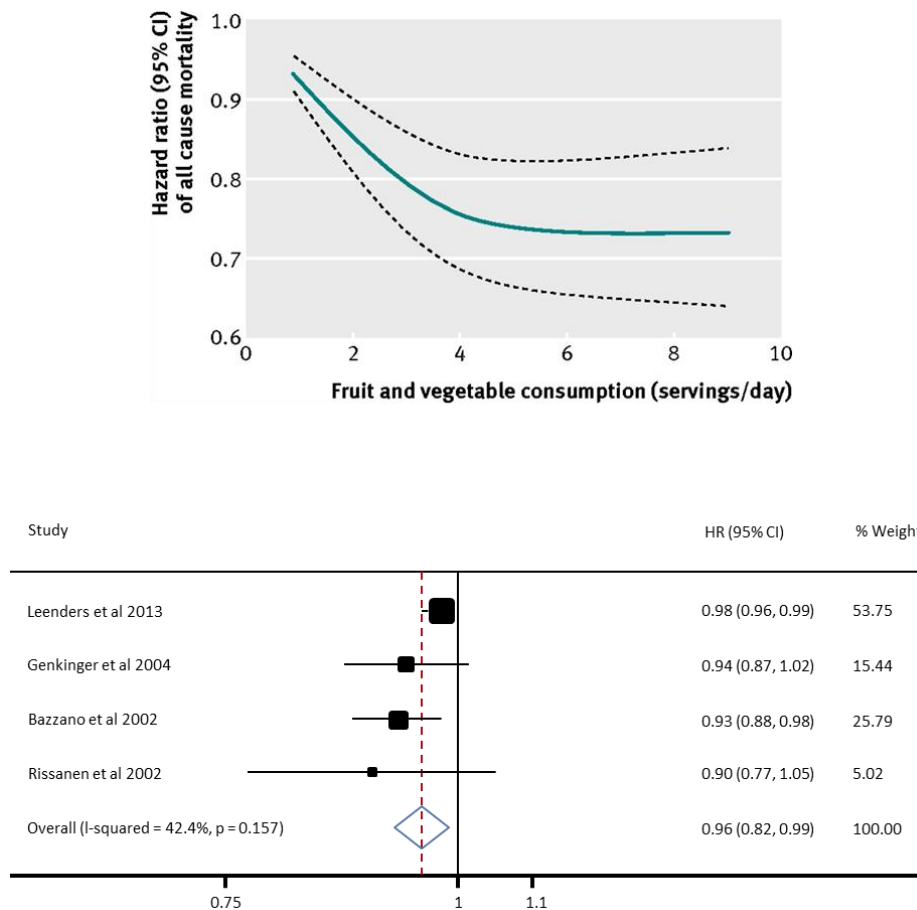
of dietary flavanols has also demonstrated their benefit in improving vascular and endothelial function (Al-Dashti et al., 2018). These results give us evidence on the significant role of diet on endothelial function and its impact on the pathogenesis of atherosclerosis

### **1.16 Diets rich in fruits and vegetables confer beneficial effects against CVD**

The UK initiative of the “5-a-day” campaign, approved by the Department of Health (DOH) and implemented within the National Health Service (NHS), underpins the drive in the UK to encourage increased intake of fruit and vegetables across the general population. This approach has been taken in response to compelling evidence pointing to a reduced incidence of many modern day diseases, namely some cancers and CVD (DOH, 2003) with increased consumption. Similar campaigns have likewise been instigated across Europe and the USA. In the latter, the campaign has focused on promoting a 7-a-day approach through their most recent campaign of ‘half a plate’ (<http://www.fruitsandveggiesmorematters.org>). More recently in a large meta-analysis, which included studies with up to 26 years follow-up, 833,234 participants and with 56,423 deaths, assessing the relationship between mortality and fruit and vegetable consumption, it was confirmed that 5 really is the ‘magic’ number (**Figure 1.9**). Furthermore, a recent meta-analysis involving 95 studies found that we should be consuming at least, up to 10 portions (800g) a day (Aune et al., 2017). However quite surprisingly, the beneficial effects of such diets relate completely to reductions in CVD and not cancer mortality (Wang X, 2014). In addition, a study by Tektonidis et al. assessed the benefit of the Mediterranean diet in a population-based cohort of 32,921 women through a self-administered questionnaire. They found that over the 10



year follow-up, better adherence to a Mediterranean diet was associated with lower risk of MI, heart failure and ischemic stroke (Tektonidis et al., 2015). Finally, a multicentre randomised trial in Spain assessed the Mediterranean diet in 7447 participants between 55 to 80 years of age. The study found that in participants that were at high cardiovascular risk, a Mediterranean diet supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events (Estruch et al., 2013).



**Figure 1.9: Graph and Forest Plot demonstrating the relationship between consumption of fruit and vegetables and all-cause mortality.** (a) Relationship between fruit and vegetable consumption and risk of all-cause mortality. (b) Risk of cardiovascular mortality associated with consumption of fruit and vegetable (servings per day). CI = confidence interval; HR = hazard ratio. Adapted from Wang et al., (Wang et al., 2014).

Diets rich in fruits and vegetables reduce cardiovascular morbidity and mortality (Joshi-pura et al., 1999; Joshi-pura et al., 2001). These beneficial effects have, in part, been attributed to well documented reductions in blood pressure (BP). The Dietary Approaches to Stop Hypertension (DASH) study (Appel et al., 1997) showed that implementation of 7 portions of fruit and vegetables a day more than a control diet (average American diet at that time) for 8 weeks resulted in a small, but significant and persistent, decrease of 2.8 mmHg in systolic BP (SBP) and 1.1 mmHg in diastolic BP (DBP) in pre-hypertensive individuals. These observations have translated into a real world primary care environment. Delivery of dietary advice of a 5-a-day fruit and vegetable intake at the start and mid-way in a 6 month intervention trial was associated with a 4/1.5 mmHg decrease in BP (John et al., 2002). Whilst such BP decreases may seem trivial, at a population level such reductions are likely to have a marked effect on cardiovascular mortality. It has been estimated that a 1 mmHg increase in SBP increases cerebrovascular incident mortality by 2% and a 1 mmHg increase in DBP increases stroke mortality by 3% (Palmer et al., 1992). In addition, a large scale-meta-analysis of 354 studies totalling 56,000 patients has demonstrated that a decrease in SBP of 5 mmHg results in a decrease in incidence of stroke of 34% in addition to a 21% reduction in incidence of acute MI (AMI) (Law et al., 2003). Similarly, in a meta-analysis of 1 million adults in 61 prospective studies a 2 mmHg increase in SBP is associated with 10% increase in stroke and 7% increase in myocardial infarction (Lewington et al., 2002). Thus, it is possible that the reduction in BP caused by fruit and vegetable rich diets accounts for the associated reported decreases in CHD and stroke. In the Health Professionals and Nurses' Health study cohorts, risk of ischaemic stroke was significantly reduced in individuals consuming 5 portions of

fruit and vegetables a day (Joshiyura et al., 1999) as well as reducing risk of CHD (Joshiyura et al., 2001). In addition, there is limited data to separate the effects of diet from changes in behaviour and weight (Foster-Schubert et al., 2012) and no large-scale clinical trial has been able to delineate this difference.

Despite substantial research efforts focusing on identifying the exact mediator of the beneficial effects of fruits and vegetables on cardiovascular health, the issue still remains unresolved. The belief that anti-oxidant vitamins may be accountable for these effects has lost favour with several large-scale clinical trials of different antioxidant vitamins failing to reproduce the effects of a diet rich in fruits and vegetables; an observation reinforced by recent meta-analyses (Bjelakovic et al., 2007; 2012). However, fruits and vegetables are also rich in a number of minerals including potassium ( $K^+$ ). Indeed,  $K^+$  supplementation exerts beneficial effects upon the cardiovascular system particularly with respect to BP lowering (He et al., 2006; Whelton et al., 1997). Although challenging this view, no significant effect of single  $K^+$  supplementation on BP in meta-analyses (Dickinson et al., 2006), and no effect in studies assessing the effect of  $K^+$  in combination with other dietary components including magnesium and fibre (Al-Solaiman et al., 2010) in hypertensive patients have been found.

Perhaps a clue to the key beneficial elements of healthy diets, was provided by Willett's group who found in The Nurses Health and Health Professionals cohorts that the greatest benefits conferred by a fruit and vegetable rich diet, in preventing complications of atherosclerosis, was provided by green leafy vegetables (Joshiyura et al., 1999; Joshiyura et al., 2001). This observation has been

supported by several further studies, most notably a meta-analysis extending the reach of benefits of fruit and vegetable rich diets from stroke and CHD to metabolic syndrome and type 2 diabetes (Carter et al., 2010; Li et al., 2014). Considering these observations, there has been recent interest in the possibility that inorganic (dietary)  $\text{NO}_3^-$  might underlie the protective effects of a fruit and vegetable rich diet (Kapil et al., 2010b; Lundberg et al., 2006). This proposed benefit is thought to stem from its capacity to elevate levels of NO in the body. Evidence suggests that this process is two step with a sequential reduction of inorganic  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and then  $\text{NO}_2^-$  to NO. This possibility has been proposed since green leafy vegetables are particularly rich in the  $\text{NO}_3^-$  anion. Indeed, all vegetables contain some inorganic  $\text{NO}_3^-$  since it is required for plant growth. Analysis of a range of foodstuffs indicate that the highest levels occur in vegetables such as cabbage, lettuce and rocket (Santamaria, 2006). In addition very high levels are also found in beetroot, with lesser amounts in other root vegetables such as carrots and potatoes (Chan, 2011). Assessment of the  $\text{NO}_3^-$  content of the DASH diet has suggested that it might contain high levels with estimates indicating potentially up to 20 mmol (1240.10 mg) of  $\text{NO}_3^-$  daily (Hord et al., 2009a).

The underlying rationale for a role for  $\text{NO}_3^-$  comes from a growing body of evidence demonstrating clear BP lowering, anti-inflammatory, anti-platelet and anti-hypertrophic effects of dietary  $\text{NO}_3^- / \text{NO}_2^-$  regimes. These observations have been made not only at the pre-clinical level but have been effectively translated in a number of phase 2 studies in patients.

### **1.17 Historical medicinal therapeutic uses of inorganic $\text{NO}_2^-$ and $\text{NO}_3^-$**

In 1880 Reichert and Mitchell published detailed writings on the physiological actions of potassium nitrite ( $\text{KNO}_2$ ) in humans and animals. It was noted that a small dose of approximately 30 mg acutely increased and then decreased BP, with larger doses resulting in pronounced hypotension. Over the following 20 years others expanded on these findings and in the early part of the twentieth century inorganic  $\text{NO}_2^-$  was marketed as a treatment for hypertension and vasospasm (Butler and Feelisch, 2008; Kevil et al., 2011). Inorganic  $\text{NO}_3^-$  also has a significant historical presence in therapeutics. It has been used in traditional Chinese medicine for over a millennium to treat angina. In a manuscript discovered at the Buddhist grotto of Dunhuang the patient is advised to take  $\text{KNO}_3$  under the tongue, then swallow the saliva (Butler and Feelisch, 2008). The advice to swallow the saliva is of particular importance as it is now known that the bioactivation of  $\text{NO}_3^-$  requires bacterial  $\text{NO}_3^-$  reductases which reside in the oral cavity (Duncan et al., 1995; Ishiwata et al., 1975; Lundberg et al., 2004). Western physicians did not realise the therapeutic worth of inorganic  $\text{NO}_3^-$  noted by the Chinese for many years, despite the fact that in 1932 Edward Stieglitz demonstrated that oral ingestion of bismuth subnitrate induced hypotension. Moreover, Stieglitz proposed that the bacteria *B. coli* (known today as *E. Coli*) found in the intestine “breaks down bismuth subnitrate to cause liberation of considerable amounts of  $\text{NO}_2^-$ ” (Stieglitz, 1936).

Irrespective of these promising early findings, the widely accepted view of  $\text{NO}_3^-$  has been that of a chemically inert product of the primary pathway of endogenous NO metabolism (Doyle and Hoekstra, 1981), that does not take part per se in any

important chemical reactions (Ignarro et al., 1993b). This is in contrast to  $\text{NO}_2^-$ . Of note the reaction of  $\text{NO}_2^-$  with oxyhaemoglobin to form methaemoglobin and  $\text{NO}_3^-$  was described by Gamgee in 1868 (Gamgee, 1868) and several others since (Doyle et al., 1981; Kosaka et al., 1979; Marshall, 1945). This reaction remains of interest with respect to organic and inorganic  $\text{NO}_2^-$ -induced methaemoglobinaemia, of which there have been a few case reports, limiting the use of drugs such as amyln  $\text{NO}_2^-$  clinically (Modarai et al., 2002; Pierce and Nielsen, 1989). However, major concerns were raised following evidence of apparent  $\text{NO}_3^-$ -induced infantile methaemoglobinaemia. In 1945 Comly reported several cases of infantile methaemoglobinaemia linked to well water contaminated with high levels of  $\text{NO}_3^-$  (Comly H.H., 1945). Further mechanistic interrogation has, however, challenged this view suggesting that it is the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  by contaminating bacteria in the water, either through environmental contamination or due to endogenous infection, that results in very high levels of  $\text{NO}_2^-$ -dependent methaemoglobin formation (Avery, 1999).

The vasodilatory properties of pharmacological doses of exogenous  $\text{NO}_2^-$  (Furchgott, 1955) have also long been known, however, it wasn't until 2001 that it was appreciated that low  $\mu\text{M}$  physiological levels of  $\text{NO}_2^-$  could relax contracted rat aorta under acidic pH (6.6) conditions (Modin et al., 2001). Subsequently, it was shown that an infusion of sodium nitrite ( $\text{NaNO}_2$ ) at doses resembling physiological concentrations (400 nmol/min for 15 min) into the human forearm resulted in vasodilatation with associated increased blood flow which was further increased with exercise (Cosby et al., 2003). This effect of  $\text{NO}_2^-$  was attributed to its chemical reduction to NO within the circulation.

The above observations have collectively heralded a renaissance in assessment of the therapeutic potential of both anions in therapeutics. Several recent clinical trials assessing the utility of both  $\text{NO}_3^-$  and  $\text{NO}_2^-$  have reported, generating substantial optimism that strategies exploiting appropriate delivery of these anions may provide important clinical benefits, through the delivery and restoring of NO levels with the circulation.

### **1.18 Levels of dietary $\text{NO}_3^-$ and $\text{NO}_2^-$ intake**

Daily  $\text{NO}_3^-$  intake in European countries is estimated to range between 0.8-2.2 mmol (50-140 mg) and approximately 80% of human  $\text{NO}_3^-$  intake is derived from eating vegetables with a minor contribution from drinking water, animal products and grain (AICR, 2007; Efsa, 2008). The acceptable recommended daily intake of  $\text{NO}_3^-$ , set by the EU, is 3.7 mg/kg/day, equivalent to ~4.2 mmol (260.42 mg) per day in a 70 kg person (Fao/Who, 2003). Maximum levels for  $\text{NO}_3^-$  in vegetables were first set in the EU in 1997 by Commission Regulation (EC) No 194/97. These regulations have been modified several times in recent years but regulated maximum levels remain. In addition, water supplies in the EU are also regulated with maximum levels set at 50 mg/L  $\text{NO}_3^-$  equivalent to 0.8 mM. These restrictions have been imposed largely due to concerns over methaemoglobinaemia and carcinogenesis (this is described in more detail in sections 2.20.1 and 2.20.2).

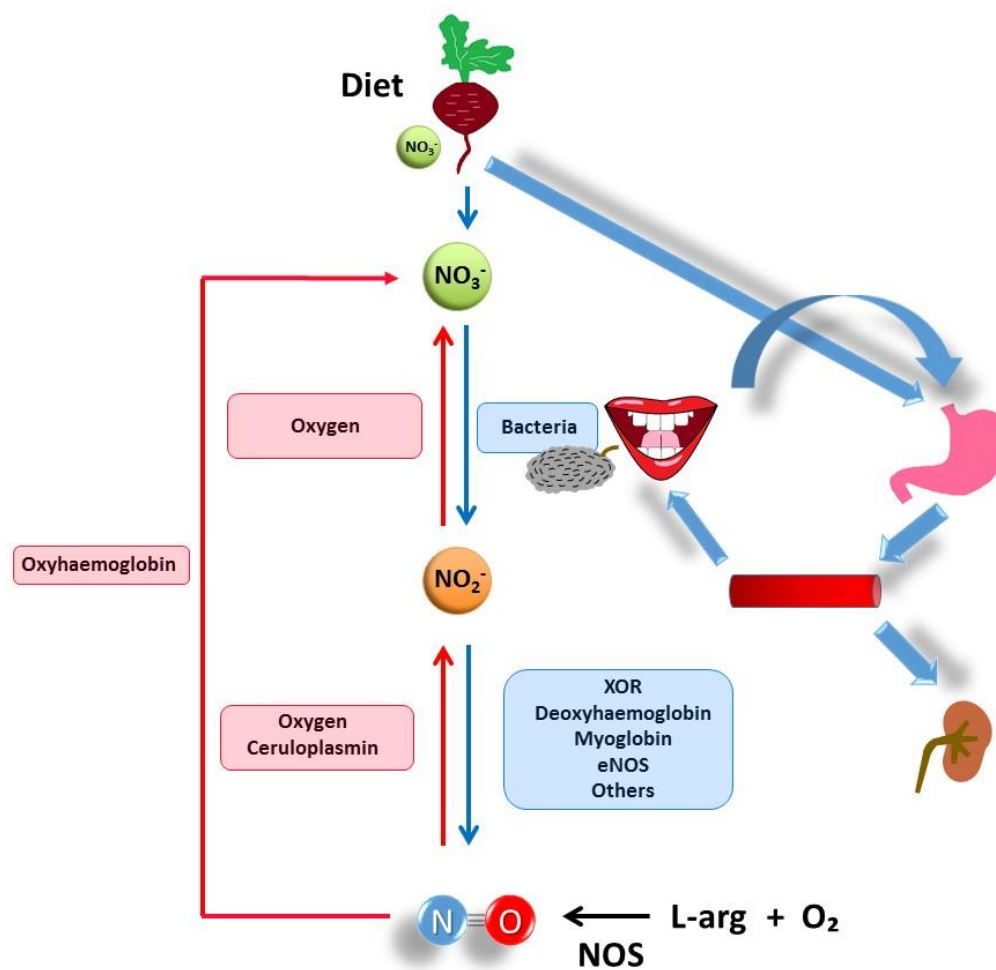
In contrast to dietary  $\text{NO}_3^-$ , dietary  $\text{NO}_2^-$  intake is not as substantial and can be attributed to cured meat, cereal and vegetable intake with drinking water contributing a negligible amount. Estimates of daily  $\text{NO}_2^-$  intake approximate to

0.3-0.9 mg/day in the Western world equating to 6-20  $\mu\text{mol NO}_2^-$  ingested daily (Schuddeboom, 1993).

### **1.19 An alternative pathway of NO generation: the enterosalivary circuit of $\text{NO}_3^-$**

*In vivo*,  $\text{NO}_3^-$  is derived from either the oxidative metabolism of endogenously generated NO from the conventional L-arginine-NOS pathway (**Figure 1.10**), or from dietary ingestion of  $\text{NO}_3^-$ , as mentioned above.  $\text{NO}_3^-$  concentrations in the plasma of healthy individuals have been reported to range between 20-40  $\mu\text{M}$  (Gladwin et al., 2000; Lundberg and Govoni, 2004; Webb et al., 2008) and are a reflection of the levels derived from both sources. Orally ingested  $\text{NO}_3^-$  is rapidly taken up into the circulation from within the gastrointestinal (GI) tract with almost 100% bioavailability, due to the bypassing of first-pass metabolism (van Velzen et al., 2008). Studies in healthy volunteers have shown that plasma  $\text{NO}_3^-$  levels rise within 15 minutes of oral ingestion with levels peaking within an hour (Lundberg and Govoni, 2004; McKnight et al., 1997; van Velzen et al., 2008; Webb et al., 2008). Following ingestion of spinach, beetroot or lettuce, all of which are rich sources of  $\text{NO}_3^-$ , the effective plasma  $t_{1/2}$  of  $\text{NO}_3^-$  has been calculated to be 5.7-6.7 hours (van Velzen et al., 2008). The majority of plasma  $\text{NO}_3^-$  is excreted in the urine (60-75%) with peak excretion occurring around 6 hours following supplementation (Pannala et al., 2003), with relatively small amounts excreted through perspiration and excreted in faeces (Bartholomew and Hill, 1984; Wagner et al., 1983).





**Figure 1.10: Schematic of the pathways for endogenous NO generation: The L-Arginine:NO synthase pathway, the  $\text{NO}_3^-$ -  $\text{NO}_2^-$  pathway of NO generation and the enterosalivary circulation.** In health circulating  $\text{NO}_3^-$  and  $\text{NO}_2^-$  levels are derived from two distinct sources. These are from the diet through a sequential *in vivo* process of chemical reduction and secondly from the oxidation of NO generated from the constitutively active conventional pathway involving L-arginine and NO synthase (NOS).

However,  $\text{NO}_3^-$  is also extracted from the circulation by the salivary glands and then secreted into the saliva resulting in approximately 10-fold greater  $\text{NO}_3^-$  levels than in plasma (Spiegelhalter et al., 1976). Salivary  $\text{NO}_3^-$  levels also increase

within 15 min following oral  $\text{NO}_3^-$  ingestion (McKnight et al., 1997; Webb et al., 2008) with approximately one quarter of any ingested  $\text{NO}_3^-$  thought to be concentrated within the salivary glands (Spiegelhalder et al., 1976; Tannenbaum et al., 1976). The pathway for this  $\text{NO}_3^-$  extraction from the circulation has been elucidated. Qin and co-workers identified the sialin (sialic acid) transporter through  $2\text{NO}_3^-/\text{H}^+$  exchange (Qin et al., 2012) as responsible for  $\text{NO}_3^-$  uptake using human salivary gland cell lines. These findings displace the previous school of thought that  $\text{NO}_3^-$  uptake was primarily facilitated by means of competitive inhibition of the anionic iodide transporter in the salivary glands (Edwards et al., 1954).

### **1.20 $\text{NO}_3^-$ reduction to $\text{NO}_2^-$**

Although, at the time, the enterosalivary circuit (**Figure 1.10**) was thought to be physiologically redundant it was noted that increases in salivary  $\text{NO}_3^-$  concentration were accompanied by increases in  $\text{NO}_2^-$ : a phenomenon thought to be important in the formation of potentially harmful nitrosamines via  $\text{NO}_2^-$ . Ishiwata revealed an absence of  $\text{NO}_2^-$  in saliva analysed directly from the salivary glands as opposed to saliva in the mouth intimating a conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  taking place within the oral cavity. These findings also suggested that the presence of  $\text{NO}_2^-$  in the saliva was likely the result of  $\text{NO}_3^-$ -reducing bacteria (Ishiwata, 1975). Subsequently, it was discovered that a single use of anti-bacterial mouthwash resulted in significantly reduced salivary  $\text{NO}_2^-$  levels following a dietary  $\text{NO}_3^-$  load in the form of celery juice, when compared to basal levels, providing further compelling evidence of oral bacterial  $\text{NO}_3^-$  reduction (Tannenbaum et al., 1976).

Studies in healthy volunteers confirmed the importance of the oral bacteria intimating that *Veilonella* strains were the most prominent bacteria contributing to oral  $\text{NO}_3^-$  reduction (Doel et al., 2005). Using rat tongue samples  $\text{NO}_3^-$  reduction was localised to the posterior third of the dorsal aspect of the tongue (Duncan et al., 1995), but significant  $\text{NO}_3^-$  reduction has also been shown to occur within dental plaque and dental biofilms (Schreiber et al., 2010). Recently further candidate  $\text{NO}_3^-$ -reducing species, in addition to *Veilonella*, have been identified in healthy volunteers (Hyde et al., 2014a). In this study 7 bacterial species never previously implicated in  $\text{NO}_3^-$  reduction were identified, including *Granulicatella adiacens*, *Haemophilus parainfluenzae*, *Actinomyces odontolyticus*, *Actinomyces viscosus*, *Actinomyces oris*, *Prevotella melaninogenica* and *Prevotella salivae*. Studies exploring oral  $\text{NO}_3^-$ -reducing potential in rodents have demonstrated some similarity with humans with respect to the relevant  $\text{NO}_3^-$  reducing bacterial strains within the oral cavity, including *Haemophilus parainfluenzae* (Hyde et al., 2014a; Hyde et al., 2014b). These observations suggest that the rat may provide a suitable laboratory model to assess the impact of dietary  $\text{NO}_3^-$  on the oral microbiome.

Some investigations have also been conducted in pigs. Feeding of inorganic  $\text{NO}_3^-$  to healthy pigs changed the diversity of the oral microbiota (Trevisi et al., 2011), with decreases in the Shannon Index (a measure of both the number of different types of bacteria and the relative abundances). This change was associated with acute increases in oral  $\text{NO}_3^-$  to  $\text{NO}_2^-$  conversion followed by decreases with high doses of  $\text{NO}_3^-$ , suggesting concentration-dependent changes in the oral microbiome (although exact identification of which bacteria were altered was not

determined). The authors suggested that the results indicate that persistent (2 week) dietary  $\text{NO}_3^-$  provision either reduces the richness of the community or causes numbers of certain species to rise, thus reducing evenness, and resulting in changes in  $\text{NO}_3^-$  processing. Interestingly in contrast, no change in the Shannon Index was observed with  $\text{NO}_3^-$  feeding in rats (Hyde et al., 2014b).

### **1.21 $\text{NO}_2^-$ reduction to NO**

Two independent groups, the Benjamin group in the UK and Lundberg & Weitzberg in Sweden, demonstrated concurrently in 1994 that following a dietary  $\text{NO}_3^-$  load NO levels in the stomach increased. In both studies it was demonstrated that following ingestion of  $\text{NO}_3^-$ , a rise in salivary  $\text{NO}_3^-$  associated with a concomitant rise in salivary  $\text{NO}_2^-$  occurred. This  $\text{NO}_2^-$  upon swallowing entered the acidic environment of the stomach where it became protonated to release free NO. This free NO was measured in air expelled from the gut using an ozone-based chemiluminescence analyser (Benjamin et al., 1994; Benjamin and Vallance, 1994; Lundberg et al., 1994). Allied *In vitro* studies demonstrated that increasing concentrations of  $\text{NO}_2^-$  caused anti-microbial activity against a range of gut and oral pathogens that was concentration and pH-dependent (i.e. the more acidic the greater NO generation including assessment at pH 2 to mimic gastric acid) (Dykhuisen et al., 1998; Xia et al., 2006). This activity has also recently been extended to clostridium difficile (Cunningham et al., 2014). It has been speculated that the high levels of NO generated under acidic conditions in the stomach, with dietary  $\text{NO}_3^-$  ingestion (Dykhuisen et al., 1998)), provides a mechanism of host defence as a consequence of the bactericidal activity of NO preventing GI infection. The critical importance of acidic pH was demonstrated by the observation that

elevations in NO levels in expelled air following dietary NO<sub>3</sub><sup>-</sup>, were attenuated by 95% in healthy volunteers following pre-treatment with omeprazole, the proton pump inhibitor (Lundberg et al., 1994). Indeed, the increased risk of infection with *Clostridium difficile* in patients on proton pump inhibitors has been attributed to loss of the anti-microbial activity of NO<sub>2</sub><sup>-</sup> due to increasing gastric pH (Cunningham et al., 2014).

However, importantly not all NO<sub>2</sub><sup>-</sup> produced in the oral cavity and swallowed is converted to NO in the gut. At least some of this NO<sub>2</sub><sup>-</sup> is able to overcome passage through the stomach, to enter the systemic circulation. The process of NO<sub>2</sub><sup>-</sup> absorption from the gastric lumen is rapid with a halving of NO<sub>2</sub><sup>-</sup> levels within 10 min (Licht et al., 1986). Curiously, it is not yet fully understood exactly how NO<sub>2</sub><sup>-</sup>, a charged anion, enters the circulation from the gut. It has been postulated that in the acidic gastric lumen passive movement of nitrous acid (HNO<sub>2</sub>) into the neutral circulation may underlie the apparent transport of NO<sub>2</sub><sup>-</sup> across the gastric wall (Webb and Ahluwalia, 2010), mirroring NO<sub>2</sub><sup>-</sup> transport across the erythrocyte membrane (Samouilov et al., 2007). Furthermore, there is now evidence to suggest that nitrosothiol formation occurs in the stomach and by lowering the gastric pH this might reduce the absorption NO<sub>2</sub><sup>-</sup> (Montenegro et al., 2017; Pinheiro et al., 2015). Similarly, except in the case of the salivary gland, uncertainty remains with respect to the pathways resulting in uptake of both anions into tissues throughout the body. In 2005 Bryan *et al.* demonstrated that within minutes of intravenous injection of NO<sub>2</sub><sup>-</sup> in rats, measurable increases in levels of the anion could be found in tissues throughout the body (Bryan et al., 2005). Similarly following ingestion of dietary NO<sub>3</sub><sup>-</sup>, elevations in tissue NO<sub>2</sub><sup>-</sup>

concentrations occur, but interestingly the magnitude of change is very tissue dependent with plasma levels tending to be the lowest *in vivo* and some of the highest levels found in blood vessels and the heart (Bryan et al., 2005) but also the erythrocyte. Indeed, the erythrocyte has been proposed to be the likely carrier of circulating  $\text{NO}_2^-$  (Dejam et al., 2005). Despite all of the uncertainties pertaining to the mechanism behind  $\text{NO}_3^-$  and  $\text{NO}_2^-$  transport from the gut into the circulation and from the circulation into the tissues, it is certain that as a consequence of ingestion and entero-salivary processing of inorganic  $\text{NO}_3^-$ , plasma  $\text{NO}_2^-$  levels rise steadily and peak within the circulation within 2.5-3 hours (Lundberg and Govoni, 2004; Webb et al., 2008) but perhaps more importantly that ingestion of dietary  $\text{NO}_3^-$  is an effective method to deliver  $\text{NO}_2^-$  *in vivo*.

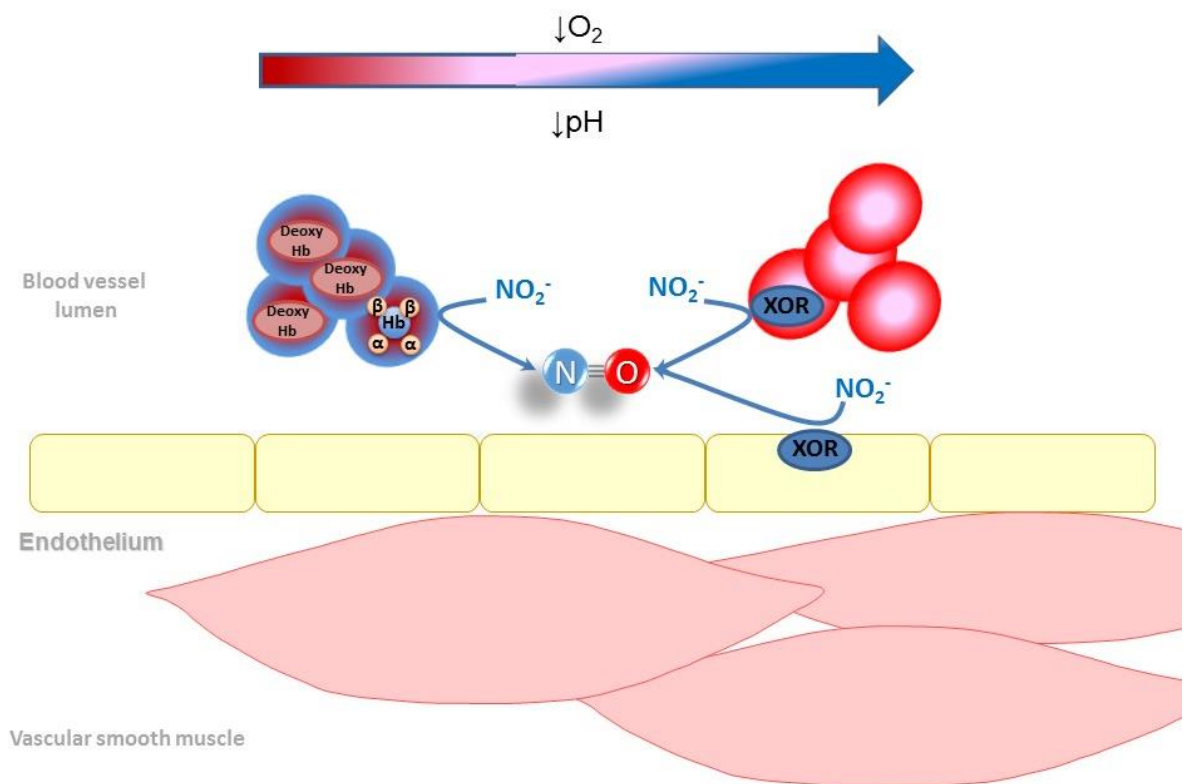
It is important to appreciate that whatever the mechanisms for uptake of  $\text{NO}_2^-$  into the circulation from the gut, the amount of the anion or nitrous acid available for uptake will also depend upon the extent of reactions of these with other substances found in the gut at the same time, and the pH of the stomach. Nitrous acid in the stomach will decompose to dinitrogen trioxide ( $\text{N}_2\text{O}_3$ ) and  $\text{H}_2\text{O}$ , but the  $\text{N}_2\text{O}_3$  is highly susceptible to hydrolysis generating nitrous acid. If, however, there are nitrosatable substrates in high concentration then it is possible that the  $\text{N}_2\text{O}_3$  will react with these substrates rather than undergo hydrolysis. In addition, the extent of nitrosation increases sharply in acidic environments with increasing  $\text{NO}_2^-$  concentrations. Both of these phenomena would result in less potential nitrous acid available to cross the gut (Habermeyer et al., 2015; Heinrich et al., 2013). The possibility that N-nitroso compounds might be formed, as a result of this nitrosation, is of potential concern due to associations with carcinogenicity

(described in more detail in sections 2.20.1 and 2.20.2), but in contrast in the presence of thiols an increase in the levels of nitrosothiols would likely occur; generating a group of compounds often associated with positive effects on physiology (Heinrich et al., 2013). Indeed several groups have recently postulated this theory (Pinheiro et al., 2015). Furthermore, we have recently demonstrated that following a 6 week ingestion of dietary  $\text{NO}_3^-$ , there was only a small increase in total N-nitroso compounds in the urine (Velmurugan et al., 2016), levels that are not associated with significant toxicity concerns.

The first demonstration that  $\text{NO}_2^-$  could be reduced to NO within the cardiovascular system came from Jay Zweier and colleagues in 1995. This group demonstrated, in the ischaemic Langendorff rat heart preparation, that despite NOS inhibition significant NO production continued to occur; implying the existence of a NOS-independent pathway for NO production. Following a series of iterative experiments Zweier demonstrated that this NO generation was attributed to the simple mechanism of disproportionation of stored tissue  $\text{NO}_2^-$ , triggered by the local acidosis (pH 5.5) that developed within the myocardium during ischaemia (Zweier et al., 1995). These observations were the first demonstrating  $\text{NO}_2^-$  reduction to NO within the cardiovascular system and heralded the birth of a now highly active area of research focussing on utilising the reductive potential of this anion as a method of NO delivery in CVD scenarios.

Since the above seminal studies it is now appreciated that in addition to simple acidic disproportionation (Zweier et al., 1995) a number of mammalian biochemical pathways exist that facilitate  $\text{NO}_2^-$  reduction (Shiva et al., 2010;

Sparacino-Watkins et al., 2014; van Faassen et al., 2009; Webb and Ahluwalia, 2010). Amongst others, deoxyhaemoglobin, myoglobin, eNOS, mitochondrial aldehyde dehydrogenase and xanthine oxidoreductase (XOR) (**Figure 1.11**) have all been identified as functional  $\text{NO}_2^-$  reductases (van Faassen et al., 2009); the role of each being dependent upon the environmental conditions, particularly pH and level of oxygenation. Of all of these pathways, currently there is a growing perception that in acidotic, hypoxic conditions and in CVD pathology, XOR predominates as the  $\text{NO}_2^-$  reductase, whilst the globins are more likely to dominate in more physiological conditions (Gladwin et al., 2005).



**Figure 1.11: Schematic of deoxyHb and XOR-facilitated reduction. XOR is expressed on both erythrocytes and the endothelium.** Under ischaemic conditions XOR is also able to reduce plasma  $\text{NO}_2^-$  to  $\text{NO}$ , this occurs in part due to the increased activity of XOR under reduced oxygen and acidotic conditions. XOR - Xanthine oxidoreductase.



## **1.22 Clinical potential of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> therapy**

### **1.22.1 NO<sub>2</sub><sup>-</sup> / NO<sub>3</sub><sup>-</sup> and blood pressure (BP)**

Whilst the BP-lowering activity of NO<sub>3</sub><sup>-</sup> has been appreciated for some time (Stieglitz, 1936); a recent renaissance of the field was ushered in by the study of Larsen *et al.* published in 2006. In this study the effect of a 3-day supplementation, with either NaNO<sub>3</sub> (at a dose of 0.1 mmol/kg/day; 8.50 mg/kg/day) or placebo (sodium chloride, at a dose of 0.1 mmol/kg/day; 8.50 mg/kg/day), on BP. was examined in 17 physically active healthy volunteers. The authors reported a statistically significant reduction in DBP of 3.7 mmHg in the NaNO<sub>3</sub> group compared to the placebo, that was associated with higher plasma NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> levels (Larsen *et al.*, 2006).

Soon after Webb *et al.* demonstrated, in healthy volunteers, that a single dose of 22.5 mmol (1395.11 mg) of a dietary source of NO<sub>3</sub><sup>-</sup> (500 mL of beetroot juice) reduced SBP by 10.4 mmHg and DBP by 8 mmHg; an effect peaking at 2.5-3 hours following ingestion. These changes in BP were correlated directly to plasma NO<sub>2</sub><sup>-</sup> but not plasma NO<sub>3</sub><sup>-</sup> levels, intimating that the effector of the BP-lowering was NO<sub>2</sub><sup>-</sup> rather than NO<sub>3</sub><sup>-</sup> (Webb *et al.*, 2008). Further studies have demonstrated that these effects of dietary NO<sub>3</sub><sup>-</sup> are dose-dependent and estimate a threshold dose of 4 mmol (248.02 mg) for significant BP-lowering in healthy volunteers (Kapil *et al.*, 2010a). This observation is of particular interest, since in a study in stage 1 hypertensive patients Ghosh *et al.* demonstrated that a dose (3.5 mmol, 217.02 mg) lower than this threshold dose caused substantial lowering of BP (11.2/9.6 mmHg): an effect comparable to any single dose of any current anti-hypertensive vasodilator therapy (Ghosh *et al.*, 2013). The authors hypothesised that these

results suggest an increased potency of  $\text{NO}_3^-$  in hypertensive individuals. Indeed, the study investigated this possibility and discovered that the  $\text{NO}_2^-$  reductase activity, localised to erythrocytes, was enhanced in hypertensive individuals (rats and humans), and that this was associated with an elevated XOR activity; a phenomenon that has been reported previously in hypertension (Laakso et al., 1998; Suzuki et al., 1998).

The acute BP effects of dietary  $\text{NO}_3^-$  have been shown to persist over a longer period. The Japanese diet, which is rich in  $\text{NO}_3^-$  has been studied in a cross-over randomised controlled trial over a 10 day period. This study of 25 healthy volunteers, demonstrated a reduction in DBP of 4.5 mmHg compared to a control group with a low  $\text{NO}_3^-$  control diet (Sobko et al., 2010). Similarly, using beetroot juice to deliver a daily  $\text{NO}_3^-$  dose (500 mL/day containing 5.2 mmol (322.43 mg) of  $\text{NO}_3^-$ ) for 15 days a significant reduction in BP compared to the control group has been demonstrated in healthy volunteers (Vanhatalo et al., 2010). Further recent studies have confirmed these findings (Ashworth et al., 2015; Bondonno et al., 2012; Joris and Mensink, 2013; Liu et al., 2013a).

Finally, in a double-blind, placebo-controlled, randomised clinical trial involving 68 patients with hypertension, daily dietary supplementation of 250 mL of beetroot juice containing approximately 6 mmol (372.03 mg) dietary  $\text{NO}_3^-$  (versus a  $\text{NO}_3^-$ -free beetroot juice placebo) for 4 weeks has been shown to produce sustained BP-lowering. The primary end-point in this study was change in clinic, ambulatory and home BPs compared to the placebo group after 4 weeks of treatment. A significant reduction in clinic BP (7.7/2.4 mmHg), 24 hour

ambulatory BP (7.7/5.2 mmHg) as well as home BP measurements (8.1/3.8 mmHg) was reported (Kapil et al., 2015). However, a recent paper by Blekkenhort and colleagues investigated whether daily consumption of NO<sub>3</sub><sup>-</sup> - rich vegetables (~150 mg nitrate/day) compared to NO<sub>3</sub><sup>-</sup> - poor vegetables (~22 mg NO<sub>3</sub><sup>-</sup>/day), or no increase in vegetables (control; ~6 mg NO<sub>3</sub><sup>-</sup>/d) over 4 weeks in would result in lower BP. They found that infact, increased intake of NO<sub>3</sub><sup>-</sup> - rich vegetables did not lower BP in prehypertensive or untreated grade 1 hypertensive individuals when compared with increased intake of NO<sub>3</sub><sup>-</sup> - poor vegetables and no increase in vegetables (Blekkerhorst et al., 2018).

### **1.22.2 NO<sub>2</sub><sup>-</sup> / NO<sub>3</sub><sup>-</sup> and vascular function**

Vascular dysfunction is thought to play a major role in the progression of CVD including in atherosclerosis (Deanfield et al., 2007). This change occurs at the very earliest stages of CVD development, preceding any evidence of disease (Celermajer et al., 1992) and is associated with most risk factors, including hypercholesterolemia and BP (Kannel et al., 1979; Steinberg et al., 1989; Vanhoutte, 1996). Indeed, measures of vascular function, particularly brachial artery FMD and arterial stiffness reflected through measurement of PWV, have been proposed as important markers of CVD. Evidence suggests that these measures inform not only upon the extent of atherosclerotic disease (Deanfield et al., 2007; Drexler and Zeiher, 1991; Halcox et al., 2009) but also of future risk of cardiovascular events (Celermajer et al., 1992; Yeboah et al., 2009). Wide-scale preventative strategies for CVD, such as statins, show improved FMD and pulse wave velocity (PWV) responses in hypercholesterolemics (Laurent et al., 2006; Masoura et al., 2011; Vlachopoulos et al., 2010).

Mechanistically, vascular dysfunction is characterised by reduced bioavailability of endothelium-derived NO. Dysfunction of the conventional L-arginine/NO synthase pathway and enhanced scavenging of NO underlie this reduced bioavailability in individuals at risk of CVD, including those with hypercholesterolemia (Hermann et al., 2006; Jeserich et al., 1992; Marin and Sessa, 2007). Thus, approaches that might restore this 'lost' NO have obvious therapeutic potential. Indeed, replacement of NO using organic nitrate improves vascular function and vice versa NOS inhibition causes reduced elastic artery distensibility and hence stiffness *in vivo* (Bellien et al., 2010; Schmitt et al., 2005; Wilkinson et al., 2002). However, the long term utility of organic NO<sub>3</sub><sup>-</sup> therapy is limited due to the rapid tolerance that develops and the eventual precipitation per se of vascular dysfunction particularly targeting the endothelium (Munzel et al., 2013). Thus, identification of strategies that deliver sustained NO elevation without tolerance or any associated negative vascular effects has been mooted as a potential useful therapeutic strategy in limiting CVD progression.

Studies of inorganic NO<sub>2</sub><sup>-</sup> supplementation, delivered in the drinking water, in mice for 3 weeks demonstrated a reversal of age-associated large elastic artery stiffness. The authors of this study speculated that this improvement likely related to a reduction in advanced glycation end-products' which have been implicated in arterial stiffness in humans (Fleenor et al., 2012; Sindler et al., 2011). Clinical studies have confirmed these findings demonstrating improvement in measures of vascular stiffness with elevation of circulating NO<sub>2</sub><sup>-</sup> levels following oral NO<sub>3</sub><sup>-</sup> delivery. In healthy volunteers an 8 mmol (496.04 mg) KNO<sub>3</sub> supplement, in the form of a capsule, caused a significant reduction in PWV at 3 hours compared to

matched KCL placebo (Bahra et al., 2012) . Similar findings in healthy volunteers have been reported using dietary sources of  $\text{NO}_3^-$  including spinach (Liu et al., 2013b) but also in patients with hypertension. Indeed, a single dose of dietary  $\text{NO}_3^-$  (3.5 mmol (217.02 mg) in the form of beetroot juice) significantly reduced PWV compared to baseline measurements in stage 1 (drug naïve) hypertensive individuals (Ghosh et al., 2013). A small study in a cohort of elderly volunteers with risk factors for CVD also found an improvement in PWV and augmentation index in the group who received  $\text{NaNO}_3$  versus  $\text{NaCl}$  control (Rammos et al., 2014). More recently, sustained daily intake of dietary  $\text{NO}_3^-$ , in the form of beetroot juice for 4 weeks, effected a persistent improvement in PWV in patients with hypertension. Thus, taken together these studies suggest that dietary  $\text{NO}_3^-$  may have a role in preventative strategies to reduce cardiovascular risk by causing a sustained reduction in arterial stiffness (Kapil et al., 2015).

Interestingly, the effects of  $\text{NO}_2^-$  or  $\text{NO}_3^-$  on FMD show a distinct pattern to that with PWV. A randomised cross-over double-blind study in 14 healthy volunteers demonstrated that, whilst an 8 mmol (496.04 mg) dose of  $\text{KNO}_3$  improved PWV and reduced BP measured 3h following ingestion, no change in the FMD response was evident (Bahra et al., 2012). More recently, Kim and co-workers in a study assessing the increase in flow and brachial artery diameter following hand-grip exercise in healthy volunteers, also demonstrated improved PWV but no effect on endothelial function with dietary  $\text{NO}_3^-$  administration (Kim et al., 2014). These observations contrast with two other published studies. In 2012, Heiss *et al.* demonstrated an increase in FMD in response to dietary  $\text{NO}_3^-$  in healthy volunteers and suggested that this effect was due to an increased mobilisation of

circulating angiogenic cells (Heiss et al., 2012). More recently the same group has shown, in studies delivering  $\text{NO}_3^-$  through spinach consumption, a dose-dependent improvement in FMD responses with doses above  $\sim 4$  mmol (248.02 mg) (Rodriguez-Mateos et al., 2015). Exactly why these differences between the studies occur is uncertain but may relate to the mode and dose of  $\text{NO}_3^-$  delivery. Although arguing against such a reason is the fact that two distinct groups, one using a  $\text{NO}_3^-$  salt and the other a dietary source, identified no effect on endothelial function in healthy volunteers. Moreover, in the studies demonstrating positive effects these were invariably evident with doses of  $\text{NO}_3^-$  that were lower than those used in the studies demonstrating no effect. Further assessments of this discord would be of value.

Interestingly, in healthy volunteers where transient endothelial dysfunction is induced by an ischaemic insult to the forearm, inorganic  $\text{NO}_3^-$  delivered either in the form of beetroot juice or through supplementation with  $\text{KNO}_3$  reversed dysfunction (Kapil et al., 2010a; Webb et al., 2008). In addition, most recently it has been demonstrated that sustained dietary  $\text{NO}_3^-$  ingestion in hypertensives (who characteristically display reduced FMD responses that have been attributed to endothelial dysfunction) results in an improvement in the FMD response; a view supported by a recent meta-analysis (Siervo et al., 2014). The exact mechanism involved in mediating this effect is uncertain. However, there is evidence to suggest that BP-lowering per se will result in improvements in vascular function and in all studies where improvements in vascular function were noted BP decreases were also reported. Further studies by Joris et al (Joris and Mensink, 2013). and Gilchrist et al (Gilchrist et al., 2013). have confirmed the

improvement of FMD following ingestion of dietary  $\text{NO}_3^-$ . Currently, it is unknown whether the improvements in both PWV and FMD are due to reductions in BP or whether other pathways might underlie these effects. More recently there has been some evidence to suggest that the beneficial effects of  $\text{NO}_2^- / \text{NO}_3^-$  in CVD may also relate to improvements in circulating lipid levels and this is discussed more fully below.

### **1.22.3 $\text{NO}_2^- / \text{NO}_3^-$ and lipids**

$\text{NO}_2^-$  has been shown to modify the oxidation of LDL-cholesterol. Atherogenic modifications of LDL by a cell-free macrophage myeloperoxidase/hydrogen peroxide/chloride system were prevented following incubation of LDL with  $\text{NO}_2^-$  (12.5-200  $\mu\text{M}$ ) *in vitro* (Carr and Frei, 2001). More recent research has shown that C57BL6 mice fed a high fat diet but supplemented with  $\text{NO}_2^-$ -containing drinking water for 3 weeks exhibited reduced leukocyte adhesion and arteriolar endothelial dysfunction together with a reduced triglyceride level compared to those fed  $\text{NO}_2^-$ -deplete water (Stokes et al., 2009). Interestingly, in a small study in patients with hypercholesterolemia a significant reduction in triglyceride levels following dietary  $\text{NO}_2^-$  has been reported (Zand et al., 2011). In this study 23 hypercholesterolemic patients were given a commercially available supplement containing a number of different herbal constituents proposed to enhance  $\text{NO}_3^-$  and  $\text{NO}_2^-$  delivery *in vivo*. After 30 days of a twice a day ingestion, triglyceride levels were significantly reduced. The authors speculated that such an effect might be useful in limiting the progression of atherosclerotic disease, highlighting a potential preventative strategy that might be utilised on a population level to control rates of atherosclerosis.

#### 1.22.4 NO<sub>2</sub><sup>-</sup> / NO<sub>3</sub><sup>-</sup> and platelet reactivity

It is widely accepted that NO plays a critical role in regulating platelet function (Gkaliagkousi et al., 2007). In health, this takes the form of a repressive effect limiting platelet activation and adhesion to the blood vessel wall. It is thought that, in CVD, dysfunction of the endothelium and the resulting loss of tonic bioavailable NO results in removal of a critical repressive influence of the endothelium on platelets (Radomski et al., 1987a; 1990). Thus, strategies that restore NO-dependent platelet regulation are of obvious potential. Studies in healthy volunteers have shown a reduction in *ex vivo* platelet aggregation following NO<sub>3</sub><sup>-</sup> ingestion. Ingestion of oral KNO<sub>3</sub> (2 mmol; 124.01 mg) in a small cohort of seven healthy volunteers resulted in inhibition of platelet aggregation assessed *ex vivo* in response to collagen (Richardson et al., 2002). This was followed in 2008 by a study, in healthy volunteers, demonstrating that platelet aggregation in response to both ADP and collagen were inhibited 2.5 hours after consumption of 500 ml of beetroot juice delivering 24 mmol (1488.12 mg) of inorganic NO<sub>3</sub><sup>-</sup> (Webb et al., 2008). In this study the anti-platelet effects of NO<sub>3</sub><sup>-</sup> were abolished when the entero-salivary circuit was interrupted, by asking the volunteers to spit all saliva out following NO<sub>3</sub><sup>-</sup> ingestion, and thus preventing the re-entry of NO<sub>2</sub><sup>-</sup> into the body.

Confirmation that NO<sub>2</sub><sup>-</sup> underlies these anti-platelet effects has been demonstrated. Early studies showed that *ex vivo* incubation of platelet-rich plasma (PRP) with very high supraphysiological concentrations of NO<sub>2</sub><sup>-</sup> (~60 μM) attenuated platelet aggregation in response to ADP, arachidonic acid and collagen (Schafer et al., 1980). However, further studies using concentrations of NO<sub>2</sub><sup>-</sup> better



reflecting the physiological range (1-100  $\mu\text{mol/L}$ ; 46.01-4600.6  $\mu\text{g/L}$ ) have shown no activity of the anion on PRP *in vitro* (Radomski et al., 1987b). However, Srihirun *et al.* have demonstrated that the anti-platelet activity of  $\text{NO}_2^-$  is critically dependent upon its bioactivation by erythrocytes. Their studies demonstrated that with reduced oxygen tension greater activity of  $\text{NO}_2^-$  became apparent as a consequence of its reduction to NO by deoxyhaemoglobin. The authors suggested that  $\text{NO}_2^-$  might have a role in altering platelet activity in the circulation, under hypoxic conditions, but also in the normal transit of blood through the circulation within the reduced oxygen tension present on the venous versus the arterial side of circulation (Akrawinthawong et al., 2014; Srihirun et al., 2012).

A follow-up study by the same group investigated how  $\text{NO}_2^-$  may affect overall clotting processes via modulating platelet function using thrombelastography. In their study, they showed that  $\text{NO}_2^-$ , as well as NO, exhibited inhibitory effects on coagulation processes resulting in increasing reaction time of fibrin formation, decreasing the rate of clot formation and reducing clot strength. These inhibitory effects of  $\text{NO}_2^-$  were greatly enhanced by the deoxygenation of blood, again implicating deoxyhaemoglobin in the reduction of  $\text{NO}_2^-$  to NO under hypoxic conditions (Park et al., 2014). In a separate study in mice, Park *et al.* found that dietary restriction of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  reduced the levels of both anions in whole blood to a greater extent than inhibition of NOS, resulting in alterations of platelet function. This group suggested that dietary  $\text{NO}_2^-$  and  $\text{NO}_3^-$  could play an important role, possibly more important than NO generated by the conventional L-arginine/NOS pathway, in regulating basal haemostasis and thrombosis by affecting platelet reactivity (Park et al., 2013). Interestingly, Emerson and co-

workers group has suggested that  $\text{NO}_3^-$  exerts an antiplatelet effect during eNOS deficiency. Hence, dietary  $\text{NO}_3^-$  may have a role in reducing platelet activity during endothelial dysfunction (Apostoli et al., 2014). Together, these data suggest that provision of dietary  $\text{NO}_3^-$ , might offer an effective strategy providing modest anti-platelet activity that could be effective in preventative strategies.

#### **1.22.5 $\text{NO}_2^-$ / $\text{NO}_3^-$ and intimal hyperplasia and restenosis**

Vascular smooth muscle cell proliferation is believed to underlie the pathogenesis of intimal hyperplasia and restenosis (Ross, 1993; Vogt et al., 2008). Pre-clinical models have shown that the delivery of NO in the form of L-arginine, pharmacological NO donors, or overexpression of NOS can inhibit proliferation of VSMCs, which in turn can reduce the injury responses within the blood vessel wall (Kibbe et al., 2000; Napoli et al., 2001; Napoli et al., 2013). Furthermore, VSMCs transfected with eNOS have demonstrated inhibition of cell proliferation and of key cell cycle regulatory molecules (Sharma et al., 1999). However, this has not been translated successfully in humans with neointimal hyperplasia. The Angioplastic Coronaire Corvasal Diltiazem (ACCORD) study found that patients undergoing PCI who received NO from intravenous linsidomine, followed by oral molsidomine for 6 months, had a reduction in angiographic luminal diameter of 10% (Lablanche et al., 1997). Conversely, another study found that patients receiving high-dose oral molsidomine for 6 months after PCI did not show any effect on restenosis rates (Wohrle et al., 2003). Recently it has been demonstrated that low dose of  $\text{NaNO}_2$  given prior to balloon injury *in vivo* prevents vascular intimal hyperplasia in both rat and mouse injury models (Alef et al., 2011). Moreover, there is also evidence to suggest that  $\text{NO}_2^-$  delivered after the

establishment of intimal hyperplasia could reverse the pathological process (Alef et al., 2011). This study demonstrated that  $\text{NaNO}_2$  was metabolised to biologically active NO by XOR and limited the proliferation of VSMC via a  $\text{p21}^{\text{Waf1/Cip1}}$  dependent-mechanism. However, a study by Vavra et al. found that neither  $\text{NO}_2^-$  or  $\text{NO}_3^-$  inhibited rat aortic VSMC proliferation *in vitro* (Vavra et al., 2011), which would suggest that  $\text{NO}_2^-$  is being reduced by a cell other than VSMC. Both these studies did suggest a benefit of  $\text{NaNO}_2$  in the treatment of restenosis *in vivo* (Alef et al., 2011; Vavra et al., 2011).

#### **1.22.6 $\text{NO}_2^-$ / $\text{NO}_3^-$ and ischaemia-reperfusion (IR) Injury**

There are a number of pre-clinical studies demonstrating beneficial effects of  $\text{NO}_2^-$  supplementation against the damaging effect of ischaemia-reperfusion (IR) injury, including improvement of left ventricular function and reduction in infarct size in the heart (Duranski et al., 2005; Gonzalez et al., 2008; Webb et al., 2004a). In the isolated Langendorff rat heart preparation, perfusion of hearts with 10 and 100  $\mu\text{M}$   $\text{NO}_2^-$ , before and throughout an IR insult, reduced infarct size by more than 60% with matching improvements in left ventricular function (Webb et al., 2004b). These findings were followed by translation into *in vivo* models of myocardial IR injury demonstrating beneficial effects of the anion administered locally in the heart in mice (Duranski et al., 2005), with similar outcomes following parenteral administration of  $\text{NO}_2^-$  in a canine model of IR injury (Gonzalez et al., 2008). Mechanistic studies have suggested that the processing of  $\text{NO}_2^-$  within the ischaemic myocardium is likely to involve both XOR (Webb et al., 2004b) and deoxymyoglobin (Hendgen-Cotta et al., 2008; Shiva et al., 2007a; Shiva et al., 2007b). Furthermore, Bryan *et al.* have shown that the effects with parenteral  $\text{NO}_2^-$

administration can be recapitulated through dietary supplementation with  $\text{NO}_2^-$  or  $\text{NO}_3^-$  for 7 days (Bryan et al., 2007).

Most interestingly these studies have now been translated into the clinical setting. Two separate phase 2 clinical trials of intravenous and intra-coronary  $\text{NO}_2^-$  have been reported (Jones et al., 2014; Siddiqi et al., 2014). Siddiqi *et al.* conducted a multi-centre randomised, double-blind, placebo controlled trial (NIAMI) where 229 patients with AMI received a continuous infusion of  $\text{NO}_2^-$  for 5 minutes prior to balloon inflation, delivering a total dose of 70  $\mu\text{mol}$  of  $\text{NaNO}_2$  or  $\text{NaCl}$  placebo (Siddiqi et al., 2014). In this study an important inclusion criteria for all patients was that the culprit vessel was occluded at the time of infusion i.e. the thrombolysis in myocardial infarction (TIMI) score was either 0 (complete block) or 1 (minimal flow). The primary endpoint in this study was the difference in infarct size (expressed as a percentage of LV myocardial mass) between the active and placebo groups at 6–8 days post-infarct assessed on cardiac magnetic resonance (CMR) (Siddiqi et al., 2014). Disappointingly, no significant difference in infarct size between the two groups was evident. However, Jones et al. conducted a randomised double-blind study of intra-coronary  $\text{NO}_2^-$  during AMI (NITRITE-AMI) where 80 patients were randomised to receive a single bolus of intra-coronary 1.8  $\mu\text{mol}$   $\text{NaNO}_2$  (124.19  $\mu\text{g}$ ) or  $\text{NaCl}$  (105.19  $\mu\text{g}$ ) placebo (Jones et al., 2014). The study did not demonstrate a reduction in infarct size (assessed by measurements of the primary outcome-creatinine kinase). However, in a subgroup analysis of patients where only patients with TIMI  $\leq 1$  flow (i.e. patients with no or little flow beyond the blocked vessel) were included, there was a significant reduction in myocardial infarct size assessed by creatinine kinase between the

NO<sub>2</sub><sup>-</sup> and control group. Furthermore, there was a statistically significant decrease in CMR-determined myocardial infarct size in the NO<sub>2</sub><sup>-</sup>-treated patients (Jones et al., 2014). Furthermore, a second manuscript from this study demonstrated reductions in the systemic inflammatory response post PCI, which were associated with nitrite treatment. They also found that in NO<sub>2</sub><sup>-</sup>-treated patients, there was a sustained long-term reduction in MACE in the nitrite group compared with the placebo group (Jones et al., 2017). Whether one might be able to use a dietary approach to recapitulate these effects is uncertain, but may prove difficult. A likely explanation for the difference between the two studies described above relates to the concentration of NO<sub>2</sub><sup>-</sup> achieved within the culprit artery prior to reperfusion. With intra-coronary administration, it was estimated that local concentrations of between 3-10 µmol/L were achieved in the patients, although direct measurement of this was not provided. In the Siddiqui study circulating levels achieved were 1.42 µmol/L. This concentration was measured 5 minutes following ceasing of anion infusion and so may not accurately reflect the peak levels. However, previous studies in pre-clinical models *in vivo* suggest that local concentrations less than 3 µmol/L of NO<sub>2</sub><sup>-</sup> are unlikely to produce substantial reduction in infarct size. In general, dietary NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> interventions achieve rises in circulating levels of NO<sub>2</sub><sup>-</sup> somewhere in the region of 0.2 to at the very most 1 µmol/L, and thus it seems unlikely that a diet-based intervention will impact sufficiently on infarct size in the AMI setting to result in measurable benefits.

## **1.23 Concerns regarding NO<sub>2</sub><sup>-</sup> / NO<sub>3</sub><sup>-</sup> therapy**

### **1.23.1 NO<sub>2</sub><sup>-</sup> / NO<sub>3</sub><sup>-</sup> and methaemoglobinaemia**

After ingestion of NO<sub>3</sub><sup>-</sup> and its subsequent reduction to NO<sub>2</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup> will bind to haemoglobin resulting in the oxidation of the ferrous iron (Fe<sup>2+</sup>) in haemoglobin to the ferric (Fe<sup>3+</sup>) state. This consequently forms methaemoglobin (Doyle et al., 1981). In this state haemoglobin cannot bind oxygen and requires methaemoglobin reductases (cytochrome b<sub>5</sub> reductase) to restore haemoglobin back to its oxygen-binding ferrous state. Methaemoglobinaemia results in insufficient oxygen-carrying capacity of the blood, and when levels exceed 5–10% clinical symptoms ensue such as cyanosis, shortness of breath, irritability and altered mental status (Greer and Shannon, 2005).

Early observational data reported in 1945 by Comly indicated that methaemoglobinaemia in infants resulted as a consequence of milk formula being made using well water with high NO<sub>3</sub><sup>-</sup> levels (Comly H.H., 1945). Shortly after, Walton in 1951 found that there were no reported cases of methaemoglobinaemia at drinking-water NO<sub>3</sub><sup>-</sup> levels <10 mg/L (Walton, 1951). Hence, currently the maximum limit of 50 mg/L (which is equivalent to < 1 mmol/L) is implemented in the UK, Europe and USA provincial water supplies. The increased susceptibility of infants to methaemoglobinaemia was proposed to be due to the fact that at less than ~6 months of age methaemoglobin reductase pathways are insufficiently developed, allowing high levels of methaemoglobin to persist.

However, the aetiological role of high NO<sub>3</sub><sup>-</sup>-containing water in the methaemoglobinaemia evident in young children has been challenged. The

original descriptions of 'blue-baby' syndrome made by Comly were in infants who also had co-existing diarrhoea, probably as a result from enteric infections caused by faecal bacteria contamination in wells (Avery, 1999). Moreover, there are numerous studies demonstrating a strong association of diarrhoea and methaemoglobinaemia in the absence of  $\text{NO}_3^-$ -contaminated water (Avery, 1999). Support for this view comes from a study where infants exposed to 157-170 mg of  $\text{NO}_3^-$  a day did not experience methaemoglobinaemia levels greater than 7.5% (Cornblath and Hartmann, 1948).

In adults, a randomised three-way crossover study in healthy volunteers has demonstrated that relatively high concentrations of  $\text{NO}_2^-$  are required before clinically significant levels of methaemoglobinaemia are achieved. Single doses of  $\text{NaNO}_2$  ranging from 150–380 mg (2.17-5.51 mmol) were administered intravenously (Kortboyer J, 1997) and resulted in methaemoglobin concentrations of 12.2% in volunteers receiving the highest dose of  $\text{NO}_2^-$  and 4.5% for those receiving the lowest dose. In a separate study up to 110mg/kg/min of  $\text{NO}_2^-$  was infused for 5 minutes into the brachial artery of healthy volunteers and achieved methaemoglobin concentrations of only 0.7% to 3.2% in adults (Dejam et al., 2007). In another 18 participant healthy volunteer study where intra-arterial infusion of  $\text{NO}_2^-$  was performed, systemic concentrations of 16  $\mu\text{mol/L}$  of  $\text{NO}_2^-$  were measured, however, methaemoglobin levels were measured at ~1% (Cosby et al., 2003). Finally, a recent study looking at the safety and feasibility of long-term intravenous  $\text{NaNO}_2$  infusion in healthy volunteers used doses up to 534  $\mu\text{g/kg/hour}$  (7.74  $\mu\text{mol/kg/hour}$ ) intravenously for 3 to 9 hours. This resulted in significant reductions of BP (up to 20 mmHg in one participant) but only a

maximum increase of methaemoglobin levels to 5% (Pluta et al., 2011). Together, these studies suggest that extremely high levels of  $\text{NO}_2^-$  are required before clinically significant methaemoglobinaemia occurs, but also that it is unlikely that the ingestion of  $\text{NO}_3^-$  poses any significant methaemoglobinaemia risk. Indeed, daily ingestion of a dietary  $\text{NO}_3^-$  dose of approximately 5 mmol for 4 weeks produced significant beneficial functional effects but was not associated with any evidence of methaemoglobin formation in adults (Kapil V, 2015).

### **1.23.2 $\text{NO}_2^-$ / $\text{NO}_3^-$ and carcinogenesis**

Although  $\text{NO}_3^-$  itself is not thought to be carcinogenic (Speijers and van den Brandt, 2003), the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and then further reaction of  $\text{NO}_2^-$  with secondary amines to form N-nitrosoamine, is thought to result in potential carcinogenesis (Hord et al., 2009b). In addition, the rate of nitrosation of amino acids is proportional to the square of the  $\text{NO}_2^-$  concentration, which would suggest that the production of nitrosation compounds in the stomach would increase suddenly with higher intake of  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . Also, previous studies have demonstrated that the most common natural amino acids are the most efficient precursors of the most powerful alkylating agents (Garcia-Santos Mdel et al., 2001). Hence, the stomach environment could potentially augment the nitrosation of common amino acids and therefore increase the risk of carcinogenesis.

Studies in rats conducted in the 1960s demonstrated that the nitrosamine, dimethylnitrosamine, produced liver carcinoma (Magee and Barnes, 1956; Sakshaug et al., 1965). A number of distinct N-nitrosoamine species have been tested and tumours have been induced in 39 species which belong to 36 genera,



25 families, 17 orders and five class of animals (Bogovski and Bogovski, 1981), indicating that these compounds show no species selectivity in terms of their carcinogenicity. *In vitro* studies have shown that N-nitrosoamines can be formed in humans by incubating gastric juice,  $\text{NO}_2^-$  and secondary amines (Sen et al., 1969). Although *in vivo* studies have demonstrated that dietary ingestion of  $\text{NO}_2^-$  containing foods can form N-nitrosamines (diphenylamine) (Fine et al., 1977), other studies have failed to demonstrate tumour formation in rats despite chronic feeding of  $\text{NO}_2^-$  (Druckrey et al., 1963). In fact, a recent study by Sodring *et al.* found that  $\text{NO}_2^-$  had a suppressive effect on colorectal cancer promotion in the small intestine of mice (Sodring et al., 2015). Moreover, recent evidence from the EPIC study suggests no link of such cancers with endogenous nitrosamine formation and  $\text{NO}_2^-$  ingestion (Loh et al., 2011).

Recent studies have also shown that  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in meats increase the risk of colorectal, bladder and prostate cancer (Cross et al., 2010; Dellavalle et al., 2014; Ferrucci et al., 2010; Sinha et al., 2009). A study by Cross *et al.* suggested an increased risk of colorectal cancer with red [HR, 1.24; 95% CI, 1.09–1.42;  $P < 0.001$ ] and processed meat [HR, 1.16; 95% CI, 1.01–1.32;  $P = 0.017$ ] (Cross et al., 2010). Worryingly, a positive association between the frequency of maternal intake of cured meat and the risk of childhood brain tumours (Dietrich et al., 2005) has also been identified. Ferrucci *et al.* showed modest support for an increased risk of bladder cancer with total dietary  $\text{NO}_2^-$  and  $\text{NO}_3^-$  but the results did not reach statistical significance (Ferrucci et al., 2010). Finally Sinha *et al.* found  $\text{NO}_2^-$  (HR = 1.24, 95% CI: 1.02, 1.51) and  $\text{NO}_3^-$  (HR = 1.31, 95% CI: 1.07, 1.61) intake was associated with advanced prostate cancer but no clear associations for fatal

prostate cancer (Sinha et al., 2009). Other epidemiological studies assessing risk of stomach, brain, oesophageal, and nasopharyngeal cancers have been inconclusive (Eichholzer and Gutzwiller, 1998). Conversely, patients with Barrett's oesophagus have an increased risk of adenocarcinoma at the gastro-oesophageal junction, which is the site where increased N-nitrosamine formation has been identified (Winter et al., 2007). Finally, studies have also demonstrated increased risk of follicular thyroid cancer with high  $\text{NO}_3^-$  levels (Kilfoy et al., 2011). Thus, collectively there does seem to be some evidence of a link between the anions and cancer. However, it is important to appreciate that all of these associations have been made with 'estimated' exposure to  $\text{NO}_3^-$  and  $\text{NO}_2^-$ . Currently there is limited data that have confirmed exposure with measurement of either anion in blood, urine or any other sample in any cohorts. Very recently, Data on 17 618 adult participants in the NHANES (National Health and Nutrition Examination Surveys), conducted between 2005 and 2014 in the United States, was analysed and published. The study found that urinary  $\text{NO}_3^-$  was significantly lower in individuals with hypercholesterolemia, diabetes mellitus, hypertension, myocardial infarction, or stroke. Interestingly, the study found that urinary  $\text{NO}_3^-$  was not associated with cancer prevalence or mortality (Mendy, 2018). Further such studies would be of great value in ascertaining whether the associations described above relate to actual *in vivo* exposure.

A review of data by the World Health Organisation Expert Committee on Food Additives found that there was no evidence that  $\text{NO}_3^-$  was carcinogenic to humans (Speijers and van den Brandt, 2003). In addition, The International Agency for Research on Cancer (IARC) reported that "There is *inadequate* evidence in humans

for the carcinogenicity of nitrate in food and in drinking water”. However, they also reported that “There is *limited* evidence in humans and animals for the carcinogenicity of NO<sub>2</sub><sup>-</sup> in food – Nitrite in food is associated with an increased incidence of stomach cancer”.....There is sufficient evidence in experimental animals for the carcinogenicity of nitrite in combination with amines or amides”.....There is limited evidence in experimental animals for the carcinogenicity of NO<sub>2</sub><sup>-</sup> per se.” (International Agency for Research on Cancer et al., International Agency for Research on Cancer). However, large prospective studies do not support the hypothesis of an association between ingestion of NO<sub>3</sub><sup>-</sup> or NO<sub>2</sub><sup>-</sup> and stomach cancer (Bryan et al., 2012). Thus, whilst it seems that the risk of cancer with NO<sub>3</sub><sup>-</sup> is negligible concerns still remain with respect to NO<sub>2</sub><sup>-</sup>.

#### **1.24 Summary**

A growing body of evidence now supports the view that the beneficial effects seen with inorganic NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> might also, at least in part, explain the benefits seen in diets that are composed of high volumes of vegetables (i.e. the Mediterranean diet). Such observations reopen the discussion of what an acceptable level of intake of these anions should be (Oyebode et al., 2014). Although there are on-going concerns regarding dietary NO<sub>3</sub><sup>-</sup> and carcinogenesis, reassuringly, these concerns to date remain unsupported. On the other hand, thus far there have been no studies exploring the beneficial effects of NO<sub>3</sub><sup>-</sup> supplementation in humans beyond 4 weeks. Hence, the NITRATE-OCT study carried out as part of this PhD will aim to identify the role of inorganic NO<sub>3</sub><sup>-</sup> in reducing restenosis in patients undergoing PCI for stable angina.

In addition, whether the beneficial effects of  $\text{NO}_3^-$  might relate to effects upon the inflammatory components of CHD is unknown and so to address this I have established two models of inflammation in healthy volunteers in the laboratory with a view to testing the effects of  $\text{NO}_3^-$  upon inflammatory responses in healthy volunteers.

### **1.25 Hypotheses**

The primary aim of my PhD is to test the hypothesis that dietary  $\text{NO}_3^-$  ingestion, in addition to conventional therapy, might improve outcome in patients with stable angina undergoing PCI.

### **1.26 Aims**

- (i) To conduct mechanistic studies in healthy volunteers in order to establish techniques and protocols for investigating anti-platelet and anti-inflammatory effects of dietary  $\text{NO}_3^-$  in a large clinical study.
- (ii) To determine whether it is safe to deliver dietary  $\text{NO}_3^-$  in patient undergoing PCI and stent implantation and whether the randomisation is effective.
- (iii) To determine whether dietary  $\text{NO}_3^-$  ingestion exerts anti-platelet effects or anti-inflammatory effects and the mechanisms involved in this effect.

## **Chapter 2.0**

# **General Methods**

## **2.0 General Methods**

### **2.1 Healthy Volunteer Study Methods**

#### **2.1.1 Volunteers**

Equal numbers of male and female healthy volunteers were recruited for each study. Volunteers were included if they fulfilled the following Inclusion criteria: 18–45 years of age, body mass index (BMI) of 18–40 kg/m<sup>2</sup>, and had not received typhoid vaccination in the previous 2 years. Exclusion criteria included: History of hypertension, diabetes or hypertensive on BP measurement, pregnant, or any possibility that a subject may be pregnant unless in the latter case a pregnancy test is performed with a negative result, history of any serious illnesses, including recent infections or trauma, subjects taking systemic medication (other than the oral contraceptive pill), subjects with self-reported use of mouthwash or tongue scrapes, subjects with recent or current antibiotic use, subjects with a history, or recent treatment of (within last 3 months) of any oral condition (excluding caries), including gingivitis, periodontitis and halitosis and subjects that have recently participated (preceding 3 months) in any clinical studies involving administration of an inflammogen. Female volunteers' appointments were scheduled to fall at a maximum of 2 weeks and no less than 1 week prior to the due-date of their next menstruation when oestrogen levels at their highest.

All clinical studies were performed in a quiet temperature-controlled laboratory (24°C to 26°C) and individuals were studied at the same time of day during each day of the study they were involved in. Volunteers refrained from caffeine consumption and strenuous exercise the day before and the day of the respective study, and were fasted overnight before all study visits.

### **2.1.2 Healthy volunteer study 1 – typhoid-induced systemic inflammation study**

A total of 24 healthy volunteers (12 males and 12 females) were consented for this 3 day study. On day 1 volunteers attended the clinic at 3 pm and measured their own BP using a portable BP device. Body temperature was measured using a standard Braun Thermoscan Ear Thermometer™. Following this PWA, PWV and FMD were determined. Blood was then collected. On day 2, the volunteers attended in the morning to receive the typhoid vaccination into the gluteal or deltoid region at 8 am in the morning and returned at 3 pm for a repeat of all the previous measurements. On day 3, volunteers attended the clinic at 3 pm for the final time and had a final repeat of all measurements (**Figure 2.1A**). In a separate study we recruited a further 16 healthy volunteers-8 males and 8 females to assess endothelium-independent vasodilation using GTN as per previous recommendations (Donald et al., 2006). Briefly, the volunteers attended the clinic as above but received a sublingual (0.4 mg) dose of GTN spray (Nitrolingual Pump Spray, Merck, UK) in the place of the reactive hyperemia stimulus. Whole blood differentials and flow cytometry were measured to assess both leucocyte and platelet numbers and activation state and plasma collected for assessment of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> (NO<sub>x</sub>) levels and CRP.

### **2.1.3 Healthy volunteer study 2 – Cantharidin-induced blister study**

A total of 32 healthy volunteers (16 male and 16 female) were consented for this 4 day study. On day 1, the volunteers measured their own BP/ using a portable blood pressure device. After this cantharidin solution was applied to the ventral aspect of a forearm. Blood, was then collected. On day 3 (48 hours after the initial

cantharidin application), the volunteer returned to have cantharidin applied to a separate area, no less than 5 cm away from the initial. Finally, on day 4 (72 hours after the initial cantharidin application), the volunteers measured their BP again and then blister fluid was sampled from both blisters and blood, urine and saliva samples were collected (**Figure 2.1B**). Blister fluid volume, cell count, flow cytometric analysis of cell type and activation state and cytokine/chemokine analysis were also run.

For the application of cantharidin solution 10  $\mu$ L cantharidin (cantharone 0.1%, Dormer Laboratories, Toronto, Canada) was applied to filter-paper discs and placed on the ventral aspects of the forearm as previously described (48-51) and dressed appropriately. Blister fluid was then harvested at 24 hours (acute phase) and 72 hours (resolution phase) by carefully piercing the side of the blister with a 25G needle and rolling a pipette tip over the surface of the blister to express blister fluid, which was then collected in a siliconised pipette and the fluid was then stored on ice until further analysis.



## Study 1-Typhoid-induced systemic inflammation study

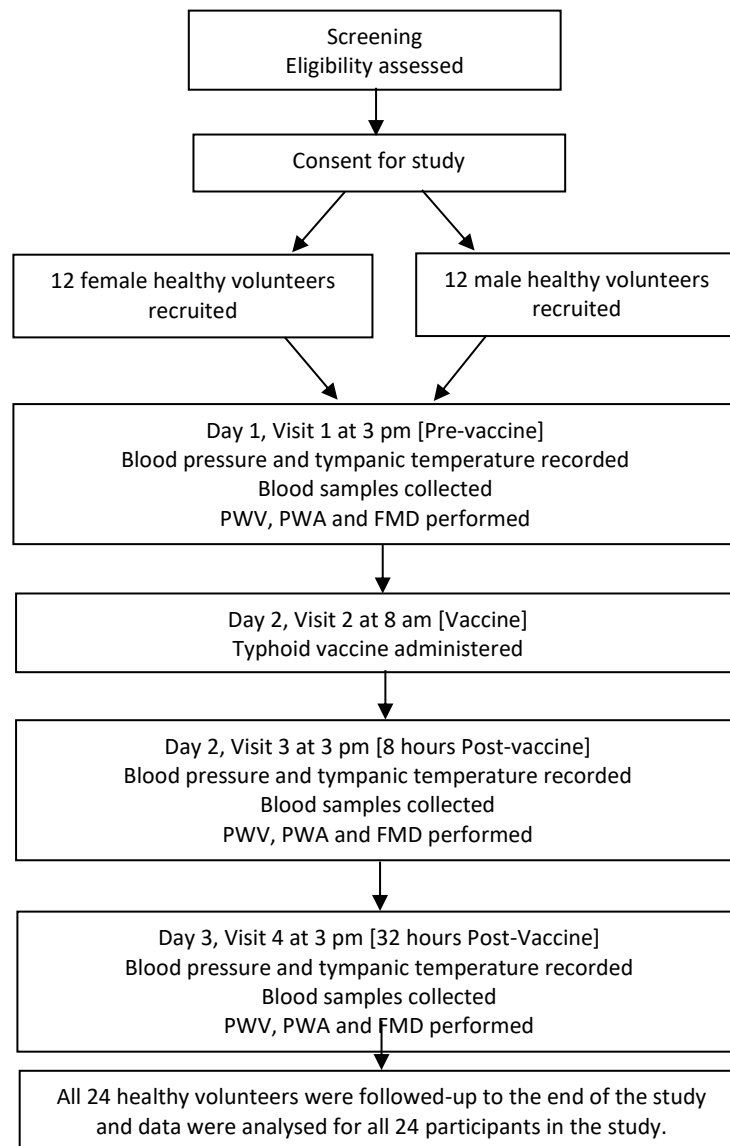


Figure 2.1 (A) Flow chart of the typhoid clinical study 1.

PWV: pulse wave velocity; PWA: pulse wave analysis, FMD: flow mediated dilatation.

## Study 2-Cantharidin-induced blister study

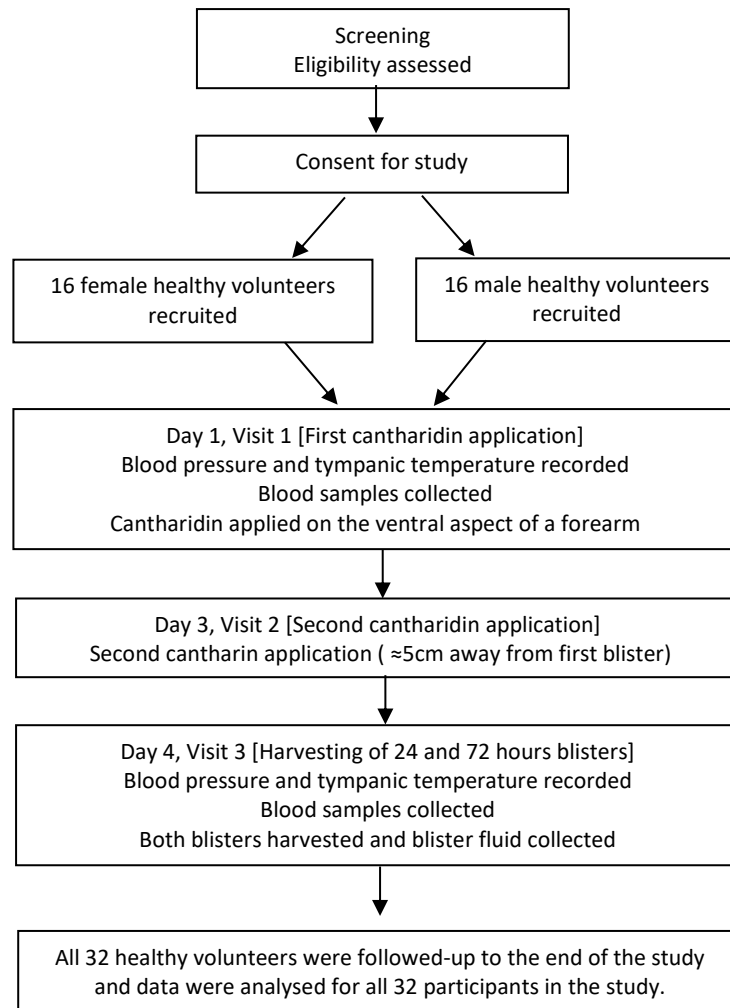


Figure 2.1 (B) Flow chart of the cantharidin clinical study .

#### **2.1.4 Power and statistical analysis**

Based upon previous observations demonstrating typhoid vaccine induced endothelial dysfunction (Hingorani et al., 2000b) and our previous studies suggesting important sex differences in responses to inflammation (Villar et al., 2011), the primary outcome measure was change in %FMD, with an expected difference between the sexes of 1.5% and a SD of 1.5 with a power of 80%. For this a total of 12 subjects are required in each group.

For the cantharidin study, based upon previous observations demonstrating that cantharidin triggers a total cell response of  $0.6 \times 10^6$  cells/ml with an SD of 0.2 (Evans et al., 2008) and in pre-clinical models where leucocyte recruitment was reduced by 50% in females (Villar et al., 2011), with a power of 0.9 a total of 12 individuals are required for each sex for statistical power. However, due to the limited sample size available for all analyses we pre-specified an n=16 for each group to enable sufficient independent samples to assess soluble mediators. Where n values shown are less than 16 this is due to insufficient sample size for full analysis.

Data are shown as mean  $\pm$  SD. Comparisons between the sexes have been made using unpaired t-tests or two way ANOVA as appropriate. With respect to the latter post-hoc comparisons have been made if the F statistic achieved  $P < 0.05$  and there was no significant variance inhomogeneity between groups using Holm-Sidak's multiple comparison tests. For multivariate statistical analysis, orthogonal partial least squares-discriminant analysis (PLS-DA) (Janes and Yaffe, 2006) was performed using SIMCA 13.0.3 software (Umetrics, Umea, Sweden) following unit

variance scaling of LM amounts. PLS-DA is based on a linear multivariate model that identifies variables that contribute to class separation of observations (Blister exudates) on the basis of their variables (LM levels). During classification, observations were projected onto their respective class model (Males vs Females). The score plot illustrates the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plot interpretation identified the variables with the best discriminatory power (Variable Importance in Projection close or greater than 1) that were associated with Males (Blue) or Females (Green) and contributed to the tight clusters observed in the Score plot.

Analysis was performed using GraphPad™ Prism software version 5.0 for Mac OsX and SPSS version 19, (SPSS Inc, Chicago, Ill). Power calculations were performed using Graphpad statmate (Graphpad Software) and are represented in the experimental protocols in each chapter.

#### **2.1.16 Trial approval and registration**

The studies were peer-reviewed by the institutional review board and were granted full ethics approval by NRES Committee London - City Road & Hampstead (11/LO/2038). Informed, written consent was taken after satisfying the inclusion criteria. The studies have been registered on ClinicalTrials.gov NCT01582321.

## **2.2 Stable Angina Patient Methods**

### **2.2.1 NITRATE-OCT Clinical Trial**

NITRATE-OCT is a randomised, double-blind, placebo-controlled study investigating the effects of inorganic NO<sub>3</sub><sup>-</sup> on vascular function, platelet reactivity and restenosis in stable angina.

#### **2.2.1.1 Study Protocol**

The full protocol was submitted for ethical approval to the National Research Ethics Service London – City Road and Hampstead (Reference: 15/LO/0555) for ethical human research and sponsored by Queen Mary University of London Research and Development (R&D) department. The trial was conducted according to Barts Health NHS Trust and Queen Mary University of London R&D guidelines and performed in agreement with the Declaration of Helsinki. Any serious adverse event or reaction during the course of the study was reported to the R&D department as per adverse event guidelines. The study was registered with the following public registries: United Kingdom Clinical Research Network (UKCRN) (Study ID 20060), <http://clinicaltrials.gov> (NCT02529189) and International Standard Randomised Controlled Trials Number (ISRCTN):17373946.

#### **2.2.1.2 Patient selection**

Commencing November 2015, we commenced a randomised double-blind placebo-controlled trial of stable angina patients referred to a single tertiary cardiac centre for PCI (**Figure 2.1**). We obtained written informed consent from all study patients. This is a single-centre trial and patients have been recruited at Barts Health NHS Trust within the Barts and The London Heart Attack Centre,

based at The Barts Heart Centre, St. Bartholomew's Hospital. In addition, we have been identifying patients from King George Hospital which is part of Barking, Havering and Redbridge NHS Trust.

### **2.2.1.3 Inclusion Criteria**

Patients were included in the study if they met the following inclusion criteria

1. Patients with stable angina diagnosed by a cardiologist on optimal medical therapy undergoing angioplasty to treat residual symptoms.
2. Aged 18-85
3. Patients able and willing to give their written informed consent.
4. Patients undergoing successful PCI procedure.

### **2.2.1.4 Exclusion Criteria**

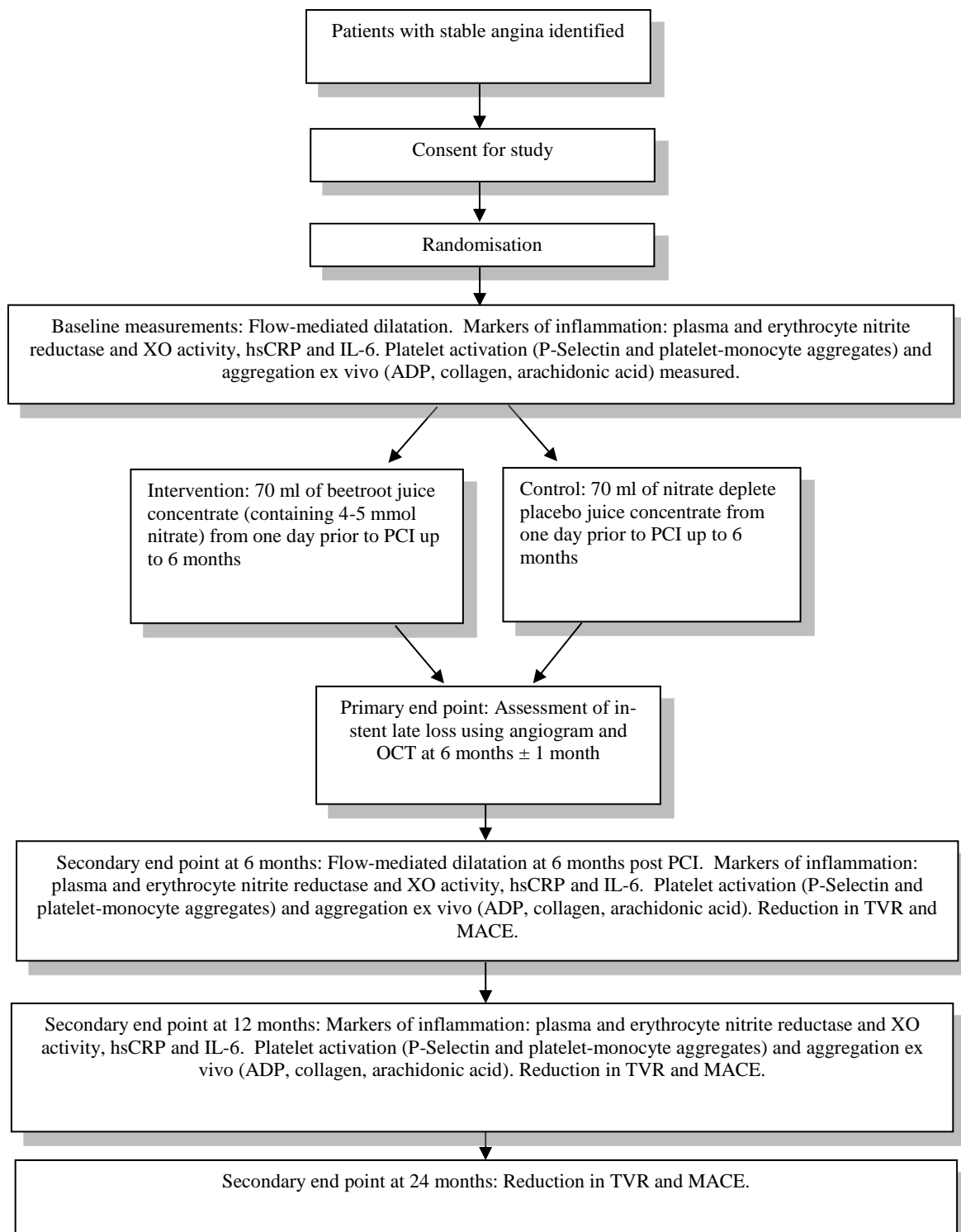
Patients were excluded if they had any of the following exclusion criteria.

1. Unstable ischaemic heart disease, with an episode of chest pain in less than 24 hours before inclusion into the study.
2. Patients who have had previous coronary artery bypass surgery (CABG), if they are undergoing angioplasty within a non-native vessel.
3. Patients undergoing angioplasty with a bio-absorbable stent since the stents will likely start dissolving prior to 6 months and therefore this would confound measures of restenosis.
4. Current diagnosis of, or treatment for, malignancy other than non-melanoma skin cancer.
5. Current life-threatening condition other than vascular disease that may prevent a subject completing the study.

6. Use of an investigational device or investigational drug within 30 days or 5 half-lives (whichever is the longer) preceding the first dose of study medication.
7. Patients considered unsuitable to participate by the research team (e.g. due to medical reasons, laboratory abnormalities, or subject's unwillingness to comply with all study related procedures).
8. Severe acute infection, or significant trauma (burns, fractures).
9. Pregnancy tested by urine HcG measurement
10. History of alcohol or drug abuse within the past 6 months.
11. A history of heart failure NYHA class 3-4 or severe LV dysfunction LVEF<30% regardless of symptom status.
12. Systemic autoimmune disease such as rheumatoid arthritis, connective tissue disease, or other conditions known to be associated with chronic inflammation such as inflammatory bowel disease.
13. Patients who have donated > 500 ml blood within 56 days prior to study medication administration.
14. Anaemia with Hb <10g/dl, or any other known blood disorder or significant illness that may affect platelet function, and coagulation.
15. A history of chronic viral hepatitis (including presence of hepatitis B surface antigen or hepatitis C antibody or other chronic hepatic disorder) or HIV.
16. Abnormal liver function due to acute or chronic liver conditions 3 x upper limit of normal at screening.
17. Renal impairment with creatinine clearance (eGFR) of 35ml/min at screening.

18. If patients are on mouthwash, they must be willing to stop using this at least 1 week before the start of the study and throughout the duration that they are involved in the study.





**Figure 2.2: Summary of NITRATE-OCT Study Scheme. hsCRP: high sensitivity C-Reactive Protein, IL-6: Interlukin 6, MACE: Major Adverse Cardiac Events, OCT: Optical Coherence Tomography, TVR: Target Vessel Revascularisation, XOR: Xanthine Oxidoreductase**

### **2.2.1.5 Procedures**

Potentially eligible patients were identified, screened and approached from pre-assessment clinic at St. Bartholomew's Hospital. Entry in to the trial was not confirmed until they had fulfilled the pre-specified inclusion criteria and did not meet any excluding criteria, as detailed in section 2.2.1.4.

### **2.2.1.6 Study Design and Intervention**

This is a prospective double-blind, placebo controlled, clinical study. A total of 246 patients (male and female, age 18-85) with stable angina as per requirements indicated above have been recruited. **Figure 2.2** shows a summary of the study scheme. Since patients who are diabetics are at high risk of developing restenosis, these patients have also been included in the study with stratification in both groups. Patients have been stratified according to the type of stent (i.e. bare metal stent or drug eluting stent) as both these groups have differing characteristics resulting in differing rates of restenosis. Follow-up has taken place in the Clinical Trials Unit, William Harvey Heart Centre. Since patients have had other co-morbidities (including hypertension, hypercholesterolemia, previous PCI etc.) these have also been recorded. Patients have been block randomised (using an on-line randomisation database) to receive 70 ml of a beetroot juice concentrate containing ~5 mmol NO<sub>3</sub><sup>-</sup> or NO<sub>3</sub><sup>-</sup>-depleted placebo juice concentrate (James White Drinks, UK) control. The volunteers have commenced taking their daily dose at home the day before the scheduled angioplasty and continued this daily for 6 months. Patients have been advised to take their dose of juice at the same time each day, preferably in the morning with their breakfast. Patients have also been provided with dietary advice in relation to the calorific content of the juice:

a daily dose of 70 ml of the juice concentrate (approximately 70 g) contains about 100 kcal.

On the day of index procedure, patients have been treated as per Barts Health Trust protocols. Here the patient arrives to the day case ward in the morning of the procedure and the senior interventional trainee registrar and consultant cardiologist have performed the coronary angiogram. At the time of the procedure, the operator has determined the route of access (either via the radial artery or femoral artery) and the consultant cardiologist has determined the size (diameter and length) and type of stent (DES or BMS) to be inserted. Following the procedure, the patient was transferred back to the day case ward and discharged by either the nurse in charge or the cardiology registrar depending on the complexity of the procedure. Patients have been followed-up according to the clinical plan suggested by the consultant cardiologist at the time of the procedure.

#### **2.2.1.7 Randomisation and Blinding Process**

Patients have been block randomised on a 1:1 basis to receive either dietary NO<sub>3</sub><sup>-</sup> or placebo, using a binary random number sequence ([www.random.org](http://www.random.org)). Treatment assignment for volunteers in both the dietary NO<sub>3</sub><sup>-</sup> and placebo groups has remained blinded until data lock and statistical analysis at the end of the study. If at any point un-blinding is required, the Chief Investigator for the study will be informed. A list of the un-blinded treatments is kept in a secure location at the The William Harvey Heart Centre. The un-blinding procedure is available at all times (24 hrs day/7 days a week).

### **2.2.1.8 Study Endpoints**

#### **2.2.1.8.1 Primary Endpoint:**

The primary endpoint is reduction of in-stent late loss assessed by angiography (quantitative coronary angiography – QCA) at  $6 \pm 1$  month. Assessment of restenosis is being made by measurement of in-stent late-loss assessed by angiography (using QCA) and OCT at  $6 \pm 1$  month.

#### **2.2.1.8.2 Secondary Endpoints:**

- (i) Improvement in endothelial function assessed by FMD of the brachial artery at 6 months compared to pre-procedure assessment.
- (ii) Reduction in target vessel revascularisation (TVR), restenosis rate (diameter >50%) and in-segment late loss at  $6 \pm 1$  month.
- (iii) Reduction in major adverse cardiac events (i.e. MI, death,) at 6, 12 and 24 months in addition to CVA and TVR.
- (iv) Reduction in plaque size as assessed using OCT at  $6 \pm 1$  month.
- (v) Reduction in inflammatory markers and changes in plasma XOR activity, hsCRP, IL-6 at 6 and 12 months.
- (vi) Reduction in platelet aggregation ex vivo at 6 and 12 months compared to pre-procedure.

### **2.2.2.3 Index angiogram**

#### **2.2.2.3.1 Percutaneous Coronary Intervention**

Angiography has been performed at baseline prior to stent placement and after stent placement prior to completion of the index PCI procedure. Either the radial or femoral vascular access has been used for the procedure. The baseline angiograms of the involved vessel have been performed in at least two near orthogonal views. Visual angiographic assessment has been used to determine if the lesion meets angiographic entry criteria. Further information collated from the index coronary angiography procedure has been collected, comprising of date of the angiogram, vessel dominance, number and location of diseased vessels, location, presence and severity of lesions for all 14 segments of the coronary tree and any complications. The PCI procedure has been completed according to normal practice by the operator.

#### **2.2.2.3.2 Index PCI**

Procedural information for the index PCI has included the date of the PCI, which coronary vessels and segments were treated, diameter, length and type of stent implanted, the procedural success for each segment, the adjunctive pharmacological therapy and any complications and information on lesions not treated have also been recorded.

#### **2.2.2.4 Repeat coronary angiography with OCT assessment of vessel treated PCI**

Repeat angiography with OCT has occurred at  $6 \pm 1$  month after the index PCI. Either radial or femoral access sites have been used and angiograms of the involved vessel has been performed in at least 2 near orthogonal views. Quantitative angiographic assessment of the involved vessel will be undertaken to determine late loss. In addition, the same angiographic images have been recorded as those obtained post-stent implantation at the time of the index procedure.

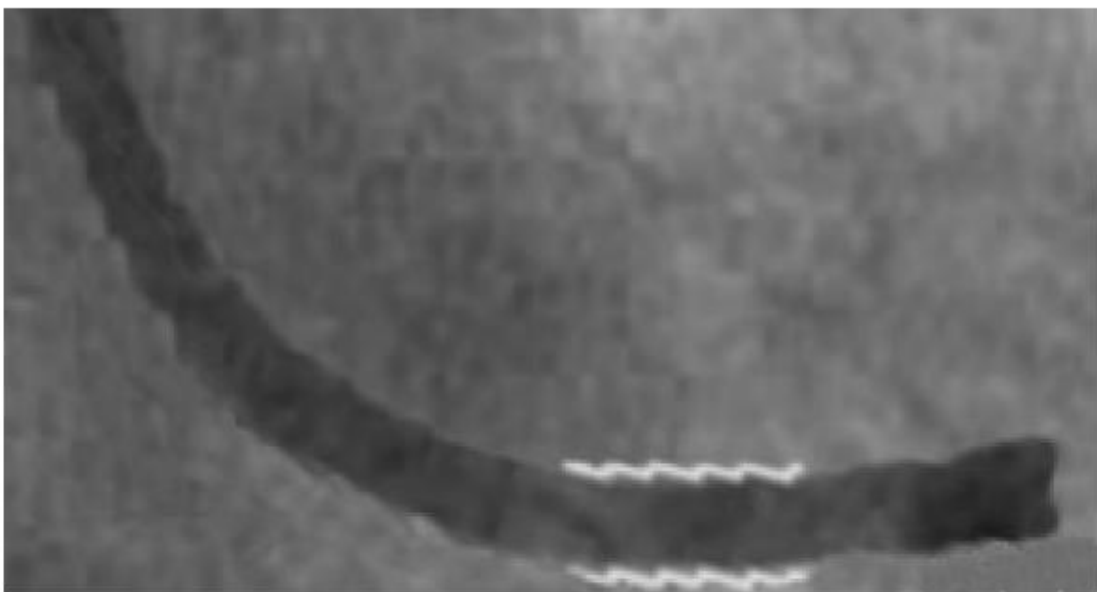
#### **2.2.2.5 Quantitative Coronary Angiography (QCA)**

Quantitative analysis will be performed using a Coronary Angiography Analysis System (Medis Inc., The Netherlands). There will be automated detection of the boundaries of a selected coronary vessel segment using digitised and optically magnified sections of the cine frame. If a wrong centreline is chosen because of large daughter branches, then correction is possible manually. The vessel diameter will be calculated in absolute values (mm). This will be done using the boundaries of a section of the contrast catheter and comparing the computed mean catheter diameter in pixels with the known catheter size in mm. To detect the contours of the vessel, the user will indicate the vessel by choosing two centre positions proximal and distal to the area of interest. QCA will be analysed in a blinded fashion by two experienced and independent cardiologists and adjudicated by a senior cardiologist if there are any discrepancies in the analysis (of >5%). For the analysis, the same Cine images of pre- and post-stent will be used as well as the same image at 6 months depicting the same part of the coronary artery in all three cases. QCA will be carried out in the same segment of the

coronary artery which contains part of the vessel free of disease before and after the stent and the whole stent itself. This will be done at baseline (pre- and post-stent) and at 6 months.

#### **2.2.2.5.1 Calibration**

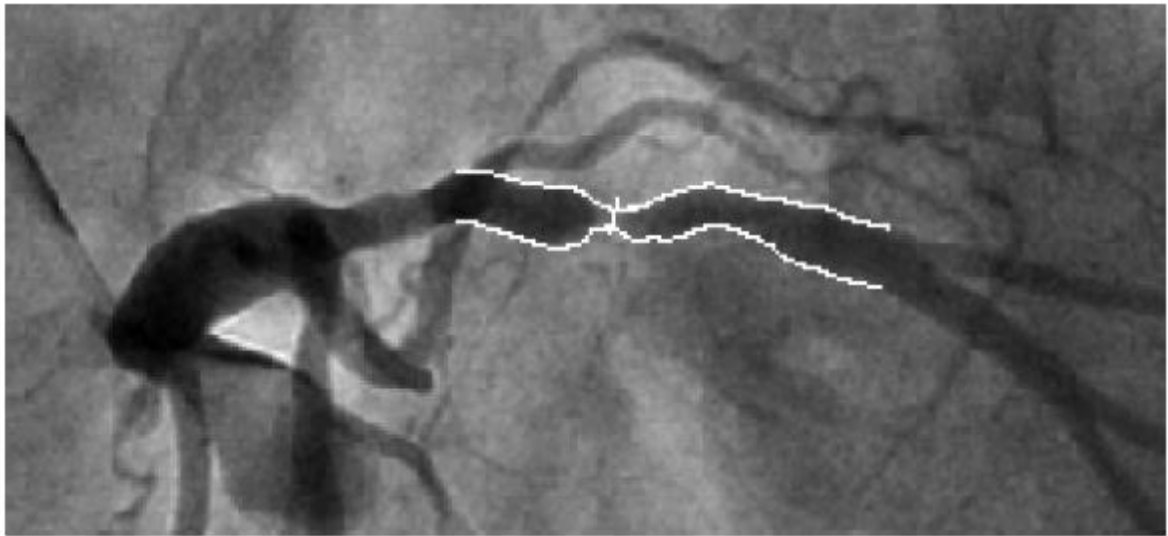
Initially, the tip of the catheter, whose diameter in French or millimetres is known, will be used as the basic tool for calibration (i.e., to estimate the proportion between pixels in the digitalised images and mm of length). Then, a central line will be drawn by hand along the stretch of the catheter tip and the software will automatically recognise its margins by using specific algorithms that, by means of digital images, recognise the change from radiopaque pixels (black or dark grey) to radio-transparent pixels (light grey or white) according to a densitometric analysis. The software will then transform every pixel into a square with sides characterised by known dimensions (mm). (**Figure 2.3**).



**Figure 2.3: Angiogram image of the Image calibration using contrast-filled injection catheter.**

### 2.2.2.5.2 Arterial automatic contour detection

The above parameter will be then employed to measure the coronary segment of interest that is focused on the stenosis or lesion undergoing treatment. The proximal and distal coronary segments will be relatively free of disease and are referred to as reference segments. Afterwards, a central line will be traced manually and the software will automatically recognise the margins of the coronary segment under consideration taking advantage of the automatic contour detection. This procedure will determine the vessel edge based on the weighted sum of the first and second derivative functions of the brightness values of scanlines perpendicular to the center-line, which in turn undergoes an analysis that determines the optimal contour path along the segment (Figure 2.4).

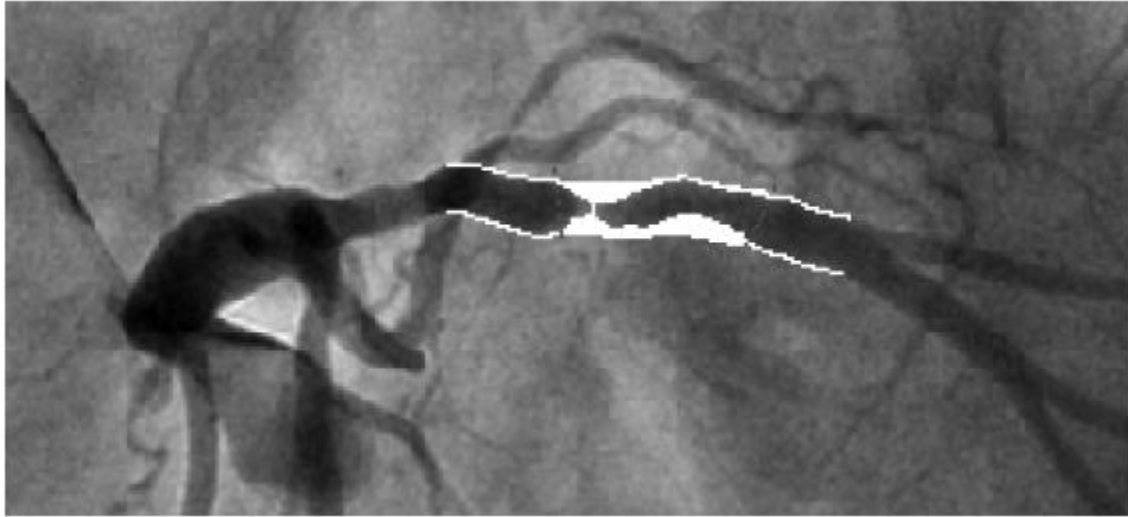


**Figure 2.4: Angiogram demonstrating that after having traced a central line, the software recognises the margins elaborating automatically the contours.**

A further step allows, always automatically, the reconstruction of the hypothetically normal coronary lumen. An algorithm then creates a line of the coronary margin that interpolates the coronary segment considered free from



illness located proximally and distally to the region of interest with the ones of the region of interest. The algorithm then reconstructs the reference coronary segments (i.e., those apparently free from disease) (**Figure 2.5**).



**Figure 2.5: Angiogram showing that software algorithm reconstructs the reference coronary segment.**

This technique is essentially based on the calculation of a mean value of the diameters of the lumen in the segments of reference located upstream and downstream to the lesion. It appears thus clear the importance of including two coronary segments in the QCA analysis, one proximal and one distal to the target segment and both angiographically-free from significant disease. These steps produce different parameters, some measured directly and others derived with interpolation techniques.

### **2.2.2.5.3 Parameters**

All angiographic measurements of the target lesion will be obtained in the “in-stent” zone, within 5 mm proximal and distal to each stent edge, and over the entire segment (“in-segment” zone). The following QCA parameters will be calculated: reference vessel diameter, minimal lumen diameter, percent diameter stenosis (difference between the reference vessel diameter and minimal lumen diameter/reference vessel diameter x 100) and late lumen loss (difference between the post-procedure and follow-up minimal lumen diameter). Binary restenosis will be defined as stenosis of 50 percent or greater in the target lesion or segment at the 6-month angiographic follow-up. The coronary parameters of major interest are summarised in **Table 2.1**.

### **2.2.2.6 Optical coherence tomography (OCT)**

OCT imaging of the previously stented segment was undertaken with motorised pull-back at 1 mm/s in all patients at  $6 \pm 1$  month after index PCI using an OPTIS™ system (St-Jude Medical, Westford, MA, USA). OCT images have been acquired at 180 frames per second (500 angular pixels x 250 radial pixels), from a 0.15mm micro-optic core, 0.36 mm image wire, displayed with an inverse gray-scale lookup table, and digitally archived.

<b>Parameter</b>	<b>Range commonly used</b>	<b>Meaning</b>
Minimal Luminal Diameter (MLD)	0–6.00 mm	The smallest lumen diameter in the segment of interest
Reference Vessel Diameter (RVD)	1.5–6.0 mm	The averaged diameter of the coronary assumed without atherosclerotic disease
Lesion length	0–60.0 mm	Length of the stenosis as measured by 2 points where the coronary margins change direction, creating a shoulder between the angiographically normal subsegment and the diseased subsegment
Acute gain	0–4.0 mm	Post-procedural MLD – pre-procedural MLD
Late loss (LL)	0.10 to 3.00	Post-procedural MLD – MLD at follow-up
Diameter stenosis (DS)	0–100%	$(RVD-MLD)/RVD$
Binary restenosis (BR)	Yes or no	DS >50% at follow-up coronary angiography in the treated coronary segment

**Table 2.1.** Main parameters obtained with QCA (adapted from Garrone, P.; Biondi-Zoccai, G. & Salvetti, I. (2009). Quantitative coronary angiography in the current era: principles and applications. *J Interv Cardiol* 22:527-536.) (Garrone et al., 2009).

### **2.2.2.6.1 Segmentation of optical coherence tomographic data**

OCT imaging analysis will be performed offline using proprietary software (QCM-CMS Leiden University Medical Center, Leiden, The Netherlands). The stented segments will be identified and the OCT frames in these segments will be analysed at 0.4 mm interval by two independent analysts blinded to stent type and patient characteristics. The lumen and the stent border will be detected, and the neointima will be defined as the tissue between the lumen and the endoluminal border of the struts. Neointima burden will be defined as 100 x neointima area vs. stent area. Neoatherosclerosis will be defined as either neointima and with a lipid or a calcific tissue (Tearney et al., 2012). The border of the fibrous cap over lipid tissue will be segmented using semi-automated software (QCM-CMS) and the minimum and mean cap thickness will be calculated (Radu et al., 2016). The borders of the lipid and calcific tissue will be detected and the lipid area, the lipid arc, and the calcific area will be estimated. The lipid/calcific burden will be defined as: 100 x lipid/calcific area vs. neointima area. Thin-cap fibroatheroma (TCFA) will be defined as lipid tissue with a fibrous cap thickness  $\leq 65 \mu\text{m}$  while this with a cap thickness  $> 65 \mu\text{m}$  will be characterised as fibroatheroma.

Neoatherosclerotic lesions will be defined as calcific tissue, TCFA, or fibroatheroma in the neointima. A gap of at least 0.5 mm will be used to define the boundary between two neoatherosclerotic lesions. In addition, the two observers will identify the presence of macrophages, neo-vessels, cholesterol crystals, thrombus and neointima discontinuities (i.e. flaps, cavities, double lumen and fissures) (Bourantas et al., 2015).

#### **2.2.2.6.2 Attenuation and backscatter analysis**

In each OCT cross-section the attenuation and backscatter indices will be estimated for the neointima tissue using established methodologies, by a module developed by the Leiden University Medical Center (Leiden, The Netherlands) (Schmitt et al., 1993; Vermeer et al., 2013). In contrast to previous reports, the analysis will be performed in the entire circumference of the neointima (Yonetsu et al., 2012). In order to exclude the strut effect in each cross section, the stent border will be moved 100  $\mu\text{m}$  inwards to the original border and the region of interest will be defined by this curve and the lumen border. The guidewire artefact will be also removed and then the 95 percentile values will be estimated for each frame. The output of this analysis will be colour coded displayed in each cross section and in spread-out stent plots. Frames portraying lipid-rich neointima are anticipated to have a higher attenuation and backscatter indices in the inner border of the lipid tissue and lower indices in the lipid tissue resulting in low mean attenuation values. Therefore the 95 percentile attenuation and backscatter values will be used to identify degenerative changes in the neointima. We will compare the 95 percentile attenuation and backscatter values: 1) between difference stent types and 2) between sub-segments with neoatherosclerosis, sub-segments located adjacent (2 mm), and sub-segments located distally (> 2 mm) to neoatherosclerotic lesions. Only frames with attenuation/backscatter values for an arc >180° of the circumference of the neointima will be included in the analysis. frames with poor flushing and thrombus will be also excluded.

Finally, OCT imaging have not be used for clinical decision making regarding need for a repeat revascularisation procedure. All repeat revascularisation decisions have been based upon a recurrence of clinic symptoms and/or non-invasive imaging evidence of myocardial ischaemia and made by the treating physician.

## **2.2.2.7 Planned recruitment and power calculations**

### **2.2.2.7.1 Statistical Analysis**

#### **2.2.2.7.1.1 Sample Size**

In order to achieve an 80% probability that the study will detect a treatment difference at a two sided 5% significance level, if the true difference in late loss between the treatments is 0.22 mm, sample size calculations determined that a total of 246 patients will be required to enter a two treatment parallel design study. This absolute difference is calculated from a mean late loss of 1.27mm with a standard deviation of the response variable of 0.550. These values being the mean and average of the standard deviations of 22 trials measuring late loss in both drug eluting and bare metal stents described in the review by Mauri et al (Mauri et al., 2005). Recruitment also takes into account an additional 10% to account for drop-out or withdrawal/non-compliance. This value is based upon previous experience in our unit.

The sample size of 246 enables sufficient power for estimation of the hard endpoint of MACE at 6 months. Very recently, in stable angina patients who have undergone elective angioplasty a remote ischaemic preconditioning intervention resulted in a significant reduction in MACE at 6 months with 4/110 (3.6%) in the

treatment group versus 13/104 (12.5%) in the control group (Davies et al., 2013). Using these data as a basis for power calculations, a total number of 230 patients are needed for 80% power using one-tailed analysis. Thus, to account for potential loss to follow-up we will recruit 246 patients in total.

Power calculations for FMD suggest that the above numbers provide sufficient power for detecting differences in FMD (n=60). To calculate power we used data provided in dietary studies including one demonstrating improvements in FMD following six weeks of artichoke juice (Lupattelli et al., 2004) a further chronic study with walnuts (Katz et al.) and one meta-analysis (Kay et al.) assessing the effects of chronic polyphenol dietary interventions published in 2012. Calculating an averaged improvement of FMD of 1.1% with no change in the control and an averaged SD of 1.45 (determined from the average of the above studies) a total of 30 volunteers would be needed in each group requiring a total of 60 volunteers. Using our own data with dietary NO<sub>3</sub><sup>-</sup> in patients with hypercholesterolemia increases of FMD of 1% with a SD of 1.5% (Velmurugan et al., 2016) a total of 74 patients are needed for sufficient power. If we assume a potential 10% drop-out rate a total of 80 patients are required.

All statistical analysis will be conducted by the trial statisticians supported by the Imperial Clinical trials unit (CTU). Data will be analysed on an intention-to-treat basis. We will also conduct further per protocol analyses and a subgroup analysis on patients who are on organic NO<sub>3</sub><sup>-</sup> as part of their routine therapy, patients on statin therapy and a comparison of DES versus BMS by incorporating these variables in a cox regression analysis model.

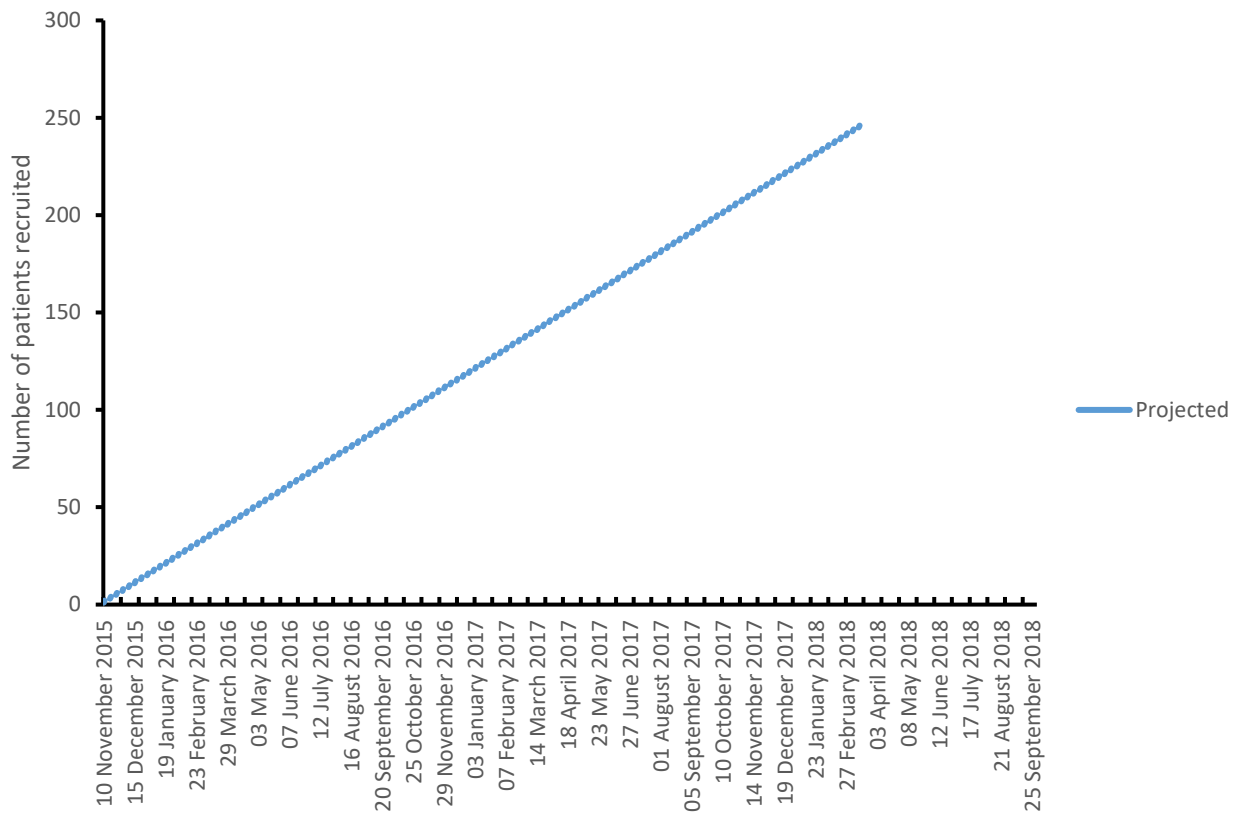
Baseline demographic and clinical variables were summarised for each arm of the study. Descriptive summaries of the distributions of continuous baseline variables are presented in terms of percentiles (e.g. median, 25th and 75th percentile), while discrete variables are summarised in terms of frequencies and percentages. Comparisons are between the nitrate- treated and placebo control-treated group for the primary and secondary outcomes. Statistical analyses were conducted blind to the treatment groups. The statistical comparisons of the treatment arms with respect to the primary endpoint was performed using an unpaired T-test. For comparisons between normally distributed data statistical comparisons were performed using unpaired T-test, for parametric data either the Mann-Whitney or Wilcoxon rank sum tests were used.

For comparisons between treatment groups assessing platelet reactivity data are expressed as mean  $\pm$  SEM and analysis was performed using two-way repeated measures ANOVA.

#### **2.2.2.7.1.2 Project timetables including recruitment rate**

The duration of the study will be 36 months. Approximately 2000 elective angioplasties are performed over 12 months, equal to 19 per week. If approximately half of these are elective stable angina patients and about half of these are suitable for the study, we would expect a recruitment rate of 2 per week, to achieve 100 patients in approximately 12 months (**Figure 2.6**).





**Figure 2.6. Graph showing the projected recruitment for NITRATE-OCT Study**

### **2.2.2.8 Ethical Considerations**

The study protocol and any subsequent amendments, along with any accompanying material provided to the patient, in addition to any advertising material, was submitted by the Investigator to an Independent Research Ethics Committee. Written Approval from the Committee was obtained and subsequently submitted to the sponsor to obtain Final Sponsorship approval and NHS permissions.

### **2.2.2.9 Safety considerations**

The intervention is 70 ml of a beetroot juice concentrate or NO<sub>3</sub><sup>-</sup> free placebo juice (James White Drinks, UK). The NO<sub>3</sub><sup>-</sup> is extracted using the same extraction technique used to remove inorganic NO<sub>3</sub><sup>-</sup> from the general drinking water supplies. There are no known harmful side effects from these interventions and this NO<sub>3</sub><sup>-</sup>-free juice is not considered to be a drug or medicine and is classified as a foodstuff. In addition, several recent publications using the placebo juice are now available (Gilchrist et al., 2013; Gilchrist et al., 2014; Lansley et al., 2011). In the unlikely event of an adverse event occurring directly as a result of the intervention, the trial would be stopped.

### **2.2.2.10 Safety reporting**

An adverse event (AE) if not defined as serious, will be documented in the participants' medical notes (where appropriate) and the CRF and followed up by the research team. Any serious adverse events (SAE) that occur will be reported to the Sponsor and main REC where in the opinion of the Chief Investigator the event was either 'related' (that is, it resulted from administration of any of the research procedures) and 'unexpected' (that is, the type of event is not listed in the protocol as an expected occurrence).

SAEs that are considered to be 'related' and 'unexpected' will be recorded in the participants' notes, the CRF, the sponsor SAE form and reported to the Research Office of the Trust within 24 hours of research staff being notified, and to the Main REC within 15 days. The co-investigators in this study will be authorised to sign the SAE forms in the absence of the Principal Investigator. Since this study will be

blinded, the treatment code for the patient will be broken in the reporting of an 'unexpected and related' SAE. This will be performed by an individual who is independent of the study and will allow the rest of the research team to remain blinded. The unblinding of single cases by the Principal Investigator in the course of this study will only be performed if necessary for the safety of the trial subject.

### **2.2.2.11 Monitoring**

#### **2.2.2.11.1 Trial Steering Committee (TSC)**

The TSC is composed of three independent experts in the fields of: pharmacology, interventional cardiology and clinical trials along with the investigators and the data monitor and medical statistician and 1 lay member. This committee has met before patient recruitment and will meet annually to assess safety, feasibility or any other arising problems (e.g. with recruitment) and their recommendations will be followed.

#### **2.2.2.11.2 Data Safety and Monitoring Board (DSMB)**

An independent DSMB has been formed to monitor patient safety as the study progresses. The DSMB has been selected by and communicates directly to the study's TSC. The committee includes a consultant cardiologist, an interventional cardiologist and a statistician. The DSMB met prior to initiation of the clinical study, after the recruitment of 10 patients and will meet at 3 monthly intervals. The DSMB will have access to unblinded patient data. If a serious concern with the safety of the patients in the trial arises, the DSMB may recommend early termination of the study.

### **2.2.2.12 Follow up**

After 2 years, the participant will be contacted by telephone for assessment of major adverse cardiac events (MACE). The whole duration of the study will be 2 years. Participants have no obligation to complete the whole study and if they decide to withdraw at any point then they are free to do so.

### **2.2.2.13 End of Study Definition**

The study will end after 2 years after the telephone follow-up of the last patients. At this time all the samples will be analysed.

## **2.3 Biochemical Measurements**

### **2.3.1 Blood sampling**

Blood was collected into EDTA tubes for differential count analysis conducted by St Bartholomew's Hospital Haematology Department. A further 4 ml of blood was collected in 1.8 mg ethylenediaminetetraacetic (EDTA) per ml of blood for NO<sub>x</sub> measurement, and another 4 ml in 3.2% buffered trisodium sodium citrate for aggregation assays and platelet flow cytometry. All blood was collected into vacutainers (BD Biosciences) through a 21-gauge butterfly needle inserted into an antecubital vein. For measurement of NO<sub>x</sub> levels, blood samples were centrifuged immediately (1300g, 4 °C, 10 min) and the supernatant was collected and stored at -80 °C pending analysis by ozone chemiluminescence as previously described (Ignarro et al., 1993a; Kapil V, 2015). Specific blood sample preparation for each protocol is described later in association with the technique used to analyse the relevant samples. With regards to the NITRATE-OCT study, the blood sampling

was performed on visit two, where patients had one blood test before starting the treatment or placebo juice. They then had a further blood test at visit three (6 months – prior to angiogram and OCT) and visit five (12 months). Blood samples were centrifuged immediately for platelet reactivity experiments and plasma and red blood cell storage only for the purposes of making biochemical measurements (e.g.  $\text{NO}_3^-/\text{NO}_2^-/\text{cGMP}$ ) and were discarded once used.

### **2.3.2 Urine and saliva samples**

Mid-stream urine samples were collected into sterile pots and an aliquot stored at  $-80\text{ }^\circ\text{C}$  pending measurement of  $\text{NO}_x$  levels at a later date. Unstimulated saliva was collected into sterile eppendorfs and centrifuged (14000g,  $4\text{ }^\circ\text{C}$ , 10 min) and the supernatant was transferred to a separate eppendorf and stored at  $-80\text{ }^\circ\text{C}$  pending measurement of  $\text{NO}_x$  levels at a later date. The pellet generated from the centrifuged saliva oral bacteria that have dislodged from the oral cavity. This pellet has been frozen for identification and analysis of the oral microbiota by second generation genome sequencing will be performed at a later date. With regards to the NITRATE-OCT study, the urine and saliva samples were taken on visit two, where patients had these taken before starting the treatment or placebo juice. They then had a further urine and saliva sample taken at visit three (6 months – prior to angiogram and OCT) and visit five (12 months).

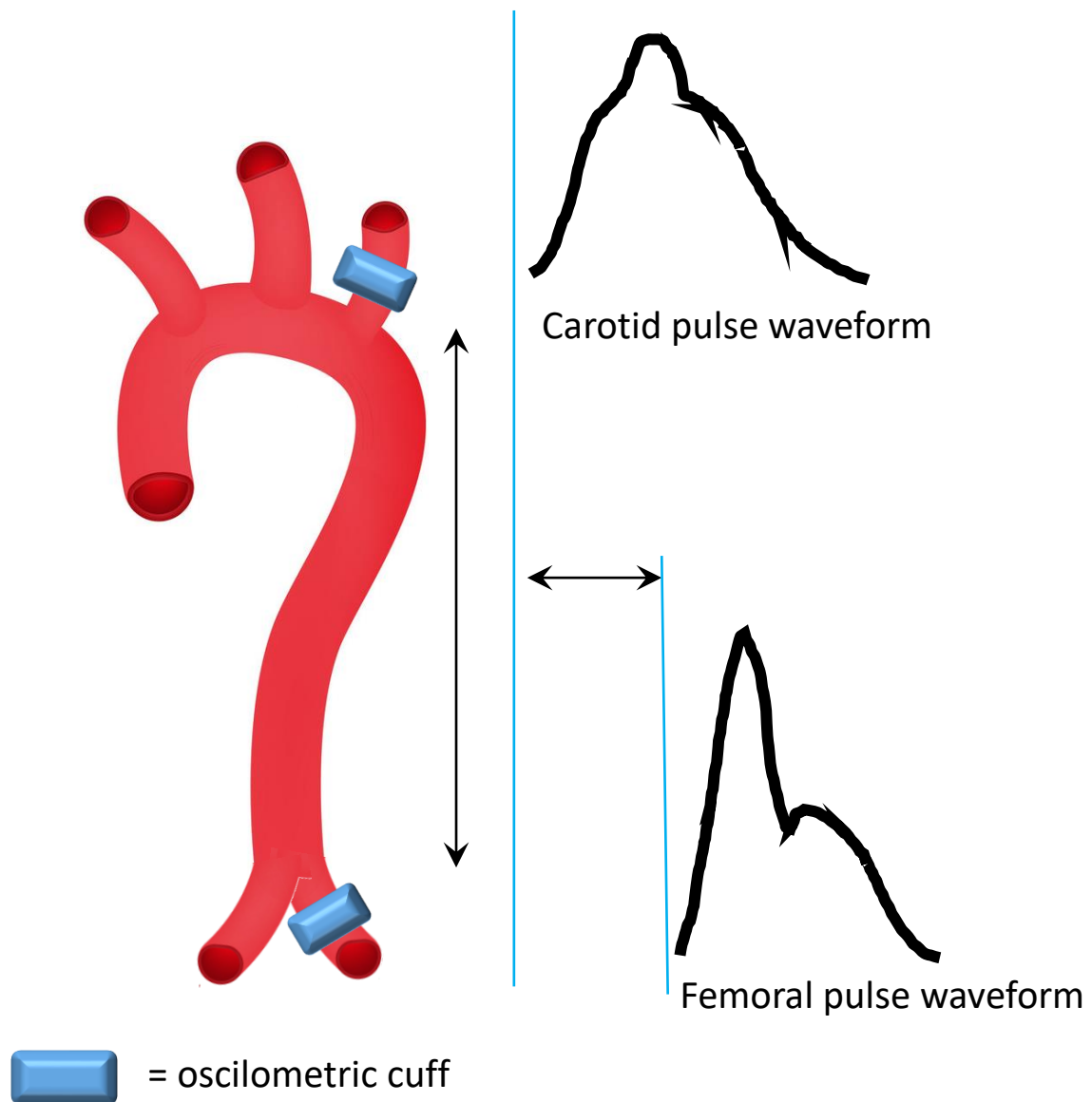
### **2.3.3 Blood pressure measurement**

Blood pressure was measured at baseline to confirm healthy volunteer status. An Omron 705IT was used for all BP measurements while participants were seated and readings were performed in triplicate according to established guidelines

(Williams et al., 2004). Laminated coverings were used for the machine and the printer so that both investigators and participants were blinded to the readings. The means of the 2nd and 3rd readings were used to calculate the final BP measurement.

#### **2.3.4 PWV and PWA measurements**

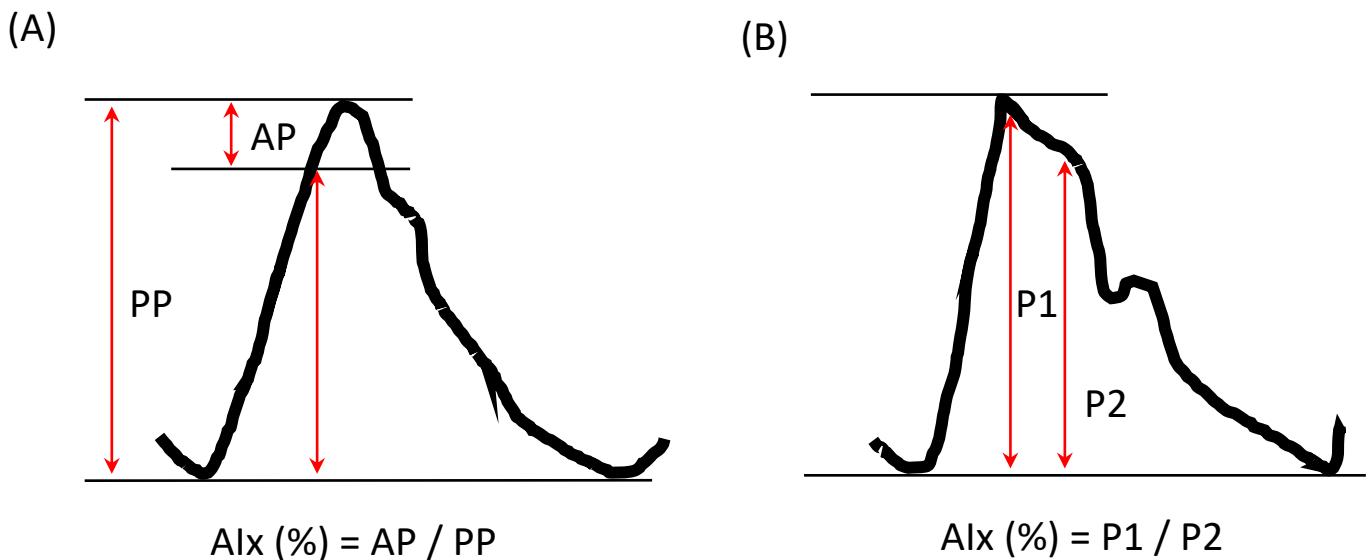
A Vicorder device (Skidmore Medical Limited, Bristol, UK) was used to simultaneously record the pulse wave from the carotid and femoral site using an oscillometric method. All arterial stiffness measurements were performed with the patient rested for 10 min in a supine position with their head at a 30 ° incline and awake. A small, inflatable neck pad is placed directly over a single carotid artery and secured around the neck by a Velcro tab. A cuff is placed around the subject's ipsilateral upper thigh. Both carotid and femoral cuffs are inflated automatically to 65 mmHg and the corresponding oscillometric signal from each cuff is digitally analysed to extract the pulse time delay. The distance between the sternal notch and the thigh cuff is measured and used as a standard estimate for the aortic length. From these measurements aortic PWV can be derived as  $PWV = \text{aortic distance} / \text{pulse time delay}$  (Hickson et al., 2009). **(Figure 2.7)**.



**Figure 2.7. Schematic showing the determination of PWV using Vicorder device.** Carotid and femoral oscillometric signals are detected and the time delay from the foot of carotid pulse waveform to foot of femoral pulse waveform is used as the pulse transit delay ( $t$ ). Estimation of aortic length by sternal-femoral distance is used as distance ( $d$ ). Aortic distance/pulse transit delay = aortic PWV. (PWV = pulse wave velocity). Adapted from Boutouyrie (Boutouyrie, 2008).

Pulse wave analysis (PWA): A mean brachial BP reading was obtained from 2 readings made using an Omron 705IT and used for calibration of peripheral waveforms. After 5 min supine rest, a 100 mm inflatable cuff was attached to the

non-dominant arm and statically inflated to 65 mmHg. Brachial artery waveforms were digitally computed by Vicorder (Skidmore Medical Limited, Bristol, UK). The vicorder software subsequently applied a brachial to aortic transfer function to calculate the waveform and values for central BP as previously described (O'Rourke, 1970). The Augmentation index (Alx) which is the difference in amplitude between the first and second systolic peak/pulse pressure x 100) was calculated automatically from the first and second central systolic peaks which were automatically defined by the software (**Figure 2.8**).



**Figure 2.8. Schematic showing the definition of Alx.** (A) Aortic pressure pulse wave. (B) Peripheral arterial pressure wave. Alx=augmentation index; AP=augmentation pressure; PP=pulse pressure; P1 represents the amplitude of the early systolic peak; P2 represents the amplitude of the late systolic peak adapted from (Shimizu and Kario, 2008).

### 2.3.5 Measurement of brachial artery diameter

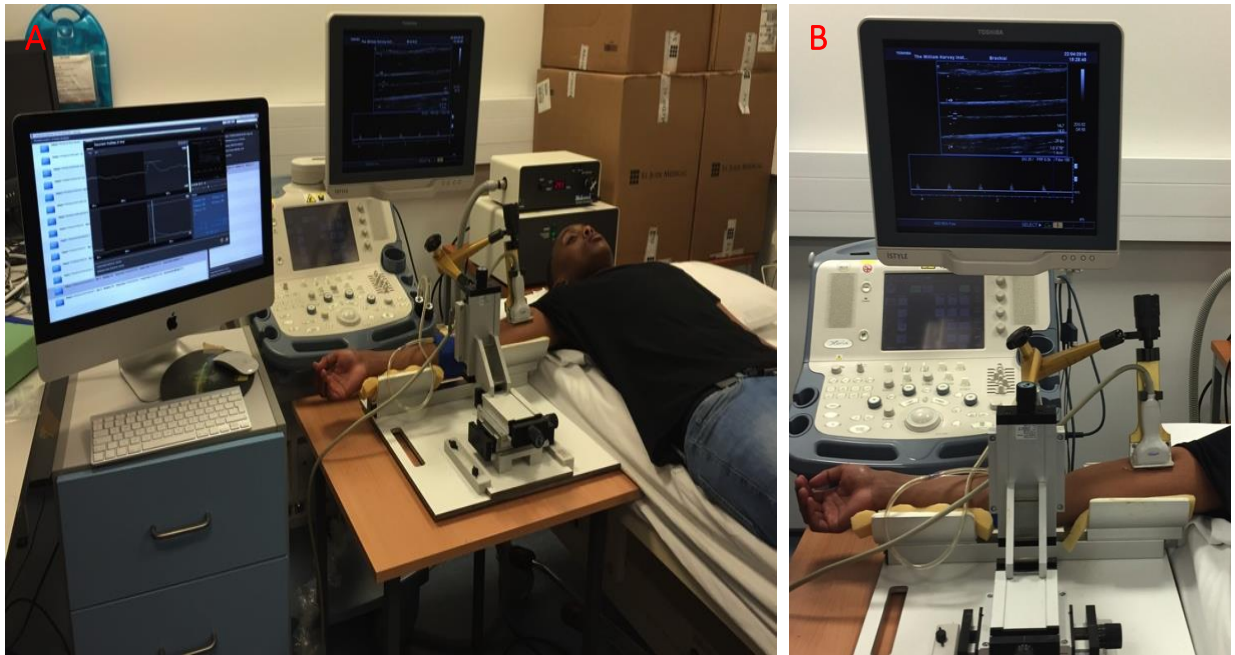
Non-invasive ultrasound assessment of endothelial function of the brachial artery in subjects has been determined by measurement of FMD. FMD is a non-invasive



method of assessing endothelial function in vivo. It utilises vascular ultrasound to measure the increase in the diameter of the brachial artery in response to increased flow (Celermajer et al., 1992) and has been conducted according to published guidelines (Corretti et al., 2002). FMD can be affected by a number of factors including temperature, food, drugs, and sympathetic stimuli. Therefore, it was essential that the conditions for each study were identical on each occasion for each participant. Thus, studies were performed in a quiet, dimly-lit, and temperature controlled room (22-24 °C). Participant skin temperature was measured by using a temperature probe placed between the thumb and forefinger and a temperature of 30°C was required prior to performing the measurements. If a temperature less than 30°C was recorded, the hand was warmed using a heated microwave wheat pack.

#### **2.1.8.1 Image acquisition**

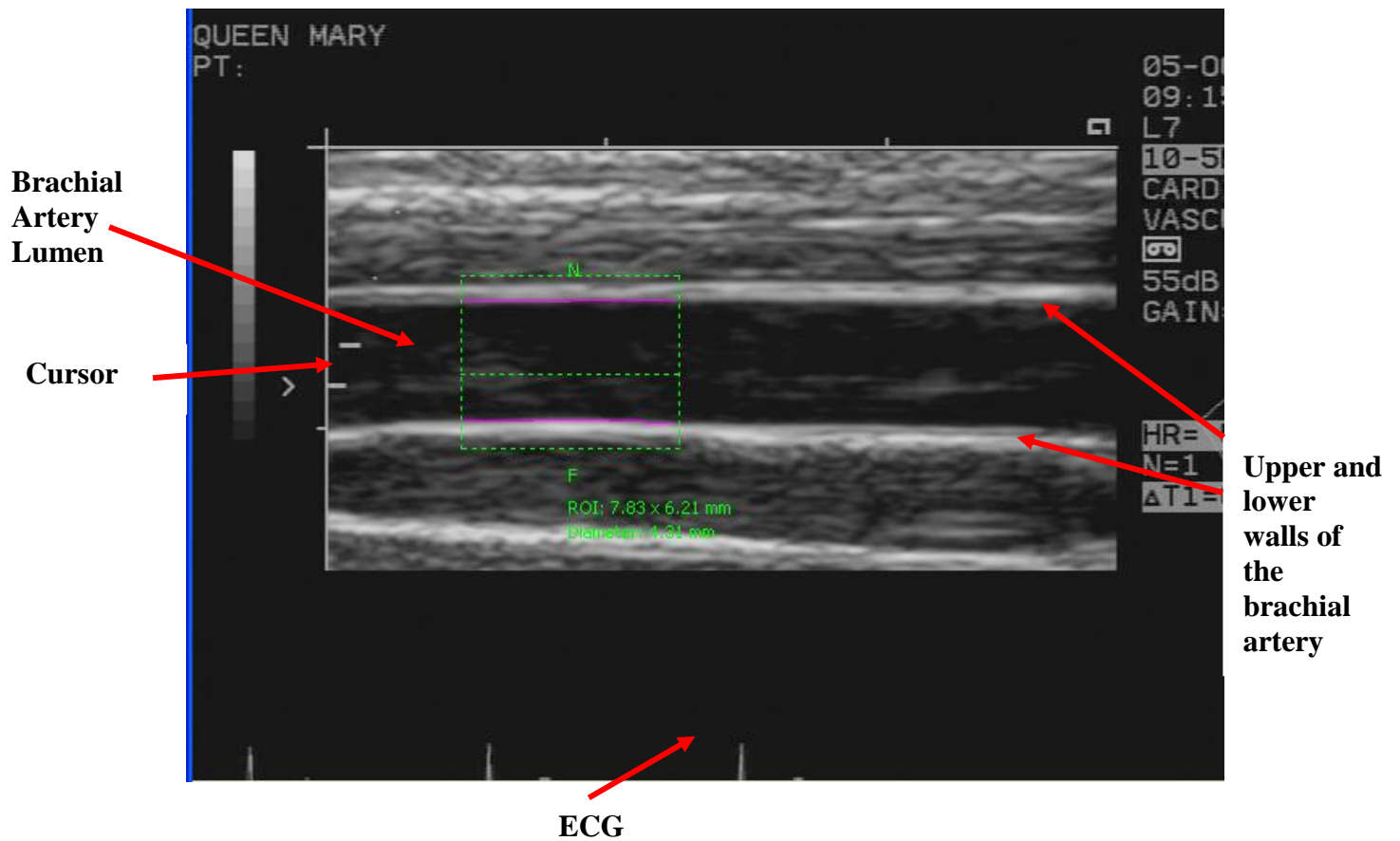
The participant would lay on a bed in a supine position with their right arm placed comfortably in a foam protected arm rest, to image the brachial artery. **Figure 2.9** depicts the FMD setup used during the study. A 7 cm-wide BP cuff was placed ~1 cm below the antecubital fossa around the forearm (one thumbs-width from the medial epicondyle.)



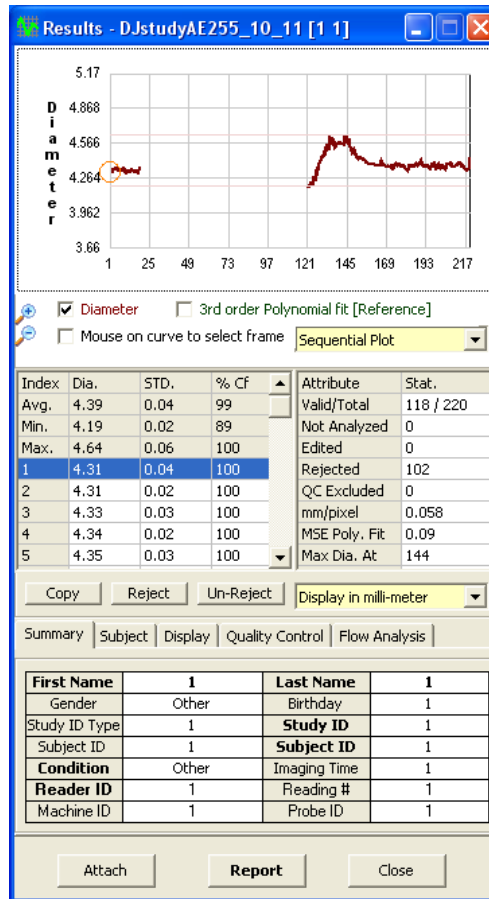
**Figure 2.9. Photograph of FMD being performed on a volunteer. A)** The volunteer was asked to lie supine. A longitudinal section of the brachial artery was obtained and FMD was determined after cuff deflation. **B)** Image showing the Doppler ultrasound device used to obtain brachial artery image for FMD measurement.

Brachial artery diameter in the right arm was measured with high-resolution external vascular ultrasound (Acuson 128XP/10 with a 7.0-MHz linear-array transducer). The vessel was scanned in longitudinal section, and the centre was identified when the clearest views of the anterior and posterior artery walls had been obtained. Images were magnified with a resolution box function and gated with the R wave of the ECG (**Figure 2.10**). End-diastolic images of the artery were acquired every 3 seconds with customized data-acquisition software and stored in digital format offline for later analysis (Mullen et al., 1997). Brachial artery diameter was measured continuously for 1 minute at baseline, during 5 minutes of reduced blood flow (induced by inflation to 300 mm Hg of a pneumatic cuff placed at a site distal to the segment of artery being analysed), and for a further 5

minutes during reactive hyperaemia after cuff release. Brachial artery diameter (over 1-2 cm section) and brachial artery dilation expressed as percentage and absolute increase from baseline diameter and time to peak dilatation were determined using automatic edge-detection software (Vascular Research Tools, Medical Imaging Applications LLC Iowa City, USA). Furthermore, for each scan, 5 analyses along the vessel length were made and the mean calculated for each of the parameters described above (i.e. baseline diameter, time to peak dilatation, percentage increase and absolute increase in diameter) to give the FMD response as a percentage (i.e. calculation of the change from the baseline diameter and expressed as a percentage) and this mean value was used as an n=1 (**Figure 2.11**).



**Figure 2.10: Ultrasound Image of the brachial artery obtained during FMD examination.** A typical image is shown above. The vessel edges and lumen of the brachial artery are clearly seen. The measurement cursor is placed in the centre of the vessel which acts as a marker for adjustment of the image if there is any distortion of the image following inflammation or deflation of the cuff. The ECG allows synchronisation (gating) of the image with the R-wave of the cardiac cycle, providing elimination of any distortion of the images due to the pulsation of the artery during the study.



**Figure 2.11: Screenshot of the Vascular Research Tools Medical Imaging Application Software for analysis of the FMD from the brachial artery.** There is a graphical analysis of the image recording over time. The x-axis has the number of frames (each frame lasting 3 seconds) and the y-axis is brachial artery lumen diameter in measured in millimetres. Following inflation of the cuff after 1 minutes (i.e. frame 20) of the baseline recording, there was induction of ischaemia, which was released at 5 minutes (i.e. frame 120). A peak response in dilatation of the artery is seen following cuff deflation within one minute of cuff deflation (i.e. within frames 120-130). This represents the FMD response following reactive hyperaemia.

### 2.3.6 Measurement of antibody titre

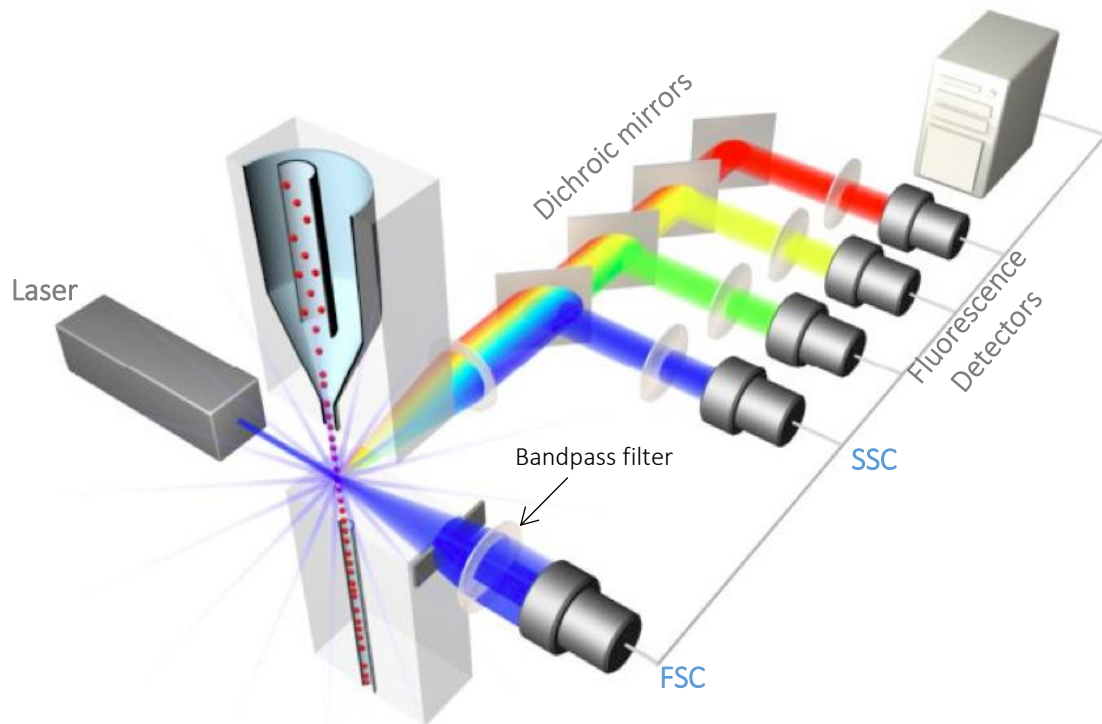
Plasma samples taken at baseline and at 32h post typhoid were analysed for antibody titre using a commercially available kit (Typhoid Vi IgM ELISA kit; Alpha

Diagnostic International, USA) as per the manufacturer's instructions. Briefly, this Typhoid Vi IgG/IgM antibody test kit is based on the principle of indirect ELISA. Typhoid Vi antigen is bound on the surface of the microtiter strips. 100  $\mu$ L of diluted sample (1:20 with distilled water) and 100  $\mu$ L of pre-diluted supplied calibrators standards were pipetted into the wells of the microtiter plate. The wells were gently mixed by tapping against the palm for 5-10 seconds. The plate was then covered with aluminium foil and incubated at room temperature (25-28  $^{\circ}$ C) for 60 mins. The contents of the wells were then aspirated and the plate was blotted on absorbent paper. The wells were then washed immediately 3 times with 300  $\mu$ L of 1 x wash buffer. 100  $\mu$ L of diluted anti-IgM-HRP conjugate was added to all the wells leaving one empty for the substrate blank. The plate was then mixed gently for 5-10 seconds. The plate was then covered and incubated for 30 minutes at room temp (25-28  $^{\circ}$ C). Vi IgG/IgM antibodies bind to the immobilised Vi antigen. The wells were then washed again with 300  $\mu$ L of 1 x wash buffer. 100  $\mu$ L the substrate (TMB) solution is added to all the wells (including the substrate blank on this occasion). The plate is then mixed gently for 5-10 seconds. The plate was then covered and incubated for 15 minutes at room temp (25-28  $^{\circ}$ C). Blue colour developed in positive controls and samples. The reaction was stopped by adding 100  $\mu$ L of stop solution to all wells. The plate was then mixed gently for 5-10 seconds to have uniform colour distribution (blue colour turns yellow). The absorbance was measured at 450 nm and a reference filter 630nm using an ELISA reader within 15 minutes.

## **2.3.7 Inflammatory cell flow cytometry**

### **2.3.7.1 Basic principles of flow cytometry**

Flow cytometry is an analytical technique that utilises light to quantify and analyse the properties of individual cells in a heterogeneous sample (Herzenberg and De Rosa, 2000). The use of fluorophore-conjugated antibodies, highly specific for cell surface antigens, enables the identification and characterisation of specific cell subpopulations in a sample (Herzenberg and De Rosa, 2000). Cells are suspended in fluid and travel in a single file through the flow chamber. When the laser beam hits each cell, light is scattered in different directions, and emitted, upon excitation of the fluorophore, at different wavelengths. Light scatter is recorded as forward scatter (FSC) and side scatter (SSC), to identify the size, and granularity and internal complexity of the cell, respectively. Light scatter and fluorescence intensities are recorded as voltages and displayed using histograms or 2D/3D dot plots.



**Figure 2.12. Picture of the flow cytometry apparatus (Cancer Research Institute. Queen's University at Kingston, 2016).** A flow cytometer is comprised of a hydrodynamic focusing system, a laser beam, filters, light detectors, and a data analysis system. Cells in suspension pass through the flow chamber in a single file stream formed as a result of hydrodynamic focusing. The laser beam hits each cell as it passes through the path of the laser, and the light scatter is detected as FSC and SSC, by light detectors. Fluorophore conjugated to antibodies are excited by the laser and emit light at different wavelengths, detected by photomultiplier tubes (PMT). Dichroic mirrors and filters placed in front of PMTs transmit a specific range of wavelength to the detectors. Signals are then sent to the computer enabling tabulation and analysis of cell characteristics in the sample.

A fluorophore molecule absorbs light over a range of wavelengths, and emits light at different, longer wavelengths (emission spectrum). As fluorophores emit light over a range of wavelengths, this may result in spectral overlap when using multiple fluorophores in an experiment. Spillover fluorescence emitted by one fluorophore can be detected by another channel for a different fluorophore. Spectral overlap can be corrected by manual fluorescence compensation, which is



crucial for accurate data analysis. Controls, comprising of an unstained and single stained samples with each individual fluorophore conjugated antibodies, were used to manually compensate for any spillover occurring at a channel (Perfetto et al., 2004). Samples were acquired and recorded on the LSR Fortessa II Cell Analyzer (BD Biosciences, San Jose, CA, USA) using BD FACSDiva Software (BD Biosciences, San Jose, CA, USA).

### **2.3.7.2 Measurement of circulating cell populations and their activation state**

The systemic inflammatory response pre and post intervention was assessed using Fluorophore conjugated monoclonal antibodies specific for leucocyte subpopulations, including neutrophils, monocytes and lymphocytes, and expression markers, CD11b, CD162 and CD62L, were used for FACS analysis (listed in **Table 2.2**).

### **2.3.7.3 Preparation of samples for flow cytometry**

18 FACS tubes were labelled in numerical order and placed on ice. The volume of conjugated antibodies pipetted into each labelled FACS tube are found in Appendix 4. 50µL of blood was immediately added to each FACS tube and incubated on ice for 30 minutes. The samples were covered with aluminium foil to avoid photo bleaching of the fluorophore-conjugated antibodies.

During incubation, the immunolyse for whole blood lysing (Beckman Coulter Inc.) was prepared in a 15mL Falcon tube. A 1:24 dilution of immunolyse solution was

prepared using the following calculation:  $380\mu\text{L}$  of immunolyse +  $9120\mu\text{L}$  of PBS ( $4560\mu\text{L} \times 2$ ).

To remove excess antibody,  $2000\mu\text{L}$  of phosphate buffered saline (PBS) solution was added to each FACS tube. Each tube was then centrifuged on  $400g$  for 5 minutes at  $4^{\circ}\text{C}$ , and the supernatant was decanted and discarded appropriately. The next step involved heamolysis, with  $500\mu\text{L}$  of the prepared immunolyse added to each FACS tube. The tubes were then vortexed thoroughly on a low setting, two at a time, whilst being incubated for 2 minutes. Samples were then fixed using  $125\mu\text{L}$  of fixative solution, containing formaldehyde. Each tube was washed again using  $2000\mu\text{L}$  of PBS and then centrifuged. The supernatant was decanted appropriately to preserve the pellet, and the wash step was repeated once more. Finally, the pellet was re-suspended in  $300\mu\text{L}$  of PBS and left incubated on ice pending FACS analysis.

All samples were then analysed using BD LSR Fortessa Cell Analyser (BD; flow cytometer) and recorded with BD FACSDiva 6.0 software.

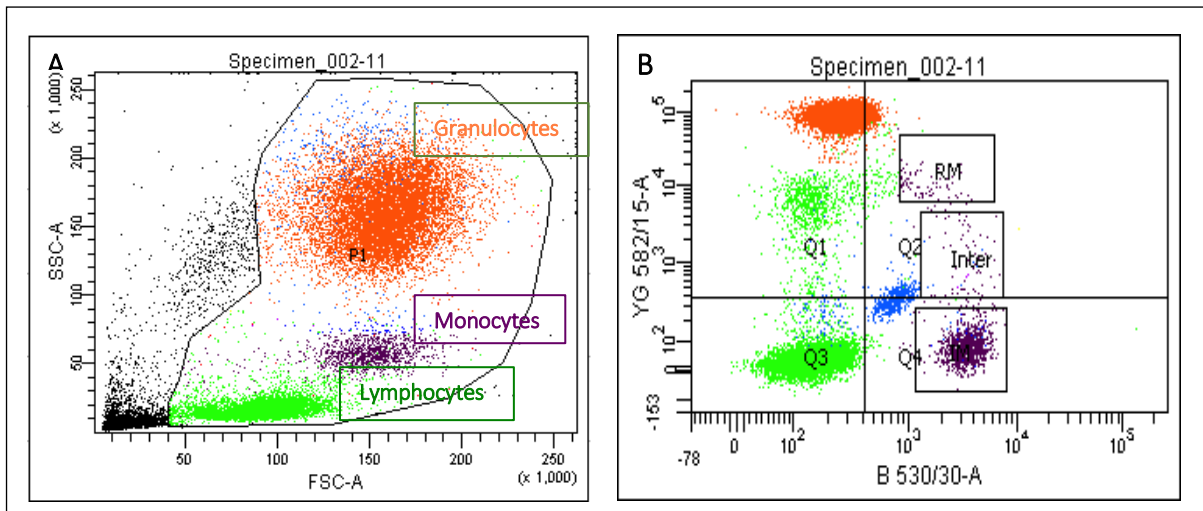
#### **2.3.7.4. Flow cytometry and analysis**

The principles of flow cytometry were described in section 2.3.7.1 and the same principles apply to the analysis of the cell types and activation markers. Combined with FSC and SSC data used to identify cell types (**Figure 2.5**) the staining pattern of each leukocyte was used to calculate the relative percentage of cells expressing each inflammatory marker. In addition, in each case the respective median fluorescence intensity (MFI) was determined and provides a measure of the

number of markers present on each cell type. To ensure that the fluorescence recorded was due to specific primary antibody binding to the desired marker, and not due to non specific binding (i.e. Fc receptors), isotype-matched control antibodies were used. MFIs were recorded in absolute values

As mentioned previously, overlapping of emission spectra between fluorochromes can occur therefore when using multiple fluorochromes, the emitted light from the leading and trailing tails from a neighbouring fluorochrome is registered. Since 7 different fluorochromes were used for cell type and activation marker analysis, spectral overlap had to be accounted for by manual compensation to allow accurate data analysis. In order to manually compensate, single staining of samples with each antibody conjugated fluorochrome was recorded on the flow cytometer.

Cell populations within a heterogeneous sample can be selected or 'gated' in order to observe the characteristics of each population. Gating is a graphical boundary that enables the removal of debris and isolation of cell subpopulations within a sample. The light scatter (FSS v SSS) dot plot, seen in **Figure 2.13**, highlights isolated leucocyte subpopulations including granulocytes, monocytes and lymphocytes. Specific subpopulations can also be 'gated' and further analysed (**Figure 2.13b**). The quadrant boundaries between cells that stained positive and negative for a particular leukocyte population was determined according to the fluorescence distribution of positively stained cell relative to the unstained sample.



**Figure 2.13. Dot plot illustrating the FSS/SSC and fluorescence intensity of leucocyte subpopulations derived from whole blood. A)** A P1 gate is used to select all leucocytes in the sample, excluding cell debris represented by black dots. The FSC/SSC plot enables the differentiation of leucocyte subtypes, according to their forward and side scatter characteristics. Lymphocytes (green) and monocytes (purple) are small in size with reduced granularity/internal complexity, whereas, granulocytes (orange) are large with increased granularity/internal complexity. **B)** Fluorescent intensity dot plot highlighting monocyte subpopulations: resident monocytes (RM), intermediate monocytes (Inter) and inflammatory monocytes (IM). Quadrant boundaries are used to identify positive and negative populations. These boundaries are placed according to an unstained control.

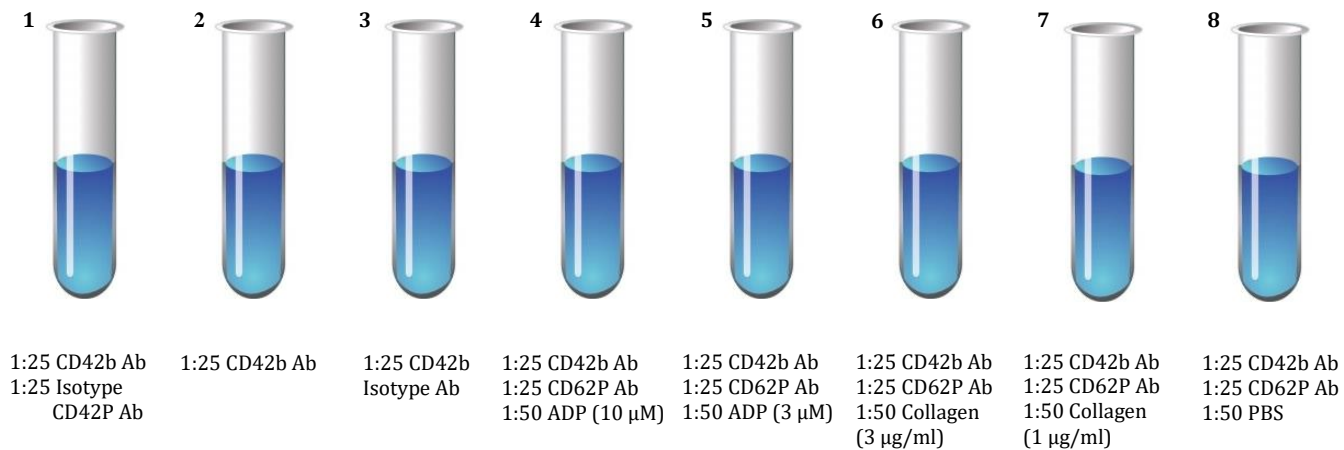
<b>Antibody</b>	<b>Function of surface marker</b>	<b>Cell types</b>	<b>Fluorophore</b>	<b>Source</b>	<b>Dilution</b>
<b>CD14</b>	Pattern recognition receptor that binds to bacterial lipopolysaccharide (LPS), mediating an innate immune response.	Monocytes	FITC	Beckton, Dickinson & company, New Jersey, USA	20 $\mu$ L
<b>CD16</b>	Low affinity Fc receptor known as Fc $\gamma$ RIIIa (CD16a) and Fc $\gamma$ RIIIb (CD16b). Receptor binds to Fc region of IgG antibodies and mediates antibody dependent cell mediated cytotoxicity.	Monocytes, Natural killer cells	PE	Beckton, Dickinson & company, New Jersey, USA	2 $\mu$ L
<b>CD16b</b>	Fc $\gamma$ RIIIb binds to the Fc region of IgG antibodies.	Neutrophils	PE	Beckton, Dickinson & company, New Jersey, USA	20 $\mu$ L
<b>CD3</b>	T cell co-receptor. Associated with T-cell receptor and $\zeta$ -chain to form TCR complex.	Lymphocytes (T cell co-receptor)	FITC	eBioscience Inc, San Diego, USA	2.5 $\mu$ L
<b>CD4</b>	Membrane glycoprotein co-receptor for TCR. Binds to MHC class II on the surface of antigen presenting cell, and initiates T cell activation and differentiation into T helper cells.	T cells, and also B cells, macrophages and granulocytes	PEcy7	eBioscience Inc, San Diego, USA	2.5 $\mu$ L
<b>CD8</b>	Transmembrane glycoprotein co-receptor for TCR expressed on cytotoxic T cells. Binds to MHC class I.	T cells	PerCy5.5	eBioscience Inc, San Diego, USA	2.5 $\mu$ L
<b>CD162 (PSGL-1)</b>	P-selectin glycoprotein ligand 1. High affinity for P-selectin but also binds to E-selectin and L-selectin. Involved in tethering and rolling of leucocytes on the endothelium.	Granulocytes, monocytes, T cells and B cells	APC	eBioscience Inc, San Diego, USA	5 $\mu$ L
<b>CD62L (L-selectin)</b>	Cell adhesion molecule expressed on leucocytes. Belongs to family of selectins, and mediates leucocyte-leucocyte interactions (secondary tethering). Also, acts as a homing receptor for lymphocytes to enter secondary lymphoid tissues.	Neutrophils, monocytes, lymphocytes	APC eflour780	eBioscience Inc, San Diego, USA	5 $\mu$ L
<b>CD11b</b>	Integrin $\alpha$ M (ITGAM) subunit that forms $\alpha$ M $\beta$ 2 (Mac1). Mediates the firm adhesion of leucocytes to endothelium.	Activated lymphocytes, monocytes, neutrophils and natural killer cells	Pacific Blue	Beckton, Dickinson & company, New Jersey, USA	10 $\mu$ L

**Table 2.2. The fluorochrome conjugated antibodies used for flow cytometry to identify specific cell surface markers**

## 2.3.8 Platelet flow cytometry

### 2.3.8.1 Flow cytometric assessment of platelet P- selectin expression

Two-colour whole blood flow cytometry was used to measure platelet P-selectin using previously published protocols and recommendations (Knight et al., 1997; Ritchie et al., 2000; Sidhu et al., 2004). There were 3 control tubes as follows: 2  $\mu\text{L}$  of P-selectin isotype control in addition to 2  $\mu\text{L}$  of CD42b were added to 41  $\mu\text{L}$  of HEPES buffer (150 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 3 mM  $\text{CaCl}_2$ , Sigma, made in MQ water). The second control tube had 2  $\mu\text{L}$  of CD42b and 43  $\mu\text{L}$  of HEPES buffer. The third and final control tube comprised of 2  $\mu\text{L}$  CD42b isotype control and 43  $\mu\text{L}$  HEPES buffer. 5  $\mu\text{L}$  of citrated whole blood was immediately added to each of the control tubes. For the samples in which P- selectin was to be measured, 2  $\mu\text{L}$  anti-CD42b and anti-CD62 were added to 40  $\mu\text{L}$  of HEPES buffer and finally 5  $\mu\text{L}$  citrated whole blood was added immediately. These samples were then treated with one of the following agonists: ADP (10  $\mu\text{M}$  or 3  $\mu\text{M}$ ), collagen (3  $\mu\text{g}/\text{ml}$  or 1  $\mu\text{g}/\text{ml}$ ) or PBS control (**Figure 2.14**) and incubated for 20 min at room temperature (21-23  $^{\circ}\text{C}$ ) after very gentle mixing and then fixed with 500  $\mu\text{L}$  of 1 % paraformaldehyde (Sigma, UK) and stored at 4 $^{\circ}\text{C}$  and then analysed using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.) on the same day.



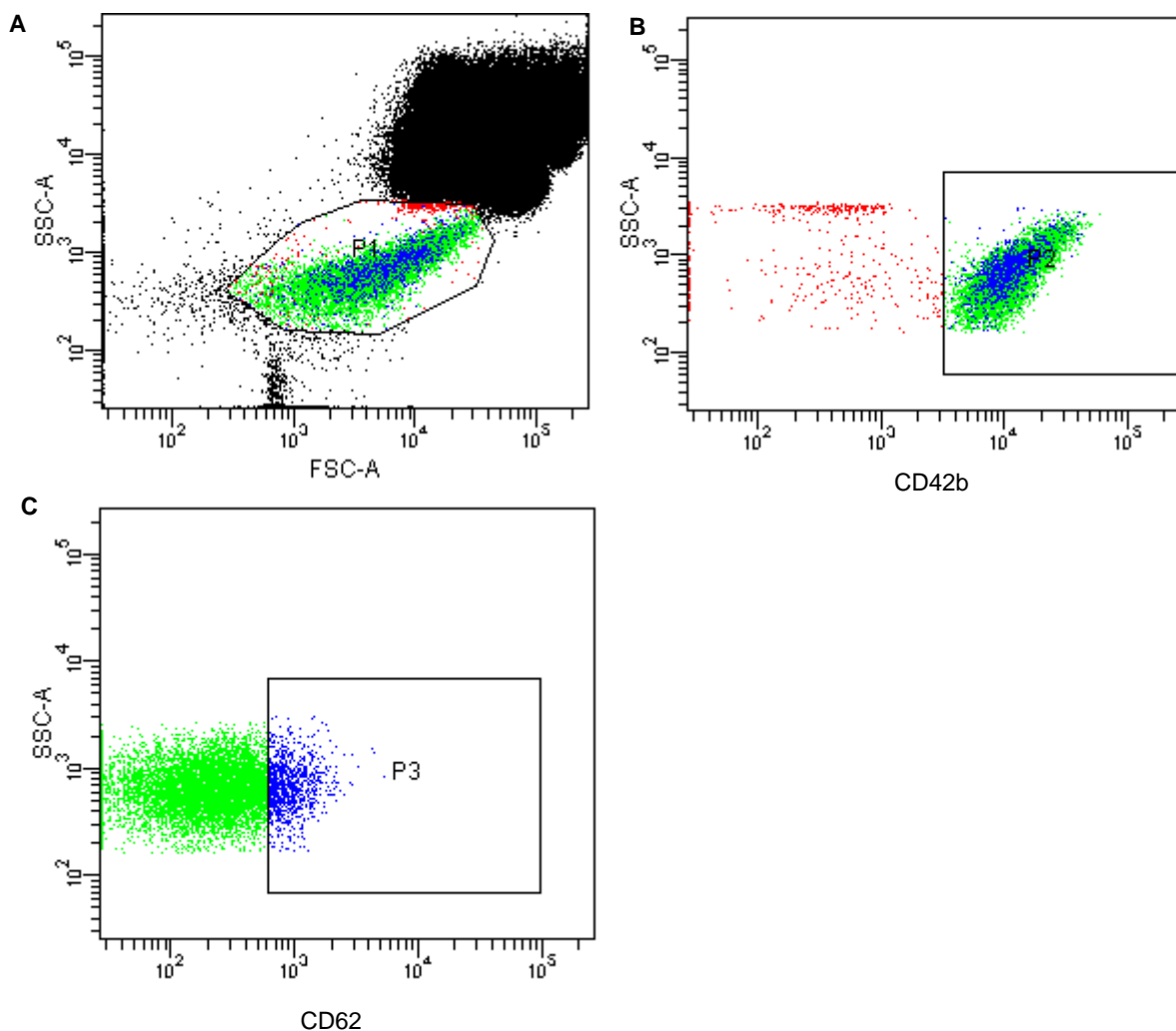
**Figure 2.14 Schematic of sample make-up for P-Selectin expression**

Lyophilised ADP (2.5 mg, Chrono-Par #384, Labmedics) was reconstituted in sterile saline to give a 1 mM stock. This stock solution was stored at -80 °C in aliquots and fresh aliquots used daily for each experiment/study. For each experiment, ADP was diluted in PBS to provide an end concentration of 10  $\mu$ M and 3  $\mu$ M. These dilutions were kept on ice until use on the day of the experiment and discarded after use.

Horm Collagen Suspension (Nycomed #1130630) was supplied as 1 mg/ml in isotonic glucose. This stock was stored at 4 °C and used as required to make fresh agonist diluted accordingly in isotonic glucose supplied by the manufacturer to provide a concentration of either 3  $\mu$ g/mL or 1  $\mu$ g/mL. Again, these dilutions were kept on ice the day of the experiment and discarded after use.

For P-selectin expression identification in blood samples, platelet population was identified preliminarily based on forward and side scatter properties, then further delineated via labelling with CD 42b monoclonal antibody conjugated to allophycocyanin (APC), (Biolegend, UK) (**Figure 2.15**). Gates were used to isolate

this population and CD62 (P-selectin; 0.4 ng/ $\mu$ L) monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) (ABD Serotec, UK) was used to determine P-selectin expression. Populations were further confirmed by use of antibody negative isotypes to P- selectin and CD42b (1:25). 10,000 platelets were acquired in the CD42b region. Results were expressed as the percentage of platelets positive for P- selectin.



**Figure 2.15. Dot plot of flow cytometry assessment of P-selectin expression**

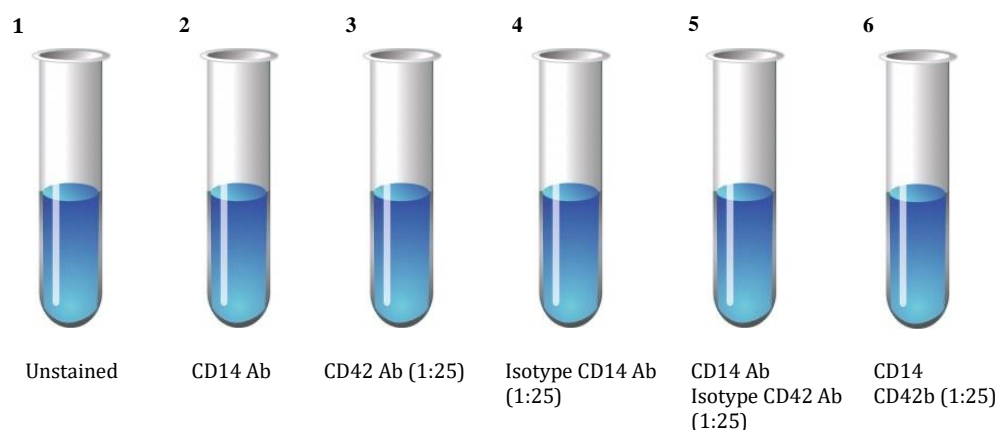
- A. Platelets were first identified based on their forward and side-scatter properties (P1)
- B. Then further delineated via labelling with CD42b monoclonal antibody conjugated to allophycocyanin (APC) (P2)
- C. CD62 (P-selectin) monoclonal antibody was used to determine P-selectin expression (P3)



### 2.3.8.2 Flow cytometric assessment of platelet monocyte aggregate expression

Platelet monocyte aggregates were measured using a modification of previously published protocols (Harding et al., 2007) with 2-colour lysed blood flow-cytometry. Initially, the monocyte population was identified based on forward and side scatter properties. This was then further delineated via labelling with CD14 monoclonal antibody (20  $\mu$ L per test as per manufacturer's instructions) conjugated to (fluoroscienisthiocyanate) FITC (BD Biosciences, UK). To isolate this population, gates were used and CD42b monoclonal antibody (1:25) conjugated to APC (Biolegend, UK) was used to determine platelets bound to the monocyte population, hence platelet monocyte aggregates.

Fluorescent antibodies selective for the platelet marker CD42b and CD14 (dilutions stated above) were incubated with 100  $\mu$ L whole blood samples in 5 ml polystyrene round bottom flow cytometry tubes (BD Falcon, UK) as per Figure below (**Figure 2.16**).

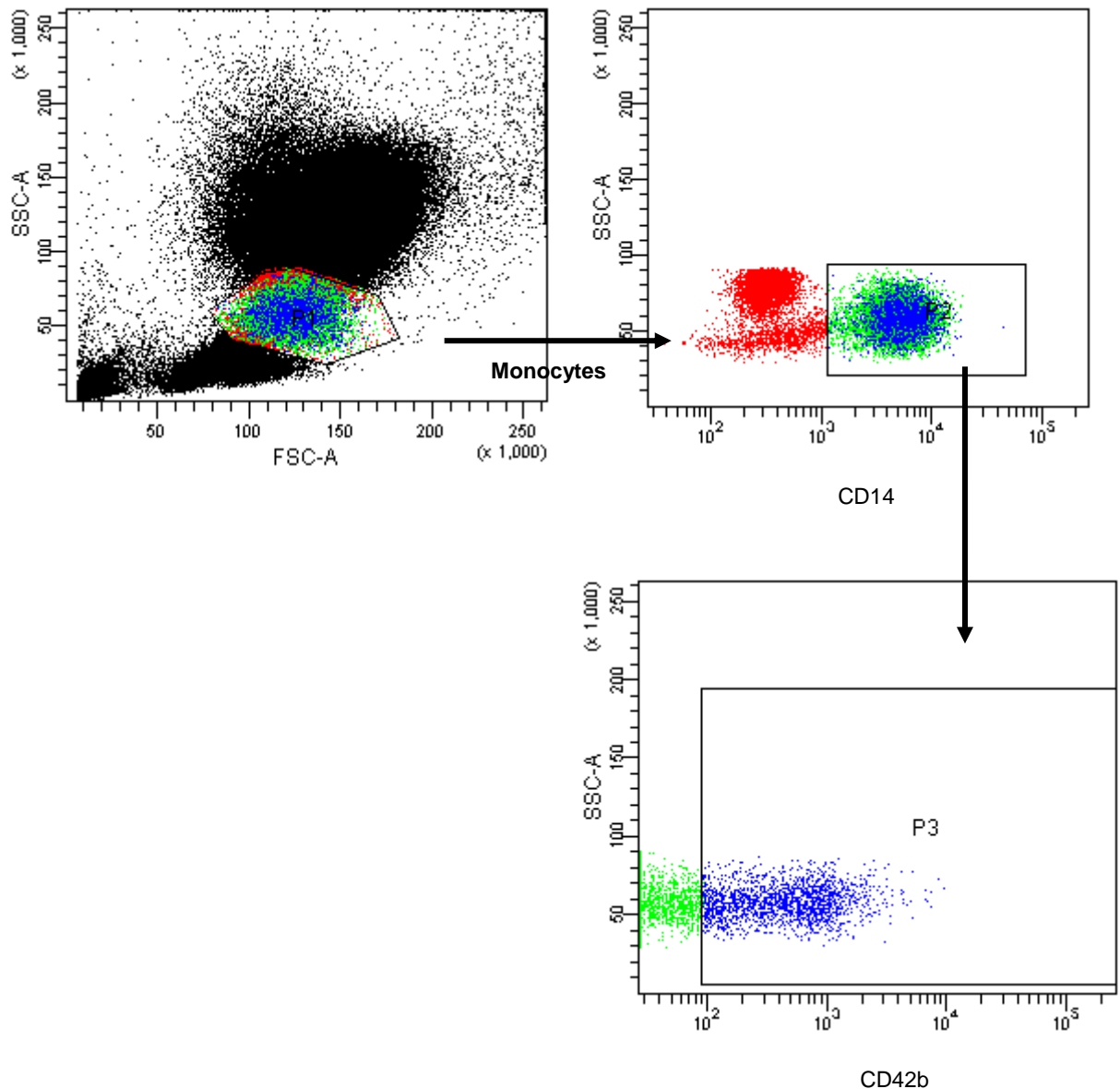


**Figure 2.16: Schematic of the antibody combinations used for platelet monocyte aggregates.**

Subsequently, 4 ml of PBS was added to each sample and all 6 samples were centrifuged at 400 g for 5 min at 21 °C. The supernatant was then discarded and 500 µL of Immunolyse solution (a whole blood lysing solution diluted in PBS (1:25)) was added to each sample. Each tube containing the samples were then vortexed twice with a 1 min interval. Following this, fixative solution was added (1:5) as supplied by the manufacturer and each sample vortexed again. Following this, 3 ml of PBS was added to each sample before centrifugation. Post centrifugation, the supernatant was discarded and 4 ml of PBS was added before a final centrifugation at 400 g for 5 min at 21°C. Finally, the supernatant was discarded and the sample was resuspended in 300 µL PBS before flow cytometry analysis. Samples were analysed immediately using a Becton Dickinson Fortessa flow cytometer (Becton Dickinson, San Jose, Calif.). Using BD-diva flow cytometry analysis software CD42b expression was measured as a percentage of the CD14+ population (see **Figure 2.17**).

### **2.3.9 Platelet aggregation**

Blood was collected into citrated blood collection tubes (3.2 % sodium citrate) (BD Biosciences, UK). Platelet aggregation was assessed in whole blood in response to PBS, ADP and collagen using impedance aggregometry (MultiplateR analyzer, Dyabyte Medical, Germany).



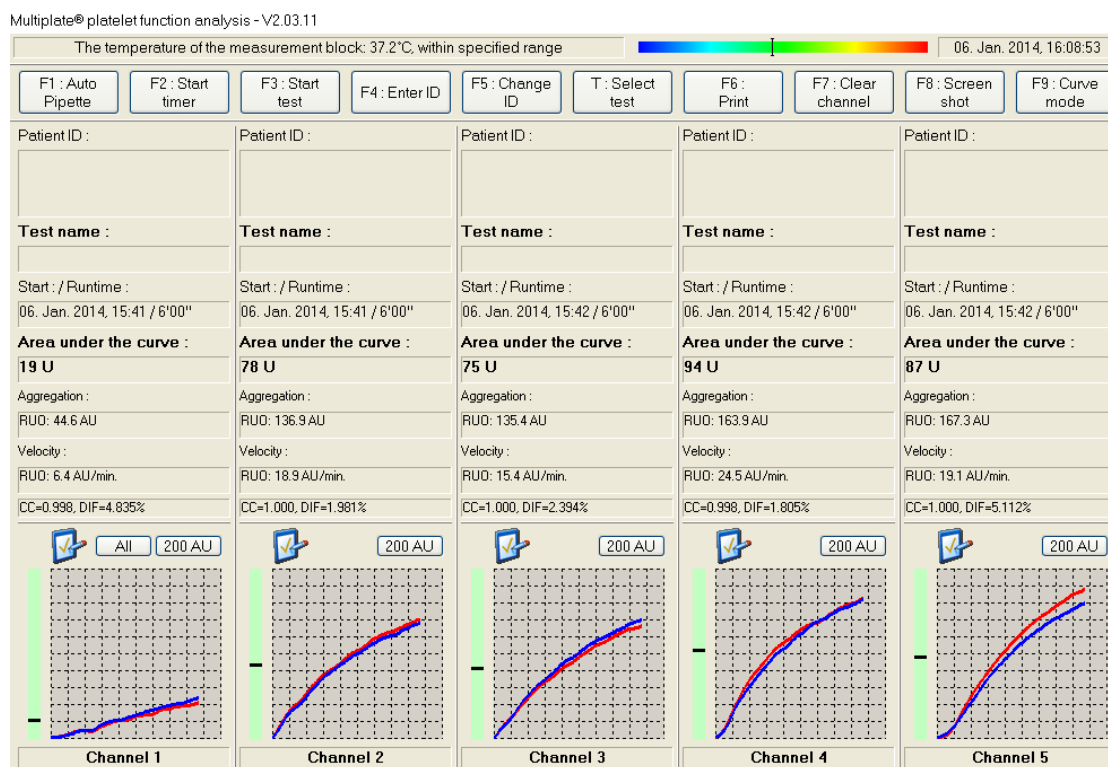
**Figure 2.17: Dot plot of typical gating of platelet population based on size and granularity (P1). P2 defines the CD14 positive population and P3 the CD42b positive subset of P2.**

### 2.3.9.1 Measurement of whole blood aggregometry

Impedance aggregometry is based on the principle that platelets are non-thrombogenic in their resting state, but expose receptors on their surface when they get activated which allow them to attach on vascular injuries and artificial surfaces. The device has five channels for parallel tests, and a single-use test cell with duplicate impedance sensors, each consisting of two straight silver coated electrode wires (blood contacting area: 3 mm length, 0.3 mm diameter for each

sensor wire). When platelets stick on the Multiplate® sensor wires, they enhance the electrical resistance (impedance) between the two electrodes, which is continuously recorded (**Figure 2.18**). This delivers automated quality control of measurements by comparison of the two simultaneously recorded single analyses. The method has been described previously (Tóth et al., 2006).

In the test cuvette, whole blood (300 µL) is diluted, 1:1, with 0.9% NaCL with 3 mmol/L CaCL<sub>2</sub> (Sigma, UK) solution maintained at 37°C for 3 minutes. At the end of this time, 12µl of agonist was added to give end concentrations of ADP of 10 µM or 3 µM (ChronoLog, Havertown, PA) and 3 µg/ml or 1 µg/ml of collagen (Nycomed, Munich, Germany). The ADP and collagen were made up as previously described in section 2.3.8.1. Phosphate buffered saline (PBS) was added to one test-cell as a control. The increase of impedance due to the attachment of platelets to the electrodes was detected for each sensor unit separately and transformed to arbitrary aggregation units (AU) that were plotted against time (AU\*min). The aggregation measured with this device was quantified as area under the curve (AUC) of AU\*min. (**Figure 2.18**)



**Figure 2.18: Screenshot of typical aggregation data.** Whole blood is incubated with PBS in channel 1, with ADP and collagen in channels 2-5 respectively.

### 2.3.10 Lipid Mediator Profiling

Blister exudates or plasma samples were added to 2 volumes of ice cold MeOH containing deuterated internal standards ( $d_4$ -LTB<sub>4</sub>,  $d_8$ -5S-HETE,  $d_4$ -PGE<sub>2</sub>,  $d_5$ -LXA<sub>4</sub> and  $d_5$ -RvD<sub>2</sub>, 500pg each). These were then kept at -20 °C for 45 minutes to allow for protein precipitation and subjected to solid phase extraction as per previous publication (Colas et al., 2014). Methyl formate fractions were then brought to dryness using a TurboVap LP (Biotage) and products suspended in water-methanol (50:50 vol:vol) for LC-MS-MS. A Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu, Kyoto, Japan), paired with a QTrap 5500 (ABSciex, Warrington, UK) were utilised and operated as described (Colas et al., 2014). To monitor each lipid mediator and respective pathways, a Multiple

Reaction Monitoring (MRM) method was developed with diagnostic ion fragments and identification using recently published criteria (Colas et al., 2014), including matching retention time (RT) to synthetic and authentic materials and at least six diagnostic ions for each lipid mediator. Calibration curves were obtained for each using authentic compound mixtures and deuterium labelled lipid mediator at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg. Linear calibration curves were obtained for each lipid mediator, which gave  $r^2$  values of 0.98 – 0.99. All analyses were conducted blind and conducted by the BartsLipidomic Unit led by Dr. Jesmond Dalli.

### **2.3.11 Cytokine/Chemokine analysis**

Cytokine/chemokine expression profiles were analysed from 50  $\mu$ L of blister fluid and plasma using a IDEXX ProCyte Dx® Haematology Analyser. This is a tool that utilises laser based flow cytometry, optical fluorescence and measurement of impedance to laminar flow to produce a readout of relative numbers and percentages of IL-6, IL-8, IL-10, CXCL1, CXCL2, CCL5 and CCL2. All analyses were conducted blind and were analysed by Labospace in Italy.

### **2.3.12.1 Measurement of nitrate and nitrite levels in plasma, saliva and urine samples [Ozone chemiluminescence]**

Following collection into EDTA tubes as described in section 2.3.1, blood samples were centrifuged immediately (1300g, 4°C, 10 min) and the supernatant then deproteinated. Deproteination was achieved by means of filtering through Microcon® Ultracel YM\3 (3kDa) (Millipore Corporation, Billerica, USA) filters via

centrifugation at 15000g for 60 min at 4°C. Prior to deproteination, the filters were washed twice with NO<sub>3</sub><sup>-</sup>- and NO<sub>2</sub><sup>-</sup>-free water to remove any possible NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> contamination. Deproteinated samples were then snap frozen until analysis using ozone chemiluminescence.

[NO<sub>x</sub>] of urine, saliva and deproteinated plasma samples was determined using ozone-based chemiluminescence as described previously (Ignarro et al., 1993b).

Ozone chemiluminescence is a highly sensitive and reproducible method of quantifying nanomolar quantities of NO and NO related species in biological fluids. The carrier gas, nitrogen, was bubbled through the sample where NO production was occurring. This was then passed on to the reaction cell and was quantified using the NO analyser, Sievers Nitric Oxide Analyser 280. In the reaction cell, NO reacted with ozone (O<sub>3</sub>), which generated nitrogen dioxide (NO<sub>2</sub><sup>\*</sup>) in an excited state (1). When nitrogen dioxide relaxes back to the ground state (NO<sub>2</sub>), a photon of a specific wavelength was emitted (2). The emitted light then goes through a filter and is intensified in a photomultiplier tube and quantified in the analyser. The intensity of the light emitted correlates to the amount of NO present initially in the reaction. Thus, using appropriate reducing agents nitrate and nitrite species can be reduced to NO and quantified. To ensure that standard curves generated were precise, with minimal variability between different days, control standards were run.



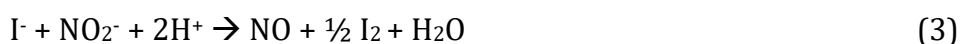
**Equation 2.1: Reaction of NO with O<sub>3</sub> to produce excited nitrogen dioxide (NO<sub>2</sub><sup>\*</sup>).**



**Equation 2.2: Stabilisation of excited nitrogen dioxide.**

### **2.3.12.1.1 Determination of nitrite levels**

NO can be formed via the reduction of nitrite. When NO is then reacted with O<sub>3</sub>, NO<sub>2</sub><sup>-</sup> is formed which can be quantified based on the intensity of light signal released when the excited species returns to ground state. The reducing agent used to measure nitrite levels in samples is 1% potassium iodide (KI, Sigma Aldrich, UK) in glacial acetic acid (C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>, AnalaR NORMAPUR, VWR, UK). 10ml of this reducing agent was injected into the purge chamber and bubbled with nitrogen for 20 minutes to eliminate any oxygen present in the vessel and for the baseline to stabilize, before standard and sample injections (**Figure 2.19**). Nitrite molecules available in samples react with the reducing agent to form NO (3).



**Equation 2.3: The overall reduction reaction of NO<sub>2</sub><sup>-</sup> to NO in the presence of iodide (I<sup>-</sup>)**

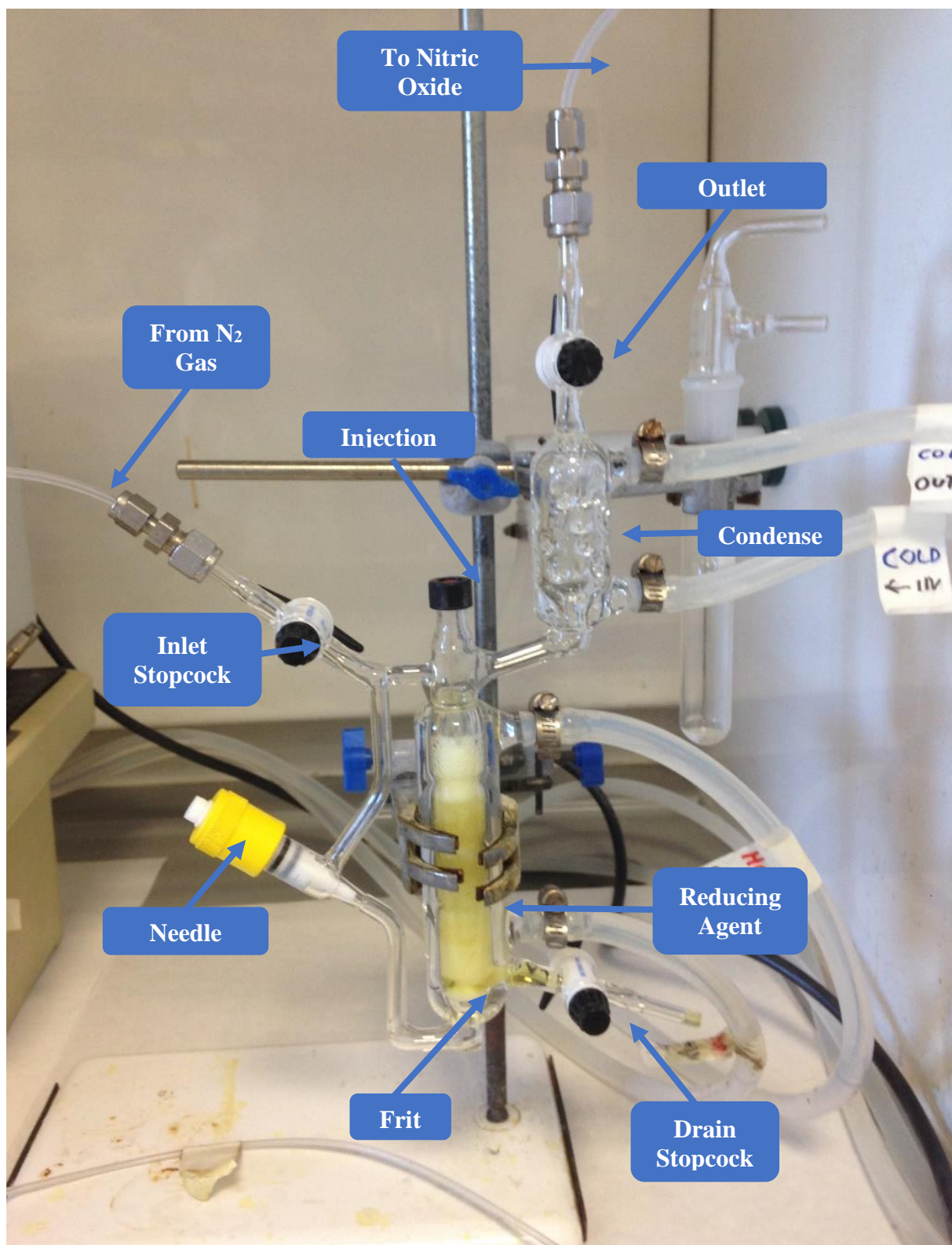
Calibration curves, using known standards of sodium nitrite, were constructed every day. This was done by dissolving 0.069g of sodium nitrite (NaNO<sub>2</sub>, Sigma Aldrich) in 1L of MQ water, which forms 1mM of stock solution. The stock solution was used to prepare dilutions of 10µl, 1µl and 0.1µl. Three MQ water samples of 25µl were injected first to ensure a baseline reading with no peaks. The nitrite standards were then run in order of the most diluted 0.1µl followed by 1µl and



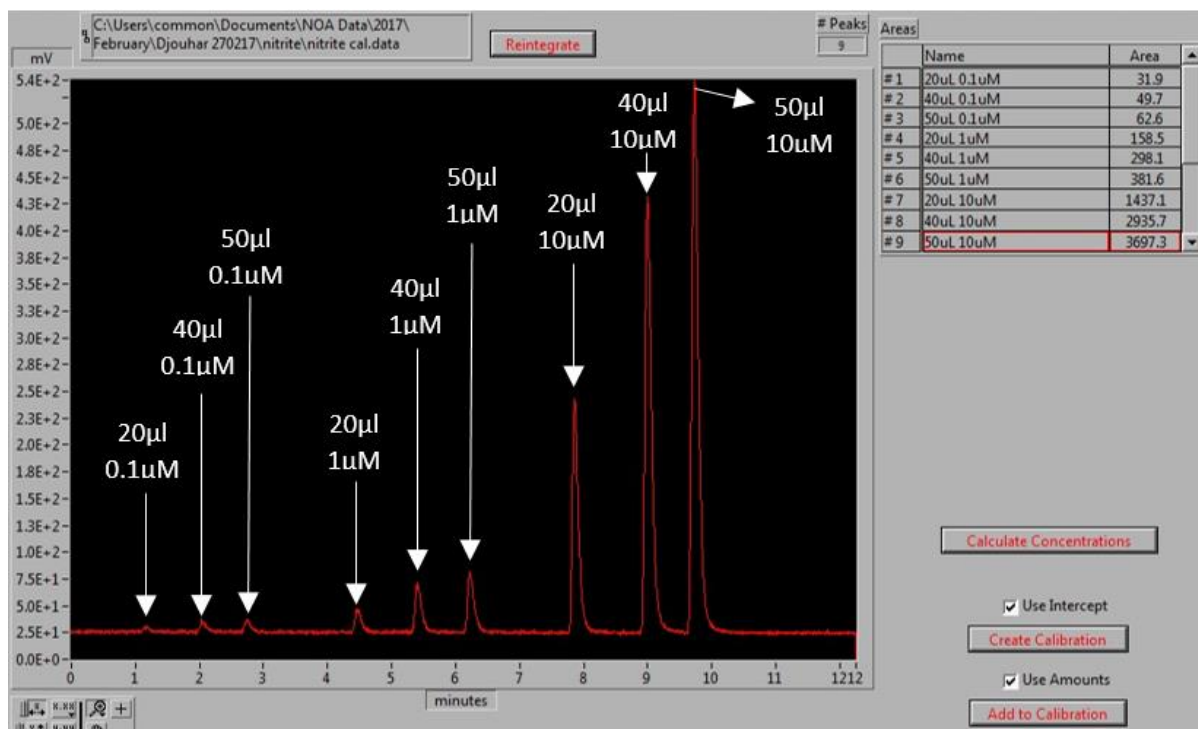
10µl. Volumes of 20µl, 40µl and 50µl were used for each of the dilutions and a sodium nitrite calibration curve was created, as shown in **Figure 2.20 & Figure 2.21**. **Table 2.3**, summarises the dilutions of each of the samples as well as volume injected. Samples were injected in duplicates to obtain mean concentrations for each sample.

<b>Sample</b>	<b>Dilution</b>	<b>Volume</b>
<b>Plasma</b>	No	25µl
	Dilution	
<b>Urine</b>	No	30µl
	Dilution	
<b>Saliva</b>	1:100	10µl

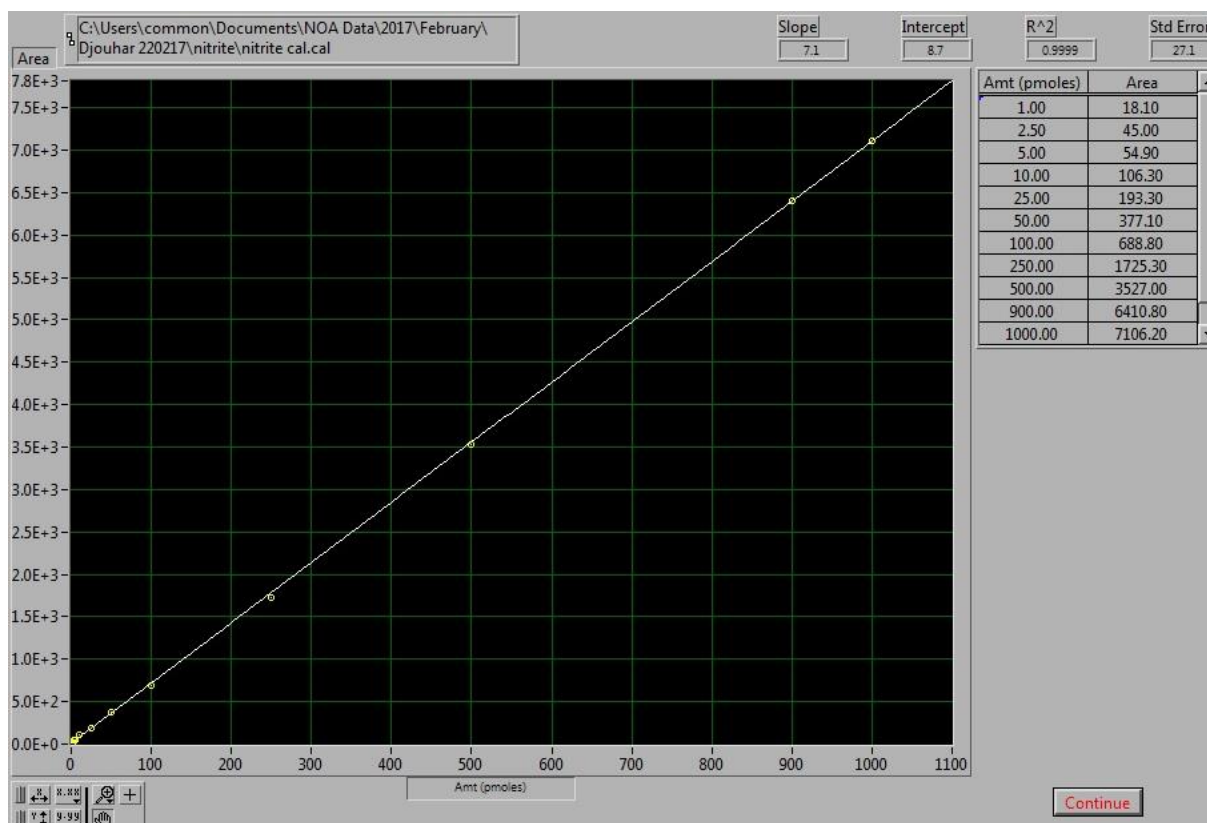
**Table 2.3: Dilutions and volumes injected of the different sample types injected into the purge vessel to measure nitrite levels.**



**Figure 2.19: Photograph of the ozone chemiluminescence setup for  $\text{NO}_2^-$  measurement.** To determine the  $\text{NO}_2^-$  concentration, samples were added to 0.09 M potassium iodide (KI) in glacial acetic acid refluxing under  $\text{N}_2$  at room temperature (equation 2.3). Initially a calibration curve is made based on the final dilutions from a stock solution of sodium nitrite ( $\text{NaNO}_2$ ). Refer to figure 2.15 for an example trace obtained from different injection volumes and concentrations of standards and figure 2.16 for a calibration curve after integration of each peak to the correct standard ( $\text{NO}_2^-$ =nitrite; KI=potassium iodide;  $\text{NaNO}_2$ =sodium nitrite).



**Figure 2.20: Graph of the generation of the NaNO<sub>2</sub> calibration curve.** The peaks represent the different volumes and concentrations of NaNO<sub>2</sub> standards, for which the area under the each peak is determined.

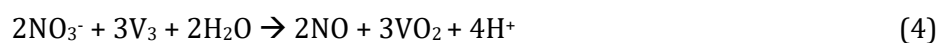


**Figure 2.21: Graph of a typical standard curve produced prior to the measurement of  $\text{NO}_2^-$  in liquid samples (slope=7.1; intercept=1.2;  $R^2=0.9999$ ) ( $\text{NO}_2^-$ =nitrite).**

### 2.3.12.1.2 Determination of nitrate levels

The reducing agent used in determining nitrate levels is a 0.1M solution of vanadium chloride ( $\text{VCl}_3$ , Sigma Aldrich, UK) dissolved in 1M hydrochloric acid (HCl, Sigma Aldrich, UK). 6ml of the solution was dispensed into the purge vessel at 95°C and nitrogen was bubbled through. This is a stronger reducing agent and reduces both nitrite and nitrate molecules present in a sample, collectively known as  $\text{NO}_x$ . A gas bubbler filled with 8ml of 1M sodium hydroxide (NaOH, Sigma Aldrich, UK), was connected between the purge vessel and NO analyser (NOA 280A, Sievers), to prevent the HCl vapour from damaging the analyser (**Figure 2.22**).

0.085g of NaNO<sub>3</sub> (Sigma Aldrich, UK) was dissolved in 1L of MQ water to prepare 1mM of stock solution. The stock solution was used to prepare the dilutions of 100µM, 10µM and 1µM. For the NO<sub>x</sub> calibration curve to be created the following injections of standards were made: 20µl, 40µl and 50µl of the 1µM, 20µl, 40µl, 50µl and 70µl of the 10µM and 10µl of the 100µM dilution; as shown in **Figures 2.23 & Figure 24**. Vanadium reacts with the nitrate available in the sample to form NO (4). As this setup determines the NO<sub>x</sub> levels in samples, by subtracting the NO<sub>2</sub><sup>-</sup> levels obtained from the previous step, the amount of NO<sub>3</sub><sup>-</sup> in each sample can be calculated (5).



**Equation 2.4 Reduction of NO<sub>3</sub><sup>-</sup> by vanadium (III) chloride to produce NO.**

$$[\text{NO}_x] - [\text{NO}_2^-] = [\text{NO}_3^-] \quad (5)$$

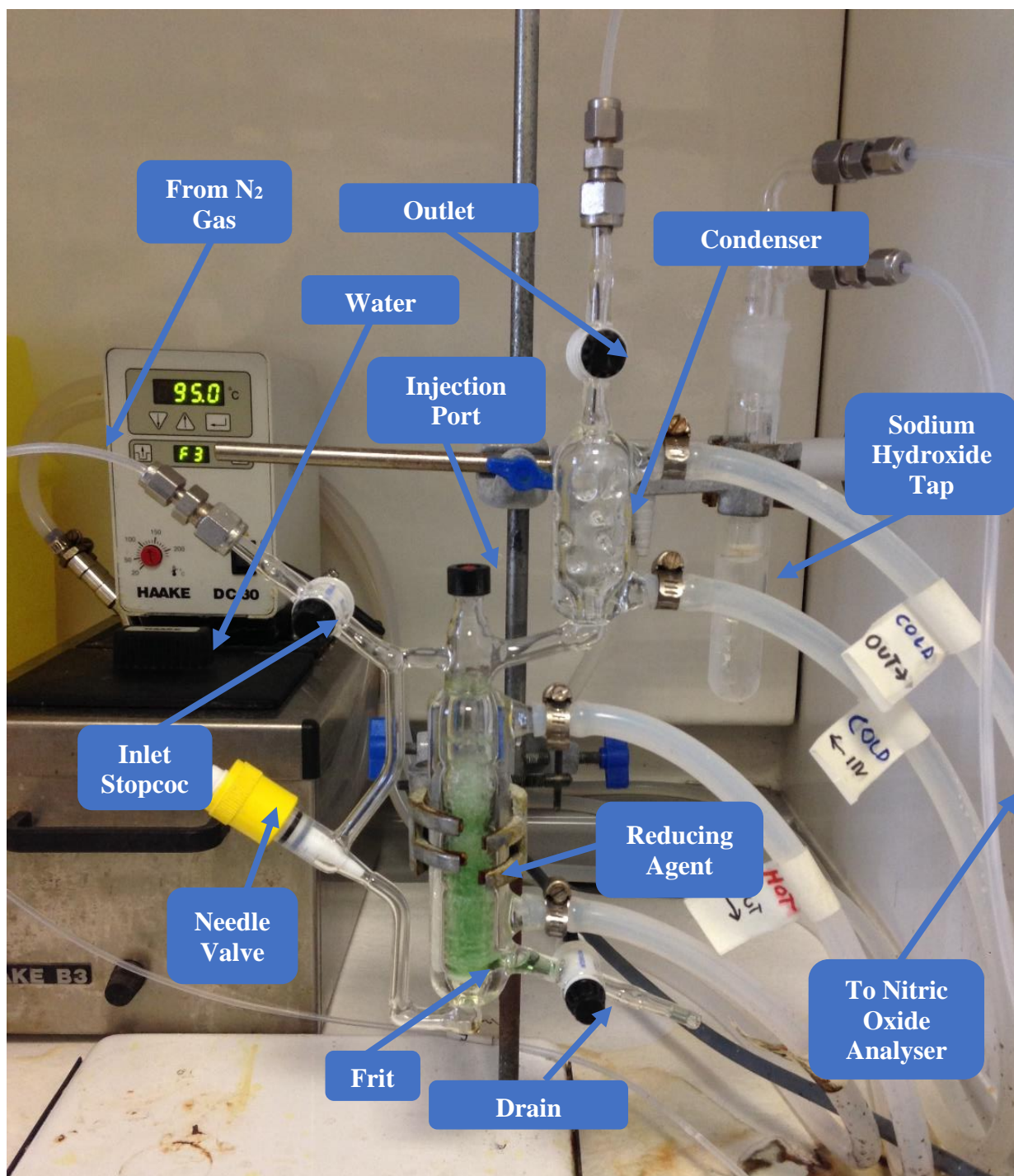
**Equation 2.5: Subtracting the NO<sub>2</sub><sup>-</sup> measurement from the NO<sub>x</sub> value gives a measure of the NO<sub>3</sub><sup>-</sup>.**

**Table 2.4**, summarises the dilutions of each of the samples as well as volume injected. Samples were injected in duplicates to obtain mean concentrations for each sample.

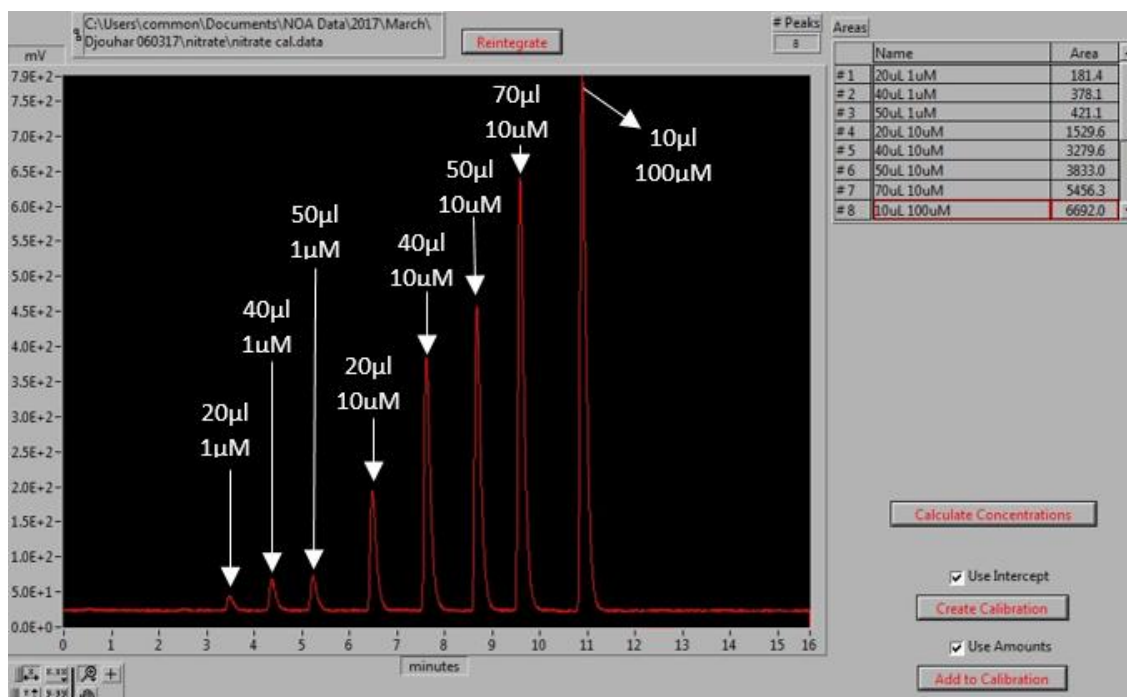
	<b>Dilution</b>	<b>Volume</b>
<b>Sample</b>		
<b>Plasma</b>	1:10	10 $\mu$ l
<b>Urine</b>	1:100	20 $\mu$ l
<b>Saliva</b>	1:100	10 $\mu$ l

**Table 2.4: Dilutions and volumes injected of the different sample types injected into the purge vessel to measure NO<sub>x</sub> levels.**



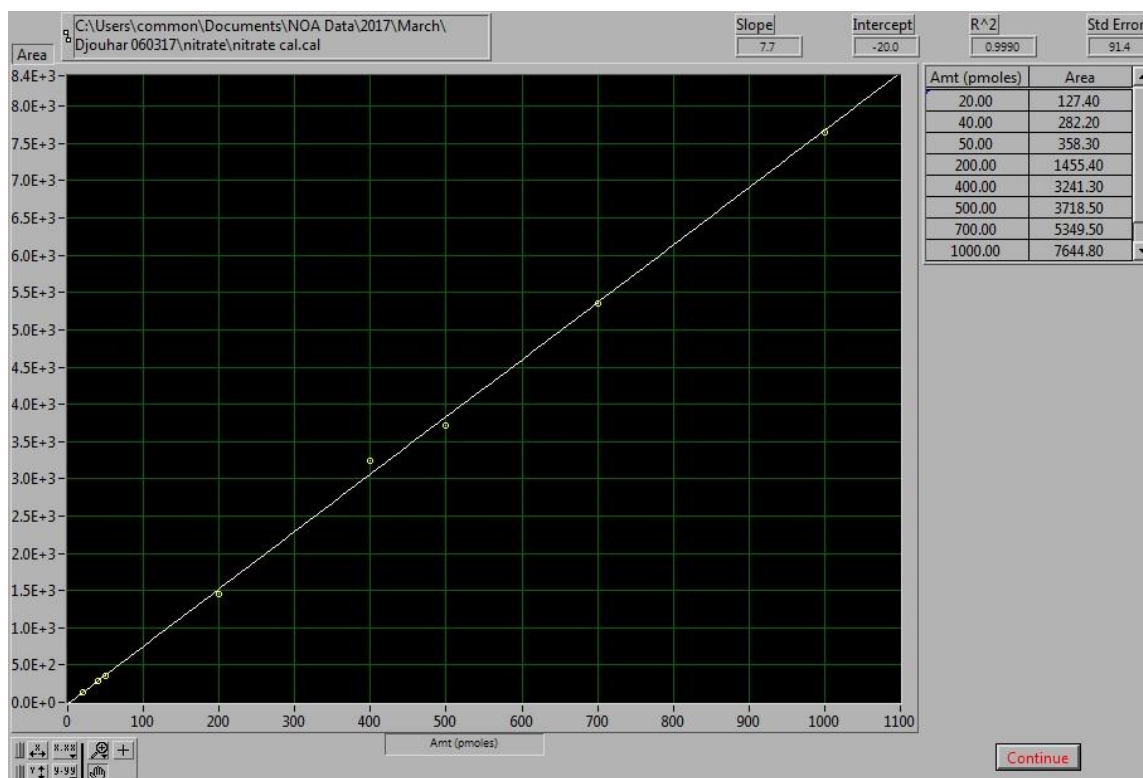


**Figure 2.22: Photograph of the ozone chemiluminescence setup for NO<sub>x</sub> determination.** A gas bubbler containing 1 M sodium hydroxide (NaOH) was installed between the purge vessel and the NOA to prevent HCl vapour damaging the NOA (NOA=nitric oxide analyser; HCl=hydrochloric acid; NaOH=sodium hydroxide).



**Figure 2.23: Graph of the generation of the NaNO<sub>3</sub> calibration curve.** The peaks represent the different volumes and concentrations of NaNO<sub>3</sub> standards, for which the area under the each peak is determined.





**Figure 2.24: Graph of the  $\text{NaNO}_3$  Calibration curve.** The calibration curve was generated from known  $\text{NaNO}_3$  standard solutions.

### 2.3.13 Measurement of plasma [cyclic GMP]

For the majority of the physiological functions that occurs due to NO, cGMP plays an important role as a second messenger. Therefore, by determining the levels of cGMP in plasma samples collected at visit 2, baseline values can be established, which theoretically should be similar in both groups. cGMP levels measured at the 6 months can then be compared to the baseline values. Thus, any changes in these levels would be due to the treatment. It is also worth noting that cGMP acts as a second messenger for atrial natriuretic factor. However, since the intervention in the study is  $\text{NO}_3^-$  (and hence NO), we can assume that the levels of atrial natriuretic factor will remain constant between the groups.

Blood was collected into tubes containing the non-competitive non-selective phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100  $\mu$ M) prior to centrifugation at 14,000 g for 10 min. Plasma was then collected and immediately snap frozen and stored at -80°C pending measurement.

### **2.3.13.1 cGMP assay**

The ELISA kit (cGMP enzymeimmunoassay Biotrak system RPN226, GE Healthcare, Little Chalfont, UK) was used to measure plasma cGMP levels and protocol followed was from the data sheet ([www.gelifesciences.com](http://www.gelifesciences.com), GE Healthcare, Little Chalfont, UK). Competitive enzymeimmunoassay is a biochemical test used in determining the concentration of specific macromolecules in a sample. In this highly specific and sensitive assay, the antigen in the sample competes for a limited antigen binding site on the anti-cGMP antibody (**Figure 2.25**). The unlabelled cGMP from the unknown sample or standard competes with fixed quantities of labelled horse raddish peroxidase conjugated cGMP. Thus, the samples with high levels of cGMP result in higher levels of unlabelled cGMP being bound to the anti-cGMP antibody, compared with the conjugated cGMP. The tetramethylbenzidine substrate added will then only bind to the labelled cGMP, developing a blue colour. Therefore, there is an inverse correlation between the intensity of the blue colour formed, after the addition of the chromogenic substrate, and the concentration of unlabelled cGMP in the sample or standard. This relationship can be used to plot a standard curve and extrapolate unknown cGMP concentrations from samples.

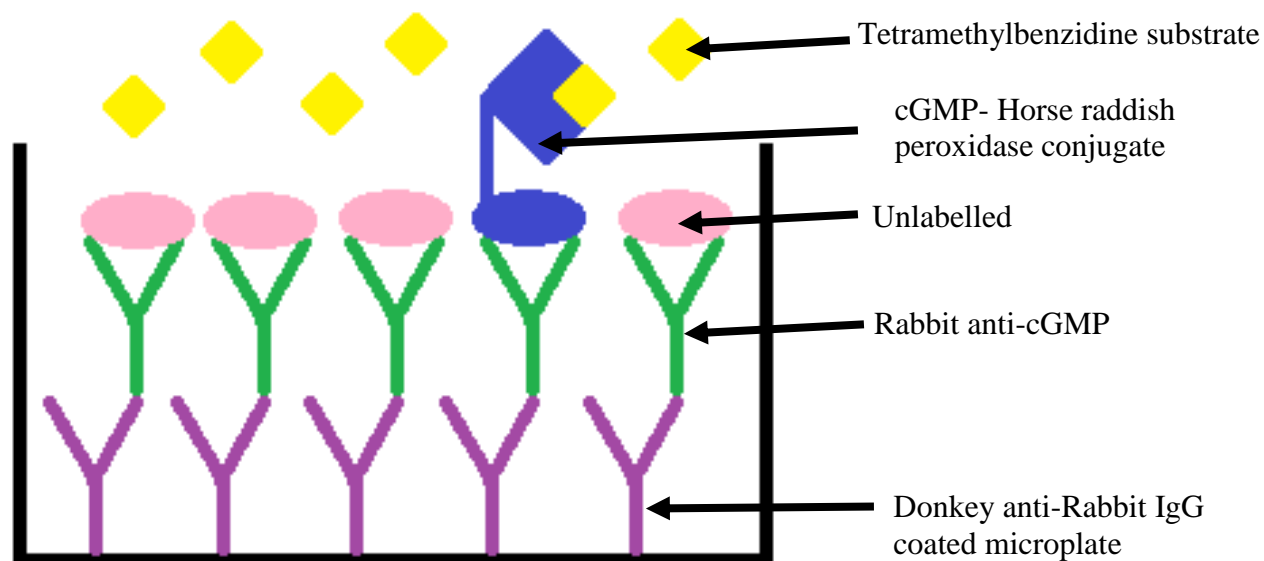


Figure 2.25: Schematic of the principle of the cGMP competitive enzymeimmunoassay system.

### 2.3.13.2 Determination of plasma cGMP levels

A 1:2 dilution of acetylation standards were prepared. The unknown plasma samples where then diluted 1:20 with assay buffer (25 $\mu$ l plasma and 475 $\mu$ l assay buffer). The assay buffer consisted of 0.05M sodium acetate buffer pH 6.0 containing 0.002% (w/v) bovine serum albumin and 0.01% (w/v) preservative. 50 $\mu$ l of acetylating reagent (2:1 of triethylamine and acetic acid) was added to each of the diluted plasma samples and standards and vortexed immediately. 100 $\mu$ l of antiserum (cGMP antibody) was dispensed into all wells in a 96-well plate (which had all been pre-coated with donkey anti-rabbit IgG) except blank and non-specific binding (NSB). 50 $\mu$ l of all the standards and unknown samples where dispensed into their appropriate wells, in duplicates. 150 $\mu$ l of assay buffer was dispensed into the NSB well. The plate was then covered, gently mixed and incubated at 4°C for 2hr. After incubation, 100 $\mu$ l of diluted conjugate of lyophilized

cGMP conjugated to horseradish peroxidase was added into all wells except blank. The plate was then covered again and incubated at 4°C for 1hr. The plate was then washed 4 times with wash buffer (0.01 M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween™ 20) and 200ul of enzyme substrate (3,3',5'5'-tetramethylbenzidine (TMB)) was added to all wells. The plate was incubated for 30 minutes at room temperature. The colourless substrate TMB is converted into blue by the peroxidase. Using a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK), the plate was read at  $\lambda=450\text{nm}$  after the addition of 100 $\mu\text{l}$  1M of sulphuric acid into all wells (which turns the blue colour into yellow). The colour change depends on the amount of enzyme linked to the well and is inversely proportional to the cGMP concentration. Each sample and standard were analysed in duplicate.

As shown in **Figure 2.26**, the standard curve was then plotted and the concentration of cGMP of unknown samples was calculated.

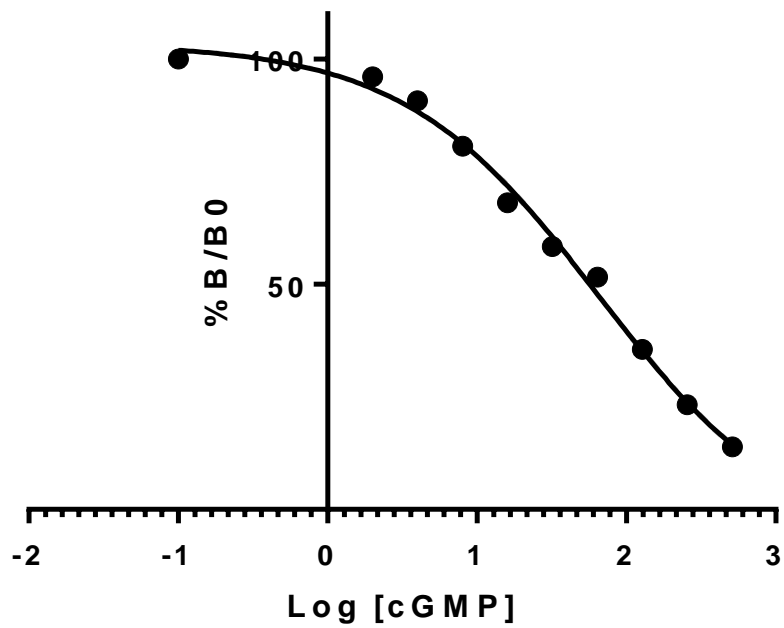


Figure 2.26: Typical standard curve generated from known standards.

### 2.3.14 Met haemoglobin measurement

A finger probe pulse oximeter (Massimo Signal Extraction technology) was used to measure Sp Met Hb level in all participants during each study visit. Pulse oximetry is based upon two physical principles. The light absorbance of oxygenated haemoglobin is different from that of reduced haemoglobin, at the oximeter's two wavelengths, which include red and near infrared light; and the absorbance of both wavelengths has a pulsatile component, which is due to the fluctuations in the volume of arterial blood between the source and the detector.

## **2.4 Clinical study design and execution**

In terms of overall design and execution of these studies, I helped to design the two healthy volunteer studies as well as the phase II clinical trial. I developed the protocol and applied for ethical approval. I recruited patients for all three studies and carried out the clinical and biochemical measurements.

The measurement and analysis of the lipid mediators were performed by Dr. Jesmond Dalli's lab at the William Harvey Research Institute. The cytokine and chemokine measurements were performed and analysed by Labospace in Italy and I am grateful to both labs for their help and guidance.

## Chapter 3.0

# **Investigating the influence of sex differences on inflammation-induced endothelial dysfunction in healthy volunteers**

### **3.0 Investigating the influence of sex on inflammation-induced endothelial dysfunction in healthy volunteers**

#### **3.1 Acute low grade systemic induced-inflammation to assess endothelium dependent dilatation**

Observational as well as epidemiological studies have suggested an association between infection or inflammation and risk of CVD (Liuzzo et al., 1994; Maseri, 1997; Quartin et al., 1997; Ridker et al., 1997; 1998a; Ridker et al., 1998b; Tillett et al., 1983; Valtonen et al., 1993). Two patterns of association have emerged: an association between an acute systemic inflammatory response and a transiently increased risk of an acute cardiovascular event (Liuzzo et al., 1994; Quartin et al., 1997; Tillett et al., 1983; Valtonen et al., 1993) and a link between chronic low-grade inflammation/infection and the slow process of atherogenesis (Ridker et al., 1997; 1998a; Ridker et al., 1998b).

##### **3.1.1 To investigate these associations**

Previously, to initiate a systemic inflammatory response, studies have used an intramuscular injection of the capsular polysaccharide typhoid vaccine Typhim Vi (Cooper et al., 1992; Plotkin and Bouveret-Le Cam, 1995). In the 8 hours after vaccination, the vaccine produced a mild leukocytosis without changes in BP or heart rate. In addition, there was an increase in IL-6 (a proinflammatory cytokine) (Hingorani et al., 2000a). In addition to the pro-inflammatory cytokine response, these studies have also demonstrated that inflammation caused by typhoid vaccination causes an increased oxidant stress and impairs forearm arterial endothelium dependent dilatation, suggesting that even a relatively mild systemic



inflammatory response is associated with significant alteration in endothelial function of a type commonly thought to be associated with increased cardiovascular risk (Calver et al., 1992; Chowienczyk et al., 1992; Gerhard et al., 1996; Panza et al., 1990). If this model accurately reflects the pathology of systemic inflammation, then this may indicate a mechanism by which inflammatory events may lead to a transient rise in the incidence of cardiovascular events.

Systemic inflammation that is far more severe and long-lasting than the insult produced by vaccination occurs in a wide variety of infective disorders and after iatrogenic procedures such as major abdominal surgery. There is growing evidence that acute systemic inflammation is associated with an increase in the risk of cardiovascular events that may persist for days or weeks (Meier et al., 1998; Tillet et al., 1983). There is also evidence that unstable angina is associated with inflammation (Liuzzo et al., 1994), which might precede the onset of the syndrome.

We therefore decided to use the typhoid-induced model of systemic inflammation to further interrogate the changes in endothelial regulation of vascular tone in the arterial circulation but also to determine whether this model might be useful ultimately in interrogating the potential beneficial effects of inorganic  $\text{NO}_3^-$  upon inflammation. Although atherosclerosis is predominantly a disease of chronic inflammation, we tested the hypothesis that endothelial dysfunction underlies the association between an acute inflammatory episode and the transiently increased risk of a cardiovascular event by examining the effects of an acute experimental

inflammatory stimulus (using the typhoid vaccine) on endothelium-dependent vasodilation as utilised previously (Hingorani et al., 2000a). To test these methodologies, we carried out studies by assessing differences between the sexes in the first place to establish the model in our laboratory I investigated the responses to typhoid in both men and women to determine whether the response to systemic inflammation is different between the sexes.

As discussed above, systemic inflammation plays a critical role in the progression of cardiovascular disease and since inflammation plays a key role in the process of restenosis, we initially developed a systemic assay of inflammatory mediators to assess endothelial function. Furthermore, to test these methodologies, we carried out studies by assessing differences between the sexes.

### **3.3 Results**

#### **3.3.1 Baseline demographics**

Healthy volunteers were recruited between 2013 to 2015. Baseline data for the 24 healthy volunteers recruited into the typhoid study demonstrates that the volunteers were well matched in terms of age. Expected differences between the sexes were observed in BMI, SBP and DBP (**Table 3.1**). For examples, whilst the volunteers had similar age, the men ha higher BMI and higher BPs. This is typical of a healthy volunteer cohort and has been well documented.

**Table 3.1.** Demographics of healthy volunteers in the typhoid study. Data are shown as mean  $\pm$  s.e.mean with statistical significance determined using Student's unpaired t-test.

	Typhoid Study		P Value
	Male	Female	
<b>Volunteers (n)</b>	12	12	
<b>Age (years)</b>	26.0 $\pm$ 5.7	24.7 $\pm$ 6.8	0.59
<b>BMI (Kg/m<sup>2</sup>)</b>	24.8 $\pm$ 3.2	21.8 $\pm$ 2.9	0.021
<b>Baseline SBP (mmHg)</b>	119.5 $\pm$ 1.8	107.5 $\pm$ 2.4	0.0005
<b>Baseline DBP (mmHg)</b>	65.6 $\pm$ 3.0	67.6 $\pm$ 1.2	0.55
<b>Heart rate (bpm)</b>	69.4 $\pm$ 2.8	65.7 $\pm$ 2.6	0.36
<b>Oral contraceptive</b>	-	4	

### 3.3.2 Typhoid vaccine induces a low grade systemic inflammation in both males and females

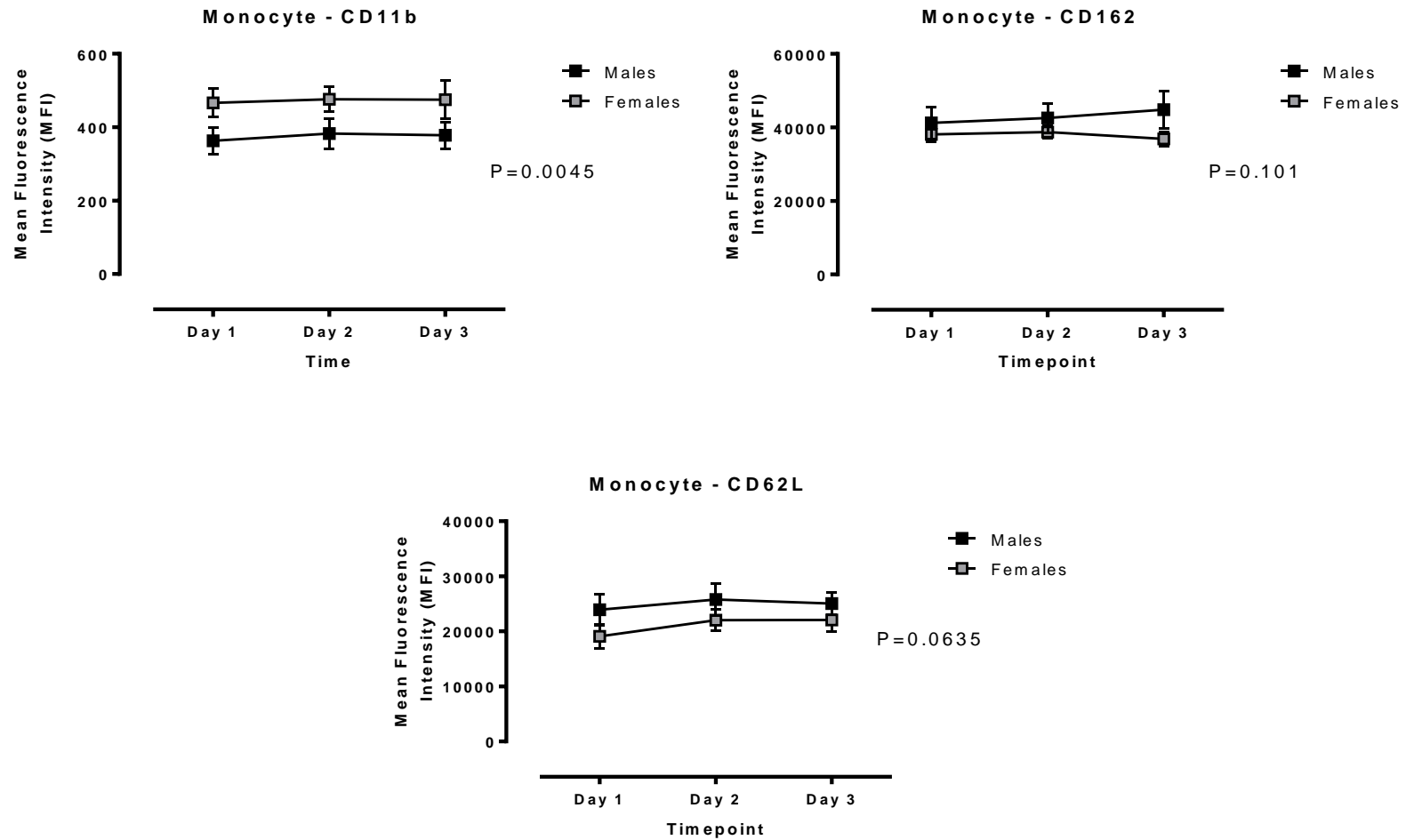
Typhoid vaccination resulted in a rise in circulating leucocyte numbers in both sexes due almost entirely to a neutrophilia (**Table 3.2**), as per previous reports (Hingorani et al., 2000b).

**Table 3.2.** Body temperature, blood differentials and CRP levels in male and female healthy volunteers at baseline and at 8 and 24 hours post typhoid vaccination. Data are shown as mean  $\pm$  s.e.mean with statistical significance determined using using two-way ANOVA with post-hoc Sidak's tests shown as \* for P<0.05 and \*\* for P<0.01 for male versus female and §§ for P<0.01, §§§ for P<0.001 and §§§§P<0.0001 for within group versus baseline comparison.

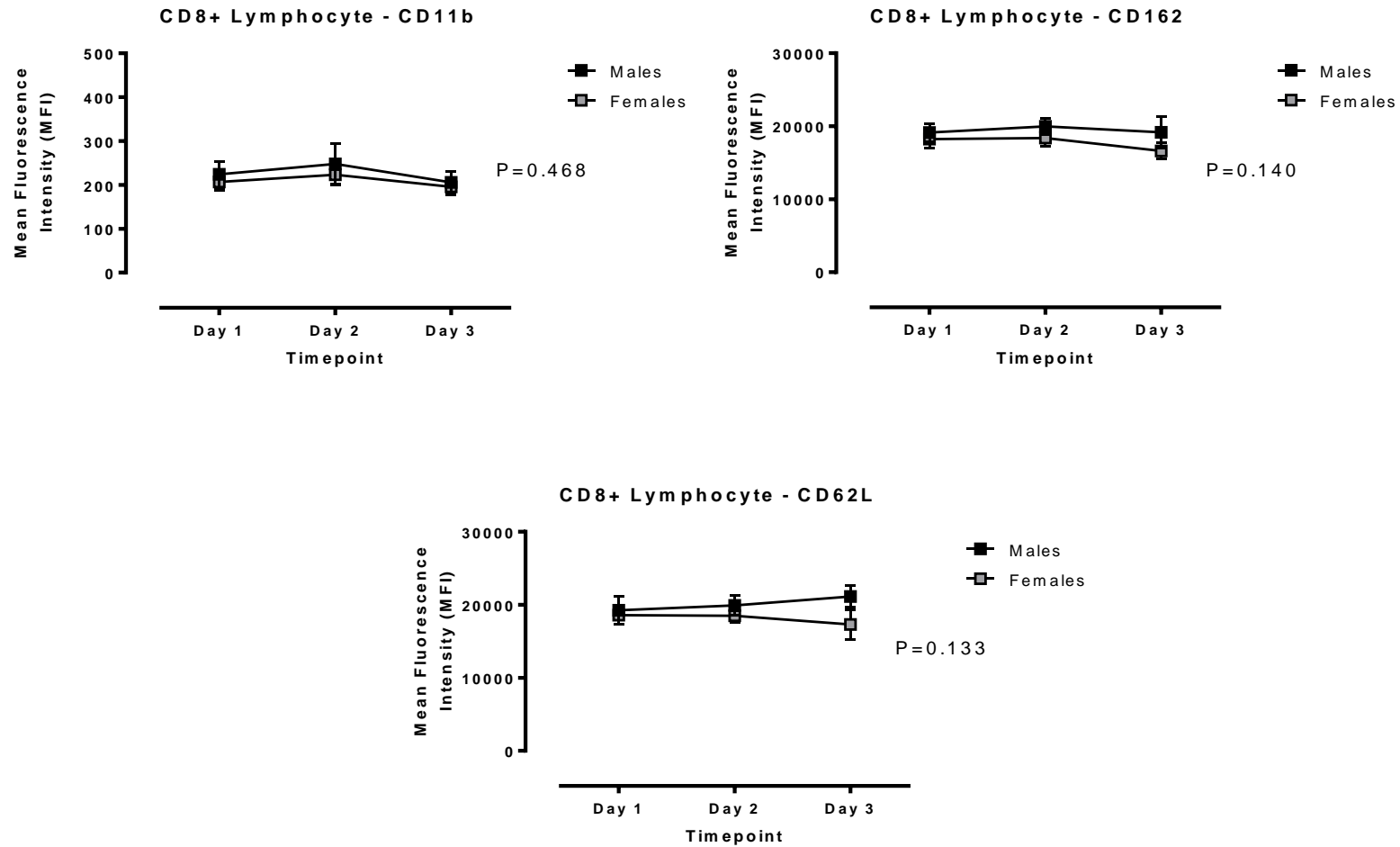
Sex	Time-point	Temp °C	Hb (g/dL)	Platelets (x10 <sup>9</sup> /L)	WBC (x10 <sup>11</sup> /L)	Neutrophils (x10 <sup>9</sup> /L)	Lymphocytes (x10 <sup>9</sup> /L)	Monocytes (x10 <sup>9</sup> /L)	CRP (mg/L)
Male	Baseline	36.0 $\pm$ 0.1**	14.8 $\pm$ 0.3**	223.4 $\pm$ 14.8	5.5 $\pm$ 0.2	2.9 $\pm$ 0.7	1.9 $\pm$ 0.4	0.5 $\pm$ 0.2	<5
	8 hours	35.9 $\pm$ 0.1*	14.5 $\pm$ 0.4*	223.6 $\pm$ 14.9	8.0 $\pm$ 0.5§§§§	5.2 $\pm$ 1.5§§§	2.1 $\pm$ 0.4	0.6 $\pm$ 0.2	<5
	32 hours	35.9 $\pm$ 0.1**	14.7 $\pm$ 0.4**	235.0 $\pm$ 15.0	5.5 $\pm$ 0.3	3.0 $\pm$ 1.1	1.9 $\pm$ 0.4	0.4 $\pm$ 0.2	<5
Female	Baseline	36.2 $\pm$ 0.1	13.3 $\pm$ 0.3	251.3 $\pm$ 12.0	6.7 $\pm$ 0.4	4.3 $\pm$ 1.3	2.2 $\pm$ 0.4	0.4 $\pm$ 0.1	<5
	8 hours	36.1 $\pm$ 0.1	13.1 $\pm$ 0.2	255.8 $\pm$ 11.2	9.2 $\pm$ 0.6§§§	6.5 $\pm$ 2.0§§	2.2 $\pm$ 0.4	0.6 $\pm$ 0.2	<5
	32 hours	36.2 $\pm$ 0.1	13.0 $\pm$ 0.2	249.0 $\pm$ 12.1	6.3 $\pm$ 0.3	3.7 $\pm$ 1.0	2.2 $\pm$ 0.4	0.5 $\pm$ 0.2	<5
<b>Sex comparison</b>		0.0073	<0.0001	0.031	0.0024	0.0005	0.024	0.864	-

Interestingly, at baseline it is notable that in females the numbers of white blood cells trend to higher values vs males. The differential counts demonstrate that this difference is due to greater numbers of neutrophils in females ( $P < 0.05$ ); all other circulating cell numbers at baseline were similar between the sexes. Use of flow cytometry to assess the relative activation state of the circulating cells demonstrated that at baseline the expression of key adhesion molecules involved in inflammatory cell recruitment (CD11b, CD162 and CD62L) were largely similar between the sexes except for an elevation in expression of activation markers on circulating monocytes in females (**Figure 3.1, Figure 3.2 and Figure 3.3**), possibly suggesting that these markers are good markers of basal activation.

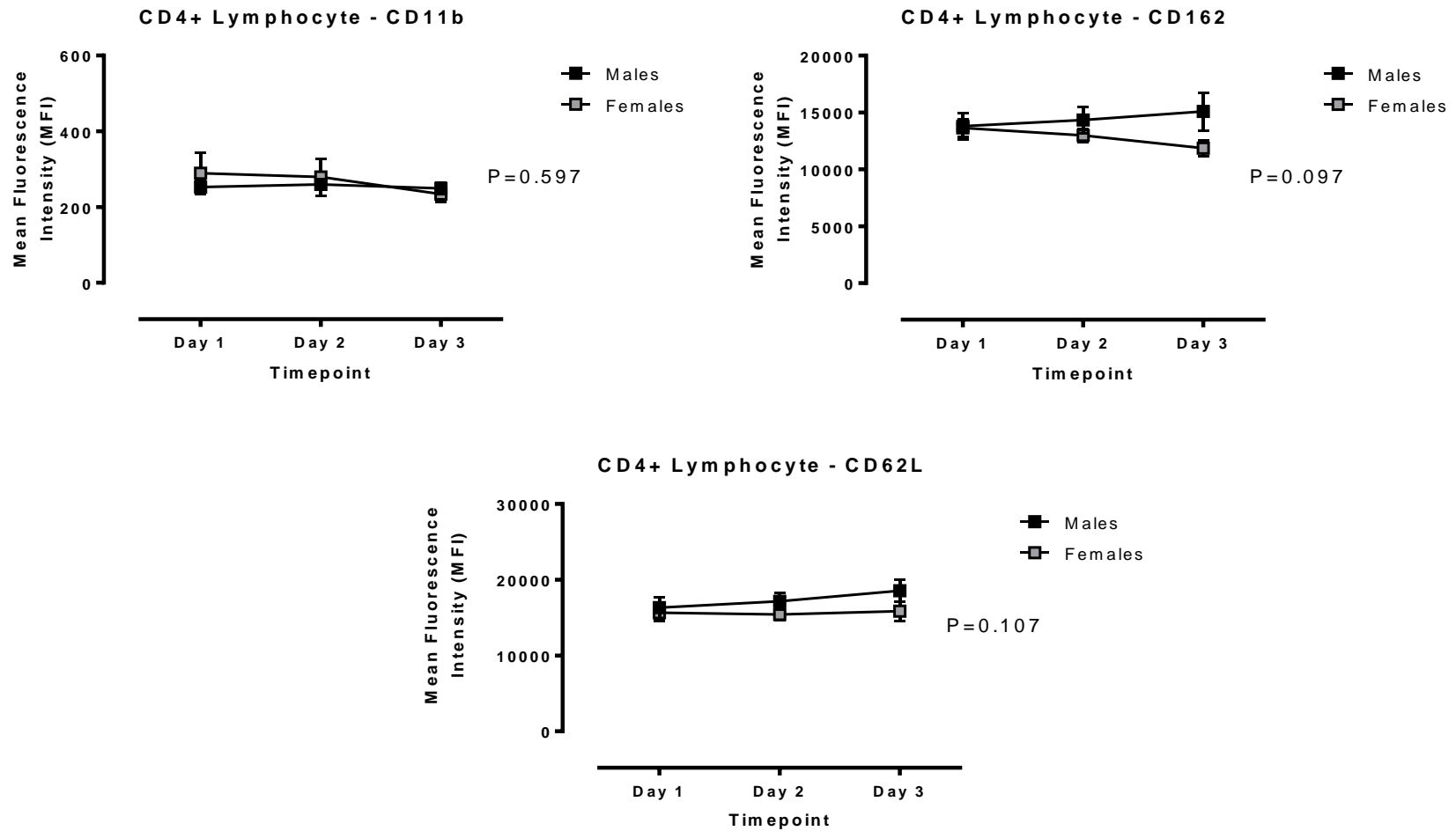
Following typhoid vaccine the activation state of circulating neutrophils relative to baseline was enhanced in males but not females. Specifically with elevations of CD162 and CD62L (**Figure 3.4**) were seen, both cellular adhesion molecules that play a pivotal role in cell homing, rolling and adhesion in an inflammatory scenario (Pillay et al.; Xu et al., 2008). The effect upon CD162 and CD62L was only evident on neutrophils and not on any other cell types interrogated (**Figure 3.1, Figure 3.2 and Figure 3.3**). There were no measureable changes from baseline in platelet reactivity in either sex and at all the time-points tested (**Table 3.3**).



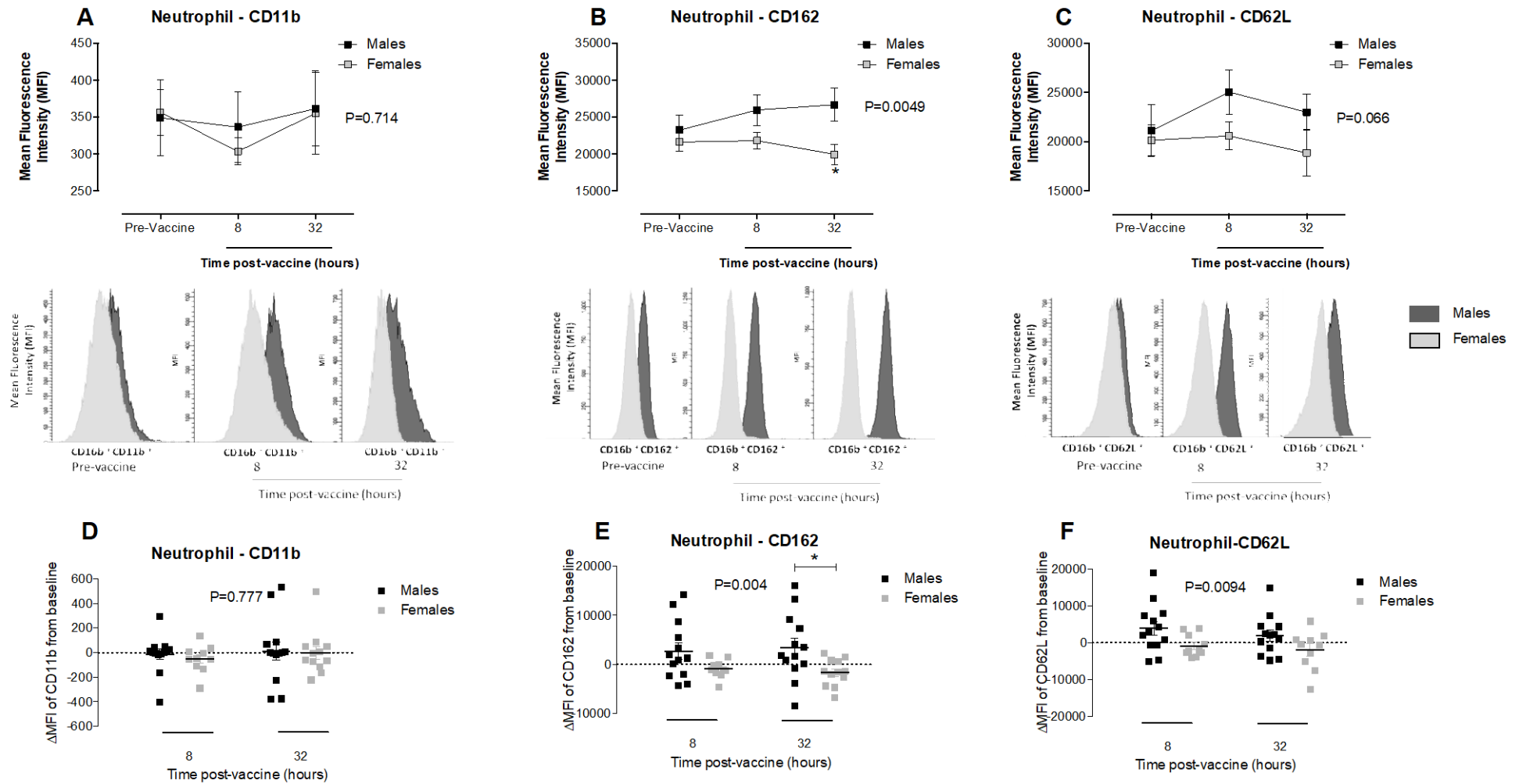
**Figure 3.1: Changes in expression of CD11b, CD62L and CD162 on monocytes, in male and female healthy volunteers measured at baseline, 8 hours and 32 hours following typhoid vaccine.** Data expressed as mean  $\pm$  s.e.mean for n=12 for each sex. Statistical significance determined using two-way ANOVA with Sidak's post-hoc analysis. MFI = Median fluorescence intensity.



**Figure 3.2: Changes in expression of CD11b, CD62L and CD162 on CD4+ lymphocytes in male and female healthy volunteers measured at baseline, 8 hours and 32 hours following typhoid vaccine.** Data expressed as mean  $\pm$  s.e.mean for n=12 for each sex. Statistical significance determined using two-way ANOVA with Sidak's post-hoc analysis. MFI = Median fluorescence intensity.



**Figure 3.3: Changes in expression of CD11b, CD62L and CD162 on CD8+ lymphocytes in male and female healthy volunteers measured at baseline, 8 hours and 32 hours following typhoid vaccine.** Data expressed as mean  $\pm$  s.e.mean for n=12 for each sex. Statistical significance determined using two-way ANOVA with Sidak's post-hoc analysis. MFI = Median fluorescence intensity.



**Figure 3.4: Low grade systemic inflammation induced by typhoid vaccination enhances neutrophil activation state in healthy male but not female volunteers.** Changes in expression of (A) CD11b (B) CD162 and (C) CD62L on neutrophils in male and female healthy volunteers at baseline, 8 hours and 32 hours following typhoid vaccine. Changes in expression of (D) CD11b, (E) CD162 and (F) CD62L from baseline in male and female healthy volunteers at 8 hours and 32 hours following typhoid vaccine. Data expressed as mean  $\pm$  s.e.mean of n=12 male and female volunteers. Statistical significance determined using two-way ANOVA with Sidak's post-hoc analysis shown as \* for P<0.05.



**Table 3.3.** The effect of typhoid on the expression of platelet activation markers and platelet activity in healthy volunteers. Data are shown for % P-selectin expression in males and females following PBS treatment and in response to ADP (3 and 10  $\mu$ M) or collagen (3 and 10  $\mu$ g/ml) at baseline, 8 hours and 32 hours post vaccine. For % platelet monocyte aggregates levels were measured at baseline, 8 hours and 32 hours post-vaccine. Data are shown as mean  $\pm$  s.e.mean and statistical analysis conducted using two-way ANOVA to determine the influence of sex differences for n=10 male and n=12 female volunteers (2 samples lost due to technical difficulties). Statistical significance was tested using two-way ANOVA with Sidak's post-hoc analysis.

	Males			Females			Sex comparison P Value
	Baseline	8 hours	32 hours	Baseline	8 hours	32 hours	
<b>% P-Selectin expression</b>							
<b>PBS</b>	2.3 $\pm$ 1.4	0.7 $\pm$ 0.2	2.2 $\pm$ 1.2	1.0 $\pm$ 0.2	0.5 $\pm$ 0.1	0.8 $\pm$ 0.3	0.517
<b>Collagen 1 <math>\mu</math>g/ml</b>	2.2 $\pm$ 0.7	1.3 $\pm$ 0.3	2.6 $\pm$ 1.1	2.3 $\pm$ 0.7	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2	0.567
<b>Collagen 3 <math>\mu</math>g/ml</b>	3.7 $\pm$ 0.9	3.2 $\pm$ 0.8	4.2 $\pm$ 1.6	3.8 $\pm$ 1.3	2.7 $\pm$ 0.6	2.2 $\pm$ 0.4	0.448
<b>ADP 3 <math>\mu</math>M</b>	9.1 $\pm$ 2.6	6.8 $\pm$ 1.3	4.2 $\pm$ 1.6	8.9 $\pm$ 2.1	12.3 $\pm$ 3.7	10.8 $\pm$ 3.3	0.723
<b>ADP 10 <math>\mu</math>M</b>	10.9 $\pm$ 3.3	7.1 $\pm$ 1.8	12.5 $\pm$ 3.3	12.0 $\pm$ 2.5	12.9 $\pm$ 3.0	13.8 $\pm$ 3.6	0.587
<b>% PMA expression</b>	31.5 $\pm$ 4.2	34.2 $\pm$ 6.4	26.8 $\pm$ 4.3	27.8 $\pm$ 5.4	34.4 $\pm$ 6.0	33.1 $\pm$ 4.7	0.611
<b>Platelet Aggregation (AU)</b>							
<b>PBS</b>	7.2 $\pm$ 2.1	8.8 $\pm$ 3.0	8.2 $\pm$ 2.3	9.2 $\pm$ 2.0	13.5 $\pm$ 2.5	8.1 $\pm$ 2.3	0.268
<b>Collagen 1 <math>\mu</math>g/ml</b>	59.5 $\pm$ 3.4	61.6 $\pm$ 5.2	54.8 $\pm$ 4.0	60.2 $\pm$ 5.3	74.6 $\pm$ 5.1	61.3 $\pm$ 5.2	0.086
<b>Collagen 3 <math>\mu</math>g/ml</b>	78.9 $\pm$ 3.7	91.5 $\pm$ 4.9	75.1 $\pm$ 3.9	83.8 $\pm$ 5.9	99.6 $\pm$ 5.9	76.8 $\pm$ 6.5	0.245
<b>ADP 3 <math>\mu</math>M</b>	35.1 $\pm$ 4.1	37.8 $\pm$ 4.1	37.7 $\pm$ 5.4	46.3 $\pm$ 5.8	54.4 $\pm$ 5.1	43.2 $\pm$ 5.5	0.008
<b>ADP 10 <math>\mu</math>M</b>	47.8 $\pm$ 4.1	49.9 $\pm$ 4.3	43.9 $\pm$ 3.7	57.0 $\pm$ 4.4	68.6 $\pm$ 3.5	56.9 $\pm$ 4.8	0.0002

Neither circulating levels of CRP or body temperature were altered by typhoid vaccine (**Table 2**). Furthermore, the differences seen between the sexes was not due to differences in dosing since typhoid (IgM) antibody titres at 32 hours following vaccination were similar (Male: 10.4  $\pm$  1.8 n=11 and female: 11.4  $\pm$  1.6 n=10 U/ml). These observations suggest that the differences in systemic inflammation relate to biological differences between the sexes in the response to an inflammatory stress and not difference in dose delivered.

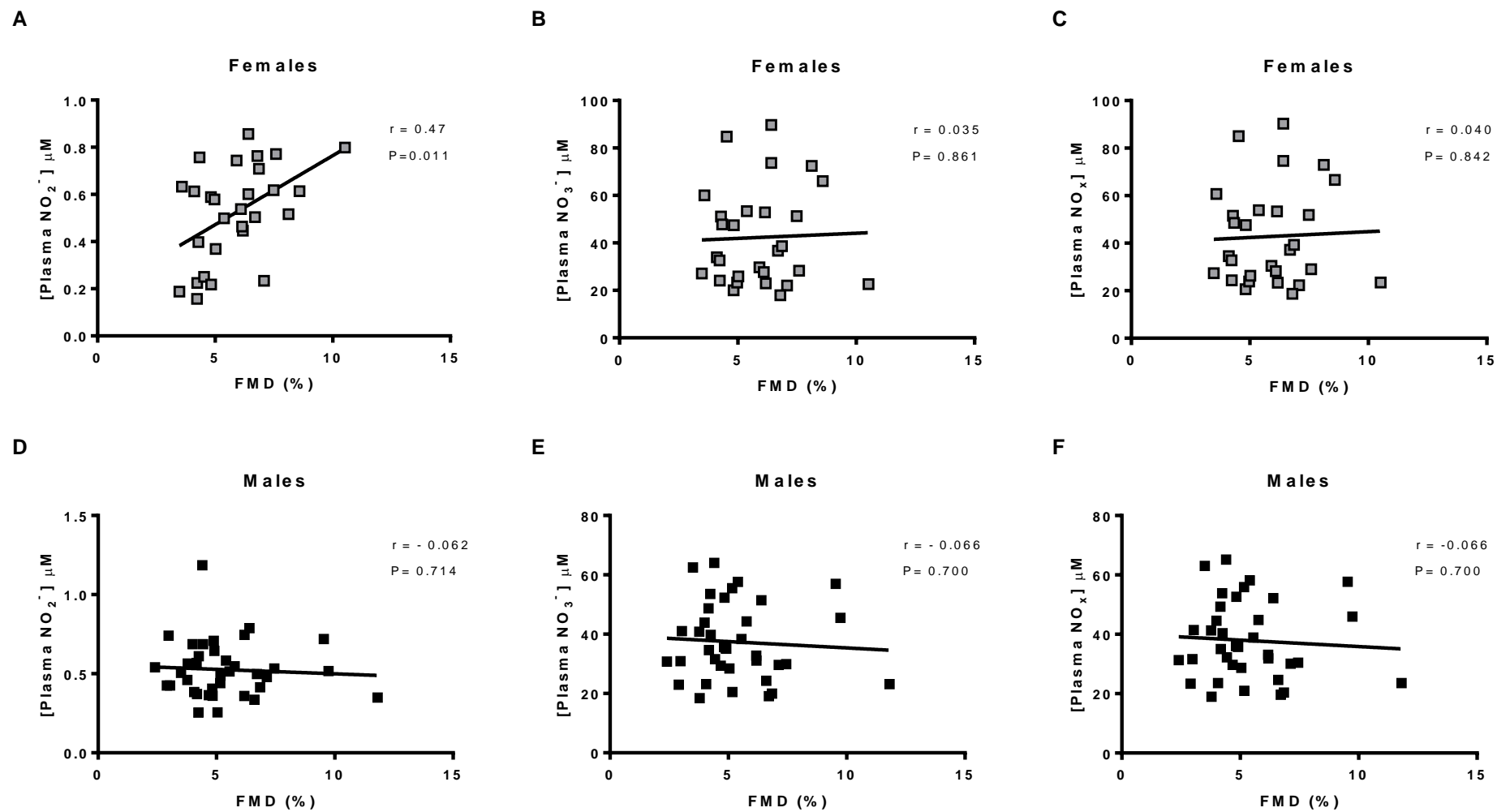
### 3.3.3 Sex differences in the vascular response to typhoid vaccination

Baseline ultrasound FMD responses were similar between the sexes, however, whilst typhoid vaccine tended to decrease the FMD response in males, in females the response actually increased at the 8 hour period. This profile of FMD response was significantly different between the sexes, with the greatest difference evident at the 8-hour time-point ( $P < 0.01$ ; **Figure 3.6 A-B**). These differences were evident despite no change over time in baseline diameter or in the time taken to reach peak diameter following release of the cuff (**Table 3.4**).

**Table 3.4.** Vascular measures at baseline and at 8 and 32 hours following typhoid vaccination. Data are shown as mean  $\pm$  SD with statistical significance determined using two-way ANOVA with post-hoc Sidak's tests shown as \* for  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  for comparison of time-points between the sexes and no significant differences for within group comparisons to baseline.

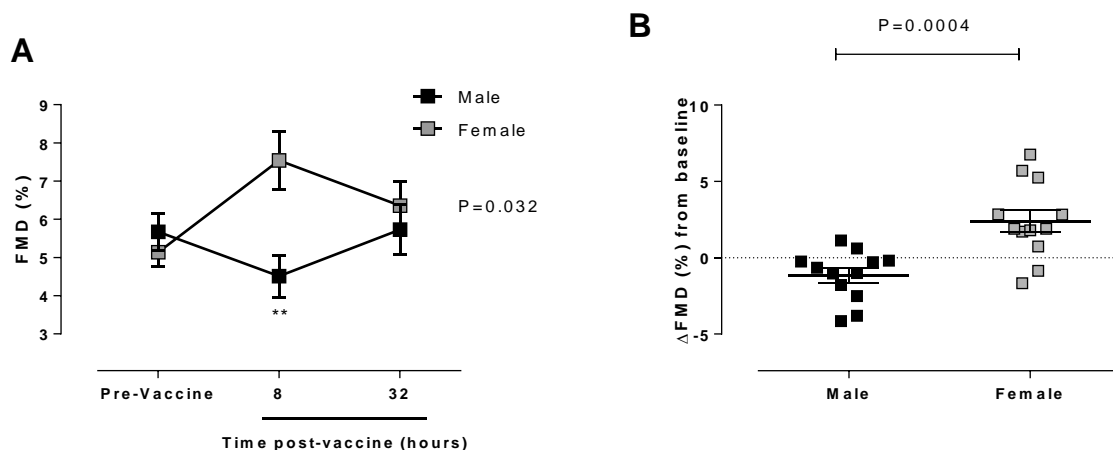
	Males (n=12)			Females (n=12)			Sex comparison
	Baseline	8 hours	32 hours	Baseline	8 hours	32 hours	
<b>Ultrasound</b>							
Baseline brachial artery diameter, mm	3.8 $\pm$ 0.5***	3.8 $\pm$ 0.5****	3.8 $\pm$ 0.4**	3.1 $\pm$ 0.3	3.1 $\pm$ 0.4	3.2 $\pm$ 0.4	<0.0001
Time to peak diameter, min	6.7 $\pm$ 0.5	6.7 $\pm$ 0.7	6.7 $\pm$ 0.8	6.7 $\pm$ 0.2	6.8 $\pm$ 0.2	6.8 $\pm$ 0.2	0.492
<b>PWA</b>							
Augmentation index, %	13.3 $\pm$ 1.9	13.8 $\pm$ 1.2	13.5 $\pm$ 1.7*	12.1 $\pm$ 1.4	11.6 $\pm$ 2.2	11.9 $\pm$ 1.9	0.24
PWV, m/s	6.7 $\pm$ 0.1	6.7 $\pm$ 0.1	6.7 $\pm$ 0.2*	6.3 $\pm$ 0.1	6.2 $\pm$ 0.2	6.2 $\pm$ 0.1	<0.0001

Evidence suggests that the FMD response is due predominantly to triggering of endothelium-dependent NO generation. To assess whether this might underlie differences in the sexes we measured nitrite and nitrate (NO<sub>x</sub>) levels. We found no significant differences in circulating NO<sub>x</sub> levels between the time-points, however interestingly post-hoc correlation analyses demonstrated that circulating nitrite concentrations correlate directly to the magnitude of the FMD response in females but not males (**Figure 3.5**). This suggests the possibility that circulating total plasma NO<sub>x</sub> levels could be reflective of localised NO production.



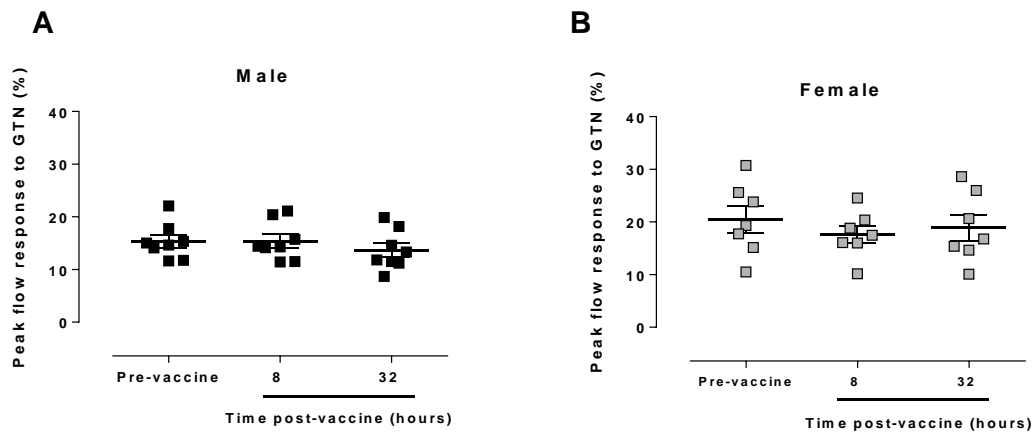
**Figure 3.5: Correlations between FMD responses in healthy volunteers and levels of plasma NO metabolites  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and  $\text{NO}_x$  in females (A-C) and males (D-F).**

Data from pre-vaccine baseline and 8 and 32 hours post-vaccine are collated. All graphs show Pearson linear regression, and statistical significance of r-value.



**Figure 3.6: Low grade systemic inflammation induced by typhoid vaccination does not cause endothelial dysfunction in healthy female volunteers.** The effect of typhoid vaccination on (A) Flow mediated dilatation (FMD) measured at baseline and 8 and 32 hours following typhoid vaccination. (B) The effect of sex differences upon the change in FMD at 8 hours following typhoid vaccination from baseline. All data expressed as mean  $\pm$  s.e.mean of N = 12 for each sex. Statistical significance determined using (A) two-way ANOVA with Sidak's post-hoc analysis shown as \*\* for  $p < 0.01$  and (B) Student's unpaired test.

In contrast to FMD, typhoid vaccination did not alter the vasodilation response to sublingual GTN in either sex (**Figure 3.7 C-D**), and did not influence PWV or augmentation index through PWA. Since the PWV and PWA are measures of arterial compliance determined predominantly by smooth muscle function (**Table 3.4**), the data collectively suggest that the differences seen in the FMD response to typhoid vaccination were not due to underlying changes in smooth muscle reactivity.



**Figure 3.7: Brachial artery responses secondary to GTN in healthy volunteers.** Maximal dilatation of the brachial artery in response to GTN in (A) male and (B) female healthy volunteers measured at baseline and 8 and 32 hours following typhoid vaccination. Data expressed as mean  $\pm$  s.e.mean of N=8. Statistical significance determined using one-way ANOVA with Sidak's post-hoc analysis.

### 3.4 Summary

- As expected, typhoid vaccination caused an expected low grade systemic inflammation that was reflected by a neutrophilia.
- The neutrophilia response to typhoid was equally evident in both men and women.
- As expected, brachial artery size was greater in men than women and this was associated with a slight lower baseline FMD and lower PWV.
- Females exhibit a significantly increased FMD response compared to baseline 8 hours following typhoid vaccination, whilst in contrast, the FMD response in men showed a trend towards a worse FMD at 8 hours post-vaccine.
- Ozone chemiluminescence reveals a trend towards sex differences in plasma  $[\text{NO}_2^-]$ ,  $[\text{NO}_3^-]$  and  $[\text{NO}_x]$  profiles following typhoid vaccination.
- Females exhibit positive correlation between FMD and plasma  $[\text{NO}_2^-]$ . However, no such correlation is observed in males.
- These results suggest that female sex protects against endothelial dysfunction induced by acute inflammation. Moreover, our data suggest such sex differences may, at least in part, reflect better utilisation of nitrite by females to improve endothelial function.

## Chapter 4.0

# **Accelerated resolution of inflammation underlies sex differences in the inflammatory response and inflammation-induced vascular dysfunction in humans**

## **4.0 Accelerated resolution of inflammation underlies sex differences in the inflammatory response and inflammation-induced vascular dysfunction in humans**

### **4.1 Assessment of the acute and resolution phases of inflammation in humans using the cantharidin blister model**

As already discussed in section 1.11, cardiovascular disorders are characterised by an underlying vascular inflammation that is considered to play a major causative role in pathogenesis (Willerson and Ridker, 2004). We used the typhoid-induced systemic inflammation model to assess whether endothelium dysfunction is different between the sexes. As shown in Chapter 3, our results indicated a resistance to the effects of typhoid in females. To study the inflammatory response in humans more closely, we explored whether a suitable model of innate inflammation might be available. There have been a number of human *in vivo* models of acute inflammation that have been developed over the last two decades. These include the negative pressure-induced skin blister model (Follin, 1999), the systemic inflammatory reaction following endotoxin inhalation (Michel et al., 1997) and the cantharidin-induced skin blister model (Day et al., 2001a). Out of all of these models, the cantharidin skin blister is relatively non-invasive, has few side-effects, and has low within-subject variability. In addition, the blisters provide immediate access to cells and supernatant to study the response more closely as well as a collection process that minimises alteration in phenotype arising from *ex vivo* processing. In addition, the blister can be maintained for several days to characterise both the immediate response and resolution of the innate inflammatory response. Following topical administration to the skin,



cantharidin (a protein phosphatase inhibitor), induces atraumatic acantholysis and blister formation (Day et al., 2001a). This is associated with an infiltration of CD16+ neutrophils into the fluid space in the first 24 hours, and CD14+ monocyte/macrophages from 24 to 72 hours (Morris et al., 2010).

Since this model of acute, localised inflammation has low within person variability (Dinh et al., 2011), we decided to use this model to characterise the leukocytes, chemokines, cytokines and resolution factors generated during an inflammatory response and to determine whether any of these may be altered between the sexes.

## **4.2 Results**

### **4.2.1 Baseline demographics**

Volunteers were recruited from 2015 to 2016. Baseline data demonstrates that the volunteers were well matched in terms of age. Expected differences were observed in BMI, SBP and DBP (**Table 4.1**). These profile of the demographics are very similar to those of the typhoid study indicating close similarities between the two cohort of participants.

**Table 4.1.** Demographics of healthy volunteers in the cantharidin study. Data are shown as mean  $\pm$  s.e.mean with statistical significance determined using Student's unpaired t-test.

	Cantharidin Study		P Value
	Male	Female	
<b>Volunteers (n)</b>	16	16	
<b>Age (years)</b>	27.4 $\pm$ 1.1	26.8 $\pm$ 1.1	0.68
<b>BMI (Kg/m<sup>2</sup>)</b>	24.6 $\pm$ 0.7	22.4 $\pm$ 0.6	0.019
<b>Baseline SBP (mmHg)</b>	115.7 $\pm$ 2.0	104.1 $\pm$ 1.7	0.0001
<b>Baseline DBP (mmHg)</b>	68.3 $\pm$ 1.7	64.6 $\pm$ 1.5	0.12
<b>Heart rate (bpm)</b>	64.1 $\pm$ 2.3	68.4 $\pm$ 1.7	0.15
<b>Oral contraceptive</b>	-	6	

#### 4.2.2 Sex differences in cantharidin-induced inflammation

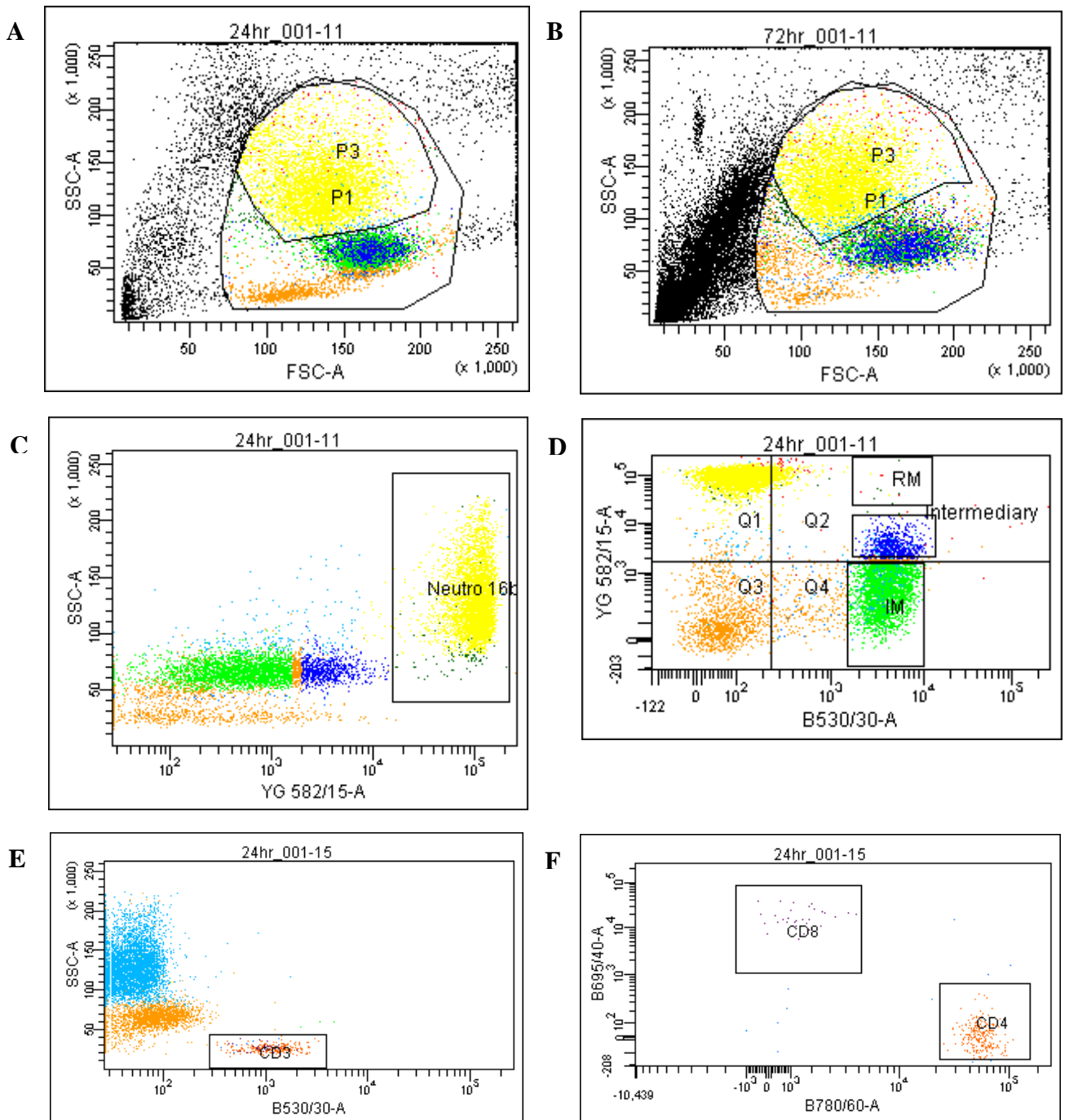
Cantharidin application at the time-points assessed was not associated with a systemic inflammatory response in either sex as reflected by no change from baseline at 72 hours after cantharidin application in peripheral blood markers of systemic inflammation such as CRP, leucocyte count, and leucocyte differential (Table 4.2).

**Table 4.2.** Blood differentials and CRP levels in male and female healthy volunteers at baseline and post cantharidin-induced blister harvest. Data are shown as mean  $\pm$  SD with statistical significance determined using two-way ANOVA with post-hoc Sidak's tests (note there were no significant differences between the sexes or on the effects of cantharidin on circulating markers or within sex).

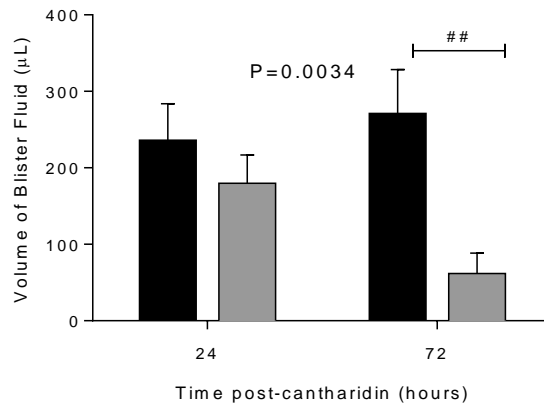
		Hb (g/dL)	Platelet count ( $\times 10^9$ /L)	WBC ( $\times 10^{11}$ /L)	Neutrophils ( $\times 10^9$ /L)	Lymphocytes ( $\times 10^9$ /L)	Monocytes ( $\times 10^9$ /L)	CRP (mg/L)
<b>Male</b>	<b>Baseline</b>	15.0 $\pm$ 0.3	234.7 $\pm$ 15.2	5.4 $\pm$ 0.3	3.0 $\pm$ 0.3	1.8 $\pm$ 0.1	0.4 $\pm$ 0.04	<5
	<b>Post-blister</b>	15.0 $\pm$ 0.3	242.2 $\pm$ 12.9	5.6 $\pm$ 0.4	3.2 $\pm$ 0.3	1.8 $\pm$ 0.1	0.4 $\pm$ 0.04	<5
<b>Female</b>	<b>Baseline</b>	13.1 $\pm$ 0.3	258.0 $\pm$ 12.0	6.3 $\pm$ 0.5	3.8 $\pm$ 0.4	1.9 $\pm$ 0.1	0.4 $\pm$ 0.02	<5
	<b>Post-blister</b>	12.7 $\pm$ 0.2	259.9 $\pm$ 12.2	6.0 $\pm$ 0.3	3.3 $\pm$ 0.2	2.0 $\pm$ 0.1	0.4 $\pm$ 0.02	<5
<b>Sex Comparison</b>		<0.0001	0.123	0.109	0.133	0.133	0.859	0

All volunteers reported that the typhoid vaccine caused minimal discomfort beyond the injection and that the cantharidin blisters were pain free other than an itching and/or tingling sensation a few hours after cantharidin application as the blisters formed. These observations are in line with other published studies (Brunner et al., 1998; Day et al., 2001b; Jenner et al., 2014), providing evidence that this is indeed a safe, reliable and reproducible technique to study acute inflammation.

Within the cantharidin-induced blister, I found significantly less oedema in terms of volume collected in females compared to males (**Figure 4.2**). More interestingly, whilst some blisters unfortunately popped prior to collection, the majority of the 72 hour blisters in the women but not the men had resolved (**Table 4.3**).



**Figure 4.1: Representative dot plots to identify leucocyte sub-populations collected in cantharidin induced blister fluid in healthy volunteers: A-B)** Characteristic FSC/SSC dot plot from a 24hrs and 72hrs blister fluid respectively; **C)** Density dot plot of neutrophils (yellow-gated cells) confirmed by anti-CD16b+ binding; **D)** Density dot plot of monocytes showing presence of CD14+/CD16- classical inflammatory (IM) monocytes (green-gated), CD14+/CD16+ intermediate monocytes (blue-gated) and a fraction of CD14lo/CD16++ resident (RM) monocytes confirmed by appropriate binding patterns with anti-CD14 and/or anti-CD16; **E)** Density dot plot of lymphocytes (red-gated cells) confirmed by anti-CD3 binding and **F)** Density dot plot showing T-cell sub-types with anti-CD4+ (orange-gated cells) and anti-CD8+ (blue-gated cells) binding



**Figure 4.2: Reduced oedema response to cantharidin in female compared to male healthy volunteers.** The total volume of fluid at 24 hours and 72 hours following application of cantharidin to the volar aspect of the forearm. Data is shown as mean  $\pm$  s.e.mean of n=16 of each sex. Statistical analysis was determined using two-way ANOVA followed by Sidak's post-tests shown as ## p <0.01.

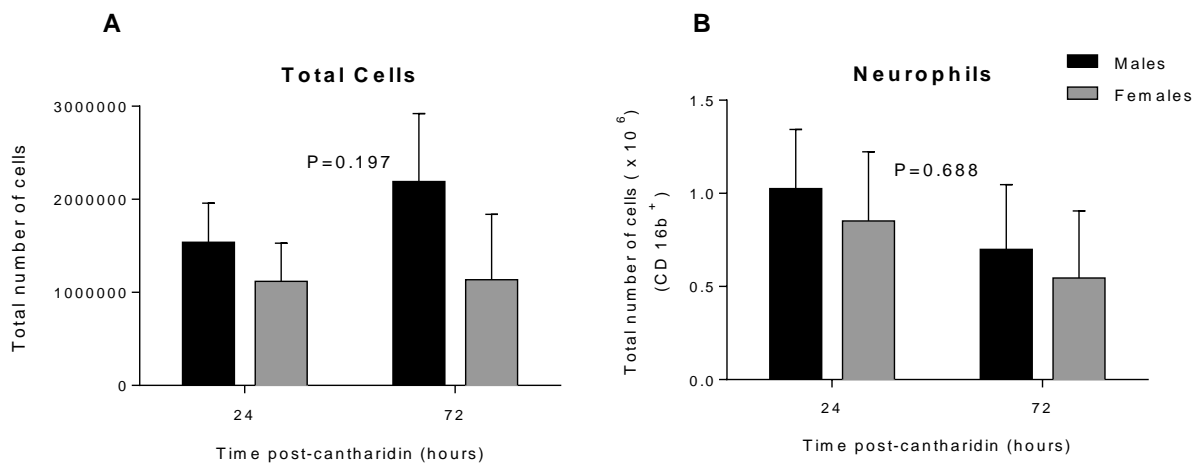
Indeed, at the 72 hour time-point blisters had completely resolved in 11 females (70%) in comparison to only 5 males (using a 4 by 2 contingency table the chi-square test demonstrated a significant difference between the sexes,  $p < 0.0019$ ) (Table 4.3).

**Table 4.3.** Number of resolved blisters at 72 hrs post-cantharadin. Statistical analysis performed using chi-squared test.

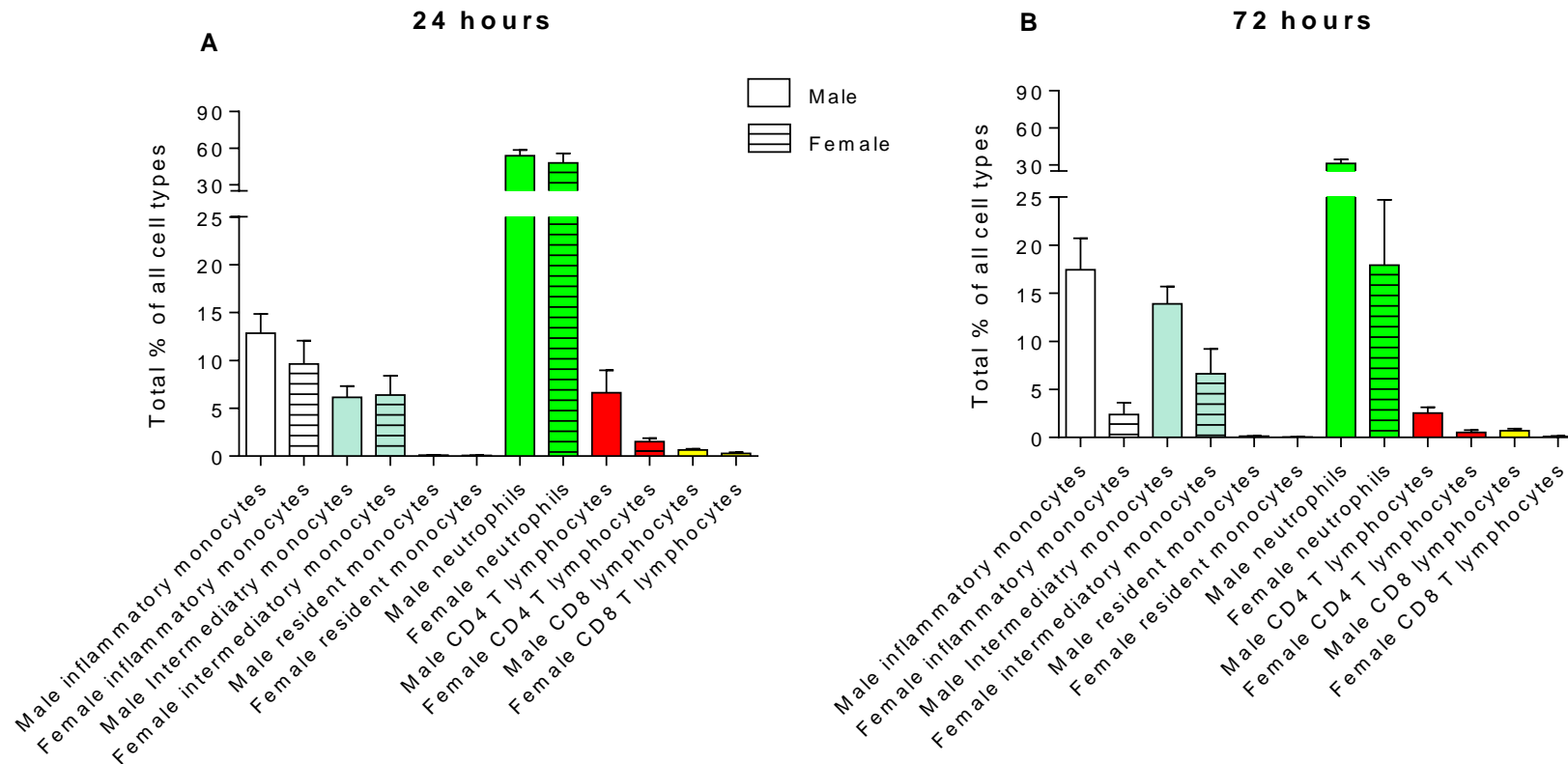
	72 hr Blisters			Total
	Blister formed and present	Resolved	Popped	
Male	12	0	4	16
Female	5	9	2	16

$p = 0.0019$

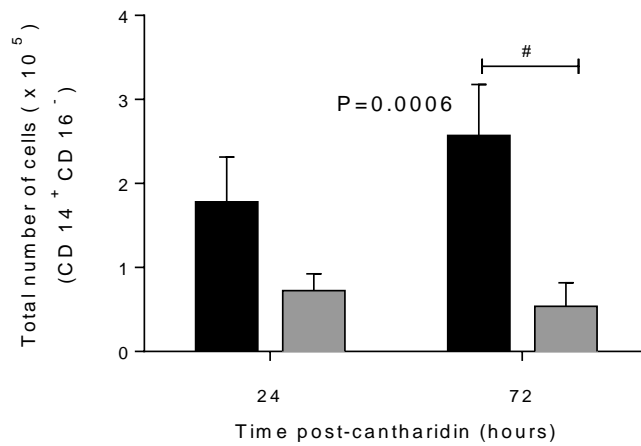
There was no significant difference (albeit a trend) in the total number of cells collected within the blisters between the sexes. Of all the cell types present, the neutrophil numbers were at least ten times higher than any other cell type. However, there were no differences in the number of neutrophils recruited into the blisters between the sexes (**Figure 4.3 A and B**). Whilst the percentage of cells were not different between the sexes (**Figure 4.4**), the assessment of levels of other key inflammatory cell types and cell activation state exposed a prominent difference between the sexes (see **Figure 4.1** for typical scatter plots).



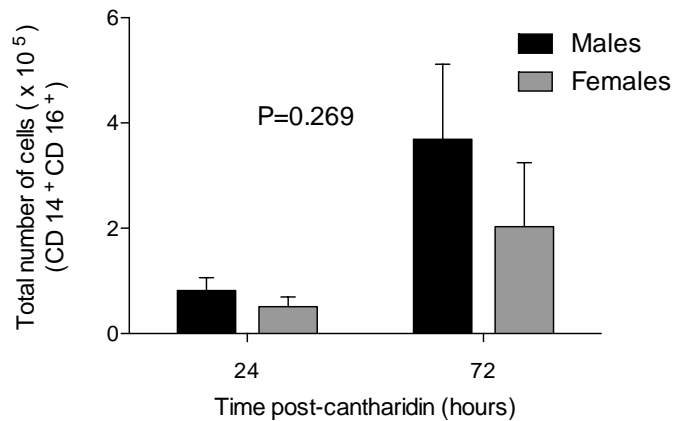
**Figure 4.3: Total number of cells and neutrophil response at 24 hours and 72 hours post application of cantharidin in female compared to male healthy volunteers.** The total number of cells at 24 hours and 72 hours (A) and neutrophil response (B) following application of cantharidin to the volar aspect of the forearm. Data is shown as mean  $\pm$  s.e.mean of n=16 of each sex. Statistical analysis was determined using two-way ANOVA followed by Sidak's post-tests.



**Figure 4.4: Total percentage of all cell types at 24 hours and 72 hours post application of cantharidin in female compared to male healthy volunteers.** The total percentage of all cell type at 24 hours (A) and 72 hours (B) following application of cantharidin to the volar aspect of the forearm. Data is shown as mean  $\pm$  s.e.mean of n=16 of each sex. Statistical analysis was determined using two-way ANOVA followed by Sidak's post-tests.

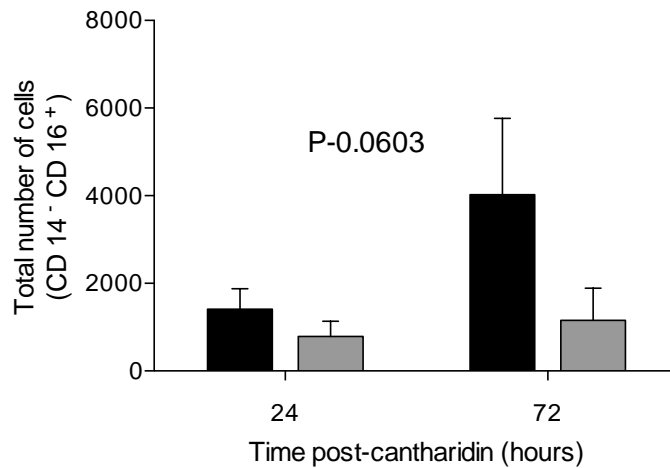


**Figure 4.5: Reduced inflammatory monocyte response to cantharidin in female compared to male healthy volunteers.** The Inflammatory Monocyte count at 24 hours and 72 hours following application of cantharidin to the volar aspect of the forearm. Data is shown as mean ± s.e.mean of n=16 of each sex. Statistical analysis was determined using two-way ANOVA followed by Sidak's post-tests shown as # p < 0.05.

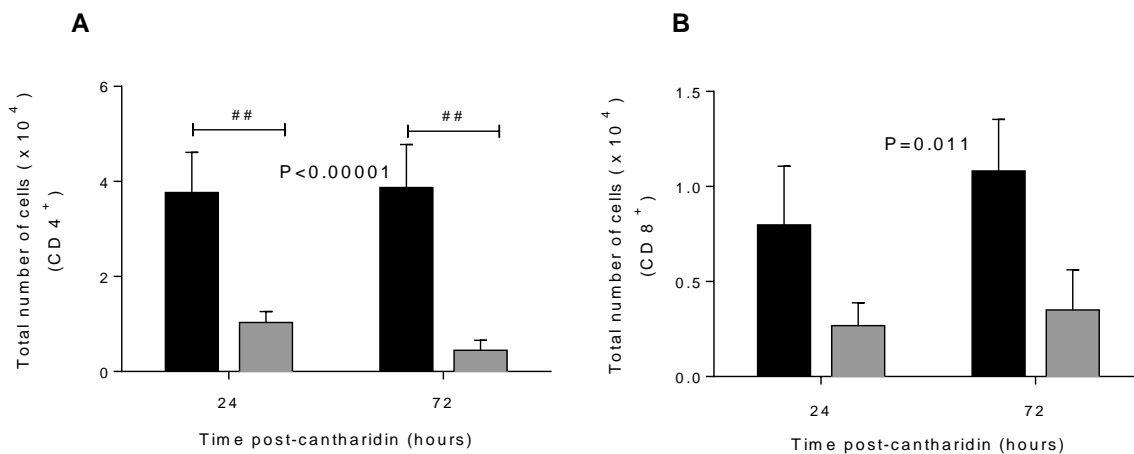


**Figure 4.6: Intermediate Monocyte count in cantharidin-induced blister fluid at 24 hours and 72 hours post application in healthy volunteers** (male n=13-15, female n=14-16). Data shown as mean ± s.e.mean with statistical significance determined using two-way ANOVA to compare between sexes on each day; not significant.





**Figure 4.7 – Resident Monocyte in cantharidin-induced blister fluid at 24 hours and 72 hours post application in healthy volunteers** (male n=13-15, female n=14-16). Data shown as mean  $\pm$  s.e.mean with statistical significance determined using two-way ANOVA to compare between sexes on each day; not significant.



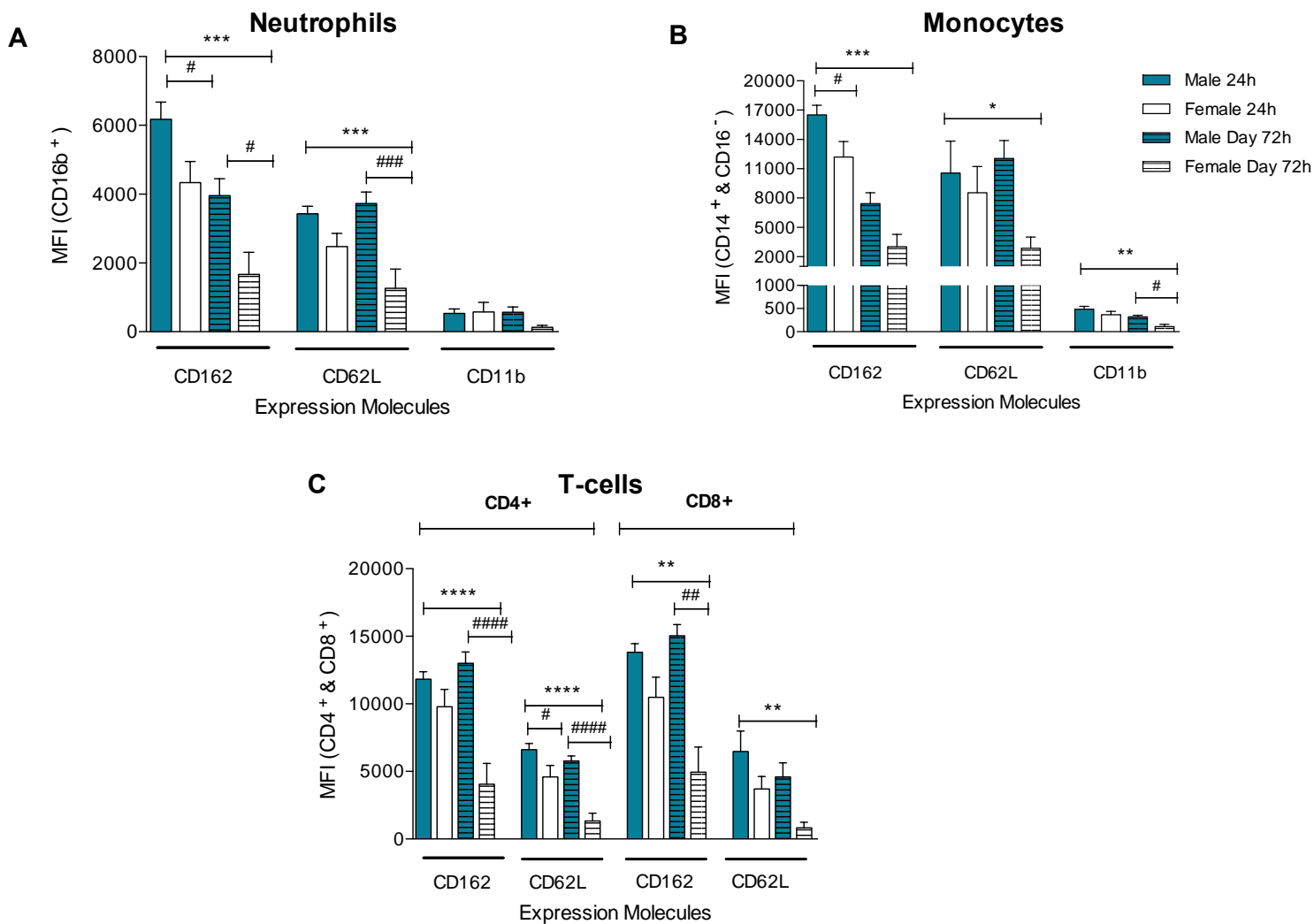
**Figure 4.8: Reduced CD4+ and CD8+ T-cell count response to cantharidin in female compared to male healthy volunteers.** The CD4+ (A) and CD8+ (B) T-cell count at 24 hours and 72 hours following application of cantharidin to the volar aspect of the forearm. Data is shown as mean  $\pm$  s.e.mean of n=16 of each sex. Statistical analysis was determined using two-way ANOVA followed by Sidak's post-tests shown as ## p < 0.01.

In addition, the proportions and total numbers of inflammatory monocytes in blisters were significantly reduced in females compared to male volunteers particularly at 72 hours (**Figure 4.5**). Of note, there were also significantly lower levels of CD4+ and CD8+ T-cells in females when compared to males at both the 24 and 72 hour time-points (**Figure 4.8 A and B**). There were no differences in the numbers of either the intermediate (inter) monocyte or the resident monocyte subtype (See **Figure 4.6 and 4.7**).

Interestingly, as shown in **Figure 4.9**, irrespective of whether there were differences in the absolute numbers of specific cell subtypes between the sexes, all cell types showed a substantially and significantly reduced expression of all 3 activation markers in females compared to males. In 24 hour blister samples, using a cytokine/chemokine bead array, we identified no differences between the sexes in the levels of the pro-inflammatory cytokines/chemokines that included IL-6, the neutrophil chemokines CCL5 and CXCL1 or the monocyte chemokine CCL-2. Interestingly, we found higher levels of IL-8 in the blisters from female volunteers compared to males and importantly a trend for higher expression of the anti-inflammatory cytokine IL-10 in females (**Table 4.4**).

**Table 4.4.** Cytokine/chemokine array of 24 hour cantharidin-induced blister supernatants in male and female healthy volunteers. Data are shown as mean  $\pm$  SD with statistical significance determined using two-way ANOVA with post-hoc Sidak's tests shown as \*\* for  $P < 0.01$  for comparison of time-points between the sexes and no significant differences for within group comparisons to baseline.

		IL-6 (pg/ml)	IL-10 (pg/ml)	CCL-5 (pg/ml)	CXCL-1 (pg/ml)	IL-8 (pg/ml)	MCP-1 (pg/ml)
<b>Male</b>	<b>24 h</b>	4361 $\pm$ 1182	6.6 $\pm$ 2.3	11.5 $\pm$ 1.8	1046 $\pm$ 606.6	5716 $\pm$ 1453	6139 $\pm$ 2141
	<b>72 h</b>	3250 $\pm$ 1178	48.1 $\pm$ 26.7	38.5 $\pm$ 26.0	224.5 $\pm$ 36.0	3675 $\pm$ 563.8	224.4 $\pm$ 26.86
<b>Female</b>	<b>24 h</b>	5087 $\pm$ 1697	18.0 $\pm$ 7.3	15.5 $\pm$ 2.7	4224 $\pm$ 1809	13383 $\pm$ 2771**	9658 $\pm$ 3113
	<b>72 h</b>	5240 $\pm$ 3007	30.6 $\pm$ 0.0	10.7 $\pm$ 0	380.4 $\pm$ 124.3	5511 $\pm$ 9376	302.6 $\pm$ 93.2
<b>Sex comparison P value</b>		0.452	0.653	0.375	0.221	0.0484	0.527



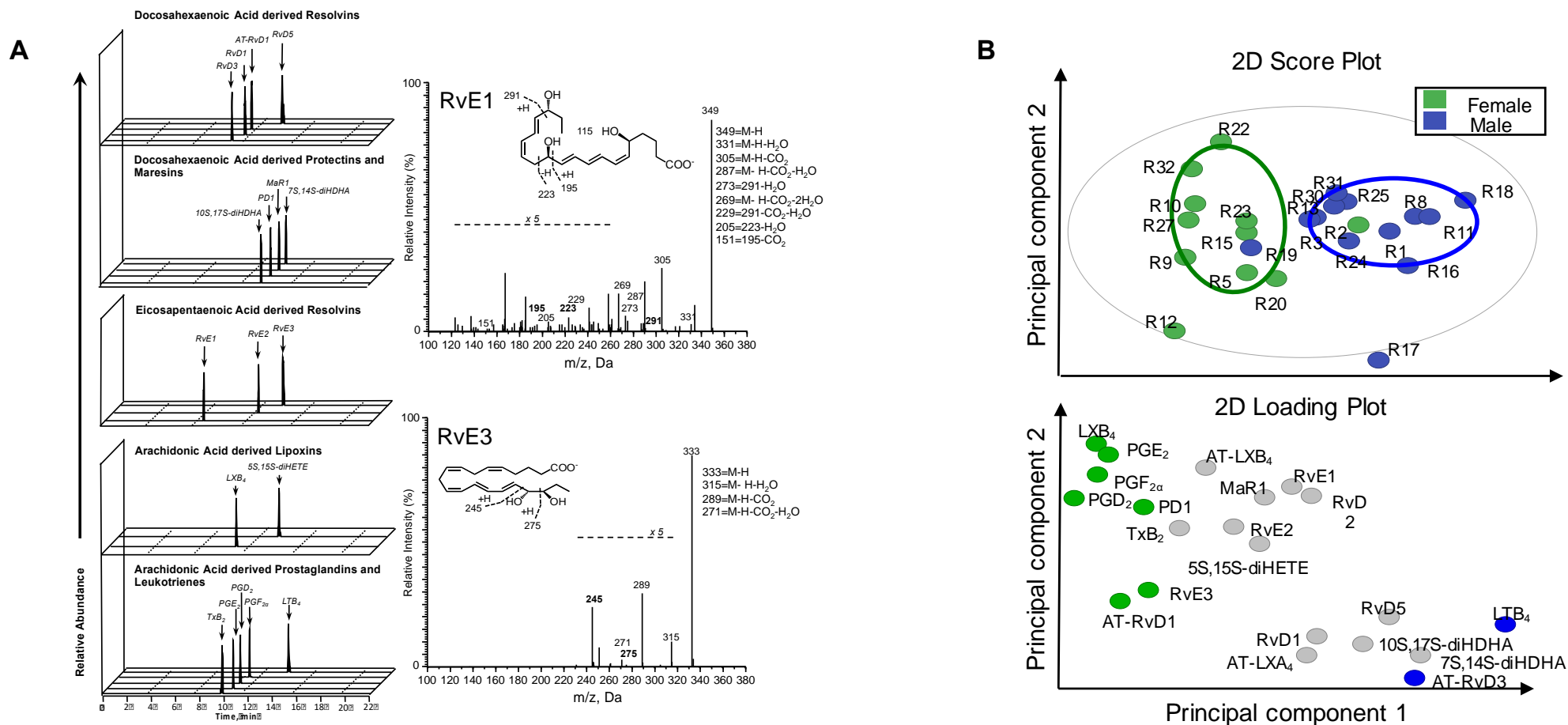
**Figure 4.9: Reduced inflammatory cell activation state in cantharidin -induced blister exudates in female compared to male healthy volunteers.** Mean Fluorescence Intensity (MFI) of the expression molecules CD162, CD62L and CD11b on **(A)** neutrophils, **(B)** inflammatory (IM) monocytes and **(C) CD4+ and CD8+ T-cells** in healthy male (n=16) and female (n=16) volunteers. Data shown as mean  $\pm$  s.e.mean. Statistical significances determined using two-way ANOVA shown as \*\*  $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\*  $p < 0.0001$  followed by Sidak's post-tests shown as #  $p < 0.05$ , ##  $p < 0.01$  and ####  $P < 0.0001$  comparing between the sexes at each time-point.

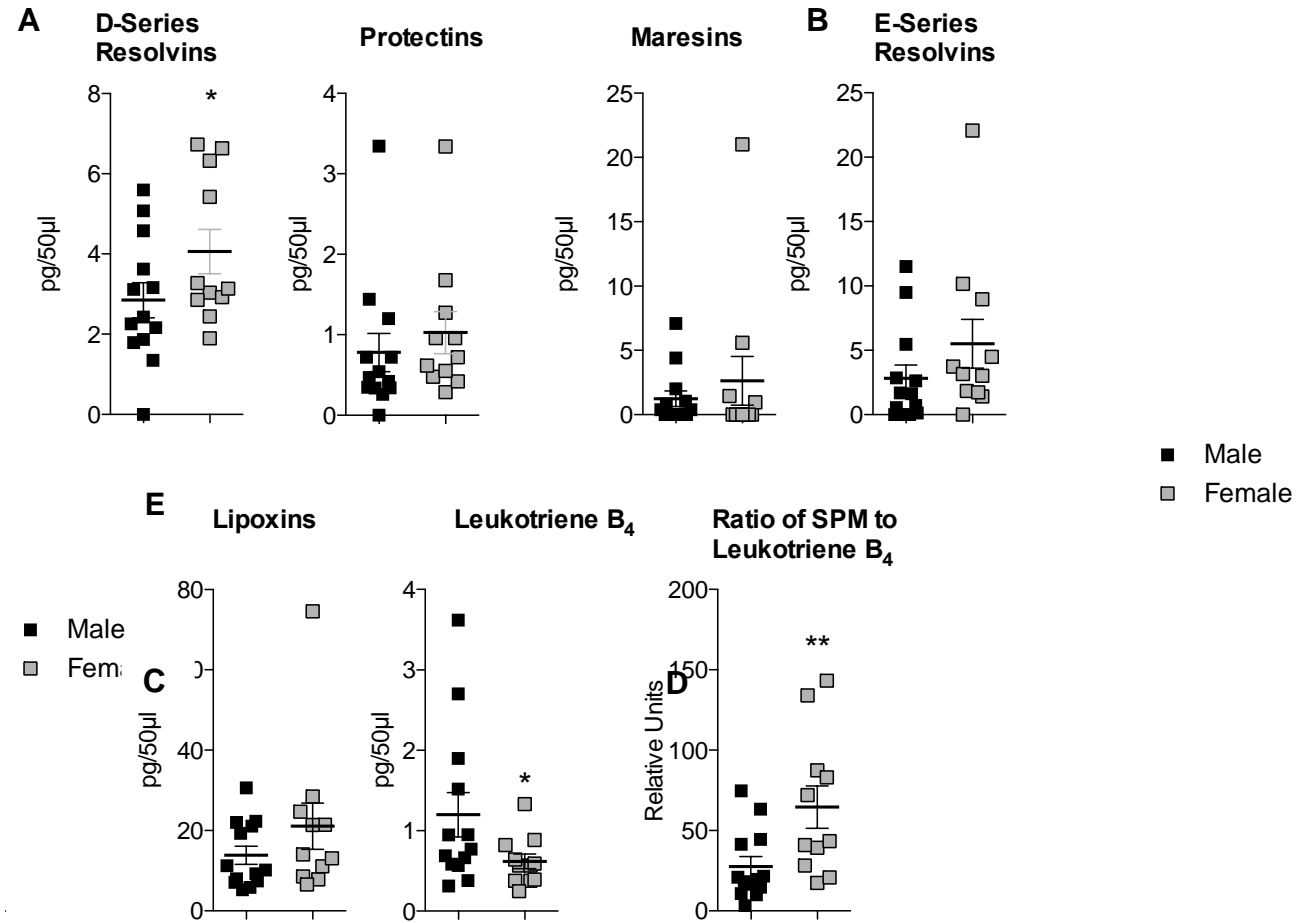
### 4.2.3 Elevated levels of resolving mediators in female blister exudates

Since lipid mediators play an important role in both the initiation and the termination of acute inflammation (Serhan, 2014; Serhan et al., 2015) we next profiled lipid mediator levels in 24 hour blister exudates to investigate whether mediator levels from the three major bioactive metabolomes were differentially regulated between males and females. Using LC-MS/MS we identified mediators from the arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) bioactive metabolomes including Leukotriene (LT) B<sub>4</sub>, Prostaglandin (PG) E<sub>2</sub>, Resolvin (Rv) D<sub>1</sub>, RvD<sub>2</sub>, RvD<sub>3</sub> and Protectin (PD) 1. These molecules were identified in accordance with published criteria (Colas et al., 2014) including matching retention times and > 6 diagnostic ions in the MS-MS to authentic or synthetic standards (**Figure 4.10 A**). Multiple reaction monitoring (MRM) was used to quantify mediators in these blister exudates. Using orthogonal-partial least squares discriminant analysis (OPLS-DA) distinct clusters for lipid mediator profiles in the exudates were evident between male and females (**Figure 4.10 B**, **Table 4.5**).

Assessment of overall levels of each of the lipid mediator families demonstrated that the specialised pro-resolving mediators (SPM) levels were elevated in female blister exudates when compared to those obtained from males (sum of all of the SPMs significantly greater in females ( $17.2 \pm 7.4$  pg/50 $\mu$ l) (SD)) versus males ( $10.5 \pm 6.3$  pg/50 $\mu$ l,  $P=0.015$ ), with particularly the D-series resolvin levels collectively demonstrating a significant increase in females compared to males (**Figure 4.11 A**). In these exudates we also found significantly higher levels of the potent

leucocyte chemoattractant LTB<sub>4</sub> in male exudates when compared to female exudates (**Figure 4.11 B**), thus indicating that female-derived exudates gave an overall pro-resolving lipid mediator profile when compared to male exudates (**Figure 4.11 C**). To determine whether similar differences were evident in volunteers subjected to typhoid vaccination we assessed the levels of lipid mediators in the plasma from volunteers 8 h following vaccination, the time-point at which the differences in endothelial function were most evident (**Chapter 3.0, Figure 3.6 A**). In plasma from these patients we identified lipid mediators from all three major bioactive metabolomes in accordance with published criteria (Colas et al., 2014). Multivariate analysis of Lipid mediator profiles obtained using MRM gave two distinct clusters. Assessment of mediators that were associated with either of the clusters demonstrated that LTB<sub>4</sub> was associated with the male cluster with a Variable in Importance Score >1, whereas RvE1 and RvE3 were found to associate with the female clusters. Statistical analysis of each of the bioactive mediator families demonstrated that in females there was a significant decrease in the LTB<sub>4</sub> metabolome and a significant upregulation of the E-series resolvins specifically, RvE1 and RvE3. (**see Figure 4.12, Figure 4.13 and Table 4.6**).





**Figure 4.11: Female blister exudates display a pro-resolving mediator profile.** Lipid mediators from exudates were extracted using C18 SPE and profiled using LC-MS-MS based lipid mediator profiling. **(A-D)** Cumulative levels for the lipid mediator from the **(A)** docosahexaenoic acid **(B)** eicosapentaenoic acid and **(C)** arachidonic acid SPM and Arachidonic acid-derived LTB<sub>4</sub> **(D)** Ratio of SPM to LTB<sub>4</sub>. Results shown are mean ± s.e.mean of n= 11 females and n=13 males. Statistical significance determined using Students unpaired t-test and shown as \* p <0.05.

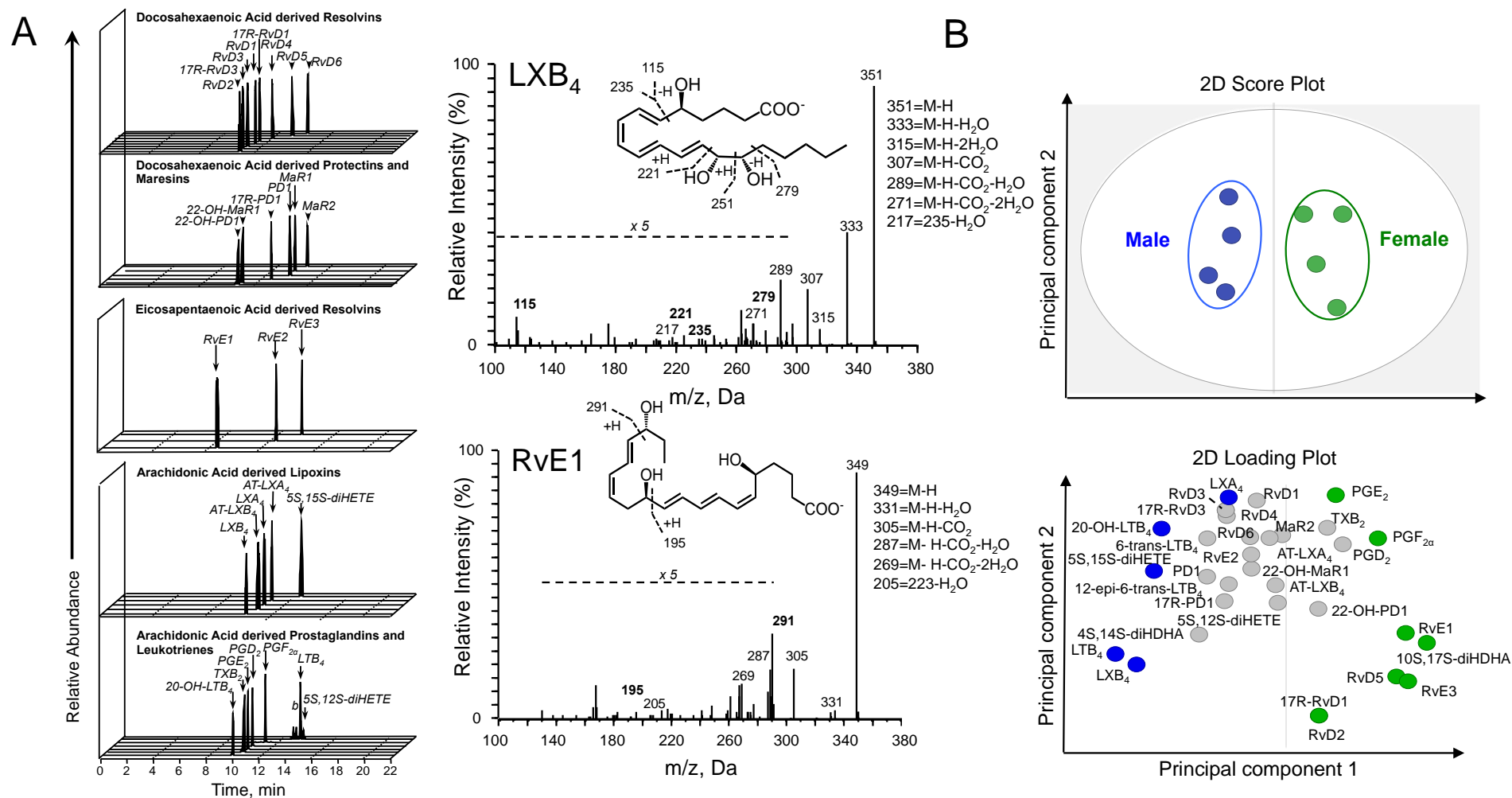


**Table 4.5.** Blister exudate LM-SPM profiles. Exudates were collected from male (n=13) and female volunteers (n=11) 24h after cantharidin application and supernatants were profiling using LC-MS/MS based profiling. Results are mean  $\pm$  s.e.mean with statistical significance determined using Student's unpaired t-test. - = below limit, limit  $\approx$  0.1 pg.

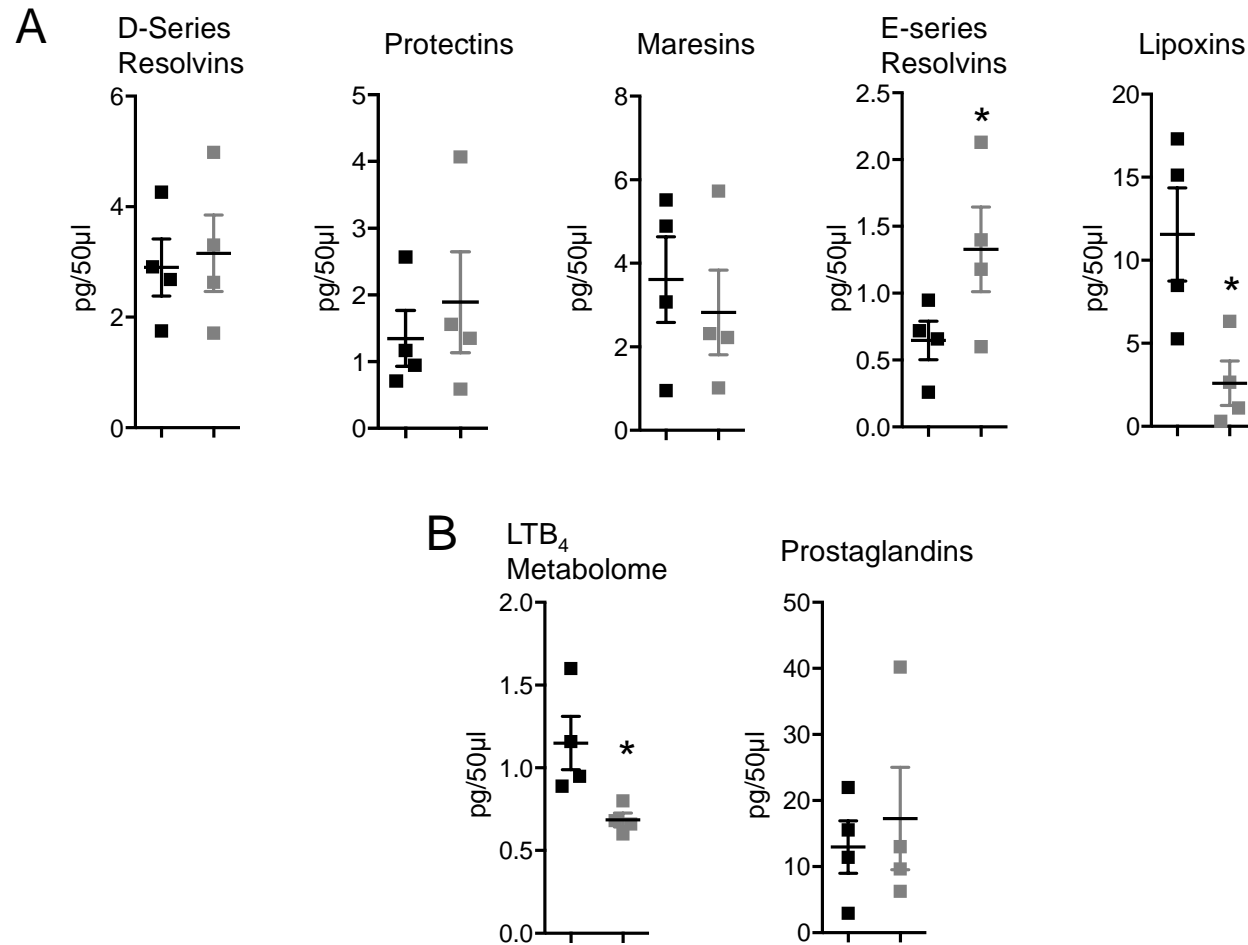
DHA bioactive metabolome	Q1	Q3	Lipid mediators levels (pg/50 $\mu$ L)						
			Female			Male			p value
RvD1	375	215	1.6	$\pm$	1.1	1.4	$\pm$	0.8	
RvD2	375	215	0.4	$\pm$	0.7	0.1	$\pm$	0.3	0.120
RvD3	375	147	0.0	$\pm$	0.1	-			0.143
RvD4	375	101	-		-	-			
RvD5	359	199	0.3	$\pm$	0.3	0.5	$\pm$	0.5	0.176
RvD6	359	159	-		-	-			
17R-RvD1	375	215	1.8	$\pm$	1.7	0.8	$\pm$	0.9	0.051
17R-RvD3	375	147	0.0	$\pm$	0.1	0.1	$\pm$	0.1	0.246
PD1	359	153	<b>0.3</b>	$\pm$	<b>0.6</b>	-			<b>0.046</b>
10S,17S-diHDHA (PDX)	359	153	0.7	$\pm$	0.6	0.8	$\pm$	0.9	0.463
MaR1	359	250	2.5	$\pm$	6.4	0.9	$\pm$	1.7	0.195
7S,14S-diHDHA	359	221	0.1	$\pm$	0.3	0.3	$\pm$	0.7	0.201
17-HDHA	343	245	22.7	$\pm$	17.5	242.6	$\pm$	589.5	0.115
14-HDHA	343	205	194.2	$\pm$	108.2	163.8	$\pm$	120.4	0.263
7-HDHA	343	141	2.7	$\pm$	1.6	3.2	$\pm$	2.7	0.282
4-HDHA	343	101	3.6	$\pm$	1.7	4.6	$\pm$	4.8	0.264
DHA	327	283	3031.3	$\pm$	1309.2	2789.6	$\pm$	1334.4	0.33
<b>EPA bioactive metabolome</b>									
RvE1	349	161	0.4	$\pm$	1.0	0.4	$\pm$	0.6	0.403
RvE2	333	199	1.0	$\pm$	1.1	0.8	$\pm$	1.2	0.344
RvE3	333	245	4.0	$\pm$	6.6	1.6	$\pm$	3.1	0.125
18-HEPE	317	259	2.1	$\pm$	4.8	4.0	$\pm$	9.6	0.288
15-HEPE	317	219	7.9	$\pm$	11.3	101.7	$\pm$	227.7	0.094
12-HEPE	317	179	20.1	$\pm$	52.2	27.3	$\pm$	70.8	0.390
5-HEPE	317	115	0.4	$\pm$	1.2	1.6	$\pm$	4.9	0.232
EPA	301	257	975.5	$\pm$	622.1	1231.0	$\pm$	857.7	0.210
<b>AA bioactive metabolome</b>									
LXA <sub>4</sub>	351	115	-		-	-			
LXB <sub>4</sub>	351	221	5.4	$\pm$	3.6	3.5	$\pm$	2.0	0.062
5S,15S-diHETE	335	115	15.0	$\pm$	19.6	9.9	$\pm$	6.6	0.193
AT-LXA <sub>4</sub>	351	115	0.2	$\pm$	0.3	0.2	$\pm$	0.3	0.493
AT-LXB <sub>4</sub>	351	221	0.6	$\pm$	1.2	0.3	$\pm$	0.5	0.198
LTB <sub>4</sub>	335	195	<b>0.6</b>	$\pm$	<b>0.3</b>	<b>1.2</b>	$\pm$	<b>1.0</b>	<b>0.037</b>
PGD <sub>2</sub>	351	189	<b>16.2</b>	$\pm$	<b>12.9</b>	<b>8.9</b>	$\pm$	<b>4.0</b>	<b>0.032</b>
PGE <sub>2</sub>	351	189	228.1	$\pm$	205.6	134.9	$\pm$	72.2	0.070
PGF <sub>2<math>\alpha</math></sub>	353	193	1116.6	$\pm$	550.0	808.9	$\pm$	468.2	0.076
TxB <sub>2</sub>	369	169	805.2	$\pm$	796.4	426.8	$\pm$	559.6	0.093
20-HETE	319	301	15.5	$\pm$	11.9	19.9	$\pm$	17.8	0.246
15-HETE	319	219	144.8	$\pm$	211.0	848.2	$\pm$	1605.3	0.082
12-HETE	319	179	551.2	$\pm$	223.4	514.5	$\pm$	311.8	0.374
5-HETE	319	115	9.3	$\pm$	5.3	13.3	$\pm$	8.6	0.098
AA	303	259	4320.9	$\pm$	1980.6	4714.8	$\pm$	2041.8	0.319

**Table 4.6. Lipid mediator profiles in plasma from male or female volunteers following typhoid vaccination** Plasma was collected and LM levels were assessed using LM-profiling. Results are expressed as pg/mL; mean  $\pm$  s.e.mean; n = 4 volunteers per group. - = below limit, limit  $\approx$  0.1 pg.

DHA bioactive metabolome	Q1	Q3	Lipid mediators levels (pg/mL)		p value
			Male	Female	
RvD1	375	233	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.280
RvD2	375	141	0.3 $\pm$ 0.1	0.4 $\pm$ 0.2	0.312
RvD3	375	147	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.187
RvD4	375	101	0.5 $\pm$ 0.5	0.1 $\pm$ 0.1	0.230
RvD5	359	199	1.2 $\pm$ 0.2	2.0 $\pm$ 0.7	0.152
RvD6	359	101	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.340
17R-RvD1	375	141	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.312
17R-RvD3	375	147	0.2 $\pm$ 0.1	0.1 $\pm$ 0.0	0.187
PD1	359	153	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1	0.296
17R-PD1	359	153	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1	0.329
22-OH-PD1	375	153	0.4 $\pm$ 0.4	0.7 $\pm$ 0.7	0.343
10S,17S-diHDHA (PDX)	359	153	0.6 $\pm$ 0.0	0.9 $\pm$ 0.3	0.098
MaR1	359	221	-	-	
MaR2	359	191	2.9 $\pm$ 1.3	2.5 $\pm$ 1.0	0.395
22-OH-MaR1	375	221	0.4 $\pm$ 0.4	0.1 $\pm$ 0.2	0.247
7S,14S-diHDHA	359	221	-	-	0.232
4S,14S-diHDHA	359	101	0.3 $\pm$ 0.3	0.1 $\pm$ 0.1	0.178
<b>EPA bioactive metabolome</b>					
RvE1	349	161	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1	0.118
RvE2	333	159	0.1 $\pm$ 0.1	-	0.369
RvE3	333	201	0.4 $\pm$ 0.2	0.9 $\pm$ 0.4	0.094
<b>AA bioactive metabolome</b>					
LXA <sub>4</sub>	351	217	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.298
LXB <sub>4</sub>	351	221	<b>5.2 <math>\pm</math> 2.6</b>	-	<b>0.028</b>
5S,15S-diHETE	335	235	4.9 $\pm$ 2.6	1.1 $\pm$ 0.8	0.082
AT-LXA <sub>4</sub>	351	217	0.2 $\pm$ 0.2	0.1 $\pm$ 0.1	0.317
AT-LXB <sub>4</sub>	351	221	1.1 $\pm$ 0.6	1.2 $\pm$ 0.8	0.440
LTB <sub>4</sub>	335	195	<b>0.6 <math>\pm</math> 0.0</b>	<b>0.5 <math>\pm</math> 0.0</b>	<b>0.006</b>
6-trans- LTB <sub>4</sub>	335	195	0.1 $\pm$ 0.0	-	0.168
12-epi-6-trans- LTB <sub>4</sub>	335	195	0.2 $\pm$ 0.1	-	0.099
5S,12S-diHETE	335	195	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.414
20-OH-LTB <sub>4</sub>	351	195	0.1 $\pm$ 0.0	-	0.085
PGD <sub>2</sub>	351	189	3.5 $\pm$ 1.8	4.4 $\pm$ 2.6	0.382
PGE <sub>2</sub>	351	189	5.7 $\pm$ 2.4	6.9 $\pm$ 3.7	0.379
PGF <sub>2<math>\alpha</math></sub>	353	193	3.8 $\pm$ 1.0	6.0 $\pm$ 3.0	0.220
TxB <sub>2</sub>	369	169	97.8 $\pm$ 36.3	108.7 $\pm$ 52.7	0.426



**Figure 4.12: Distinct plasma LM-SPM profiles in males and females following typhoid vaccination.** Plasma was collected for healthy volunteers following typhoid vaccine administration and LM levels were assessed using LM-profiling (see methods for details). **(A)** *left panel* Representative Multiple Reaction Monitoring chromatograms for identified mediators. *a*= $\Delta$ 6-trans-LTB<sub>4</sub>, *b*=12-epi,  $\Delta$ 6-trans-LTB<sub>4</sub>. *right panel* MS-MS fragmentation and diagnostic ions employed for LXB<sub>4</sub> and RvE1 identification. m/z, mass-to-charge ratio; M-H, molecular ion. **(B)** *top panel* 2-dimensional score plot of human plasma LM-SPM profiles. *Bottom panel* 2-dimensional loading plot. Grey ellipse in the score plot denotes 95% confidence regions. Results are representative n=4 male and n=4 female healthy volunteers. Results are mean $\pm$ s.e.mean n=4 volunteers per group. \* p<0.05 vs Male volunteer plasma values.



**Figure 4.13: Distinct plasma LM-SPM profiles in males and females following typhoid vaccination.** Plasma was collected for healthy volunteers following typhoid vaccine administration and LM levels were assessed using LM-profiling (see methods for details). **(A-B)** Cumulative levels for each of the bioactive LM families identified. Results are mean±s.e.mean n=4 volunteers per group. \* p<0.05 vs Male volunteer plasma values.

### 4.3 Summary

- Cantharidin application to the skin induced blister formation in both sexes. Whilst the exudate volume generated at 24 hrs was similar between the sexes, at 72 hrs the volume was substantially less in females compared to males.
- The total number of cells recruited into blisters showed a trend for reduction in females – although this did not reach statistical significance.
- After 72 hrs, blisters had resolved in 56% of females and 0% in male volunteers, suggesting a faster rate of resolution in females.
- Flow cytometry analysis exposed a prominent sex difference in the proportions of leucocyte subsets recruited into blisters, with significantly reduced numbers of monocytes and lymphocytes in females compared to males.
- There was a generalised reduction in the activation state of all major leucocytes populations as demonstrated by reduced fluorescence intensity of activation markers in females compared to males.
- Lipid mediator profiling exposed a prominent sex difference with a pronounced shift in balance towards a pro-resolving profile in females compared to males.
- Our findings suggest that differences in female sex either suppresses inflammatory leucocyte recruitment in inflammation or promotes leucocyte exit from blisters, with reduced numbers of both inflammatory monocytes and lymphocytes and a reduced activation state of all leucocyte subsets. This difference is associated with a lipid mediator profile indicating that the reduction in cell recruitment in females is likely due to a more efficient resolution of the inflammatory response.
- Both the cantharidin and typhoid vaccine (described in Chapter 3.0) models of inflammation in healthy volunteers enabled us to establish cell assessing techniques in our laboratory and use similar techniques for larger phase II clinical trials such as the NITRATE-OCT study.

## Chapter 5.0

# **A randomised, double-blind, placebo-controlled study investigating the effects of inorganic nitrate on vascular function, platelet reactivity and restenosis in stable angina: Protocol of the NITRATE-OCT study**

## **5.0 A randomised, double-blind, placebo-controlled study investigating the effects of inorganic nitrate on vascular function, platelet reactivity and restenosis in stable angina: Protocol of the NITRATE-OCT study**

### **5.1 Introduction to NITRATE-OCT**

The mainstay treatment for reducing the symptoms of angina and long-term risk of heart attacks in patients with heart disease is stent implantation in the diseased coronary artery. Whilst this procedure has revolutionised treatment the incidence of secondary events remains a concern. These repeat events are thought to be due, in part, to continued enhanced platelet reactivity, endothelial dysfunction and ultimately restenosis of the stented artery. The NITRATE-OCT trial is investigating whether a once a day inorganic nitrate ( $\text{NO}_3^-$ ) administration might favourably modulate platelet reactivity and endothelial function leading to a decrease in restenosis. This is a double-blind, randomised, single-centre, placebo-controlled phase II trial that aims to enrol 246 patients with stable angina due to have elective PCI procedure with stent implantation. Patients are randomised to receive 6 months of a once a day dose of either  $\text{NO}_3^-$ -rich beetroot juice or  $\text{NO}_3^-$ -deplete beetroot juice (placebo) starting up to one week before their procedure (**Figure 5.1**). As recruitment for this is still ongoing, the results in this chapter contains the baseline data for 200 participants that have been recruited upto 30<sup>th</sup> of April 2018.

#### **5.1.1 Introduction to the effect of inorganic nitrate on the inflammatory response following PCI**

Inorganic  $\text{NO}_3^-$ , when converted to  $\text{NO}_2^-$  via the enterosalivary circuit, has been shown to reduce smooth muscle cell proliferation and promote endothelial cell

growth in our laboratory. This study contains a sub-analysis of our large Phase II double blinded randomised controlled trial (NITRATE-OCT) that is investigating whether there is a reduction in the growth of smooth muscle cells (preventing restenosis within the stent), following ingestion of inorganic  $\text{NO}_3^-$  in patients undergoing percutaneous coronary intervention (PCI) with stent implantation for stable angina. If this hypothesis is found to be correct, then the exact mechanisms by which this benefit might be occurring are not yet clear.

The aim of this part of the thesis was to explore the possibility that the beneficial effects of inorganic  $\text{NO}_3^-$  with regards to reducing restenosis in stable angina patients might relate to effects on inflammation, by assessing the levels of specific pro-inflammatory components and circulating cell subtypes as exploratory endpoints in the NITRITE-OCT cohort.

To ensure that the trial remained blinded from the investigators of the trial, the samples have been grouped as the 'whole cohort' at baseline compared to 6 months post intervention. We analysed peripheral blood leukocyte subsets including markers of activation and the release kinetics of high-sensitivity CRP (hs-CRP), and the neutrophil chemokines CX3CL-1 and CCL-2 in patients who received either inorganic  $\text{NO}_3^-$  in the form of beetroot juice containing 4-5 mmol of inorganic nitrate (N = 78 in total). Blood samples were collected at baseline and at 6 months following ingestion of the juice.



## 5.2 Aims

The primary aim of this study is to test whether dietary NO<sub>3</sub><sup>-</sup> ingestion in addition to conventional therapy has beneficial effects in patients with stable angina. To date, I have recruited 200 patients with 130 patients who have completed their primary endpoint. In this chapter, I will present all the baseline data so far.

Specific aims are:

- 1) To determine whether it is safe to deliver dietary NO<sub>3</sub><sup>-</sup> in patient undergoing PCI and stent implantation and whether the randomisation is effective.
- 2) To determine whether dietary NO<sub>3</sub><sup>-</sup> ingestion exerts anti-platelet effects or improvement of endothelial function and the mechanisms involved in this effect.

## 5.3 Methods

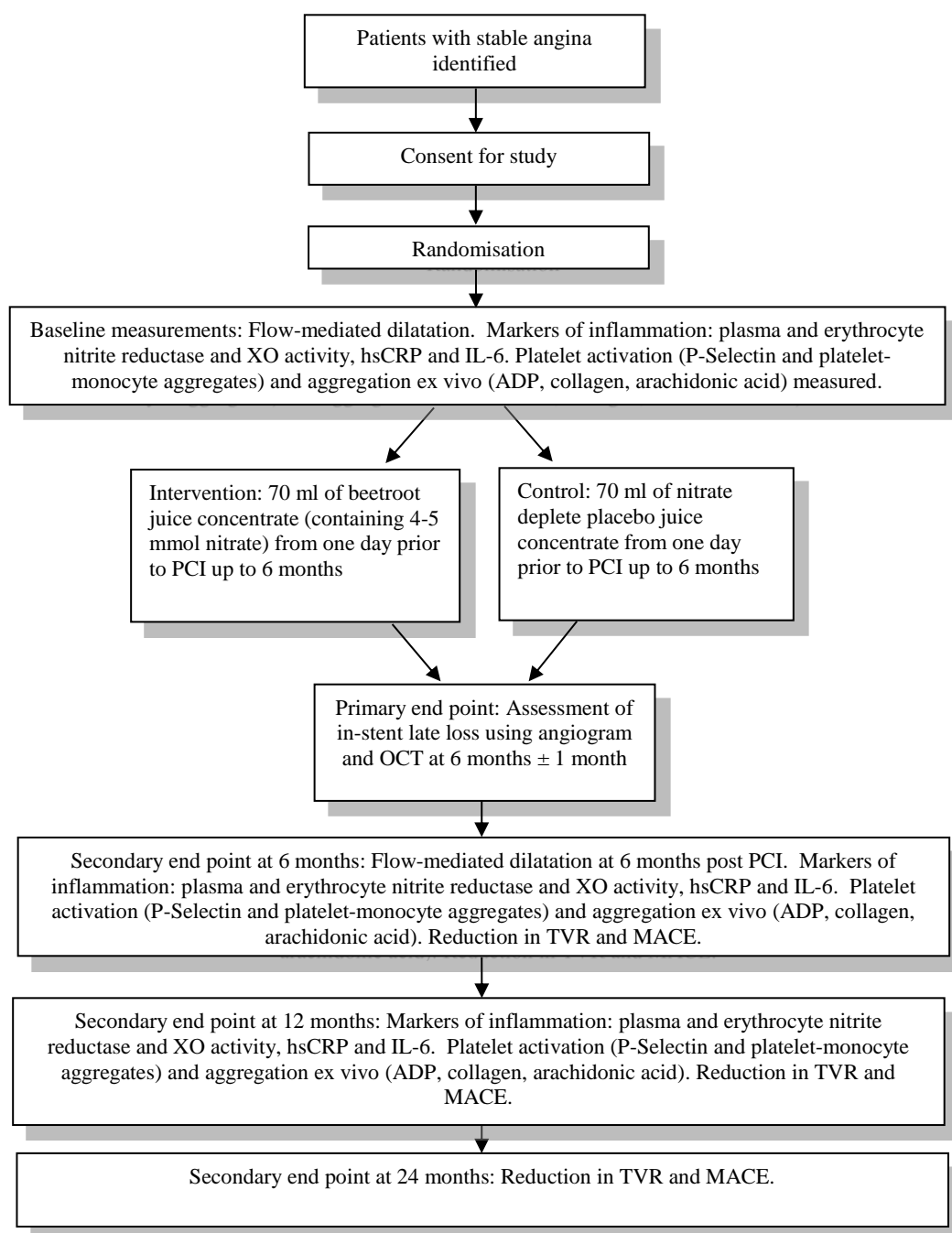
The study was carried out according to the detailed methods described in Chapter 2 section 2.2 and the flow chart shown in **Figure 5.1**.

### 5.3.1 Sample size calculation

Sample size was determined for a total of 246 patients for a two treatment parallel-design study. We calculated that with a probability of 80 percent we are able to detect a treatment difference at a two sided 5% significance level, if the true difference in late loss between the treatments is 0.22 mm (i.e. ~50% of the effect size seen with PDE inhibition which resulted in a 35% reduction in late loss) (Mauri et al., 2005). This absolute difference is calculated from a mean late loss of 1.27mm with a standard deviation of the response variable of 0.550. These values

being the mean and average of the standard deviations of 22 trials measuring late loss in trials of both drug eluting and bare metal stents described in the review by Mauri et al (Mauri et al., 2005). The recruitment of 246 also takes into account an additional 10% for drop-out or withdrawal/non-compliance. This value is based upon previous experience in our unit. All sample calculations were done using G\*Power 3.0 <sup>TM</sup>.

In addition, the sample size of 246 enables sufficient power for secondary outcome measure of estimation of the hard endpoint of MACE at 6 months. The number is based on a study, in stable angina patients who underwent elective angioplasty and a remote ischaemic preconditioning intervention which resulted in a significant reduction in MACE at 6 months with 4/110 (3.6%) in the treatment group versus 13/104 (12.5%) in the control group (Davies et al., 2013). Using these data as a basis for power calculations, a total number of 230 patients are needed for 80% power using one-tailed analysis. Thus, to account for potential loss to follow-up 246 patients will be recruited in total. The data will be analysed on an intention to treat basis. In addition, further per protocol analyses and a subgroup analysis of patients on organic nitrates as part of their routine therapy and a comparison of DES (drug-eluting stents) versus BMS (bare-metal stents) will be conducted.



**Figure 5.1: Flow diagram of Study design**

hsCRP: high sensitivity C-Reactive Protein, IL-6: Interleukin 6, MACE: Major Adverse Cardiac Events, OCT: Optical Coherence Tomography, TVR: Target Vessel Revascularisation, XOR: Xanthine Oxidoreductase.

## 5.4 Data analysis

The data analysis plan for this study is as detailed in the methods (sections 2.2.2.5 and 2.2.2.6). However, since the study is still underway, for this thesis all available baseline data is shown according to the randomisation group. The study has been maintained blind by an unrelated individual dividing the patients recruited into gamma and delta groups. For all analysis comparing baseline with completed, the two groups are combined and an overall assessment of baseline with 6 months conducted for only those patients that have completed the primary outcome at 6 months. For the sake of this thesis, paired analysis has been conducted.

The data are shown for 200 patients for demographics, 200 patients for discharge and 130 patients have reached the primary end point. For the biochemical analysis, there is data available for 83 plasma  $\text{NO}_3^-/\text{NO}_2^-$  analysis, 74 saliva  $\text{NO}_3^-/\text{NO}_2^-$  analysis and 66 urine  $\text{NO}_3^-/\text{NO}_2^-$  analysis. With regards cGMP measurement, we have analysed 77 paired samples to date. For the inflammation sub-group analysis, we have 78 paired data for total leukocyte and urate levels, 66 paired analysis for the activations markers from flow cytometry and 78 paired data for the cytokine and chemokine analysis. **Table 5.1** describes this attrition and reasons in more detail.

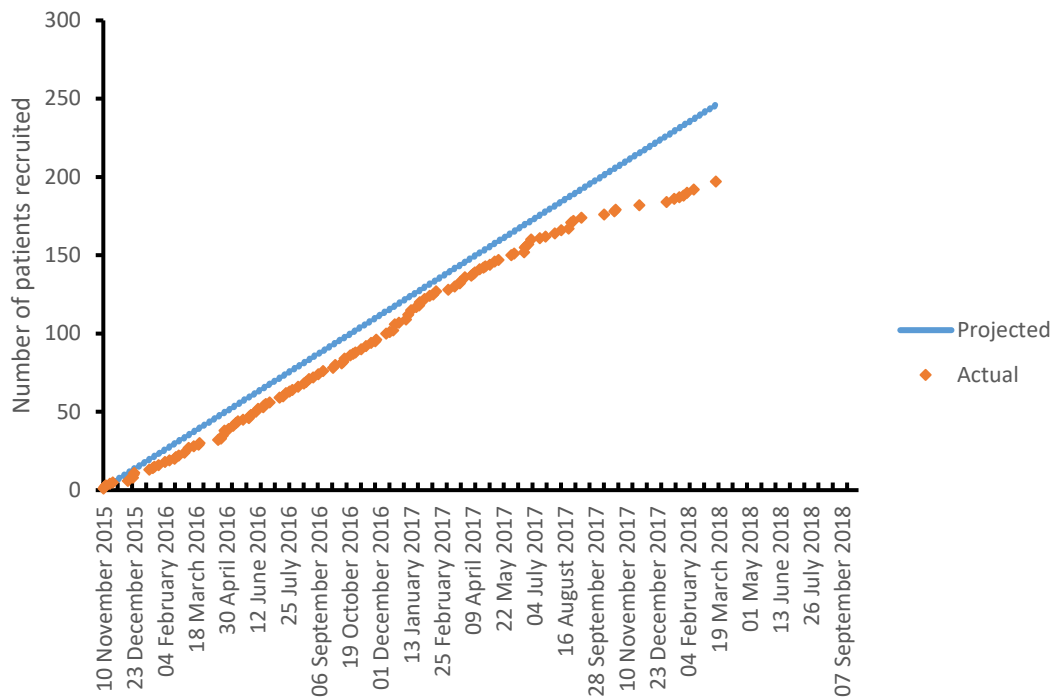
	Total Number so far	Target	Reasons
<b>Data collected</b>	<b>(n=200)</b>		
Demographics	200 (100.0%)	246	Study is ongoing collecting data
Discharge data	200 (100.0%)	246	Study is ongoing collecting data
Primary end point	130 (65.0%)	220	Study is ongoing collecting data
Biochemical Analysis			
Plasma NO <sub>3</sub> <sup>-</sup> /NO <sub>2</sub> <sup>-</sup>	83 (41.0%)	246	Study is ongoing collecting data
Saliva NO <sub>3</sub> <sup>-</sup> /NO <sub>2</sub> <sup>-</sup>	74 (37.0%)	246	Study is ongoing collecting data
Urine NO <sub>3</sub> <sup>-</sup> /NO <sub>2</sub> <sup>-</sup>	66 (33.0%)	246	Study is ongoing collecting data
cGMP	77 (38.5%)	246	Study is ongoing collecting data
Inflammation Sub-group			
Total leukocyte	78 (39.0%)	80	Loss of data due to drop-outs
Urate levels	78 (39.0%)	80	Loss of data due to drop-outs
Activation marker data	66 (33.0%)	80	Loss of data due to drop-outs
Chemokine/cytokine data	78 (39.0%)	80	Loss of data due to drop-outs

**Table 5.1. Attrition rate of data from the NITRATE-OCT Study.** Values shown as number (%) unless otherwise stated.

## 5.5 Clinical Trial Results

### 5.5.1 Clinical Trial recruitment to date

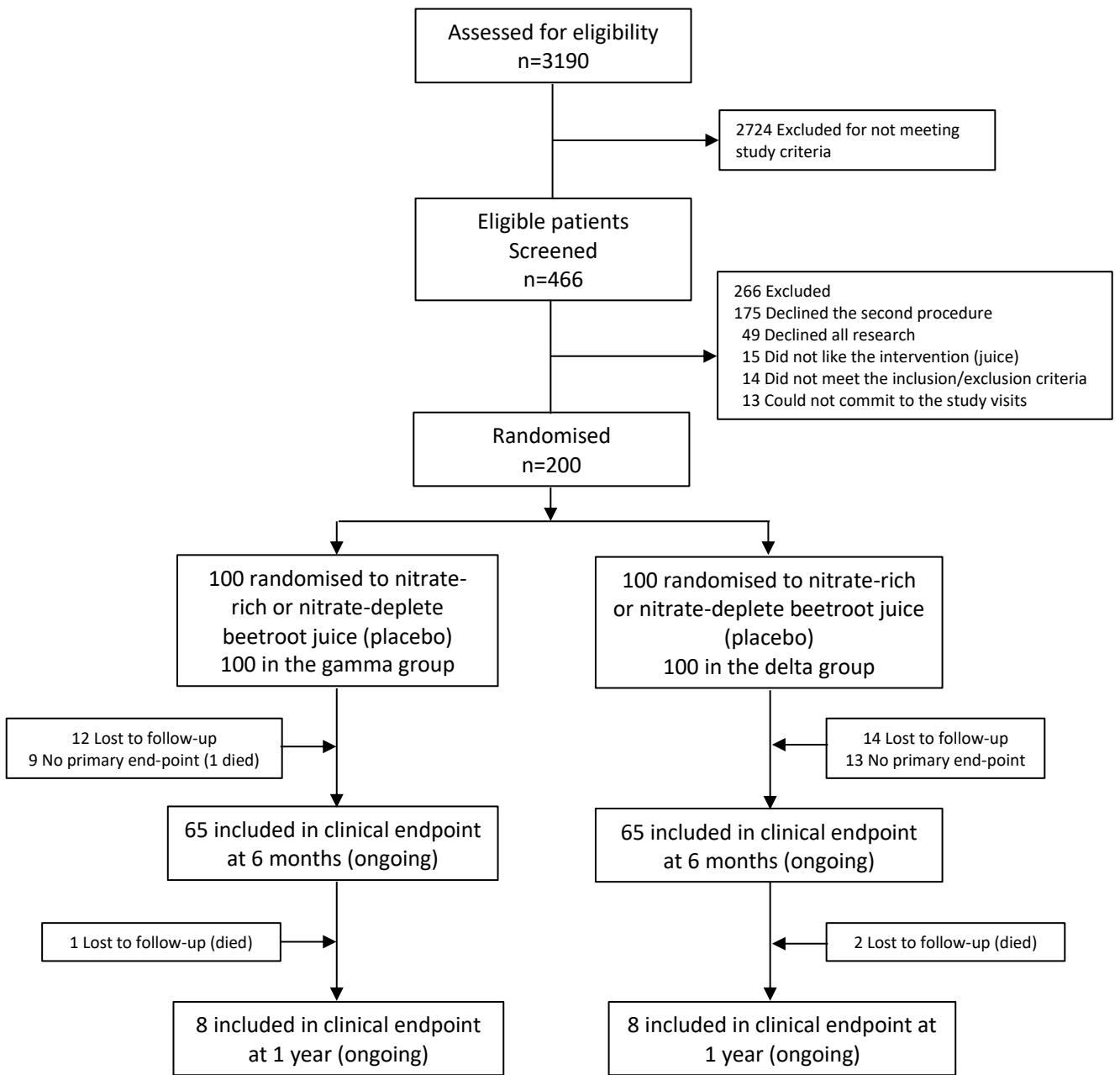
200 patients have been recruited to date (from the 10<sup>th</sup> of November 2015 to the 30<sup>th</sup> of April 2018) and 130 have completed the primary endpoint as of 30<sup>th</sup> April 2018, (**Figure 5.2**).



**Figure 5.2: Recruitment to NITRATE-OCT (As of 30<sup>th</sup> April 2018)**

Between November 2015 and April 2018, 3190 patients were admitted for an elective angioplasty and stent insertion for symptoms of stable angina at The Barts Health Heart Attack Centre. Of these patients 466 were eligible for this study and 2724 were excluded for not meeting study criteria. Among these 466 patients, 266 (57.1%) were excluded for the following reasons: 175 (37.6%) patients declined to have the second procedure in the study, 49 (10.5%) patients declined to take part in any research, 15 (3.2%) patients did not like the taste of beetroot juice (the intervention), 14 (3.0%) patients did not meet the inclusion/exclusion criteria and 13 (2.8%) patients could not commit to the study visits involved in the study. This left 200 patients suitable who were randomised. In the unit, many studies in both healthy volunteers and CVD patients have been conducted and this is the first that we have witnessed taste as a criteria for refusal to participate. The 3% rate

suggests that whilst this is more than previous, this would still not be a major deterrent for therapeutics. Nevertheless, this data suggests that large-scale trials are needed to discern the true potential of this dietary approach in terms of compliance. Certainly, this represents the largest and longest trial to date of such a dietary intervention, with previous studies lasting no more than 6 months and at most in 126 patients (Mills et al., 2016). From the patients recruited so far, baseline data is available for 200 patients in total (100 patients in both groups). Furthermore, we have data collected for the primary endpoint for 130 (**Figure 5.3**).



**Figure 5.3: Consort diagram.**



### 5.5.3 General characteristics of Study population

As the NITRATE-OCT study is still ongoing, the treatment given to each group remains blinded to avoid bias in analysis. The groups have been labelled gamma and delta to ensure that the trial remains blinded from the investigators.

This study is a randomised trial and as such the baseline characteristics demonstrate good matching of the groups. All baseline characteristics were similar between the treatment groups (**Table 5.2**) except for mean baseline heart rate (HR), which was higher in the gamma group compared to the delta group ( $69.85 \pm 17.00$  bpm vs  $64.93 \pm 12.71$  minutes). This difference in HR in 1 in 30 of the different baseline characteristics is likely to be reflective of a chance observation. The mean age of the trial participants was 62 years, with 83.5% male. This split is typical of such cohorts and reflects the prevalence as discussed previously of increased rates of coronary artery disease in men versus women. In general, rates of coronary artery disease in women catch up to those in men post 65 years (Maas and Appelman, 2010). The two study groups were similar with respect to NYHA and CCS classification of angina and Previous MI/PCI/CABG (**Table 5.2**).

	Gamma (n=100)	Delta (n=100)	P Value
Age (yr) (Mean±SD)	61.5±9.1	63.1±9.6	0.257
Sex (M/F)	84 (84.0)	82 (82.0)	0.981
Ethnicity			
Caucasian	84 (84.0%)	82 (82.0%)	1.000
Afro-Caribbean	3 (3.0%)	5 (5.0%)	0.444
Asian	10 (10.0%)	10 (10.0%)	1.000
East Asian	3 (3.0%)	3 (3.0%)	1.000
Diabetes mellitus	26 (26.0%)	26 (26.0%)	1.000
Type I	4 (4.0%)	4 (4.0%)	1.000
Type II	22 (22.0%)	22 (22.0%)	1.000
Body-mass index (kg/m <sup>2</sup> ) (Mean±SD) <sup>a</sup>	28.8±4.3	29.7±5.2	0.185
Hypertension	75 (75.0%)	75 (75.0%)	1.000
Hypercholesterolaemia	73 (73.0%)	84 (84.0%)	0.106
Previous MI	20 (20.0%)	22 (22.0%)	0.855
Previous PCI	28 (28.0%)	34 (34.01%)	0.422
Previous CABG	3 (3.0%)	3 (3.0%)	1.000
Previous Smoker	62 (62.0%)	64 (64.0%)	0.878
PVD	7 (7.0%)	11 (11.0%)	0.433
CVA/TIA	3 (3.0%)	6 (6.0%)	0.720
NYHA			
Class I	3 (3.0%)	6 (6.0%)	0.720
Class II	3 (3.0%)	4 (4.0%)	0.541
CCS			
CCS I	15 (15.0%)	20 (20.0%)	0.442
CCS II	42 (42.0%)	33 (33.0%)	0.283
CCS III	42 (42.0%)	46 (46.0%)	0.654
Asthma	9 (9.0%)	9 (9.0%)	1.000
COPD	11 (11.0%)	5 (5.5%)	0.281
Previous History of CAD	47 (47%)	53 (58.2%)	0.181
Heart rate (BPM) (Mean±SD)	69.9±17.0	64.9±12.7	0.030
Systolic BP (mmHg) (Mean±SD)	135.3±18.0	136.5±16.2	0.652
Diastolic BP (mmHg) (Mean±SD)	76.9±8.8	75.8±10.5	0.745
Culprit Vessel			
Left main stem	1 (1.0%)	0 (0.0%)	1.000
Left anterior descending	33 (33.0%)	39(39.0%)	0.536
Circumflex	21 (21.0%)	17 (17.0%)	0.569
Right coronary	34 (34.0%)	30 (30%)	0.633

**Table 5.2. Baseline characteristics of patients in NITRATE-OCT by blinded treatment group.**

Values shown as number (%) unless otherwise stated. (PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES drug-eluting stent; MI, myocardial infarction; BPM, beats per minute; BP, blood pressure; CCS, Canadian classification system; NYHA, New York Heart Failure Association Classification; CABG, Coronary artery bypass grafting; COPD, chronic obstructive pulmonary disease).

<sup>a</sup>The body-mass index is the weight in kilograms divided by the square of the height in metres.

There were no differences in the type of stent deployed, length of stent or diameter of stent between the two groups. Use of drug-eluting stents and anti-platelet therapy were similar between the 2 groups (**Table 5.3**).

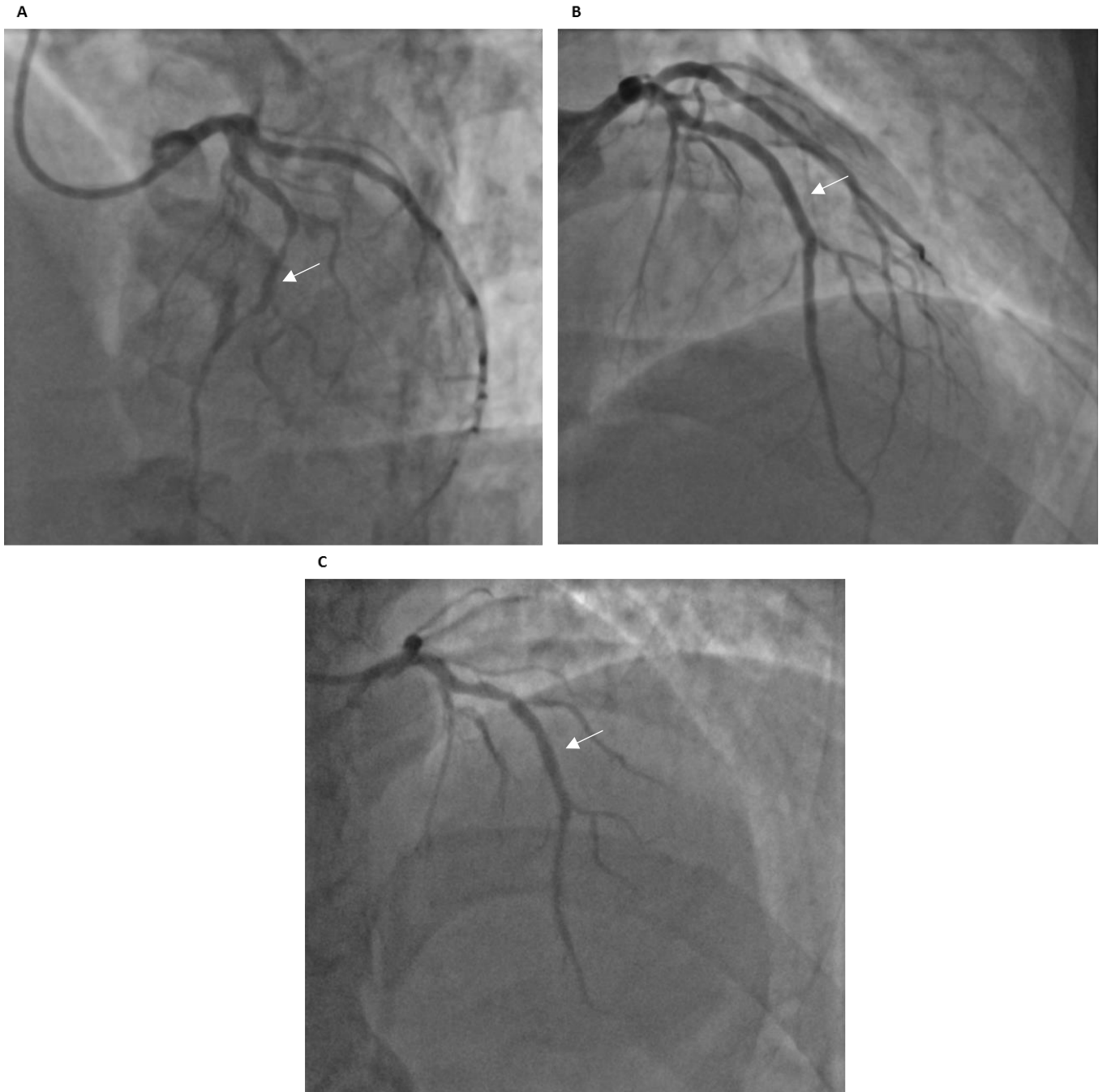
There were three main types of drug eluting stents used over the period of recruitment. These included Xience (Everolimus-eluting stent), Promus (Everolimus-eluting stent) and Resolute Integrity (Zotarolimus-eluting). Overall, majority of the patients had a Xience stent implanted mainly based on the operator preference. Since there were a number of patients who did not undergo a stent implantation during the procedure, which was a decision made by the cardiologist at the time, the sum of all the stents do not add up to 100%. This was documented as a protocol deviation for the study.

	Gamma (n=100)	Delta (n=100)	P value
Access site			
Radial	76 (76.0%)	76 (76.0%)	1.000
DES use	87 (87.0%)	88 (88.0%)	0.778
Number of stents used (Mean±SD)	1.51±0.82	1.56±0.75	0.847
Stent Type			
Xience	74 (74.0%)	76 (76.0%)	0.865
Resolute Integrity	6 (6.0%)	3 (3.0%)	0.598
Promus Premier	2 (2.0%)	4 (4.0%)	0.368
Biofreedom	0 (0%)	1 (1.0%)	1.000
Stent length (Mean±SD)			
First Stent	25.2±9.1	25.24±8.7	0.983
Second Stent	23.1±8.2	22.06±9.6	0.662
Third Stent	20.0±5.8	20.25±8.1	0.945
Fourth Stent	26.5±16.3	23.00±13.4	0.781
Stent diameter (Mean±SD)			
First Stent	3.1±0.5	3.0±0.5	0.145
Second Stent	3.2±0.6	3.0±0.5	0.119
Third Stent	3.0±0.6	2.9±0.4	0.815
Fourth Stent	3.0±0.7	3.5±0.8	0.458
Treatment at time of PCI			
Verapamil	43 (46.0%)	43 (43.0%)	0.842
Heparin	100 (100%)	100 (100%)	1.000
Heparin Dose (Mean±SD)	8926.5±2770.1	9198.5±3126.9	0.698
Aspirin	100 (100%)	100 (100%)	1.000
Clopidogrel/Ticagrelor	100 (100%)	100 (100%)	1.000
Procedural Success	100 (100%)	100 (100%)	1.000

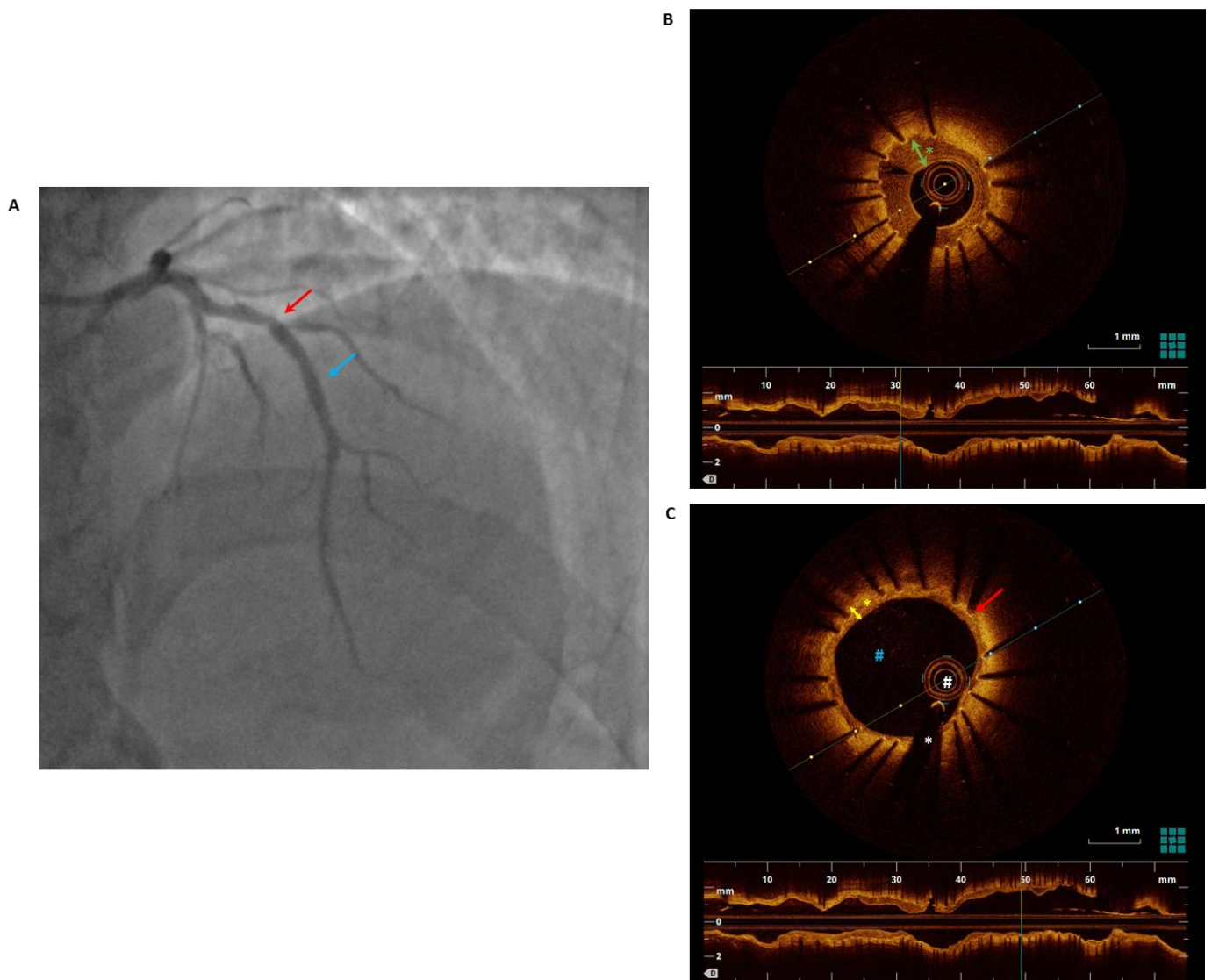
**Table 5.3. Procedural characteristics by treatment group.** Values shown as number (%) unless otherwise stated. (DES; Drug-eluting stent)

#### 5.5.4 Primary End-point

Out of all patients recruited to date, 130 have achieved the primary endpoint of Angiography and for QCA analysis and OCT. An example of the angiogram (at baseline and 6 month) for patient 040 is shown in **Figure 5.4** and **Figure 5.5**.



**Figure 5.4: Angiogram images of the LAD (indicated by the white arrow) at A) baseline before the stent was implanted, B) at baseline after the stent and C) at 6 months after the stent.**



**Figure 5.5: Representative images of: A) An angiogram of the LAD artery, B) OCT imaging of the proximal LAD stent showing moderate to severe in-stent restenosis and C) OCT imaging of the distal LAD stent showing normal endothelialisation and virtually no in-stent restenosis.**

Panel A demonstrates an angiogram of the LAD in the RAO Cranial view. There is moderate to severe stenosis within the proximal portion of the stent. Panel B shows moderate to severe ISR (represented by the green \*). Panel C shows normal endothelialisation within the stent (represented by the yellow \*). The red arrow

shows the stent strut and there is drop-out of light behind the stent strut. The blue is showing the lumen of the LAD artery. The white # is showing the OCT catheter and the white \* represents the guide wire, again there is drop-out of light behind the wire due to wire artefact.

### 5.5.5 Acute safety and tolerability of nitrate across the whole cohort

There were no significant differences between the groups regarding heart rate, systolic BP or diastolic BP at 6 months compared to baseline (**Table 5.4**).

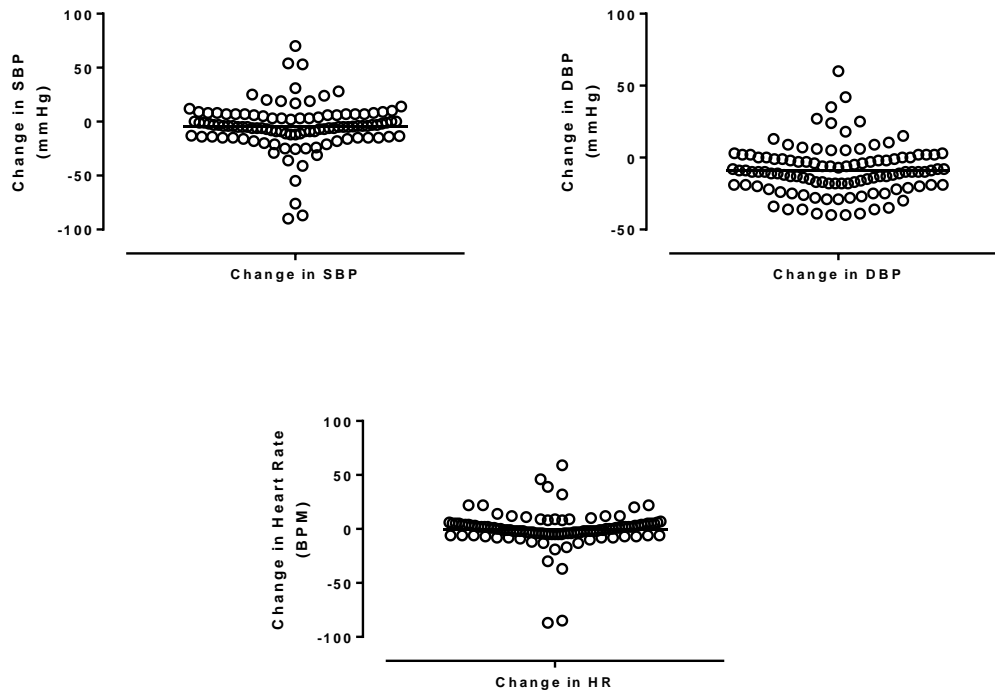
Baseline	Baseline (n=130)	6 months (n=130)	P value
Heart rate (BPM) (Mean±SD)	67.38±15.16	66.71±16.48	0.276
Systolic BP (mmHg) (Mean±SD)	135.91±17.05	129.78±21.00	0.385
Diastolic BP (mmHg) (Mean±SD)	76.316±9.67	73.68±10.11	0.686
Methaemaglobinemia level (Mean±SD)	0.41±0.27	0.54±0.31	0.839

**Table 5.4. Baseline and 6 month blood pressure, heart rate and methamaglobinaemia levels are shown** (BP, blood pressure; BPM, beats per minute).

Assessment of the change in each parameter over the 6 month indicated an overall reduction in SBP at 6 months compared to baseline of -4.50 mmHg (IQR: -14.00 – 6.00) and an overall reduction in DBP at 6 months compared to baseline of -10.00 mmHg (IQR: -19.00 – 0.00). In contrast, there does not appear to be any significant change in heart rate at 6 months compared to baseline (1.00 BPM (IQR: -4.50 – 4.50) (**Figure 5.6**).

Met-Hb levels were measured to assess the possibility that raising circulating NO<sub>2</sub><sup>-</sup> levels may result in complications with oxygen delivery due to the interaction of NO<sub>2</sub><sup>-</sup> with oxy-Hb (Greer and Shannon, 2005). Overall, there appears to be no

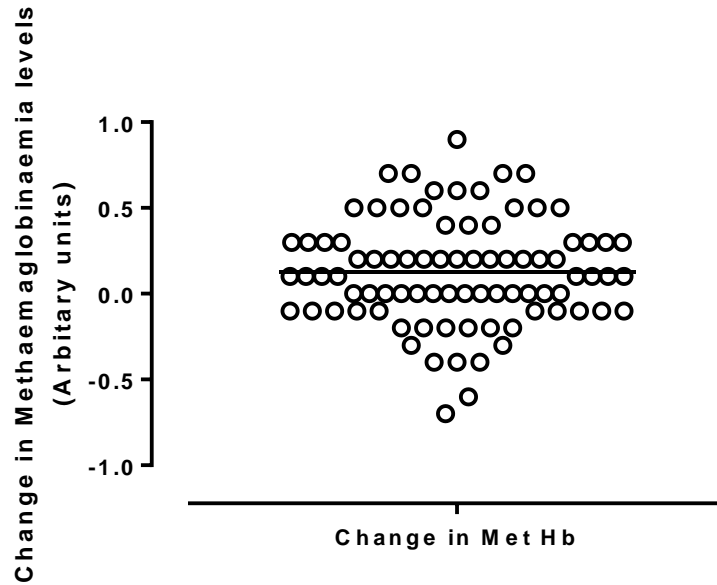
clinically significant rise in met-Hb levels at 6 months compared to baseline (median 0.10 IQR: -0.10 - 0.30). (Figure 5.7).



**Figure 5.6: Assessment of the effect of beetroot juice (both inorganic nitrate and placebo) on systolic blood pressure, diastolic blood pressure and heart rate.**

The change in BP (A & B) and heart rate (C) following 6 months of the intervention compared to baseline is shown.





**Figure 5.7: Assessment of the effect of beetroot juice (both inorganic nitrate and placebo) on methaemaglobinaemia levels.**

Overall change in methaemaglobinaemia levels at 6 months compared to baseline in (n=130) are shown. This suggests no significant change in methaemaglobin levels.

### 5.5.6 Medication

Data was available on prescribed medication for 100% of patients at discharge following the angioplasty. Overall the majority of both patient groups were optimised on medical therapy at discharge (dual anti-platelet agents, statin, beta-blocker and angiotensin-receptor blocking drugs). (**Table 5.5**).

	Gamma	Delta	P value
<b>Medication at discharge</b>	<b>(n=100)</b>	<b>(n=100)</b>	
ACE inhibitor	55 (55.0%)	45 (45.0%)	0.307
ARB	11 (11.0%)	17 (17.0%)	0.195
Diuretic	9 (9.0%)	12 (12.0%)	0.626
Amlodipine	23 (23.0%)	19 (19.0%)	0.585
β Blocker	63 (63.0%)	74 (74.0%)	0.152
Statin	95 (95.0%)	92 (92.0%)	0.767
Aspirin	100 (100%)	100 (100%)	1.000
Clopidogrel	78 (78.0%)	75 (75.0%)	0.621
Ticagrelor	22 (22.0%)	25 (25.0%)	0.725
Nicorandil	9 (9.0%)	13 (13.0%)	0.478
Organic Nitrate	23 (23.0%)	20 (20.0%)	0.718
GTN	55 (55.0%)	66 (66.0%)	0.172
Ranolazine	2 (2.0%)	2 (2.0%)	1.000
Warfarin	1 (1.0%)	3 (3.0%)	0.621
NOAC	5 (5.0%)	5 (5.0%)	1.000

**Table 5.5. Prescribed drugs at baseline** Values shown as number (%) unless otherwise stated.

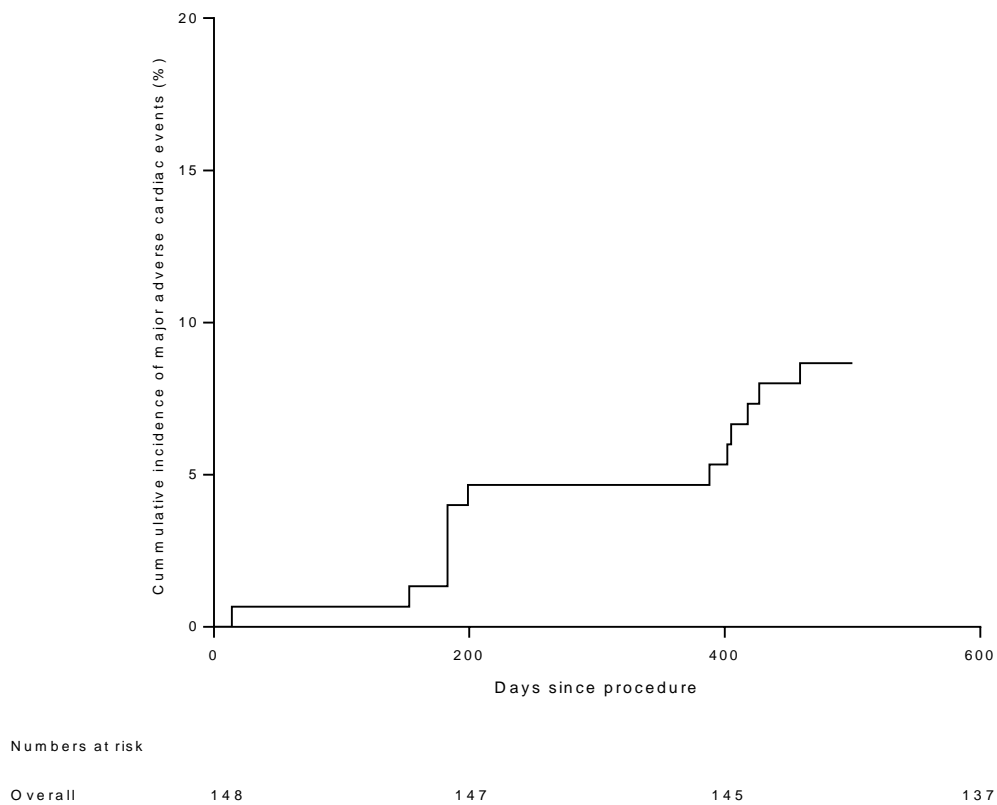
Abbreviations: ACEi: Angiotension-converting enzyme; ARB: Angiotensin receptor blocker.

### **5.5.7 Serious adverse events and adverse events**

Of the 200 patients recruited, all of the patients were discharged successfully following their procedures with no events during their stay. On average, the stay duration was  $1.3 \pm 0.6$  days. There were no differences between the groups in the length of stay. There have been 15 serious adverse events (SAE) (4 deaths – (1 following mitral valve surgery 5 months following the index procedure, 3 further deaths beyond 1 year from recruitment, which means they were 6 months following their end of the intervention), 3 re-hospitalisations for myocardial infarction (2 NSTEMIs and 1 STEMI), 2 re-hospitalisations (1 for right sided chest pain following a mechanical fall), 1 prolonged hospitalisation for left ventricular failure, 6 patients underwent target vessel revascularisation at their 6 month angiogram and 1 patient was diagnosed with prostate carcinoma. There have been 37 adverse events (16 patients had gastrointestinal upset resulting in 13 patients withdrawing from the study, 1 patient had light-headedness at 5.5 months into the study, 1 patient had light-headedness 3 months into the study, 1 patient had an unplanned diagnostic angiogram due to shortness of breath – angiogram was unremarkable, 1 patient had PCI to by-standing disease in another vessel due to angina, 1 patient was diagnosed with leukaemia but is not life-threatening, 2 patients had high blood sugars which was managed by increasing the doses of diabetic medications and 1 patient broke their ankle at work (mechanical in nature). There have been no suspected, unexpected serious adverse reactions. All four deaths in the study have been reviewed by the Chief Investigator and Principal Investigator of the study and we have concluded that this unfortunate event was not related to the intervention and therefore the study was not unblinded at the time.

### 5.5.8 Major adverse cardiac events

MACE is a powered secondary endpoint in this study. The events shown have been recorded but not unblinded. Overall, at a 2 year time-point after being recruited into the study, 13 patients have suffered MACE (4 deaths, 3 recurrent myocardial infarction and 6 unscheduled revascularizations) (**Figure 5.8**).

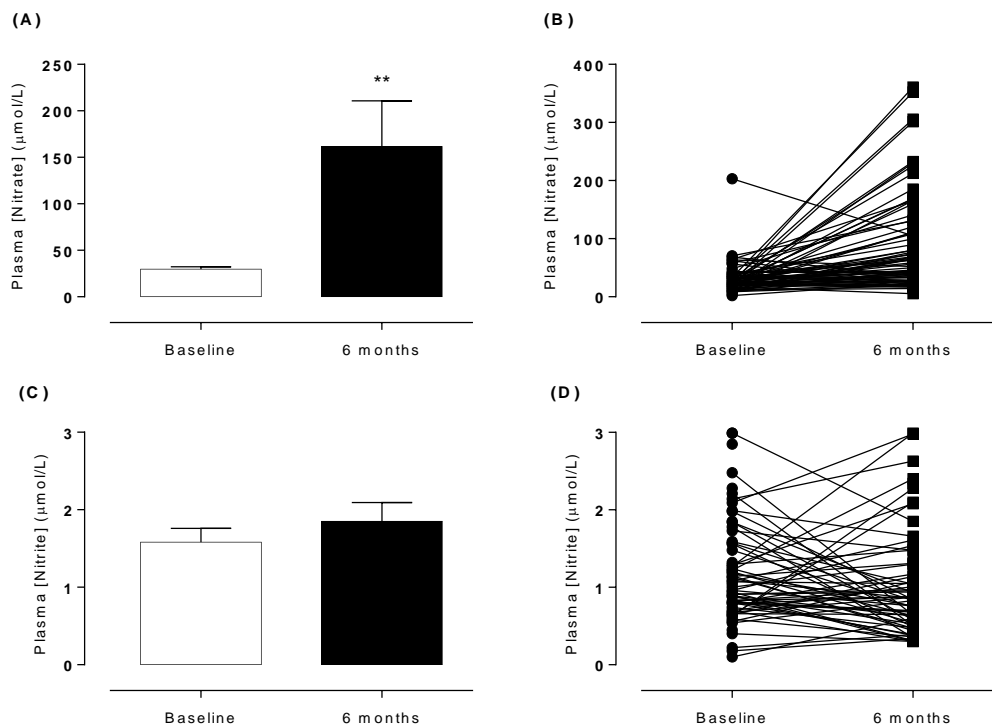


**Figure 5.8: Kaplan Meier curve.** Survival curve showing cumulative probability of major adverse cardiac events (MACE) after PCI for stable angina in the overall population.

## **5.6 Comparison of baseline and 6 month plasma [cGMP] and plasma/urine/saliva [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>] in patients with stable angina**

### **5.6.1 Effect of 6 month intervention on [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] in plasma samples from patients with angina**

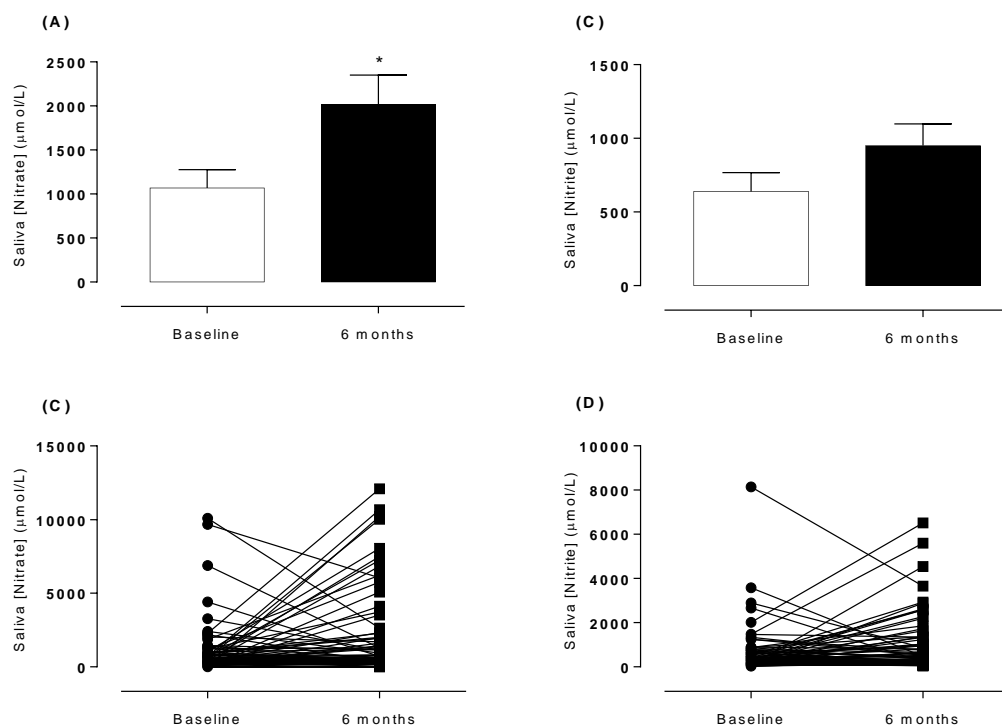
There was a significant increase in the plasma [NO<sub>3</sub><sup>-</sup>] concentration from baseline of approximately 3.7 - fold (**Figure 5.9A**). In contrast, there was a trend towards an increase approximately 0.6 - fold in plasma [NO<sub>2</sub><sup>-</sup>] concentration but this did not reach significance when comparing 6 months to baseline (**Figure 5.9B**).



**Figure 5.9: Plasma (A) and (B) [NO<sub>3</sub><sup>-</sup>] and (C) and (D) [NO<sub>2</sub><sup>-</sup>] of all recruited patients that had completed the study, at baseline and after 6 months of treatment.** The treatment was with either dietary nitrate (nitrate-rich juice 70 mL daily) or placebo (nitrate-depleted juice 70 mL daily), in patients with stable angina. Data expressed as mean ± SEM. n = 83. Statistical analysis was performed using paired t-test. \*\*P = 0.0087.

### 5.6.2 Effect of 6 month intervention on [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>] in saliva samples from patients with angina

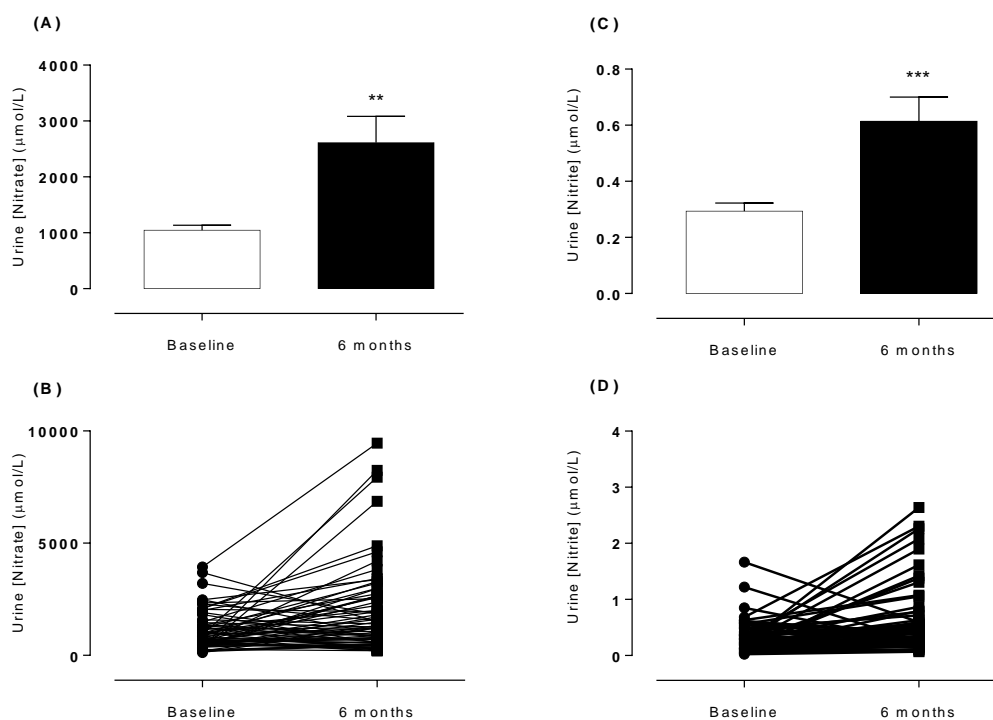
There was a significant increase in the saliva [NO<sub>3</sub><sup>-</sup>] concentration at 6 months compared to baseline of approximately 2.5 - fold (**Figure 5.10A**). There was also a trend towards an increase in the saliva [NO<sub>2</sub><sup>-</sup>] concentration at 6 months compared to baseline of approximately 1.5 – fold but this did not reach statistical significance (**Figure 5.10B**).



**Figure 5.10: Saliva (A) and (B)  $[\text{NO}_3^-]$  and (C) and (D)  $[\text{NO}_2^-]$  of all recruited patients that had completed the study, at baseline and after 6 months of treatment.** The treatment was with either dietary nitrate (nitrate-rich juice 70 mL daily) or placebo (nitrate-depleted juice 70 mL daily), in patients with stable angina. Data expressed as mean  $\pm$  SEM.  $n = 74$ . Statistical analysis was performed using paired t-test. \* $P = 0.0120$ .

### 5.6.3 Effect of 6 month intervention on $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$ in urine samples from patients with angina

There was a significant increase in the urine  $[\text{NO}_3^-]$  concentration at 6 months compared to baseline of approximately 2.5 - fold (**Figure 5.11A**). Similarly, there was a significant increase in the urine  $[\text{NO}_2^-]$  concentration at 6 months compared to baseline of approximately 2.1 - fold (**Figure 5.11B**).

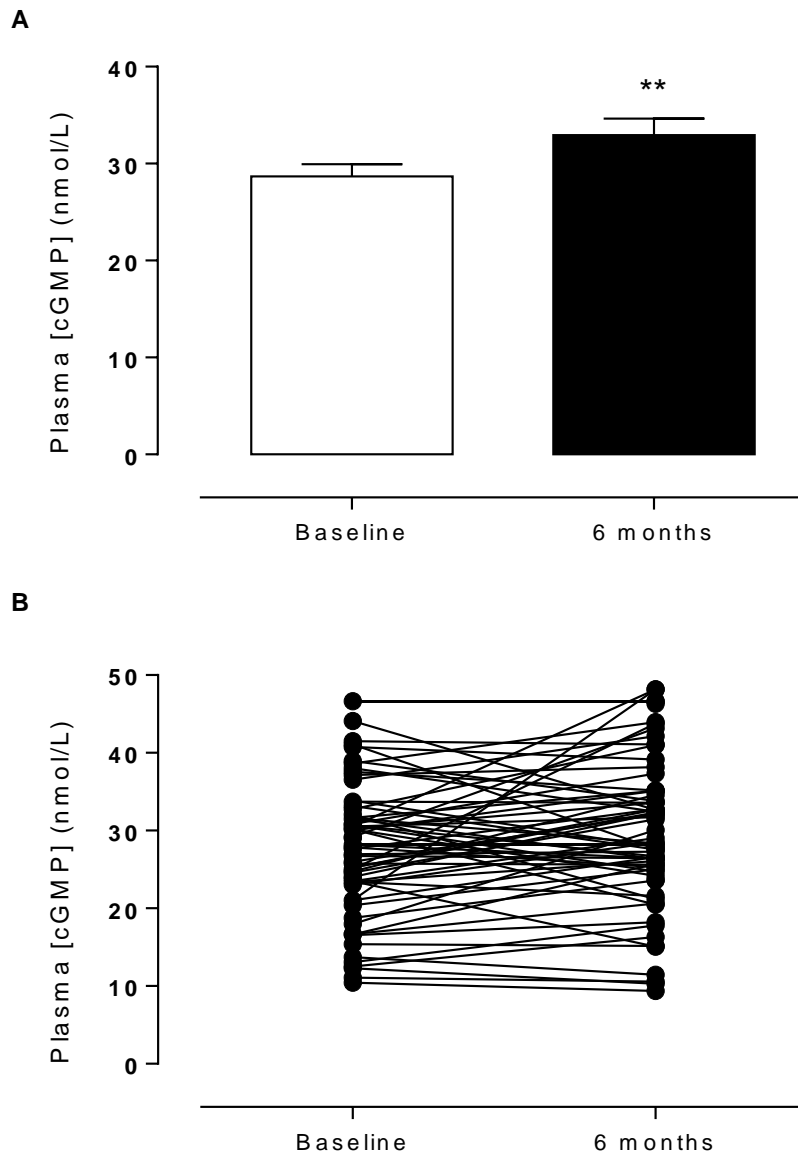


**Figure 5.11: Urine (A) and (B)  $[\text{NO}_3^-]$  and (C) and (D)  $[\text{NO}_2^-]$  of all recruited patients that had completed the study, at baseline and after 6 months of treatment.** The treatment was with either dietary nitrate (nitrate-rich juice 70 mL daily) or placebo (nitrate-depleted juice 70 mL daily), in patients with stable angina. Data expressed as mean  $\pm$  SEM. n = 77. Statistical analysis was performed using paired t-test. \*\*\*P = 0.0012; \*\*\*P = 0.0007.

#### 5.6.4 Plasma [cGMP] in patients with angina

Overall, after being treated for 6 months, plasma [cGMP] was significantly increased 1.15-fold (Figure 5.9).





**Figure 5.12: Plasma (A) and (B) [cGMP] of all recruited patients that had completed the study, at baseline and after 6 months of treatment.** The treatment was with either dietary nitrate (nitrate-rich juice 70 mL daily) or placebo (nitrate-depleted juice 70 mL daily). Data expressed as mean  $\pm$  SEM.  $n = 77$  in the whole cohort. Statistical analysis was performed using paired t-test. \*\*  $P = 0.0027$ .

## **5.7 Exploratory investigations of the effect of inorganic nitrate on the inflammatory response following primary percutaneous coronary intervention for stable angina**

### **5.7.1 Effect of nitrate on the systemic inflammatory response in patients with stable angina**

#### **5.7.1.1 General characteristics of inflammation study subpopulation**

Looking at the subpopulation of patients from the NITRATE-OCT study where we have inflammation data, the treatment given to each group was still kept blind to avoid bias in analysis. The groups have again been labelled gamma and delta to ensure that the trial remains blinded from the investigators. Again, the baseline characteristics demonstrate good matching of the groups. All baseline characteristics were similar between the treatment groups in this sub-analysis (**Table 5.6**).

	Gamma (n=40)	Delta (n=40)	P Value
Age (yr) (Mean±SD)	61.7±8.8	62.1±10.1	0.421
Sex (M/F)	31 (77.5%)	37 (92.5%)	0.115
Ethnicity			
Caucasian	33 (82.5%)	29 (72.5%)	0.211
Afro-Caribbean	1 (2.5%)	3 (7.5%)	0.615
Asian	5 (12.5%)	7 (17.5%)	0.378
East Asian	0 (0.0%)	1 (2.5%)	1.000
Diabetes mellitus	9 (22.5%)	9 (22.5%)	1.000
Type I	1 (2.5%)	1 (2.5%)	1.000
Type II	8 (20.0%)	8 (20.0%)	1.000
Body-mass index (kg/m <sup>2</sup> ) (Mean±SD) <sup>a</sup>	28.8±4.5	29.0±4.7	0.889
Hypertension	32 (80.0%)	32 (80.0%)	1.000
Hypercholesterolaemia	27 (67.5%)	35 (87.5%)	0.059
Previous MI	6 (15.0%)	4 (10.0%)	0.737
Previous PCI	8 (20.0%)	9 (22.5%)	1.000
Previous CABG	2 (5.0%)	2 (5.0%)	1.000
Previous Smoker	24 (60.0%)	27 (67.5%)	0.642
PVD	2 (5.0%)	4 (10.0%)	0.675
CVA/TIA	0 (0.0%)	0 (0.0%)	-
NYHA			
Class I	3 (7.5%)	1 (2.5%)	0.842
Class II	2 (5.0%)	1 (2.5%)	0.958
CCS			
CCS I	9 (22.5%)	11 (27.5%)	0.797
CCS II	13 (32.5%)	14 (35.0%)	1.000
CCS III	17 (42.5%)	14 (35.0%)	0.647
Asthma	4 (10.0%)	3 (7.5%)	1.000
COPD	5 (12.5%)	1 (2.5%)	0.201
Previous History of CAD	18 (45.0%)	23 (57.5%)	0.371
Heart rate (BPM) (Mean±SD)	68.3±19.7	61.9±10.7	0.279
Systolic BP (mmHg) (Mean±SD)	132.4±20.7	135.0±16.5	0.388
Diastolic BP (mmHg) (Mean±SD)	75.4±8.3	76.0±11.4	0.084
Culprit Vessel			
Left main stem	1 (1.0%)	0 (1.0%)	1.000
Left anterior descending	13 (32.5%)	15 (37.5%)	0.815
Circumflex	13 (32.5%)	9 (22.5%)	0.453
Right coronary	9 (22.5%)	12 (30%)	0.612

**Table 5.6. Baseline characteristics of inflammation sub-study patients in NITRATE-OCT by blinded treatment group.**

Values shown as number (%) unless otherwise stated. (PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES drug-eluting stent; MI, myocardial infarction; BPM, beats per minute; BP, blood pressure; CCS, Canadian classification system; NYHA, New York Heart Failure Association Classification; CABG, Coronary artery bypass grafting; COPD, chronic obstructive pulmonary disease).

<sup>a</sup>The body-mass index is the weight in kilograms divided by the square of the height in metres.

Again, there were no differences in the type of stent deployed, length of stent or diameter of stent between the two sub-groups. Use of drug-eluting stents and anti-platelet therapy were similar between the 2 groups (**Table 5.7**). There were three main types of drug eluting stents used over the period of recruitment. These included Xience (Everolimus-eluting stent), Promus (Everolimus-eluting stent) and Resolute Integrity (Zotarolimus-eluting). Overall, the majority of patients had a Xience stent implanted mainly based on the operator preference. Again, since there were a number of patients who did not undergo a stent implantation during the procedure, which was a decision made by the cardiologist at the time, the sum of all the stents do not add up to 100%. This was documented as a protocol deviation for the study.

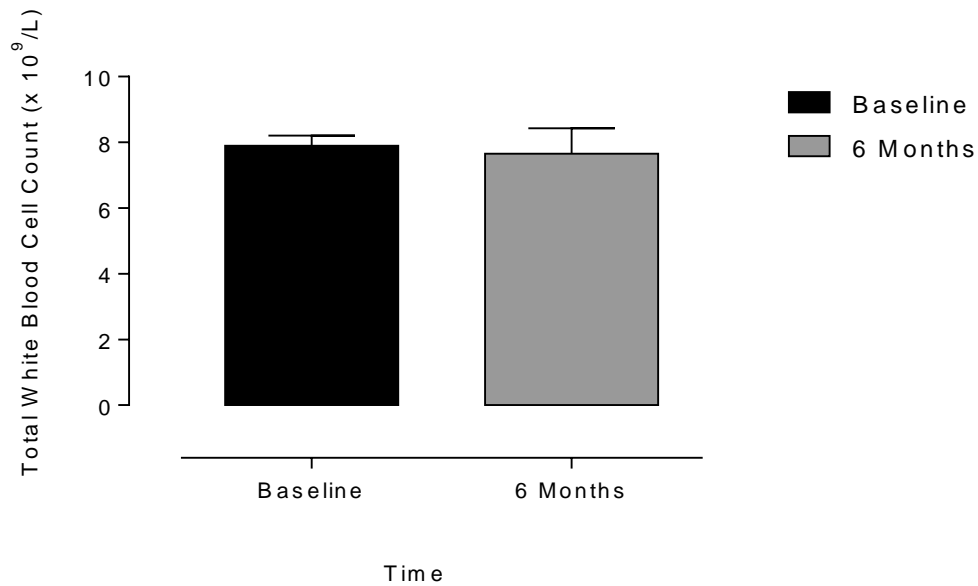
	Gamma (n=40)	Delta (n=40)	P value
Access site			
Radial	32 (80.0%)	31 (77.5%)	1.000
DES use	37 (92.5%)	35 (87.5%)	0.712
Number of stents used (Mean±SD)	1.52±0.83	1.58±0.81	0.910
Stent Type			
Xience	31 (77.5%)	29 (72.5%)	0.782
Resolute Integrity	5 (12.5%)	3 (7.5%)	0.677
Promus Premier	4 (10.0%)	8 (20.0%)	0.348
Biofreedom	0 (0%)	0 (0%)	-
Stent length (Mean±SD)			
First Stent	23.1±9.5	25.0±8.8	0.643
Second Stent	22.0±8.1	27.6±9.4	0.553
Third Stent	23.0±5.7	20.8±11.8	0.240
Stent diameter (Mean±SD)			
First Stent	3.0±0.5	2.9±0.4	0.122
Second Stent	3.3±0.6	3.2±0.6	0.726
Third Stent	2.9±0.5	2.9±0.5	0.877
Treatment at time of PCI			
Verapamil	14 (35.0%)	12 (30.0%)	0.691
Heparin	100 (100%)	100 (100%)	1.000
Heparin Dose (Mean±SD)	8166.7±2301.9	8285.7±2212.9	0.919
Aspirin	100 (100%)	100 (100%)	1.000
Clopidogrel/Ticagrelor	100 (100%)	100 (100%)	1.000
Procedural Success	100 (100%)	100 (100%)	1.000

**Table 5.7. Procedural characteristics of the inflammation sub-study by treatment group.**

Values shown as number (%) unless otherwise stated. (DES, Drug-eluting stent)

### 5.7.1.2 Total leukocyte cell count

Total circulating leukocyte cell counts were measured at baseline and 6 months after PCI in all patients. Total leukocyte cell counts were similar between the two timepoints at 6 months compared to baseline (P = 0.822) (**Figure 5.13**).



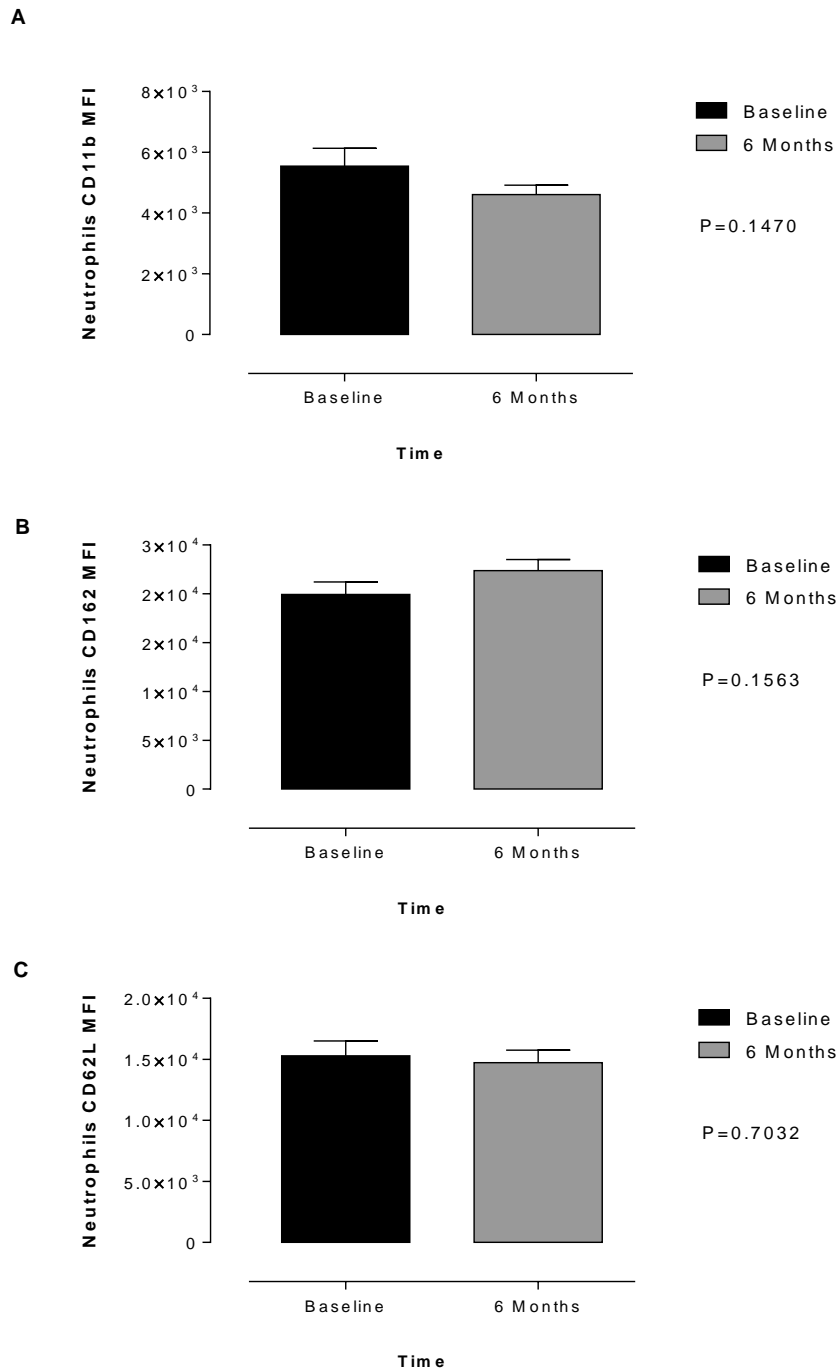
**Figure 5.13: Total leukocyte count pre and post PCI.** Total leukocyte count was measured at baseline and 6 months after PCI. Leukocyte count assessed over time (n=78). Data expressed as mean  $\pm$  SEM. Statistical analysis was performed using paired t-test.

### 5.7.1.3 Leukocyte inflammatory marker expression

Inflammatory marker expression (CD11b, CD62L and CD162) on each leukocyte population: neutrophils, monocytes (inflammatory, intermediate and resident), and T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>) were measured. Measurements were made at baseline and 6 months post-PCI in 66 patients in total (33 patients in each group).

### 5.7.1.4 Neutrophil activation marker expression

Neutrophil CD11b, CD62L and CD162 changed from baseline to 6 months but not this did not reach statistical significance. (**Figure 5.14 A, C and D**).



**Figure 5.14: Neutrophil Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.

### **5.7.1.5 T Lymphocytes**

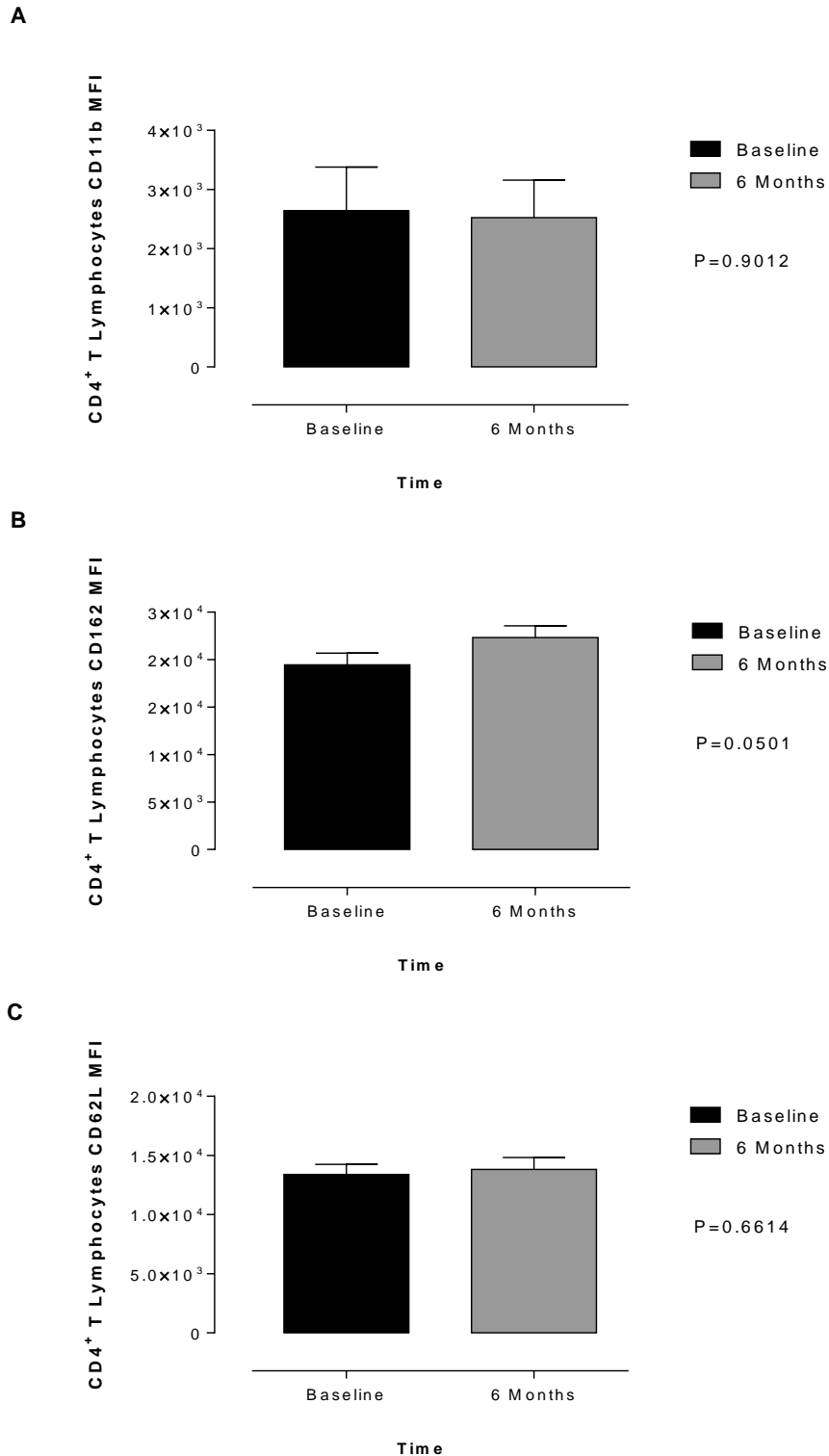
#### **5.7.1.5.1 CD4<sup>+</sup> T lymphocytes**

Expression levels of CD11b and CD62L on CD4<sup>+</sup> lymphocytes did not change from baseline to 6 months (**Figure 5.15 A and C**). There was a trend towards an increase in expression levels of CD162 at 6 months compared to baseline, however this did not reach statistical significance ( $p=0.0501$ , **Figure 5.15B**).

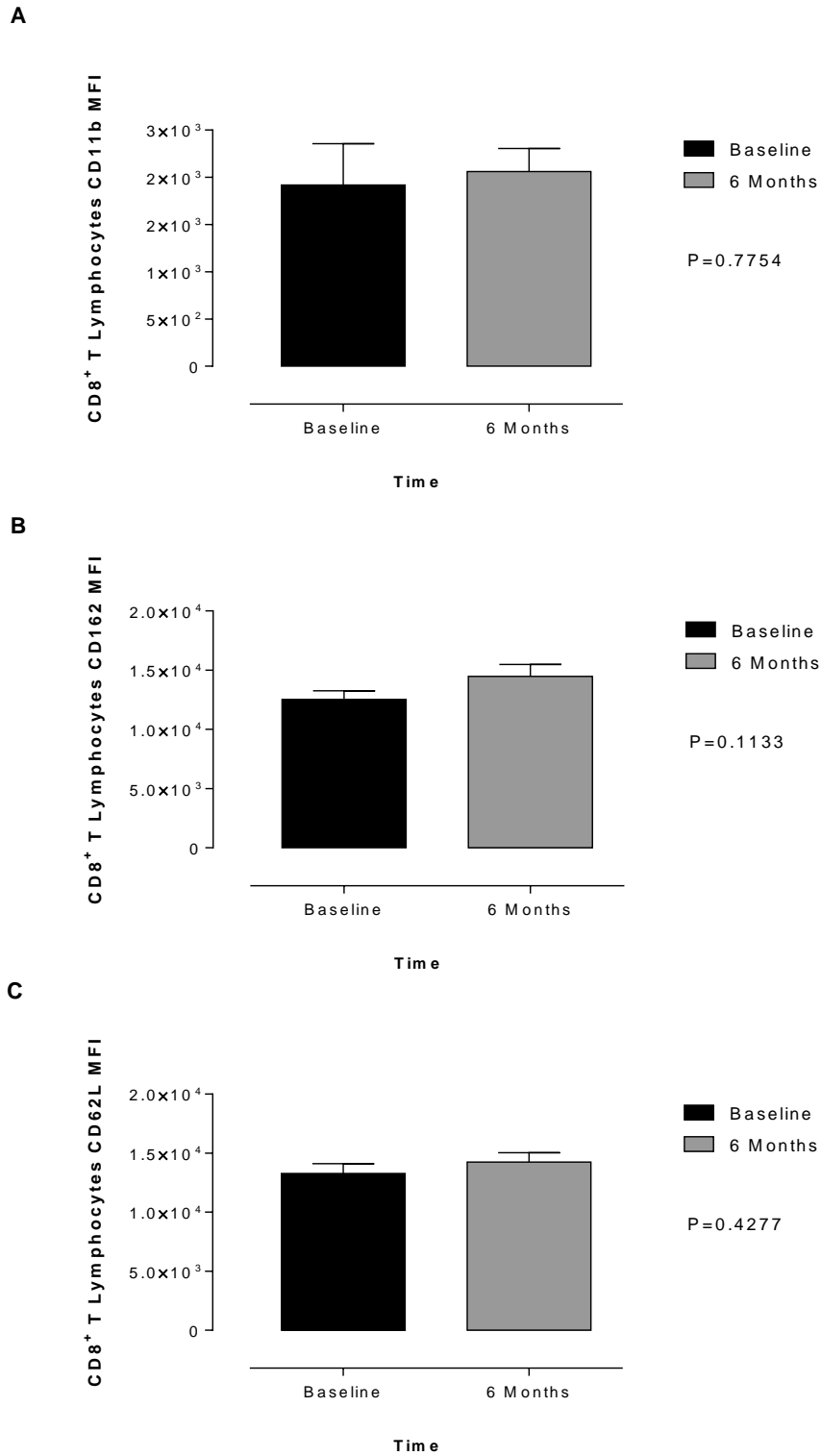
#### **5.7.1.5.2 CD8<sup>+</sup> T lymphocytes**

CD8<sup>+</sup> T lymphocyte CD11b, CD162 and CD62L levels did not change from baseline to 6 months. (**Figure 5.16 A, B and C**).





**Figure 5.15: CD4<sup>+</sup> T Lymphocyte CD11b/CD62L/162 Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.



**Figure 5.16: CD8<sup>+</sup> T lymphocyte Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.

### **5.7.1.6 Monocytes**

Monocytes were split into inflammatory (classical) monocytes (CD14<sup>+</sup>/CD16<sup>-</sup>), intermediate monocytes (CD14<sup>+</sup>/CD16<sup>+</sup>) and non-classical/resident monocytes (CD14<sup>+</sup>/CD16<sup>++</sup>) (Passlick et al., 1989; Ziegler-Heitbrock et al., 2010; Rogacev et al., 2012)

#### **5.7.1.6.1 Inflammatory monocytes (CD14<sup>+</sup>/CD16<sup>-</sup>)**

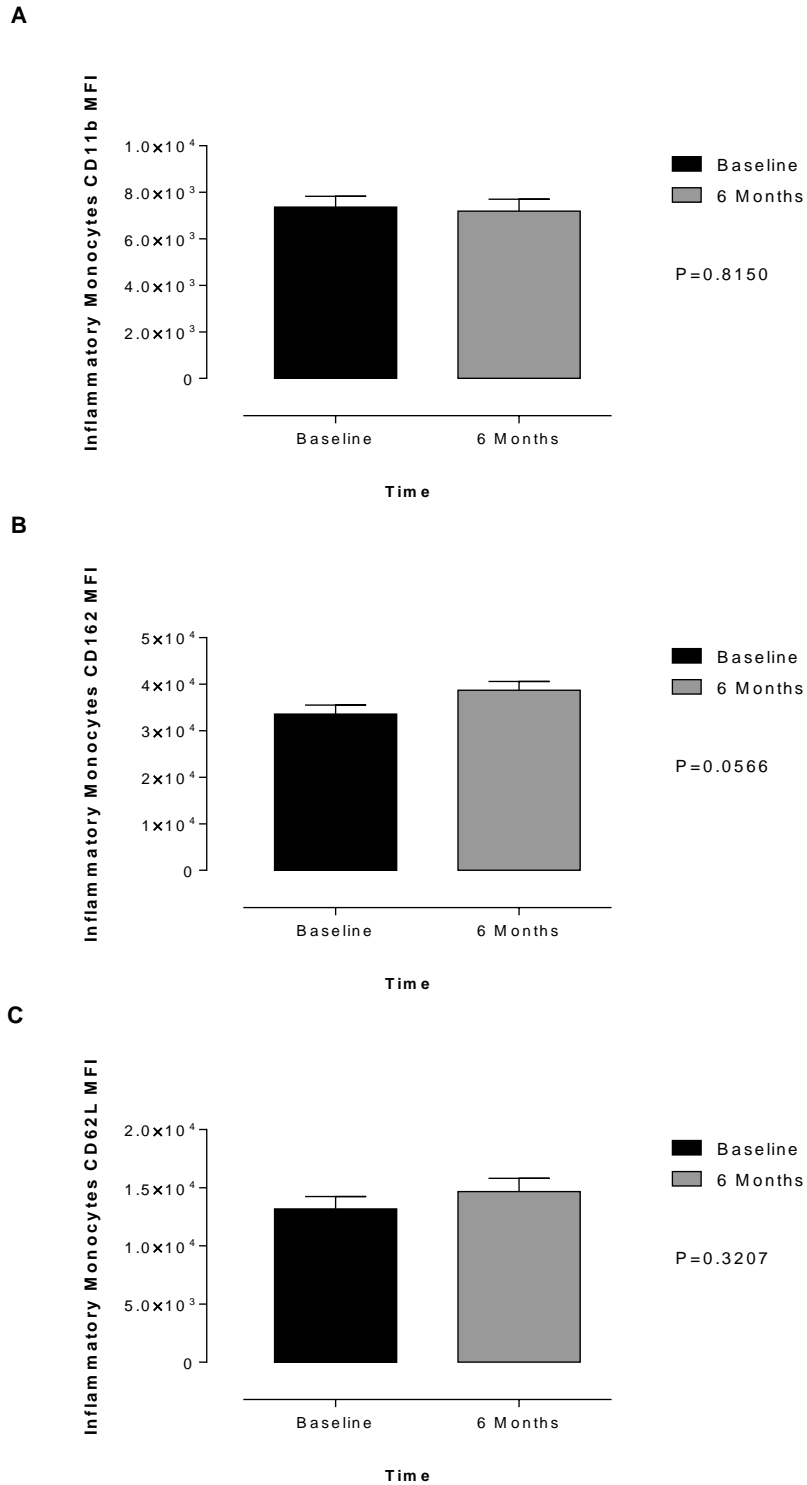
Inflammatory monocyte CD11b and CD62L did not change from baseline to 6 months (**Figures 5.17 A and C**). However, CD162 expression increased from baseline to 6 months but this did not reach statistical significance. (**Figure 5.17B**).

#### **5.7.1.6.2 Intermediate monocytes (CD14<sup>+</sup>/CD16<sup>+</sup>)**

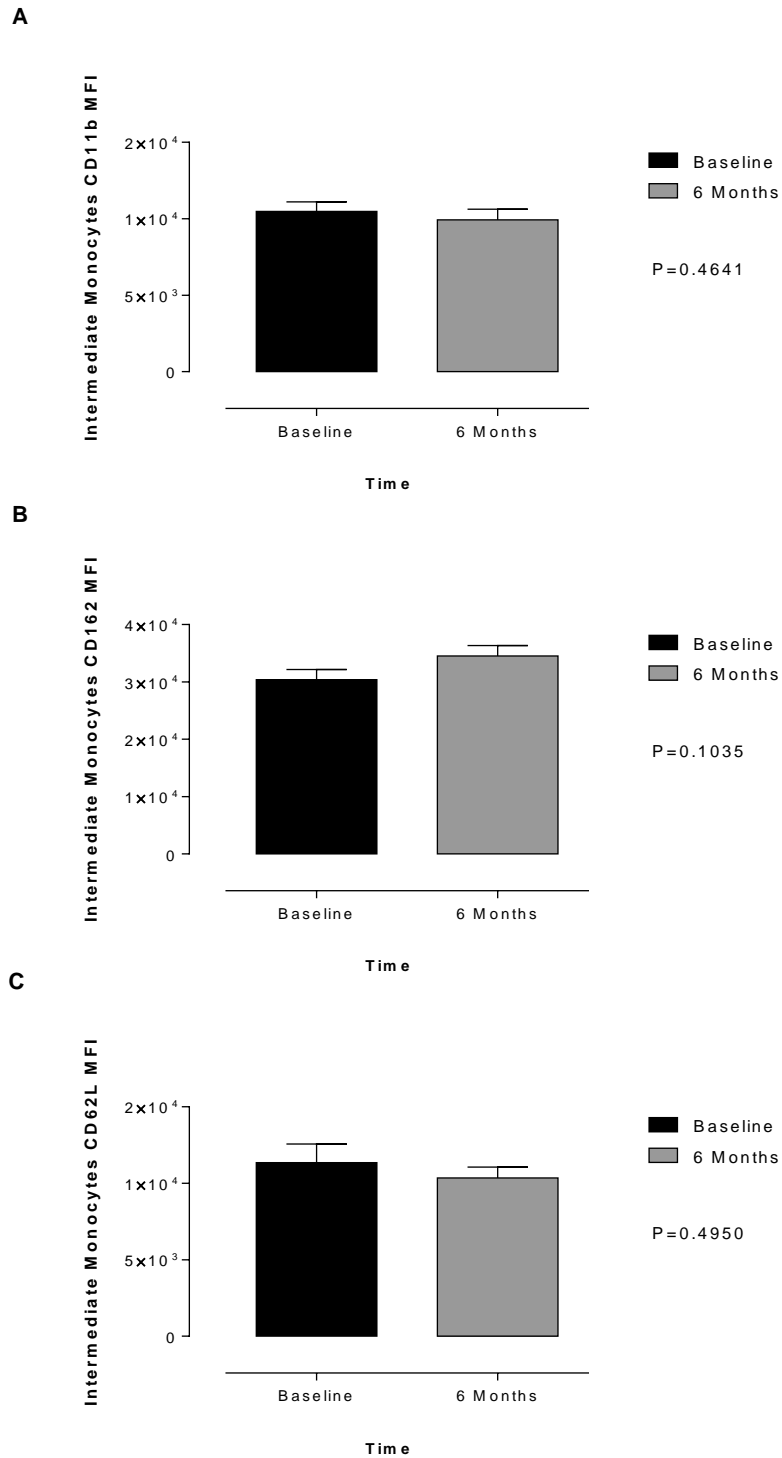
Intermediate monocyte CD11b, CD162L and CD62L expression did not change from baseline to 6 months (**Figures 5.18 A, B and C**).

#### **5.7.1.6.3 Resident monocytes (CD14<sup>+</sup>/CD16<sup>+</sup>)**

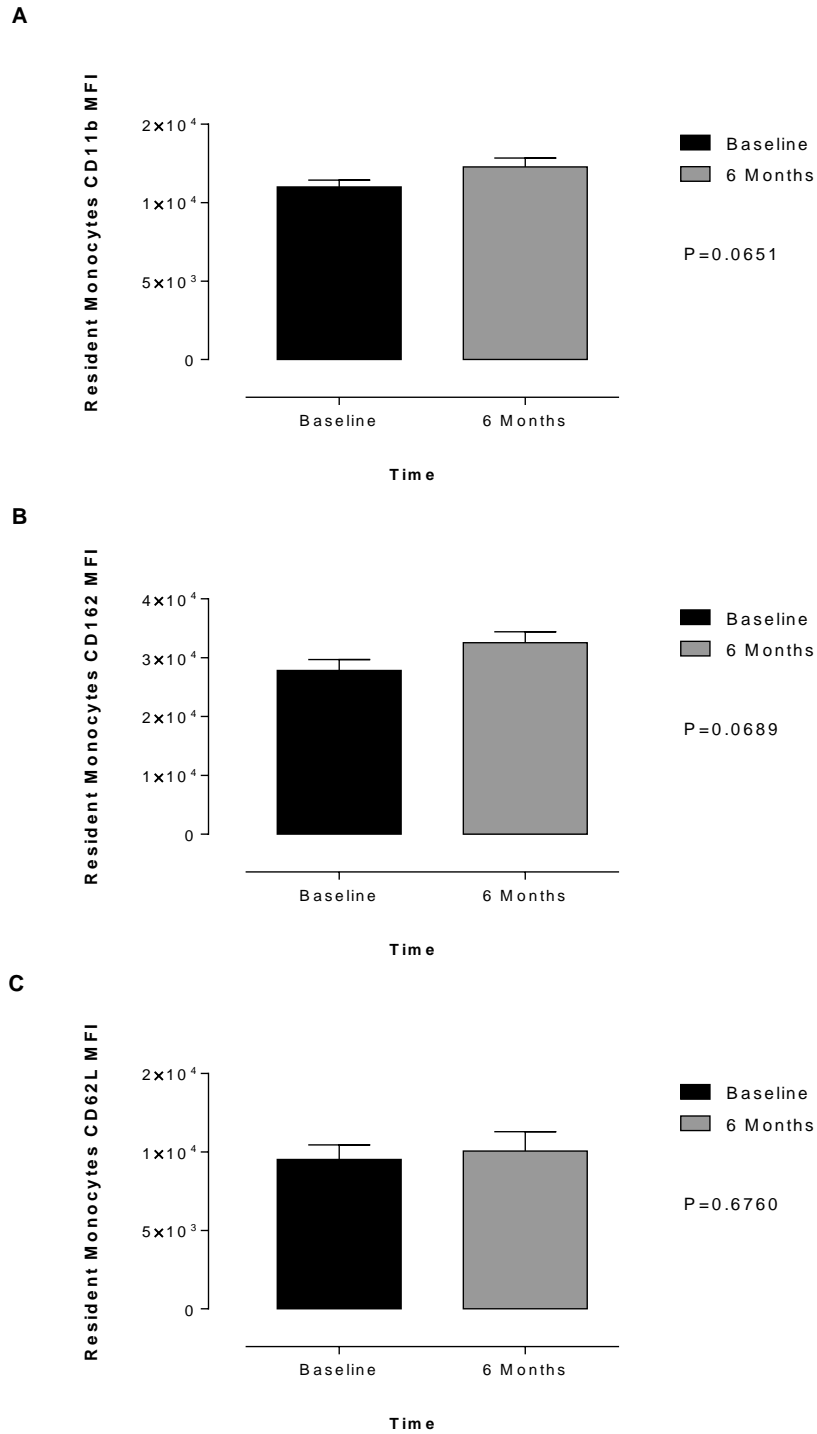
Resident monocyte CD11b and CD162 expression increased from baseline to 6 months but this did not reach statistical significance (**Figure 5.19 A and B**). CD162 expression did not change over the time period (**Figure 5.19C**).



**Figure 5.17: Inflammatory monocyte Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.



**Figure 5.18: Intermediate monocyte Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.



**Figure 5.19: Resident monocyte Median Fluorescence Intensity.** Median Fluorescence Intensity (MFI) for CD11b/CD62L/CD162 was measured at baseline and 6 months after PCI as shown in panels A-C in 66 patients. Panel A shows CD11b MFI assessed over time. Panel B shows CD162 MFI over time. CD62L MFI was assessed over time as shown in panel C. Data expressed as mean ± SEM. Statistical analysis was performed using paired t-test.

## **5.7.2 Effect of nitrate on the inflammatory mediators in patients with stable angina**

Plasma levels of high-sensitivity C-reactive (hs-CRP) protein, urate, interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) were measured at baseline and 6 months post-PCI.

### **5.7.2.1 High-sensitivity C-reactive protein**

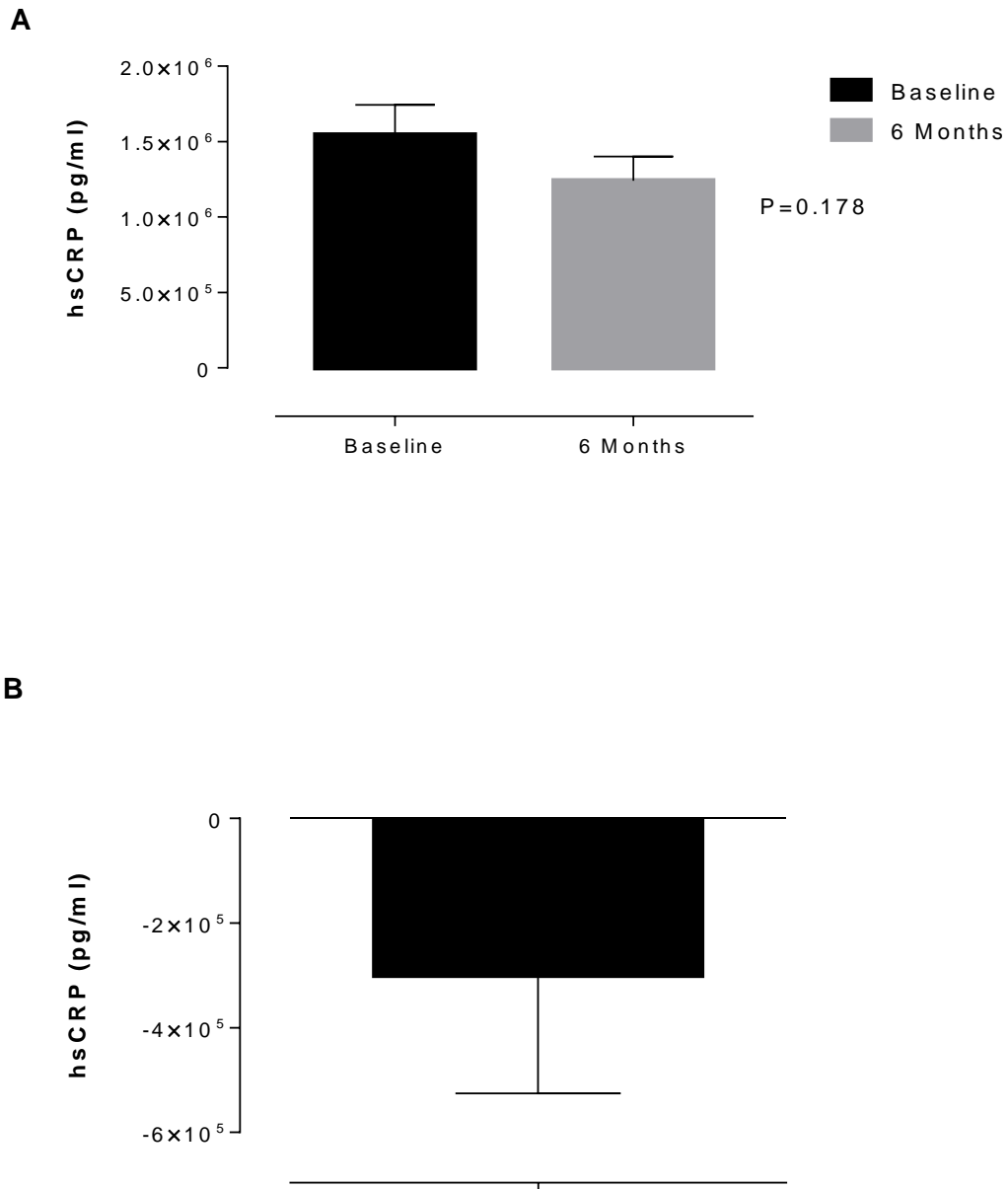
Overall, plasma hs-CRP levels showed a trend towards a reduction at 6 months compared to baseline (**Figure 5.20A**,  $p=0.1780$ ). After measuring changes from baseline, there was an overall reduction in hs-CRP level compared to baseline (**Figure 5.20B**).

### **5.7.2.2 Interleukin1- $\beta$ (IL-1 $\beta$ ), Interleukin-6 (IL-6) and Tumor necrosis factor $\alpha$ (TNF $\alpha$ )**

Overall, plasma TNF $\alpha$  showed a trend towards a reduction at 6 months compared to baseline (**Figure 5.21A**). IL-1 $\beta$  levels were not statistically different at 6 months compared to baseline (**Figure 5.21B**). However, IL-6 levels were statistically significantly lower at 6 months compared to baseline (**Figure 5.21C**) ( $p=0.0106$ ).

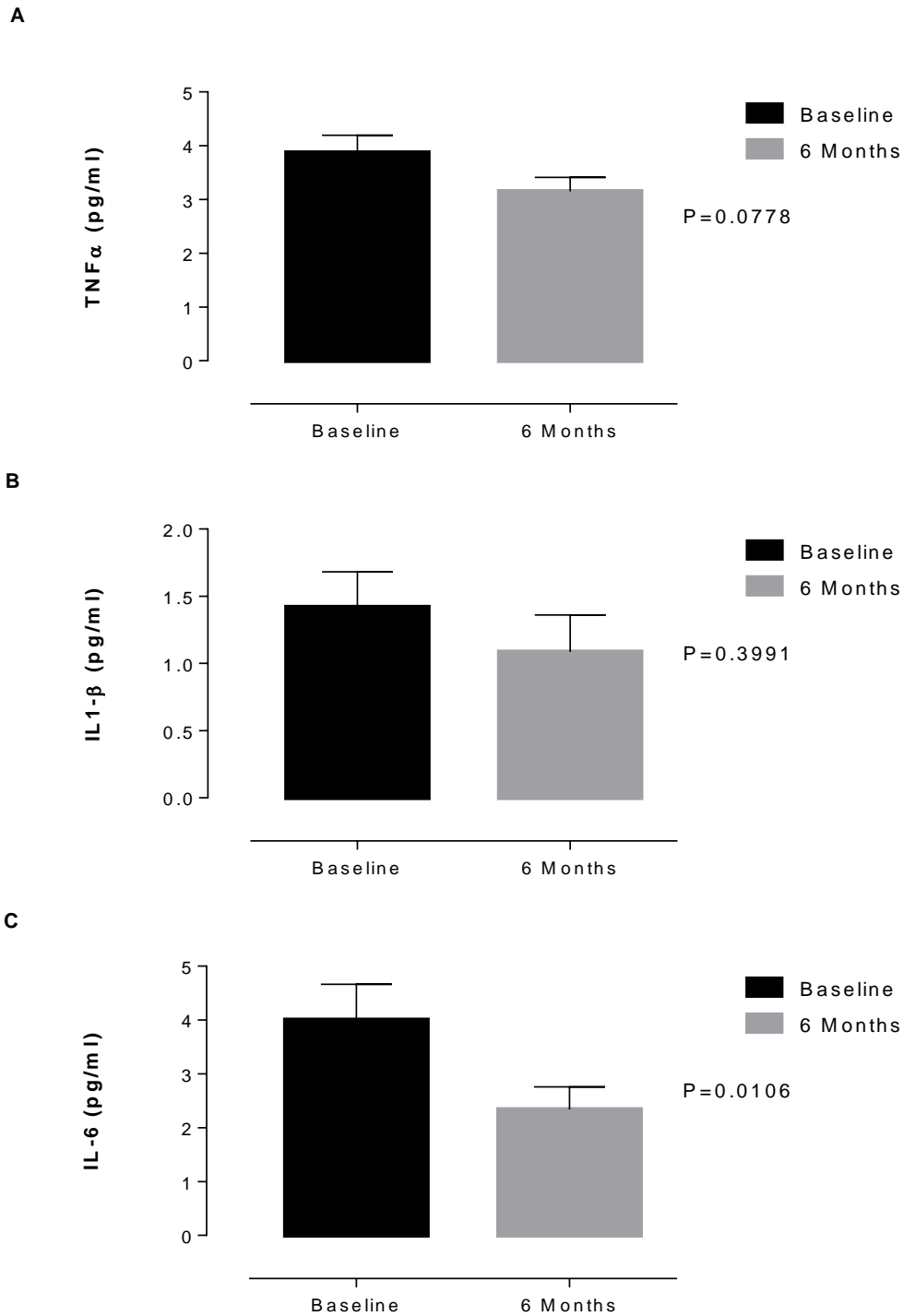
### **5.7.2.3 Plasma Urate levels**

Plasma urate did not change significantly from baseline at 6 months. (**Figure 5.22**,  $p=0.854$ ).

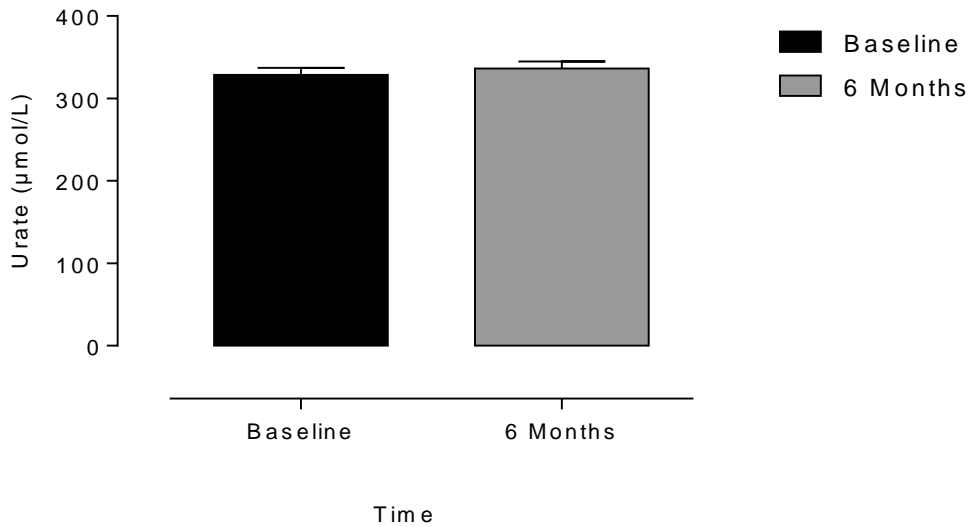


**Figure 5.20: Effect of time and treatment on plasma hs-CRP.** High-sensitivity C-reactive protein (hs-CRP) was measured at baseline and 6 months after PCI in the whole cohort (n=78) (panel A). Panel B shows the change in hs-CRP at 6 months compared to baseline in the whole cohort. Data expressed as mean  $\pm$  SEM. Comparison performed using paired t-test for panel A. (Hs-CRP= High-sensitivity C-reactive protein).





**Figure 5.21: Effect of time and treatment on plasma TNF $\alpha$ , IL-1 $\beta$  and IL-6.** Plasma levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) were measured at baseline and 6 months post-PCI shown in panel A, B and C, respectively in 78 patients. Data expressed as mean  $\pm$  SEM. Comparison performed using paired t-test.



**Figure 5.22: Serum urate levels pre and post PCI.** Plasma urate levels were measured at baseline and 6 months after PCI in the whole cohort (n=78) (panel A). Data expressed as mean  $\pm$  SEM. Statistical analysis was performed using paired t-test for both panels.

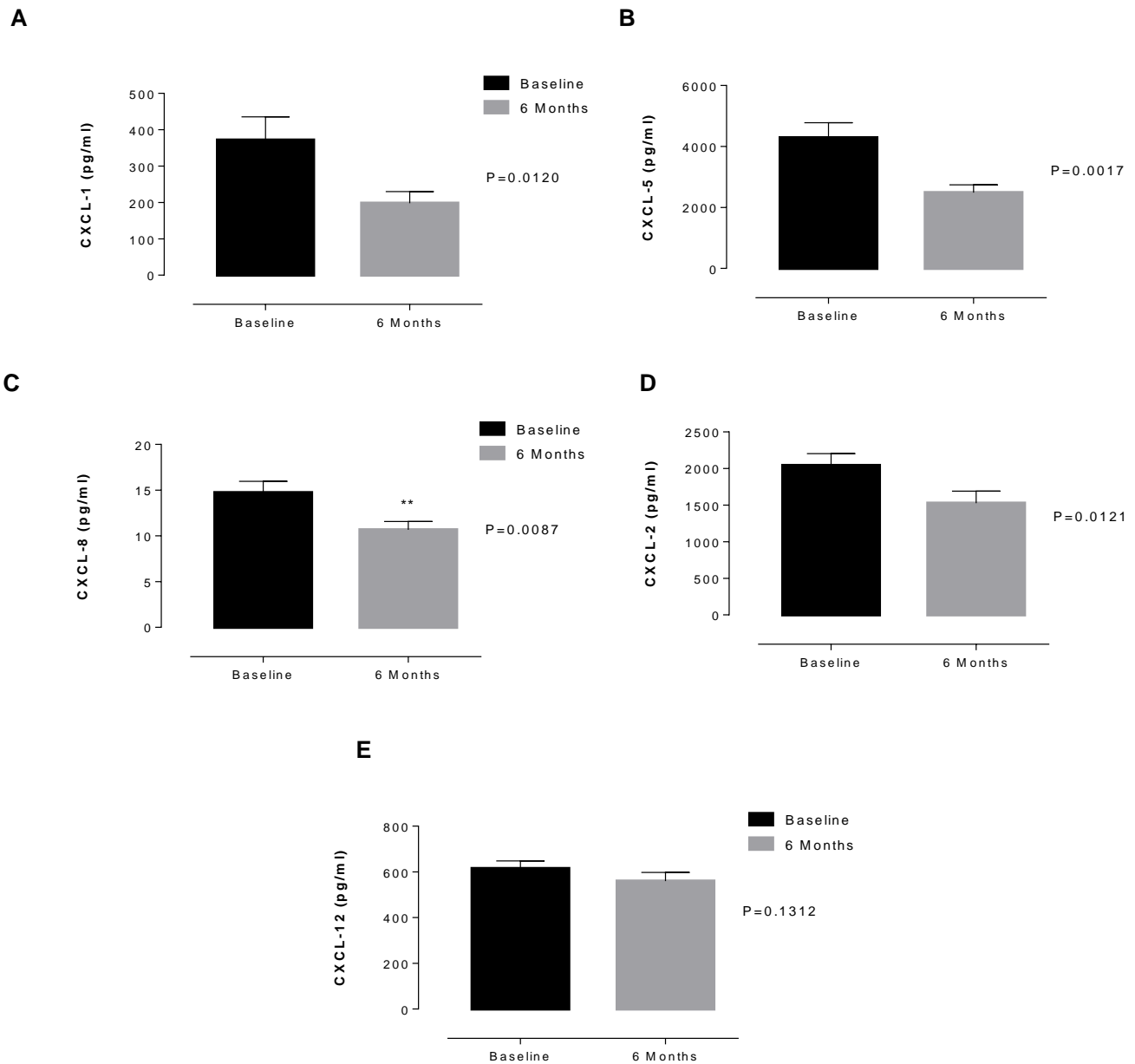
#### 5.7.2.4 Neutrophil chemokines

We investigated the levels of CXCL-1, CXCL-5, CXCL-8, CXCL-2 and CXCL-12 measured in 78 patients at baseline and 6 months post-PCI.

##### 5.7.2.4.1 CXCL-1, CXCL-5, CXCL-8, CXCL-2 and CXCL-12

Plasma CXCL-1 levels were significantly lower at 6 months compared to baseline (**Figure 4.11A**) (p=0.0120). CXCL-5 levels were significantly lower at 6 months compared to baseline (**Figure 5.23B**) (p=0.0017). CXCL-8 levels were significantly lower at 6 months compared to baseline (**Figure 5.23C**) (p=0.0087). CXCL-2 were significantly lower at 6 months compared to baseline (**Figure 5.23D**) (p=0.0121).

However, CXCL-12 levels were comparable in the two groups at baseline and at 6 months (**Figure 5.23E**) ( $p=0.1312$ ).



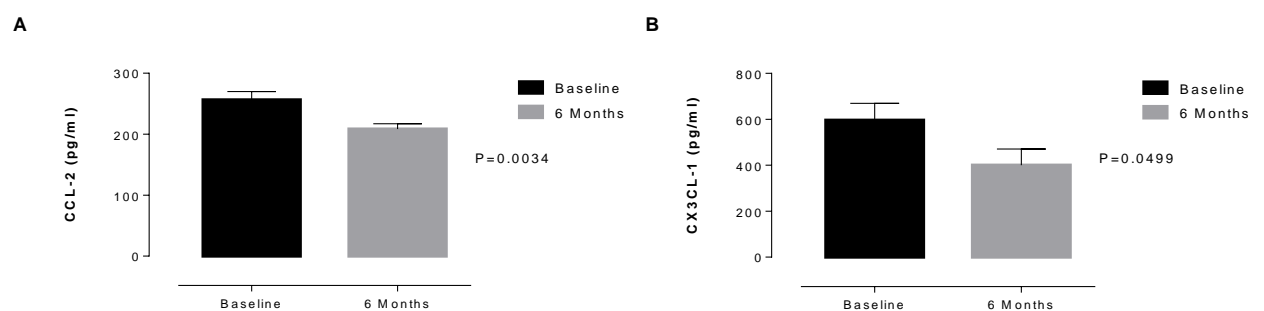
**Figure 5.23: Effect of time and treatment on plasma CXCL-1 CXCL-5, CXCL-8, CXCL-2 and CXCL-12.** Plasma levels of CXCL-1 (panel A), CXCL-5 (panel B), CXCL-8 (panel C), CXCL-2 (panel D) and CXCL-12 (panel E) were measured at baseline and 6 months post-PCI over time in 78 patients. Data expressed as mean  $\pm$  SEM. Comparison performed using paired t-test.

### 5.7.2.5 Monocyte chemokines

We also measured levels of CCL-2 and CX3CL-1 in 78 patients at baseline and 6 months post-PCI.

#### 5.7.2.5.1 CCL-2 and CX3CL-1

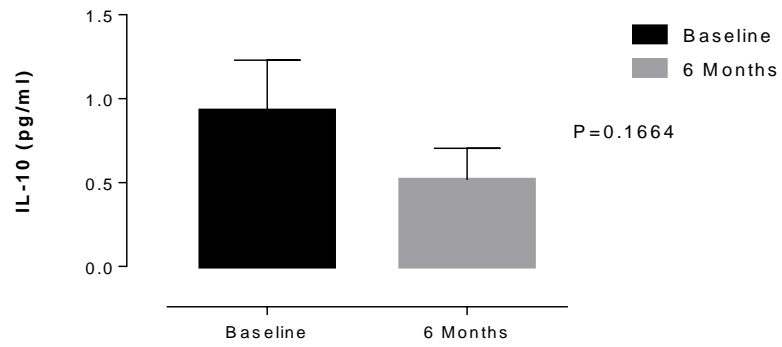
Plasma CCL-2 levels were significantly lower at 6 months compared to baseline (**Figure 5.24A**,  $p=0.0034$ ). CX3CL-1 levels were significantly lower at 6 months compared to baseline (**Figure 5.24B**,  $p=0.0499$ ).



**Figure 5.24: Effect of time and treatment on plasma CCL-2 and CX3CL-1.** Plasma levels of CCL-2 and CXCL-1 were measured at baseline and 6 months post-PCI. CCL-2 as shown in panel A and CX3CL-1 (panel B) were assessed over time in 78 patients. Data expressed as mean  $\pm$  SEM. Comparison performed using paired t-test.

#### 5.7.2.6 IL-10, anti-inflammatory mediator

Overall, there was a trend towards a reduction in IL-10 at 6 months compared to baseline (**Figure 5.25**,  $p=0.1664$ ).



**Figure 5.25: Effect of time and treatment on plasma IL-10.** IL-10 levels were measured at baseline and 6 months after PCI (N = 78). Data expressed as mean  $\pm$  SEM. Comparison performed using a paired t-test. (IL-10=interlukin-10).

## 5.8 Summary

1. For the 200 patients recruited, baseline characteristics were similar indicating adequate randomisation of the study so far.
2. To date, no safety issues with respect to the placebo or active juice have been identified.
3. In the whole cohort, total circulating WCC numbers and hs-CRP did not change significantly, however, there was reduction in IL-6 levels at 6 months compared to baseline.
4. There was an overall combined reduction in the levels of neutrophil specific chemokines (CXCL-1, CXCL-5, CXCL-8 and CXCL-2) at 6 months compared to baseline.
5. There was also an overall combined reduction in the levels of the monocyte specific chemokines (CX3CL-1 and CCL-2) at 6 months compared to baseline.
6. There were no differences in the number of other leukocyte populations (neutrophils, inflammatory monocytes and lymphocytes) or activation markers expressed by these cells.
7. These results suggest an increase in NO<sub>x</sub> metabolite and a reduction in systemic inflammatory marker over time. Whether this is due to nitrate is currently unknown and will become clear following unblinding at the end of the trial.

## **Chapter 6.0**

# **Discussion**

## 6.0 Discussion

Despite significant advances in the management of CVD, CHD still represents an enormous burden in terms of morbidity and mortality and there is no doubt that further improvement in treatment is needed in this field particularly in the management of stable angina. PCI is a procedure that is used to 're-open' patients' narrowed coronary arteries with the view to reduce or completely relieve the patients' angina symptoms. Since the birth of PCI in 1977, there has been significant growth in stent technology, however, one of the main issues surrounding both BMS and DES is the accelerated neo-intimal proliferation at the site of the stent placement, resulting in luminal restenosis. Currently there is a growth of evidence suggesting that inflammation may play an important role in restenosis (Libby et al., 2003; Toutouzas et al., 2004), raising the possibility that measurement of inflammation may improve our ability to predict those at increased risk.

It is thought that systemic inflammation is a key driver of CVD and particularly atherosclerosis and CHD. This view has recently been corroborated with the outcome of the CANTOS trial. In CANTOS, Ridker and colleagues assessed whether targeting inflammation might reduce CVD in patients at risk of events following the use of canakinumab, a therapeutic monoclonal antibody targeting IL-1 $\beta$ . The study recruited 10,061 patients with previous MI and a hsCRP level of  $\geq 2$  mg/litre. The study found that anti-inflammatory therapy targeting the IL-1 $\beta$  innate immunity pathway resulted in a significantly lower rate of recurrent cardiovascular events than placebo, which was independent of lipid-level lowering (Ridker et al., 2017). This confirms that targeting inflammation provides



options for therapeutics in CVD and CHD. The work in this thesis has been carried out to assess whether inflammation is the target for  $\text{NO}_2^-/\text{NO}_3^-$  and whether the effects are sufficient to lead to benefit

However, here are many issues surrounding the use of biologics including cost of development (which results in high marketing costs), route of administration (often requiring hospital admission due to need for intravenous administration), side effects (including immunosuppression) and lack of long-term data in terms of efficacy and safety. In addition, one of the issues with the approach taken in CANTOS is that biologics are expensive and so identifying perhaps easier to deliver therapeutics that overcome the issues with biologics is desirable. One such possibility of course is inorganic  $\text{NO}_3^-$ , the testing of which has been a key aim in this thesis and discussed in the latter sections of this discussion. In addition, the fact that altering inflammation impacts upon CVD raises the question of whether differences in inflammatory responses between distinct groups of individuals might also underlie differences in CVD susceptibility. Of particular relevance to this PhD is the question of whether differences in inflammation between the sexes might relate to differences in response to inflammatory stressors. Chapters 3 and 4 of this thesis focused upon testing whether this might be the case.

### **6.1 Differences in responses to inflammatory stimuli between the sexes could underlie differences in CVD pathogenesis**

It is accepted that pre-menopausal women have reduced levels of CVD compared to their age-matched male counterparts (Lerner and Kannel, 1986). The exact mechanisms involved in mediating this protection are uncertain but have been

attributed to an effect of female sex hormones (Mendelsohn and Karas, 2005). Separately we know that whilst women tend to experience increased autoimmune disease, they have lower rates of inflammation in the context of chronic disease such as CVD and infection (Beagley and Gockel, 2003; Gubbels Bupp, 2015). Since good evidence links innate immune responses with CVD (Ross, 1999b) and inflammation is particularly thought to be causative in endothelial dysfunction (a critical pathogenic step in CVD progression) we speculated that reduced acute inflammatory responses in females may contribute to the protection of women against endothelial dysfunction and ultimately CVD development. Using experimental models in healthy volunteers, we have shown that female sex protects against the endothelial dysfunction induced by a mild systemic inflammatory response and that this protection likely relates to an enhanced leucocyte surveillance in the female circulation and an accelerated resolution of inflammation. We speculate that this enhanced capacity to deal with and then recover from an inflammatory stress likely plays a crucial role in the reduced rates of inflammatory CVD in premenopausal females.

### **6.1.1 Low grade systematic inflammation results in transient endothelial dysfunction in males but not females**

Administration of typhoid vaccine to healthy volunteers causes a transient endothelial dysfunction reflected by a reduced response to reactive hyperaemia (Hingorani et al., 2000b). In Chapter 3.0, I have shown that the FMD response was reduced following typhoid vaccination in males but, if anything, in females FMD was greater following vaccine administration. Comparison of the change in FMD from baseline between the sexes (the primary outcome of this study)

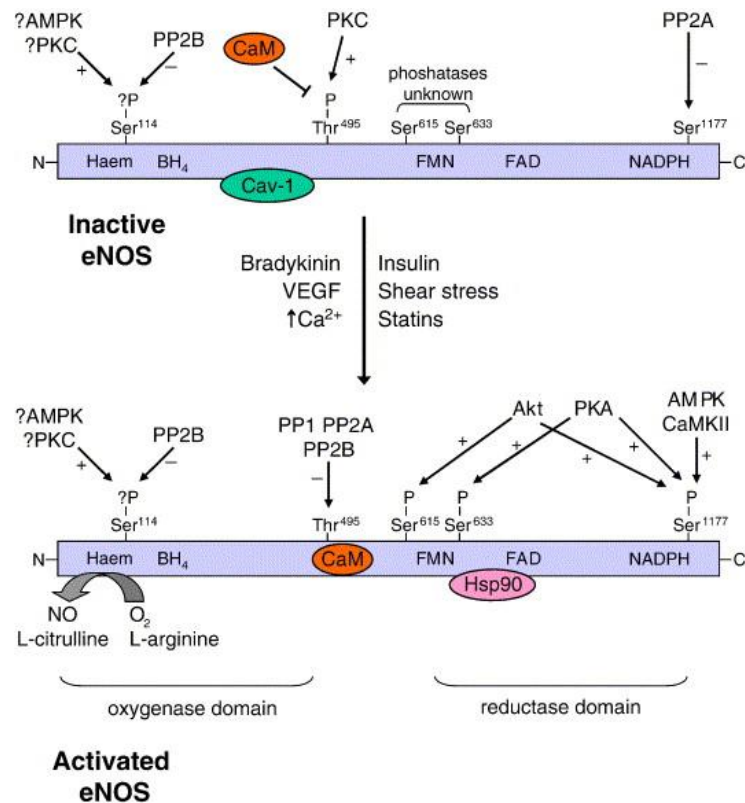
demonstrates a clear protection in females from the damaging effects of systemic inflammation induced by typhoid vaccination. Since I also demonstrated that the responses to the endothelium-independent vasodilator, GTN, in both sexes were unchanged over the course of the study and neither were there any changes in arterial stiffness measures of PWV and PWA, my findings suggest that protection against the damaging effects of typhoid vaccination evident in females, relates to alterations in endothelial function and not changes in smooth muscle reactivity. This latter observation agrees with previous findings demonstrating no effect of typhoid vaccination upon NO donor-induced brachial artery dilator response either to sublingual GTN (Hingorani et al., 2000b) or intra-arterial infusions of sodium nitroprusside (Chia et al., 2003). It is worth noting that although previous studies have demonstrated a significant reduction in FMD in healthy male volunteers following the administration of the typhoid vaccine, I only demonstrated a trend. One possible explanation for this finding could be that in our group, the absolute FMD measurements have been much lower (Ranging between 5-8% in healthy participants compared to that published in the literature (ranging between 10-15%) (Nowicki et al., 2018; Peretz et al., 2007). Hence, the relative change is much smaller., which would mean we would require a larger sample size to see a significant reduction in FMD following administration of the typhoid vaccine.

Plasma  $\text{NO}_2^-$  levels are thought to reflect endothelial NO generation, and NO is considered to mediate at least part of the shear stress-induced FMD response (Kleinbongard et al., 2003; Lauer et al., 2001; Mullen et al., 2001; Mullen et al., 1997). Post-hoc correlation analyses show that whilst FMD responses in females

correlated directly with circulating  $\text{NO}_2^-$  levels there was no such relationship in males. The primary pathway implicated in upregulation of beneficial NO levels in health is phosphorylation of endothelial nitric oxide synthase (eNOS), and perhaps in particular via an AKT-dependent pathway (**Figure 6.1**); and importantly this pathway also underlies oestrogen-induced enhanced NO bioavailability (Garcia-Cardena et al., 1998; Russell et al., 2000). It is likely that this pathway is responsible for the enhanced FMD responses in this study too. In pre-clinical studies oestrogen-induced upregulation of the AKT-eNOS phosphorylation pathway in blood vessels in females is resistant to repression by pathological stimuli that cause profound endothelial dysfunction in males (Taguchi et al., 2012). Additionally, stimuli activating the AKT pathway procure greater activation of this pathway in females compared to males (Kane et al., 2009). Of relevance to this study acute low grade inflammation whilst activating pro-inflammatory pathways also triggers reflex protective effects particularly through upregulating AKT phosphorylation (Ha et al., 2008; Jones and Bolli, 2006). These observations together support the view that the enhanced FMD response in females following typhoid is likely secondary to a phosphorylation of eNOS resulting in greater shear-stress induced NO generation.

The lack of a correlation between plasma  $\text{NO}_2^-$  levels and FMD in males suggests that the FMD response in males is not entirely dependent upon NO (Green et al., 2014). This view is supported by a recent meta-analysis of studies assessing the contribution of NO in mediating FMD, where it was estimated that 47-67% of the dilator response to flow could be attributed to NO. The wide range possibly being due to differences in the methodology used (Green et al., 2014) but also possibly

due to the high inter-individual variability that exists in the FMD response (Parker et al., 2011), but also now our data suggest that this variability may also be a reflection of the influence of sex differences.



**Figure 6.1 The role of kinases and phosphatases in the activation of eNOS by multi-site phosphorylation.** Activation of NO synthesis by eNOS involves multiple and coordinated phosphorylation events and protein–protein interactions. Important activators of eNOS include bradykinin, vascular endothelial growth factor (VEGF), shear stress and pharmacological stimuli such as statins. Numerous kinases and phosphatases contribute to the regulation of eNOS phosphorylation, although the specific kinases and phosphatases involved can vary depending on either the activating stimulus or the tissue bed. + indicates kinase-mediated phosphorylation. – indicates phosphatase-mediated dephosphorylation. Adapted from Mount *et al.* (Mount et al., 2007).

As expected a number of demographic variables were distinct between the sexes despite being age-matched and healthy (Vitale et al., 2009). These include BMI and BP but also WCC. The elevated WCC at baseline in females has been attributed to higher neutrophil numbers (Bain and England, 1975a) and indeed in my study different neutrophil numbers accounted for the differences in WCC between men and women. Importantly, all women recruited into these studies attended experimental days at the mid-point of their menstrual cycles when oestrogen levels are generally at their highest and so also are the leucocyte and neutrophil counts (Bain and England, 1975b). This raised WCC/neutrophil number in females may be due to oestrogen-induced margination of neutrophils from the bone marrow. In healthy women asked to conduct controlled exercise, as a trigger for changes in granulocyte distribution, the response remained unchanged at different stages of the menstrual cycle (Bain and England, 1975b). However, studies in mice suggest that low dose oestrogen inhibits rather than triggers bone marrow margination (Josefsson et al., 1992). Exactly why these diametrically opposing results occur is uncertain but may simply reflect important species differences.

Interestingly, whilst we saw no significant differences in monocyte numbers at baseline my data suggest a generalised raised activation state of circulating neutrophils and monocytes in females compared to males. In particular, we saw enhanced expression of both CD162 and CD62L adhesion molecules on these cell types in men at 8h, with recovery of baseline expression levels by 32h following typhoid vaccine, but no significant changes in expression levels in women. If anything, there was some suggestion of a slight but non-significant decrease in

females in the expression of the adhesion molecules relative to baseline. With respect to CD62L it is possible that this reflects the shedding of CD62L which occurs during inflammatory responses and is thought to enhance leukocyte transmigration (Hafezi-Moghadam et al., 2001), however further experiments powered against CD62L levels are needed to investigate this. Both cellular CD162 and CD62L have been identified as critical mediators of the homing, rolling and adhesion of both neutrophils and monocytes to sites of inflammation through interaction with the endothelium and thus the enhanced cell expression in the males is likely to reflect an increased cell activation (Pillay et al.; Xu et al., 2008).

### **6.1.2 Acute localised inflammatory response is reduced in females compared to males**

Irrespective of which mechanisms might underlie the sex differences in cell numbers and activation state, the outcome of such a response would be to reduce exposure of the vasculature to the potentially long-term detrimental effects of a sustained inflammatory response, and offers possible explanations for the absence of vascular dysfunction in the female sex. Interestingly, recent evidence suggests that the sex differences in leucocyte count and activation state evident in healthy volunteers are lost in individuals with raised CVD risk. Although it is worth noting that the average age of the women in this particular study was 62, an age when the majority of the women must have been post-menopausal (although whether this was the case or not is unclear) (Gomez-Sanchez et al., 2015). Our observations in typhoid-induced systemic inflammation suggest that there is an enhanced surveillance and readiness to deal with inflammatory stimuli in women resulting, probably, in a reduced detrimental impact upon the vasculature. In

order to probe the possible mechanisms further we assessed acute inflammatory responses using the cantharidin based model of innate inflammatory response (Day et al., 2001b).

Cantharidin is a protein phosphatase 1 and 2 alpha inhibitor (Honkanen, 1993). When applied to the skin it results in acantholysis and blister formation which has been characterised as the detachment of tonofilaments from desmosomes (Bertaux et al., 1988). This in turn causes leucocyte extravasation, cytokine release and clinical inflammation. During inflammation leucocytes and proteins traverse the blood vessel into the extravascular space. Recruitment of cells to inflammatory sites is dependent on the release of vasoactive and chemotactic factors that increase regional blood flow, increase microvascular permeability and promote the exudation of leucocytes from the circulation into the tissues (Suffredini et al., 1999). Cantharidin was used in this study to trigger this response to enable quantification of and, therefore, comparison between the sexes of this inflammatory response. This was achieved through assessment of the volume of fluid collecting into the blister as well as characterising the number and types of inflammatory cell collecting within the exudate. In this study, cantharidin elicited a similar magnitude of blister response, in terms of oedema (i.e. volume) and total number of cells, in males and females; and a response that was similar in magnitude to that reported previously, (Jenner et al., 2014). Indeed, at the 24 hour time-point the number of neutrophils, the first cell recruited to a site of inflammation, was very similar between the sexes suggesting that female sex does not suppress the capacity to respond to an inflammatory stimulus per se. Assessment of the pro-inflammatory cytokine/chemokine profile in the main



supports this view. Our analyses suggest no differences between the sexes of some of the key mediators previously implicated in the cantharidin blister response in humans, including IL-6 (Jenner et al., 2014), neutrophil CXCL1, CCL5 and monocyte chemokines (CCL2) (Viola and Luster, 2008). However, at 72 hours there was a pronounced reduction in the blister volume with a slight trend for reduced cell numbers in females; a reduction accounted for by a pronounced and significant reduction in inflammatory monocyte numbers. Importantly, in many of the women (~70% vs 30% in men) the blisters had resolved by 72 hours resulting in no volume collection at all. Since the immediate (24 hour) response was similar between the sexes, we speculated that the reduced cell number and volume at 72 hours likely reflects an enhanced rate of resolution of inflammation.

This suggestion of enhanced resolution is in line with recent evidence demonstrating the key role of resolvins in clearing oedema following an inflammatory insult, where oedema in the lung induced in mice with hydrochloric acid was reduced in mice treated with aspirin-triggered RvD3 (Colby et al., 2016). To explore this possibility further we assessed the level of IL-10, a pivotal cytokine released during the resolution stage of an inflammatory response that is derived from the anti-inflammatory intermediate (M2) monocyte (Chinetti-Gbaguidi et al., 2015). We did observe a trend for enhanced IL-10 in the blisters of females at 24 hours, however this did not reach statistical significance, likely due to the fact that the study was not powered for this measurement. However, it is thought that IL-10 represses further cell recruitment in part by down-regulating inflammatory cell activation. Indeed, the levels of CD62L, CD11b and CD162 expression, were all substantially reduced supporting the view that whilst the response to the

inflammatory stimulus was similar between the sexes that in females this inflammatory response was likely cleared and 'resolved' at a much faster rate.

### **6.1.3 Elevated pro-resolving lipid mediator profile in blister exudates from females**

It is now accepted that the resolution of acute inflammation is an active process that is initiated when the very first leucocytes arrive at the inflammatory site. This process of resolution is triggered by the recruited cells themselves and is mediated by a switch in the local production of lipid mediators from the pro-inflammatory eicosanoids, including prostaglandins and leukotrienes, to the pro-resolving and tissue reparative SPM including the lipoxins and resolvins (Serhan, 2014). Using targeted lipid mediator profiling we found a 1.4- to 2-fold higher level for each class of SPM in females. In addition, SPM amounts relative to those of the pro-inflammatory/chemotactic lipid mediator LTB<sub>4</sub> exposed a 3 times higher ratio of SPMs to LTB<sub>4</sub> in the females compared to the males, suggesting that the balance of pro-resolving to pro-inflammatory lipid mediators was tipped in favour of resolution in females.

SPMs share a number of key defining bioactions including their ability to limit neutrophil recruitment to a site of inflammation, counterregulate the production and actions of pro-inflammatory mediators and promote macrophage phagocytosis of cellular debris and apoptotic cells. In addition, each SPM displays unique biological action. For example MaR1 is produced in the later stages of the resolution phase and promotes tissue repair as well as displays potent antinociceptive actions (Dalli and Serhan, 2016). RvD1, RvD5 and PD1 are

produced during self-limited infections and promote the clearance of bacterial infections (Chiang et al., 2012), while Resolvin D2 potentially regulates endothelial NO production (Spite et al., 2009). In the present study, we found a statistically significant reduction both in neutrophil and monocyte/macrophage activation state in females compared to males. This is in line with recent findings demonstrating that RvD1 potentially regulates neutrophil recruitment and adhesion molecule expression (Norling et al., 2012) as well as with the findings that RvD1 regulates LTB<sub>4</sub> formation by regulating 5-lipoxygenase phosphorylation and translocation to the nuclear membrane (Fredman et al., 2014). This balance in favour of the resolving SPMs in the blisters of females compared to males was also evident in the plasma of females 8 h following typhoid vaccine. This observation supports the view that active resolution may also underlie the protection against vascular dysfunction in females. In line with such a proposal are my findings that in plasma samples collected from volunteers at 8 h post-typhoid vaccination we found an upregulation in the women compared to the men of molecules from the E-series resolvins that carry potent cardiovascular protective actions (Dona et al., 2008). The differential regulation of EPA vs DHA derived resolvins in plasma and inflammatory exudates may reflect the activation of different biosynthetic pathways by the different stimuli in line with published findings (Dalli and Serhan, 2012), as well as a differential utilisation of precursor fatty acids in plasma versus tissues (Colas et al., 2014; Kasuga et al., 2008).

Interestingly, alterations in pathways influencing SPM levels has also been proposed to be a target for sex steroid activity. Studies in vitro using whole blood and isolated neutrophils collected from healthy male and female volunteers

demonstrated that following stimulation with a pro-inflammatory stimulus (lipopolysaccharide plus N-formyl-methionyl-leucyl-phenylalanine or with Ca<sup>2+</sup>-ionophore A23187) the levels of the 5-lipoxygenase products, including LTB<sub>4</sub>, were reduced in males compared to females. Moreover, this study suggested that this effect was related to activity of testosterone and the inhibition of nuclear localisation of 5-lipoxygenase (Pergola et al., 2008). Indeed, treatment of neutrophils, collected from healthy women, with 5- $\alpha$ -dihydrotestosterone lowered the levels of 5-lipoxygenase products produced following stimulation compared to those evident in the cells isolated from males (Pergola et al., 2008). Of note, treatment of neutrophils from either males or females with female sex hormones, 17 $\beta$ -oestradiol or progesterone, did not alter the levels of the pro-inflammatory lipid mediators. However, it is worth noting that for the female cells the additional female sex hormones may not have been effective since the cells will have been exposed to physiological levels in vivo, and in males the lower levels of the 5-lipoxygenase-derived mediators per se may have meant further inhibition unlikely. Treatment with additional 5- $\alpha$ -dihydrotestosterone had no further effect in cells isolated from males suggesting this possibility likely.

Exactly why in our studies we see higher SPM:LTB<sub>4</sub> ratios in females whilst in the study mentioned above higher ratios were evident in males is uncertain. An important difference between the study by Pergola et al and our work, is that whilst in our study all assessments reflect the in vivo setting, in the work of Pergola all hormone treatments were conducted in vitro. Thus the differences may simply reflect the difference between in vivo and in vitro assessment. To tease apart these issues is difficult until the field matures further and drugs that target

the molecular SPM synthetic pathways are developed. Further studies exploring the regulation of SPM receptors on inflammatory cells themselves, the impact of inhibiting SPM formation on resolution of inflammatory responses, as well as studies assessing the impact of raising SPM levels will be important to confirm the sexual dimorphism described and awaits the further delineation of the molecular pathways and receptor targets of the SPMs. Development of selective drug tools but also pharmacological approaches to ascertain whether delivery of SPMs, for example through dietary provision of substrate (Buckley et al., 2014; Serhan et al., 2015), will be important to determine whether targeting the SPMs might offer a therapeutic approach that could be useful in limiting inflammatory responses.

#### **6.1.4 Limitations of inflammatory models in humans**

There are a number of limitations of this work in healthy volunteers. Firstly, both models of inflammation provide an approximation of the systemic and local inflammatory scenarios. It is possible that the specific cellular profile induced by typhoid or cantharidin are not the same as those induced in a CVD scenario.

Since a large number of female blisters had resolved at the 72 hour time-point, a full profile of the resolution time-course was not possible. Future experiments including time-points mid-way between 24 and 72 hours might provide a more detailed window on the time-course of the resolution between the sexes, although this may prove difficult and may offer only limited advances if the volumes of fluid are small. In addition, recording of the time of resolution in males would also provide an improved comparison of the time scale between the sexes but the

difficulties with this relate to needing the blister to remain open and uncovered for assessment and thus possibly prone to rupture.

#### **6.1.5 Inflammatory experimental models in humans as tools to study the impact of dietary nitrate interventions**

These studies in healthy volunteers demonstrate that the damaging effects of systemic inflammatory stimuli on the vasculature are suppressed in females compared to males, and that this is likely due to a more rapid resolution of the local inflammatory response. In this era of growing research in translational medicine, and particularly relevant for this thesis, the evidence demonstrating the critical role of immune responses and inflammation in CVD, identifying useful experimental models that enable testing of new therapeutic approaches that might dampen inflammation, are of major value. My experiments have shown that the skin blister represents a reproducible model to tease out the processes that drive immune responses in humans and thus may also provide a useful model to use to ask whether defective pathways in the evolution of the response or its resolution contribute to the aetiology of chronic inflammatory conditions such as CVD. I have also re-confirmed that the low-grade inflammation caused by typhoid vaccination causes a pro-inflammatory cytokine response that impairs forearm arterial endothelium-dependent dilatation in male volunteers but interestingly not in females. By demonstrating that the same resolution pathways are altered between the sexes, my data indicates that combining studies using the blister model with typhoid vaccination to interrogate potential anti-inflammatory agents for assessing potential in CVD is feasible. Indeed, these studies have provided a framework with which experiments assessing the impact of dietary

NO<sub>3</sub><sup>-</sup> on inflammation have been structured. As a result of my work we have designed and are mid-way through assessment of the impact of dietary NO<sub>3</sub><sup>-</sup> on cantharidin-induced inflammation and typhoid-induced endothelial dysfunction.

We have initiated a double-blinded randomised, placebo- controlled parallel study to investigate the effect of dietary NO<sub>3</sub><sup>-</sup> on vascular dysfunction and inflammation in healthy volunteers. This study aims to assess whether dietary NO<sub>3</sub><sup>-</sup> (in the form of beetroot juice) can reduce systemic inflammation and endothelial dysfunction that arises following administration of typhoid vaccine. The principal research objective in this study is to determine whether circulating plasma NO<sub>2</sub><sup>-</sup> levels can be raised sufficiently to prevent the systemic inflammation that underlies typhoid vaccine-induced endothelial dysfunction. The study aims to recruit 31 participants in each arm of the study (i.e. placebo and intervention). We are comparing FMD measurements between the intervention and placebo groups in healthy volunteers who have the typhoid vaccine. The study involves healthy participants attending for measurements of PWV/PWA, FMD, BP and collection of blood, urine and saliva samples. They then undergo randomisation to receive either 8 mmol of NO<sub>3</sub><sup>-</sup> - rich beetroot juice or NO<sub>3</sub><sup>-</sup> - deplete beetroot juice (placebo). They then attend 4 days later at 8 am to receive the typhoid vaccine and then on the same day, attend 8 hours later to undergo the same measurements as those performed at baseline on day 1. Finally, they attend on Day 5 to undergo the same measurements as the previous day to assess recovery allowing measurements of the resolution phase of inflammation. A key component of the design of this study, based upon my observations, is that assessment of dietary NO<sub>3</sub><sup>-</sup> is conducted in males only, since inflammation in females resolves rapidly. To assess whether

dietary nitrate alters resolution we will be assessing resolving lipid mediators using LC/MS-MS as described in section 2.3.10. Importantly our recent studies in animal models of atherosclerosis suggests that dietary  $\text{NO}_3^-$  treatment raises IL-10 levels (Khambata et al., 2017). As mentioned there is a close link between this cytokine and the SPMs. Studies have shown that resolvin E2 displays proresolving actions. It does this by potently enhancing nonphlogistic phagocytosis as well as promoting increased IL-10 production by macrophages. Therefore, these studies indicate that RvE2 has proresolving actions in accordance with and characteristic of the SPMs (Oh et al., 2012).

Simultaneously we have embarked upon a second study using the cantharidin model of inflammation. This study is also a double-blind, randomised, placebo-controlled parallel study assessing the influence of dietary  $\text{NO}_3^-$  on skin inflammation in healthy volunteers. Here we aim to assess whether dietary  $\text{NO}_3^-$  might reduce localised recruitment of leucocytes and/or the activation state of leucocytes or perhaps alter the processes of resolution using the cantharidin model of inflammation. The principal research objective similarly to the typhoid study is to determine whether inorganic  $\text{NO}_3^-$  in the form of beetroot juice compared to placebo controls can raise circulating plasma  $\text{NO}_2^-$  levels sufficiently to modulate the inflammatory response. The study aims to recruit 12 healthy volunteers in each arm of the study (i.e. placebo and intervention and thus a total of 24 volunteers). Again, due to the findings in my previous study, the rapid resolution of inflammation in females compared to males has driven our decision to conduct this study in males participants only. Within this study, we are comparing the mechanisms of resolution in localised inflammation induced by



cantharidin using flow cytometry. In addition, we will also measure both the cytokines and chemokines involved in this form of inflammation, such as IL-6, IL-8, IL-10, CXCL1, CXCL2, CCL5 and CCL2. The reasons for selecting this group of cytokines and chemokines is that we have already observed a rise in levels during our first study as described in Chapter 3. . Again, we will assess the impact of dietary NO<sub>3</sub><sup>-</sup> on the resolution phase of localised inflammation using LC/MS-MS to measure SPMs in order to investigate the relative levels of the various lipids so that an estimation of the resolving power can be made. Our hypothesis is that the male participants who are taking the NO<sub>3</sub><sup>-</sup> rich juice are more likely to have a faster resolution of inflammation compared to the males participants on the placebo.

## **6.2 Effect of dietary nitrate on the enterosalivary circuit in patients with stable angina**

Studies have shown that dietary NO<sub>3</sub><sup>-</sup> has positive effects upon vascular function; including reduction of BP, leukocyte activation and platelet reactivity as well as improvement of endothelial dysfunction (Kapil et al., 2015; Velmurugan et al., 2016). Given the importance of NO in vascular function and its anti-inflammatory actions, it is logical to hypothesise that enhancing NO bioavailability might prove to be an effective strategy in improving vascular function, platelet reactivity and restenosis in patients with stable angina after PCI particularly since inflammatory pathways play a key role in progression of pathology.

As the NITRATE-OCT study is still ongoing, the treatment given to each group has remained blinded, which is key to avoid bias in the final analysis. The groups have

been labelled gamma and delta for the baseline demographic data analysis to enable determination of whether the randomisation has been effective but the blinding has remained since the study has not yet completed recruitment of the target number of 246. Therefore, to avoid the introduction of selection bias and analysis bias the investigators in the study have remained blinded and will do so until the primary endpoint has been completed.

With regards to the baseline demographics, there were no significant differences between the two groups in the study, except for mean baseline HR, which was higher in the gamma group compared to the delta group. This difference in HR in 1 of 30 of the different baseline characteristics is likely to be reflective of a chance observation. Overall, the similarities between the two groups suggests good matching. Furthermore, there were no differences between the two groups with regards to procedural characteristics, particularly the type of stent used, and medications at discharge. This means that any differences that we see in the rates of restenosis between the two groups are likely to be due to the intervention. In addition, patients will be stratified according to the type of stent BMS or DES as both these groups have differing characteristics resulting in differing rates of restenosis. Finally, since patients with diabetes are at high risk of developing restenosis, these patients are included in this study with stratification in both groups to ensure even number of diabetic patients in both groups.

When comparing the concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  at baseline particularly in plasma, between the patients with stable angina with previous observations in healthy volunteers published in the literature my results suggest some potentially

important differences. The overall mean baseline plasma  $\text{NO}_2^-$  concentrations at baseline was  $1.58 \mu\text{mol/L}$ , which is approximately 4 times higher than the baseline levels measured in healthy volunteers (Bahra et al., 2012). Previous studies by Lauer et al have suggested that plasma levels of  $\text{NO}_2^-$  are a more sensitive indicator of *in vivo* NO production. The raised level in this study suggests that in the patients with angina, NO levels may have been elevated even before treatment with dietary  $\text{NO}_3^-$  (Lauer et al., 2001). This result is consistent with the theory that perhaps in these patients with angina there is an enhanced inflammatory state that has led to the expression of iNOS, iNOS is not normally expressed in health but can be rapidly induced in response to inflammation and cytokine production (Libby, 2005). A related point to consider is that, NO produced by iNOS is not region specific, hence the increased and widespread production of NO reacts with superoxide and produces higher levels of peroxynitrite. This contributes to further deterioration of endothelial function despite the paradoxical high levels of  $\text{NO}_2^-$  that would be expected after treatment (Ridker et al., 2002). Furthermore, iNOS is mostly expressed in macrophages (Kajita et al., 2011; Panaro et al., 2003) and vascular smooth muscle cells (Fries et al., 2003; Ginnan et al., 2008) rather than the endothelial cell, although there is also some evidence suggesting that iNOS is present in endothelial cells (Binion et al., 1998; Leifeld et al., 2002; Yu et al., 2004) which can all account for the elevation of NO in patients with stable angina. This NO results in continuously elevated NO generation which is not stimulus specific and not requiring rises in intracellular calcium.

Based on the results of the whole cohort it is clear that after 6 months there is on average a significant increase in the concentrations of cGMP,  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in the

whole group in the different compartments measured. This supports the assumption that at least some of patients received a daily dietary  $\text{NO}_3^-$  treatment. If one looks at the paired data it is clear that the patients separate in to two groups and this likely reflects the difference in the  $\text{NO}_3^-$  content of the 2 interventions. This will be confirmed upon unblinding.

The discovery of the  $\text{NO}_3^-$ - $\text{NO}_2^-$ -NO pathway, which relies on an intact enterosalivary circuit, has provided scope for the development of novel disease prevention strategies through dietary  $\text{NO}_3^-$  supplementation. Results from this project suggest that perhaps a once daily ingestion of 70ml of  $\text{NO}_3^-$  rich beetroot juice increases the conversion of  $\text{NO}_3^-$  to NO and increases the levels of cGMP in patients with stable angina. This highlights the possibility that the enterosalivary circuit is intact in these patients and that the disease per se does not interfere with the functioning of this circuit.

Overall, providing 4-5mmol daily of dietary  $\text{NO}_3^-$  for 6 months increased plasma  $\text{NO}_3^-$  concentrations by 3.7-fold. This is a similar peak fold increase to those achieved in previous studies (Velmurugan et al., 2016). The increase from baseline in  $\text{NO}_2^-$  plasma concentration after 6 months was of approximately 1.2-fold. This lower fold increase in  $\text{NO}_2^-$  concentrations, compared to  $\text{NO}_3^-$ , highlights the fact that this step relies on the bioconversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  by oral bacteria (Salvatore Davide Tomasello, 2011). Inorganic  $\text{NO}_3^-$  from the diet is extracted from the blood after absorption by the salivary glands and enters the saliva where it is concentrated approximately 10-fold.  $\text{NO}_3^-$  is reduced to  $\text{NO}_2^-$  by the commensal bacteria in the oral cavity when it is secreted into saliva (Webb et al., 2008). The

levels of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in the saliva of the patients with stable angina increased by 2.5-fold and 1.5-fold respectively. Since we know that at least half of the subjects received the placebo and thus assume that upon unblinding the groups would be separated, this theoretically should result in a doubling of these values in the dietary  $\text{NO}_3^-$  limb compared the placebo. This finding is in line with the results in healthy volunteers who were administered once daily doses of  $\text{NO}_3^-$  supplementation (Velmurugan et al., 2013). This further confirms the hypothesis that the enterosalivary circuit is intact in patients with stable angina and remains functional over the 6 months of once daily  $\text{NO}_3^-$ -treatment. Furthermore, the baseline  $\text{NO}_3^-$  levels were  $\sim 27$ -fold higher in the saliva compared to plasma at baseline. This finding suggests that patients with stable angina could either have greater capacity to extract  $\text{NO}_3^-$  from the blood or have better ability to concentrate the  $\text{NO}_3^-$  in saliva compared to healthy volunteers.

The ratio of  $\text{NO}_2^-$  to  $\text{NO}_3^-$  in saliva samples of healthy volunteers is approximately 0.3 (Efsa, 2008; Velmurugan et al., 2016). In this project, the  $\text{NO}_2^-:\text{NO}_3^-$  after 6 months was 0.5 and at baseline was 0.6. Hence, this finding perhaps suggests that in patients with stable angina, there is a baseline increased capacity to reduce  $\text{NO}_3^-$  and it is possible that this is sustained with the increased uptake of inorganic  $\text{NO}_3^-$  for a long duration. Again, once the groups are separated after unblinding it is possible that the ratio will be different between the groups and possibly even higher than 0.5 in the  $\text{NO}_3^-$  limb. This observation would be in agreement with recent findings by our lab that have demonstrated that following 6 weeks of  $\text{NO}_3^-$  rich beetroot juice intake, alterations in the oral microbial community occurred with a significant shift in the structure of the bacterial community after dietary

$\text{NO}_3^-$  intake that was not evident in the placebo group (Velmurugan et al., 2016). Therefore, it will be of value to analyse the salivary microbiome and assess for any changes after 6 months of treatment.

Inorganic  $\text{NO}_3^-$  has been considered as a prodrug for the production of  $\text{NO}_2^-$  with advantages to the use of  $\text{NO}_3^-$  over  $\text{NO}_2^-$  for treatment. For instance,  $\text{NO}_3^-$  has a longer half-life in plasma of about 6h, whereas the half-life of  $\text{NO}_2^-$  is 20-40min (Bhatt and Topol, 2003). Moreover, the relative rapid time to peak plasma  $\text{NO}_3^-$  concentration is between 1-3 hours after ingestion. The plasma  $\text{NO}_3^-$  then recirculates contributing to a sustained release of  $\text{NO}_2^-$  that slowly reaches a peak after 3 hours which then remains elevated for a further 3 hours, with levels remaining slightly raised at 24h after a single dose (de Winter et al., 2002).

Another potential is the possible involvement of xanthine oxidoreductase (XOR), which is a source of reactive oxygen species (ROS) but also has  $\text{NO}_2^-$  reductase activity. The activity of XOR becomes upregulated in conditions like CHD, where there is increased oxidative stress and reduced activity of eNOS (Albert and Ridker, 2006). As shown in previous studies, XOR expression and activity is increased following dietary  $\text{NO}_3^-$  supplementation. This results in switch of XOR function into a favourable increase in the production of  $\text{NO}_2^-$  and NO with a concurrent reduction in ROS generation (Garrone et al., 2009). XOR works by either generating NO directly from the reduction of  $\text{NO}_2^-$  by catalysing the reaction at the molybdenum site where  $\text{NO}_2^-$  accepts electrons. Hence, fewer electrons are donated to oxygen so fewer ROS are produced that can scavenge NO, leading to an

increased bioavailability of NO (Garrone et al., 2009). This could also explain the increased baseline  $\text{NO}_2^-$  levels observed. This also highlighted the fact that NO produced by XOR after  $\text{NO}_3^-$  supplementation would be generated specifically to the area of ischaemia and endothelial dysfunction to help improve vascular haemostasis, as opposed to the NO generated by iNOS that is widespread.

Finally, the plasma concentrations of cGMP increased by 1.15-fold after  $\text{NO}_3^-$  treatment, which could reflect an increase in NO bioavailability. This was a similar fold increase as measured in hypertensive patients after  $\text{NO}_3^-$  treatment (Kapil V, 2015). An elevation in cGMP levels after an acute dose of  $\text{NO}_3^-$  occurs around 3-24 hours after ingestion (Kapil et al., 2015). The overall mean cGMP concentration at baseline was 28.5 nmol/L. This was 4 times higher than the values recorded in previous studies, but was consistent with the 4 times higher plasma  $\text{NO}_2^-$  measured in this study (Kapil et al., 2015). As mentioned previously, iNOS and XOR generation of NO does possibly contribute to the higher cGMP levels measured. However, this could also be due to the fact that in the management of angina, organic  $\text{NO}_3^-$  such as isosorbide mononitrate and nitroglycerin are commonly used. These drugs are NO donors, which result in a stimulated production of cGMP that cause vasodilation, thus relieving symptoms of chest pain (Cohn and Rittinghausen, 1985). Interestingly, in our study, 21.5 % of the patients recruited so far are regularly taking isosorbide mononitrate and 60.5% of patients are taking nitroglycerin 'when required during episodes of angina'. Hence, these high doses of regular and 'when required' doses of organic  $\text{NO}_3^-$  could explain the high levels of cGMP found in our study so far.

Inorganic and organic  $\text{NO}_3^-$  both generate NO, but their bioavailability and metabolic profiles are different. The acute administration of organic  $\text{NO}_3^-$  has potent immediate vasodilation effects and these compounds have a relatively short half life. There are two main pathways for bioactivation of organic  $\text{NO}_3^-$ . Firstly, enzymes of the cytochrome P450 system (McDonald and Bennett, 1990) mediate the low potency pathway which produces measurable NO from nitroglycerin (Kleschyov et al., 2003). The second pathway, which is a high potency pathway, involves the enzyme aldehyde dehydrogenase-2 (ALDH-2). This pathway was identified following the use of ALDH-2 inhibitors, cyanamide and chloral hydrate (Chen et al., 2002). These inhibitors blocked the production of  $\text{NO}_2^-$  and glycerol dinitrate from nitroglycerin, which had previously been shown to be intermediates in production of NO-related vasodilation by organic nitrate compounds (Sage et al., 2000). Inorganic  $\text{NO}_3^-$  in contrast produces more sustained and subtle effects throughout at 24h period. In addition, another key difference is that a major limitation of the organic  $\text{NO}_3^-$  is that tolerance develops with chronic use resulting in the attenuation of their pharmacological effects. There are several causes of this effect. Firstly, the organic  $\text{NO}_3^-$  require metabolism to generate NO. Nitroglycerin is metabolised by mitochondrial ALDH-2 generating NO and ROS simultaneously. ROS scavenges bioavailable NO, uncouples eNOS and inhibits aldehyde dehydrogenase activity (Sage et al., 2000; Shu et al., 2015). This thus impairs further nitroglycerin metabolism, further aggravating  $\text{NO}_3^-$  tolerance. In addition, the organic  $\text{NO}_3^-$  have been shown to cause endothelial dysfunction per se with chronic use. The mechanism behind this process is not completely understood, although ROS generation with  $\text{O}_2^{\cdot-}$  seems to be an important factor. In a model with rabbits, there was twice as much  $\text{O}_2^{\cdot-}$



generation compared to the controls after a 3 day application of transdermal GTN (Munzel et al., 1995). In patients undergoing CABG who were pre-treated with GTN, there was an increase in  $O_2^-$ , which was associated with endothelial dysfunction (Schulz et al., 2002). In contrast, inorganic  $NO_3^-$  improves endothelial function, with no reports of tolerance (Dejam et al., 2007; Haverkate et al., 1997).

Thus, a dietary  $NO_3^-$  approach could prove to be a cost-effective strategy in reducing the rates of adverse effects in patients with stable angina and could also improve treatment compliance of patients, although completion of the trial and additional investigation is warranted.

### **6.2.1 Future analysis for this part of the study**

Even though there is a significant rise in [cGMP], [ $NO_3^-$ ] and a trend in plasma and saliva [ $NO_2^-$ ] from baseline to 6 months, it is important to complete the measurements of [cGMP], [ $NO_3^-$ ] and [ $NO_2^-$ ] to ensure accurate interpretation of the trends. As the  $NO_2^-:NO_3^-$  ratio found in this study was higher than previous studies it would be interesting to analyse the oral microbiota from saliva samples. This is because, our lab recently conducted a clinical trial in patients with hypercholesterolemia and detected a significant shift in microbiome structure following 6-weeks of  $NO_3^-$  – rich beetroot juice. In this study, there were significant increases in both *Neisseria flavescens* and *Rothia mucilaginosa* ( $NO_3^-$ -reducing bacteria) associated with improvements in  $NO_3^-$  reduction which suggests that dietary  $NO_3^-$  supplementation “pushes” the oral microbiome composition towards one characterised by higher presence of  $NO_3^-$  –reducing bacteria (Velmurugan et al., 2016). Whether this is a characteristic of dietary  $NO_3^-$  treatment that might be

used to promote cardiovascular health in healthy volunteers or improve outcome in patients with CVD is unknown. In NITRATE-OCT, we have collected similar saliva samples to assess the microbiome but it would be useful to collect additional samples from age-matched healthy volunteers to determine whether differences in oral microbiome composition occur between health and disease, whether dietary  $\text{NO}_3^-$  - feeding might be useful in enhancing the efficacy of microbial  $\text{NO}_3^-$  metabolism and whether shifts in microbiome composition return to baseline with removal of the dietary intervention. We intend to use both next generation sequencing and where possible whole genome sequencing approaches adapted for microbiome analyses to assess these possibilities.

### **6.3 Determining whether dietary $\text{NO}_3^-$ might improve intimal hyperplasia and thereby restenosis rates post PCI and stent implantation**

To date there have been no clinical studies investigating the role of orally ingested  $\text{NO}_3^-$  in reducing restenosis in patients undergoing PCI for stable angina. NITRATE-OCT study is the first clinical study assessing the safety and efficacy of oral  $\text{NO}_3^-$  in a dietary form (beetroot juice) in this group of patients. Recruitment in my trial to date is only slightly behind the projected schedule and should be completed at a provisional date of December 2019. Hopefully this study will translate to humans the impressive effects of inorganic  $\text{NO}_2^-$  on reducing hyperplasia in animal studies (Alef et al., 2011).

The proposed mechanisms of these benefits of inorganic  $\text{NO}_3^-$  are thought to be through a number of mechanisms with effects demonstrated on both platelet reactivity, and inflammation. However, this thesis has demonstrated that

inorganic  $\text{NO}_3^-$  influences the expression of adhesion molecules on monocytes, and subsequent monocyte activation, and thus potentially reducing the inflammatory component of restenosis. This issue and were several other aims set out by the NITRATE-OCT Study, which are ongoing, are discussed below.

### **6.3.1 Ingestion of dietary nitrate has no clinical adverse effects**

The ingestion of dietary  $\text{NO}_3^-$  has not resulted in any adverse effects in the cohort so far. Due to the known vasodilator (Cosby et al., 2003) and BP lowering effects (Dejam et al., 2007; Pluta et al., 2011) of raised circulating  $\text{NO}_2^-$  levels, BP was measured in all our patients. In NITRATE-OCT so far there is a trend towards a reduction in BP at 6 months compared to baseline but no evidence of any negative hypotension effects. This suggests that some of the patients are likely to be taking the  $\text{NO}_3^-$  - rich beetroot juice confirming findings from our previous studies (Kapil et al., 2015; Velmurugan et al., 2016).

Levels of methaemoglobin were also measured in NITRATE-OCT because of the known interaction of  $\text{NO}_2^-$  with oxyhaemoglobin to generate methaemoglobin (Beck, 1909; Roe, 1933). Levels of methaemoglobin were not clinically significant indicating that the circulating levels of  $\text{NO}_2^-$  that are likely to be required to achieve beneficial functional effects, from the dose we have used in this study, upon the cardiovascular system are much lower than the levels associated with their detrimental effects.

#### **6.4 Determining whether dietary NO<sub>3</sub><sup>-</sup> ingestion exerts anti-inflammatory effects and the mechanisms involved in this effect following PCI for stable angina**

The second key aim of the NITRATE-OCT study is to investigate the potential anti-inflammatory mechanisms by which dietary inorganic NO<sub>3</sub><sup>-</sup> may reduce restenosis. In this study, patients showed a trend towards a reduction in hsCRP at 6 months compared to baseline in the whole cohort. We are yet to see whether there is a statistically significant difference between the two treatment arms at 6 months compared to baseline and this will be analysed once the whole study is completed and unblinding.

Epidemiological studies have demonstrated that serum CRP levels can be a tool to detect low-level systemic inflammation and are a strong predictor of the occurrence of cardiovascular events such as MI in apparently healthy subjects (Ridker et al., 1997; Ridker et al., 2000) and in patients with a recent coronary event (Ridker et al., 2005). Although there is a suggestion that “the overall and local inflammatory status at the time of PCI plays a significant role in the development of restenosis” (Toutouzas et al., 2004), previous studies of inflammatory marker levels and restenosis have shown conflicting results. There may be several explanations for this, including differences in the population studied (stable angina versus unstable angina), concurrent use of medications that may blunt the inflammatory response (statins, glycoprotein IIb/IIIa inhibitors, thienopyridines), timing of inflammatory marker measurement (pre-PCI versus post-PCI), PCI procedure (balloon angioplasty versus stent deployment), and outcomes studied (angiographic restenosis versus clinical restenosis or events)

(Bhatt, 2004). The relevance of statin use is of particular importance as a number of patients had their dose of statins increased following PCI, which could explain the generalised reduction in majority of cytokines and chemokines (IL-6, CXCL-1, CXCL-5, CXCL-8, CXCL-2, CCL-2 and CX3CL-1) at 6 months compared to baseline in the overall groups. I will be able to check this because I can analyse the change in the dose of statins at discharge and 6 months compared to baseline as we are collecting this data as part of the study.

The mechanisms that underlie coronary events following PCI are distinct from the mechanisms that underlie restenosis (Bhatt and Topol, 2003), so it is possible that inflammatory marker levels could be predictive of clinical events following PCI but not of restenosis. Larger studies (de Winter et al., 2002; Dibra et al., 2003) have shown that pre-PCI CRP levels predict 1-2 year cardiac event rates in stable angina patients undergoing PCI. Therefore, although CRP is not predictive of restenosis per se in stable angina patients, it does appear to be predictive of plaque instability and, consequently, of clinical manifestations of ischaemia. This is consistent with our current understanding of atherosclerosis and the pathogenesis of ACS (Libby, 2005), and with observations that CRP levels are also predictive of medium to long term cardiac events in asymptomatic individuals (Ridker et al., 2002), in patients with stable angina (Haverkate et al., 1997), and in patients with ACS not undergoing PCI (Lindahl et al., 2000).

#### **6.4.1 Inflammatory marker response to PCI**

Previous studies that have confirmed that in stable angina patients, PCI leads to a systemic inflammatory response. CRP levels can be higher 48 hours post-PCI than

pre-PCI, which then return to pre-PCI levels somewhere between one week and one month post-PCI (Gomma et al., 2004; Segev et al., 2004). There is also some evidence to suggest that that post-PCI inflammatory marker levels may be more predictive of restenosis than pre-PCI levels (Bhatt, 2004). and that higher post-PCI CRP levels may be associated with angiographic restenosis in stable angina patients (Gottsauer-Wolf et al., 2000). In the largest study to date (which included 1,321 stable angina patients), the association between the post-PCI CRP rise and angiographic restenosis was significant in patients with stable angina ( $p=0.05$ ) (Dibra et al., 2005). In addition, there are a number of studies that have reported a significant increase in inflammatory marker levels after PCI (Azar et al., 1997; Cipollone et al., 2003; Quinn et al., 2004; Segev et al., 2004). Our assessments at this stage in the study have found that there was trend towards a reduction in hsCRP levels at 6 months compared to baseline in the overall cohort of patients. It might be the case that once all the patients have been recruited and the data is unblinded, there could be a significant reduction in hsCRP in the  $\text{NO}_3^-$  - rich beetroot juice group compared to the placebo group, which in term could help to explain a reduction in restentosis rates in this group compared to the placebo if our hypothesis is confirmed.

#### **6.4.2 Effects of the ingestion of inorganic $\text{NO}_3^-$ on circulating immune cells**

The reduced number of macrophages within plaque regions could be related to lower monocyte recruitment to the vessel wall, which is a direct effect on the endothelial cell or the monocyte, or an indirect effect of reduced neutrophil accumulation. We also know that the recruitment of neutrophils in atherosclerosis occurs in the early stages of atherogenesis before monocyte recruitment (van

Leeuwen et al., 2008; Zerneck et al., 2008) and it is believed that these neutrophils play an important role in instructing other key leukocytes, especially monocytes, to accumulate at vascular sites with a preference for atheroma formation (Doring et al., 2015). Once our study has been unblinded, our data assessing inflammatory cell numbers and activation state will indicate whether inorganic  $\text{NO}_3^-$  treatment results in a reduction in circulation leukocyte numbers at 6 months compared to baseline, as well as whether treatment influences the activation state of these cells (CD11b, CD62L and CD162). However, so far with the data grouped together, there is no evidence of a change in expression of any of the activation markers at 6 months compared to baseline in any other cell subtype.

As mentioned pre-clinical studies from our lab have demonstrated that inorganic  $\text{NO}_3^-$  therapy of ApoE KO mice reduces neutrophil recruitment followed by consequent reduction in the number of inflammatory monocytes in a model of peritonitis. We speculated that this effect was due to an elevation of IL-10 levels (Khambata et al., 2017). These studies also demonstrated that within lesions in the ApoE KO mice there was a reduction in inflammatory load in inorganic-  $\text{NO}_3^-$  fed ApoE KO mice associated with elevated IL-10 mRNA levels. We speculated that this effect could result in a reduction in neutrophil-dependent recruitment of monocytes into the atherosclerotic plaque and potentially an acceleration of the resolution of inflammation, driven by elevations in IL-10 expression and consequent activity (Couper et al., 2008; Mosser and Zhang, 2008). Although not statistically significant, in the patients to date in NITRATE-OCT there is an overall reduction in IL-10 levels at 6 months compared to baseline. It will be interesting to see if the levels of IL-10 are significantly lower in the treatment arm compared

to the placebo arm at 6 months compared to baseline, once the study has been completed and the results are unblinded.

#### **6.4.3 Improved vascular health at 6 months in nitrite treated patients**

We hypothesise that patients in the placebo arm are more likely to have endothelial dysfunction compared to the treatment arm at 6 months, which in turn would support the concept of impaired vascular health and endothelial dysfunction in these patients. Higher levels of MCP-1 have also been shown to predict adverse events and reflect elevated levels of inflammation (Martinovic et al., 2005). A study by Huang and colleagues has shown that endothelial vasodilator dysfunction in patients with suspected coronary artery disease is associated with elevated hs-CRP and MCP-1 compared to patients with a normal chronotropic response to exercise and no endothelial dysfunction (Huang et al., 2006). Therefore, it would be interesting to see how this combination of factors are affected in the whole cohort of patients at 6 months compared to baseline in the NO<sub>3</sub><sup>-</sup>-rich group. We would predict that there would be reduced platelet reactivity, lower levels of hs-CRP and MCP-1 levels coupled with higher levels of NO<sub>2</sub><sup>-</sup>/ NO<sub>3</sub><sup>-</sup> and cGMP, which are likely to reflect reduced inflammation, improved endothelial function and vascular health in these patients and therefore could result in a reduced rate of major adverse cardiac events in the cohort at this time-point.

#### **6.5 Future Work from the NITRATE-OCT Clinical Trial**

Recruitment for NITRATE-OCT, will be completed by the end of 2018 (December). The primary endpoint will be completed at 6 months after the last patient is



recruited with 1 year's follow-up (visit 5) by December 2019. Over the next few months, the remaining stored samples will be analysed, which will include plasma samples being analysed for cGMP, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> levels, plasma markers of inflammation; hs-CRP and urate. Analysis of flow cytometry data for each subjects' PMA and p-selectin expression will be performed. QCA analysis of the angiographic data and OCT analysis will be performed.

All patients will be telephoned at 1 year (visit 5) after their 1 year follow-up visit to assess for any symptoms, hospitalisations and major-adverse cardiac events.

#### **6.5.1 Study limitations**

So far, the low clinical event rate is unlikely to demonstrate a significant difference between the two groups to detect associations between the intervention and MACE. However, once this study is completed, our assessments suggest that it will be adequately powered to detect a difference between the intervention and placebo groups in terms of MACE as well as powered to assess restenosis (through late lumen loss) using quantitative coronary angiography.

In some patients, missing inflammatory marker levels and quantitative coronary angiographic results may have introduced unappreciated bias, although the baseline clinical and angiographic characteristics of these patients were similar to those of the main study cohort. Furthermore, our study included a relatively homogeneous population of patients with stable coronary disease undergoing elective PCI for a de novo native coronary lesion, limiting the likelihood of confounding or systematic bias. Other strengths of this study include

randomisation with even distribution of the use of statins as well as stratification to diabetes status, both of which allowed us to exclude the possibility that use of these agents and/or diabetes masked a positive association between inflammatory markers and possible restenosis between the groups. We are also using an objective measurement of restenosis using late lumen loss on QCA angiography at 6-month angiographic follow-up with assessment of angiographic restenosis. This avoids the potential for observer bias that arises with use of less objective outcome measures such as clinical restenosis (Buffon et al., 1999). In addition, results from the platelet markers is yet to be analysed as the data collection is still ongoing.

This study has been powered for a sample size of 246 but since patient recruitment is still ongoing, the results of only 200 participants at baseline have been included in this thesis. Also, as the study remains blinded, the basis of the discussion was limited to an overall combined effect of the two arms and therefore this has limited the depth of data that can be analysed until the end of the trial.

## **6.6 Conclusions**

This study demonstrated that the enterosalivary circuit remains intact throughout the 6 months of treatment, in patients with stable angina. There is a clear increase in the concentrations of  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and cGMP in the overall group, likely to be due to half the patients being treated with  $\text{NO}_3^-$ . The increase in cGMP levels after treatment in the intervention arm reflected the overall increased NO bioavailability. Moreover, the raised levels of baseline plasma cGMP and  $\text{NO}_2^-$  has been attributed to the use of isosorbide mononitrate and upregulation of iNOS and

XOR activity. We have also found that patients with stable angina have a generalised baseline systemic inflammatory response with high circulating levels of the inflammatory marker hsCRP. In addition, there was a trend towards a reduction in hsCRP levels at 6 months compared to baseline, likely to be due to half the patients being on the intervention of inorganic  $\text{NO}_3^-$  (beetroot juice). Finally, in addition to completing the study with analysis of the primary endpoint of late lumen loss via angiography and OCT, further work needs to be conducted to categorically explain the mechanisms involved with dietary  $\text{NO}_3^-$  treatment. Consequently, this will provide further evidence of the benefits of inorganic  $\text{NO}_3^-$  and specifically with the aim of improving endothelial function and reducing rates of restenosis in patients with stable angina.

## **Chapter 7.0**

# **Bibliography**

## 7.0 Bibliography

- Ahrens I, Peter K and Bode C (2003) Use of GPIIb/IIIa inhibitors in cardiovascular medicine. *Expert review of cardiovascular therapy* **1**:233-242.
- AICR (2007) Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective, in *AICR* (Research WCRFAIfC ed), Washington DC.
- Ait-Oufella H, Taleb S, Mallat Z and Tedgui A (2011) Recent advances on the role of cytokines in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **31**:969-979.
- Akin I, Schneider H, Ince H, Kische S, Rehders TC, Chatterjee T and Nienaber CA (2011) Second- and third-generation drug-eluting coronary stents: progress and safety. *Herz* **36**:190-196.
- Akrawinthawong K, Park JW, Pikhova B, Sibmooh N, Fucharoen S and Schechter AN (2014) A flow cytometric analysis of the inhibition of platelet reactivity due to nitrite reduction by deoxygenated erythrocytes. *PloS one* **9**:e92435.
- Al-Dashti YA, Holt RR, Stebbins CL, Keen CL and Hackman RM (2018) Dietary Flavanols: A Review of Select Effects on Vascular Function, Blood Pressure, and Exercise Performance. *J Am Coll Nutr*:1-15.
- Al-Solaiman Y, Jesri A, Mountford WK, Lackland DT, Zhao Y and Egan BM (2010) DASH lowers blood pressure in obese hypertensives beyond potassium, magnesium and fibre. *J Hum Hypertens* **24**:237-246.
- Albert MA and Ridker PM (2006) C-reactive protein as a risk predictor: do race/ethnicity and gender make a difference? *Circulation* **114**:e67-74.
- Alderman EL, Kip KE, Whitlow PL, Bashore T, Fortin D, Bourassa MG, Lesperance J, Schwartz L and Stadius M (2004) Native coronary disease progression

- exceeds failed revascularization as cause of angina after five years in the Bypass Angioplasty Revascularization Investigation (BARI). *Journal of the American College of Cardiology* **44**:766-774.
- Alef MJ, Vallabhaneni R, Carchman E, Morris SM, Jr., Shiva S, Wang Y, Kelley EE, Tarpey MM, Gladwin MT, Tzeng E and Zuckerbraun BS (2011) Nitrite-generated NO circumvents dysregulated arginine/NOS signaling to protect against intimal hyperplasia in Sprague-Dawley rats. *The Journal of clinical investigation* **121**:1646-1656.
- Allahverdian S, Chehroudi AC, McManus BM, Abraham T and Francis GA (2014) Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation* **129**:1551-1559.
- Andre P, Nannizzi-Alaimo L, Prasad SK and Phillips DR (2002) Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. *Circulation* **106**:896-899.
- Anonymous (1958) Hormones and atherosclerosis. Meeting in Utah. . *BMJ (Clinical research ed)* **1**:1059-1060.
- Apostoli GL, Solomon A, Smallwood MJ, Winyard PG and Emerson M (2014) Role of inorganic nitrate and nitrite in driving nitric oxide-cGMP-mediated inhibition of platelet aggregation in vitro and in vivo. *Journal of thrombosis and haemostasis : JTH* **12**:1880-1889.
- Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin PH and Karanja N (1997) A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *The New England journal of medicine* **336**:1117-1124.

- Ashworth A, Mitchell K, Blackwell JR, Vanhatalo A and Jones AM (2015) High-nitrate vegetable diet increases plasma nitrate and nitrite concentrations and reduces blood pressure in healthy women. *Public health nutrition* **18**:2669-2678.
- Aukrust P, Muller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Froland SS and Gullestad L (1999) Enhanced levels of soluble and membrane-bound CD40 ligand in patients with unstable angina. Possible reflection of T lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. *Circulation* **100**:614-620.
- Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum N, Norat T, Greenwood DC, Riboli E, Vatten LJ and Tonstad S (2017) Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-a systematic review and dose-response meta-analysis of prospective studies. *International journal of epidemiology* **46**:1029-1056.
- Avery AA (1999) Infantile methemoglobinemia: reexamining the role of drinking water nitrates. *Environmental health perspectives* **107**:583-586.
- Azar RR, Aoun G, Fram DB, Waters DD, Wu AH and Kiernan FJ (2000) Relation of C-reactive protein to extent and severity of coronary narrowing in patients with stable angina pectoris or abnormal exercise tests. *The American journal of cardiology* **86**:205-207.
- Azar RR, McKay RG, Kiernan FJ, Seecharran B, Feng YJ, Fram DB, Wu AH and Waters DD (1997) Coronary angioplasty induces a systemic inflammatory response. *The American journal of cardiology* **80**:1476-1478.

- Babapulle MN, Joseph L, Belisle P, Brophy JM and Eisenberg MJ (2004) A hierarchical Bayesian meta-analysis of randomised clinical trials of drug-eluting stents. *Lancet* **364**:583-591.
- Bahra M, Kapil V, Pearl V, Ghosh S and Ahluwalia A (2012) Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **26**:197-202.
- Bain BJ and England JM (1975a) Normal haematological values: sex difference in neutrophil count. *British Medical Journal* **1**:306-309.
- Bain BJ and England JM (1975b) Variations in leucocyte count during menstrual cycle. *British Medical Journal* **2**:473-475.
- BARI Investigators (2007) The final 10-year follow-up results from the BARI randomized trial. *Journal of the American College of Cardiology* **49**:1600-1606.
- Barrett-Connor E and Bush TL (1991) Estrogen and coronary heart disease in women. *Jama* **265**:1861-1867.
- Bartholomew B and Hill MJ (1984) The pharmacology of dietary nitrate and the origin of urinary nitrate. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **22**:789-795.
- Barthwal MK, Anzinger JJ, Xu Q, Bohnacker T, Wymann MP and Kruth HS (2013) Fluid-phase pinocytosis of native low density lipoprotein promotes murine M-CSF differentiated macrophage foam cell formation. *PloS one* **8**:e58054.
- Bayes-Genis A, Conover CA, Overgaard MT, Bailey KR, Christiansen M, Holmes DR, Jr., Virmani R, Oxvig C and Schwartz RS (2001) Pregnancy-associated



- plasma protein A as a marker of acute coronary syndromes. *The New England journal of medicine* **345**:1022-1029.
- Beagley KW and Gockel CM (2003) Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. *FEMS immunology and medical microbiology* **38**:13-22.
- Beck EG (1909) Toxic effects from bismuth subnitrate with reports of cases to date. *JAMA : the journal of the American Medical Association* **LII**:14-18.
- Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *The American journal of physiology* **271**:C1424-1437.
- Bellien J, Favre J, Iacob M, Gao J, Thuillez C, Richard V and Joannides R (2010) Arterial stiffness is regulated by nitric oxide and endothelium-derived hyperpolarizing factor during changes in blood flow in humans. *Hypertension* **55**:674-680.
- Bendall JK, Douglas G, McNeill E, Channon KM and Crabtree MJ (2014) Tetrahydrobiopterin in cardiovascular health and disease. *Antioxidants & redox signaling* **20**:3040-3077.
- Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M and McKenzie H (1994) Stomach NO synthesis. *Nature* **368**:502-502.
- Benjamin N and Vallance P (1994) Plasma nitrite as a marker of nitric oxide production. *Lancet* **344**:960.
- Bennett MR (2003) In-stent stenosis: pathology and implications for the development of drug eluting stents. *Heart (British Cardiac Society)* **89**:218-224.

- Bentzon JF and Falk E (2010) Atherosclerotic lesions in mouse and man: is it the same disease? *Current opinion in lipidology* **21**:434-440.
- Bertaux B, Prost C, Heslan M and Dubertret L (1988) Cantharide acantholysis: endogenous protease activation leading to desmosomal plaque dissolution. *The British journal of dermatology* **118**:157-165.
- Bhagat K and Vallance P (1997) Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo. *Circulation* **96**:3042-3047.
- Bhatt DL (2004) Inflammation and restenosis: is there a link? *American heart journal* **147**:945-947.
- Bhatt DL and Topol EJ (2003) Periprocedural myocardial infarction and emboli protection.
2003. p. 251-66., in *Textbook of interventional cardiology* (EJ T ed), Saunders, Philadelphia.
- BHF (2016) Cardiovascular Disease Statistics 2016, British Heart Foundation, BHF Website.
- Binion DG, Fu S, Ramanujam KS, Chai YC, Dweik RA, Drazba JA, Wade JG, Ziats NP, Erzurum SC and Wilson KT (1998) iNOS expression in human intestinal microvascular endothelial cells inhibits leukocyte adhesion. *The American journal of physiology* **275**:G592-603.
- Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG and Gluud C (2007) Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* **297**:842-857.
- Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG and Gluud C (2012) Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev* **3**:CD007176.

- Blann AD, Lip GY, Beevers DG and McCollum CN (1997) Soluble P-selectin in atherosclerosis: a comparison with endothelial cell and platelet markers. *Thrombosis and haemostasis* **77**:1077-1080.
- Blekkenhorst LC, Lewis JR, Prince RL, Devine A, Bondonno NP, Bondonno CP, Wood LG, Puddey IB, Ward NC, Croft KD, Woodman RJ, Beilin LJ and Hodgson JM (2018) Nitrate-rich vegetables do not lower blood pressure in individuals with mildly elevated blood pressure: a 4-wk randomized controlled crossover trial. *The American journal of clinical nutrition* **107**:894-908.
- Bogaty P, Poirier P, Simard S, Boyer L, Solymoss S and Dagenais GR (2001) Biological profiles in subjects with recurrent acute coronary events compared with subjects with long-standing stable angina. *Circulation* **103**:3062-3068.
- Bogovski P and Bogovski S (1981) Animal Species in which N-nitroso compounds induce cancer. *International journal of cancer Journal international du cancer* **27**:471-474.
- Bombeli T, Mueller M and Haeberli A (1997) Anticoagulant properties of the vascular endothelium. *Thrombosis and haemostasis* **77**:408-423.
- Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC, Rich L, Puddey IB, Swinny E, Mubarak A and Hodgson JM (2012) Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. *Free radical biology & medicine* **52**:95-102.
- Bourantas CV, Serruys PW, Nakatani S, Zhang YJ, Farooq V, Diletti R, Ligthart J, Sheehy A, van Geuns RJ, McClean D, Chevalier B, Windecker S, Koolen J,

- Ormiston J, Whitbourn R, Rapoza R, Veldhof S, Onuma Y and Garcia-Garcia HM (2015) Bioresorbable vascular scaffold treatment induces the formation of neointimal cap that seals the underlying plaque without compromising the luminal dimensions: a concept based on serial optical coherence tomography data. *EuroIntervention : journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology* **11**:746-756.
- Boutouyrie P, Briet, M., Collin C., Vermeersch S, Pannier B. (2008) Assessment of Pulse Wave Velocity. *Artery Research* **3**:3-8.
- Brar SS and Stone GW (2009) Advances in percutaneous coronary intervention. *Current cardiology reports* **11**:245-251.
- Brasselet C, Garnotel R, Perotin S, Vitry F, Elaerts J, Lafont A, Metz D and Gillery P (2007) Percutaneous coronary intervention-induced variations in systemic parameters of inflammation: relationship with the mode of stenting. *Clinical chemistry and laboratory medicine* **45**:526-530.
- Brayton KM, Patel VG, Stave C, de Lemos JA and Kumbhani DJ (2013) Same-day discharge after percutaneous coronary intervention: a meta-analysis. *Journal of the American College of Cardiology* **62**:275-285.
- Bredt DS and Snyder SH (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proceedings of the National Academy of Sciences of the United States of America* **87**:682-685.
- British Heart Foundation (2009a) *Coronary Angioplasty.*, BHF.
- British Heart Foundation (2009b) *Returning to work with a heart condition.*, BHF.
- British Heart Foundation (2014) *Angioplasty: Your quick guide.*

- Brunner M, Schmiedberger A, Schmid R, Jager D, Piegler E, Eichler HG and Muller M (1998) Direct assessment of peripheral pharmacokinetics in humans: comparison between cantharides blister fluid sampling, in vivo microdialysis and saliva sampling. *British journal of clinical pharmacology* **46**:425-431.
- Bryan NS, Alexander DD, Coughlin JR, Milkowski AL and Boffetta P (2012) Ingested nitrate and nitrite and stomach cancer risk: an updated review. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **50**:3646-3665.
- Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY and Lefer DJ (2007) Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proceedings of the National Academy of Sciences of the United States of America* **104**:19144-19149.
- Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J and Feelisch M (2005) Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *NatChemBiol* **1**:290-297.
- Buckley Christopher D, Gilroy Derek W and Serhan Charles N (2014) Proresolving Lipid Mediators and Mechanisms in the Resolution of Acute Inflammation. *Immunity* **40**:315-327.
- Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F and Maseri A (2002) Widespread coronary inflammation in unstable angina. *The New England journal of medicine* **347**:5-12.
- Buffon A, Liuzzo G, Biasucci LM, Pasqualetti P, Ramazzotti V, Rebuffi AG, Crea F and Maseri A (1999) Preprocedural serum levels of C-reactive protein

- predict early complications and late restenosis after coronary angioplasty. *Journal of the American College of Cardiology* **34**:1512-1521.
- Butler AR and Feelisch M (2008) Therapeutic uses of inorganic nitrite and nitrate: from the past to the future. *Circulation* **117**:2151-2159.
- Calver A, Collier J, Moncada S and Vallance P (1992) Effect of local intra-arterial NG-monomethyl-L-arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *Journal of hypertension* **10**:1025-1031.
- Calvert PA, Obaid DR, O'Sullivan M, Shapiro LM, McNab D, Densem CG, Schofield PM, Braganza D, Clarke SC, Ray KK, West NE and Bennett MR (2011) Association between IVUS findings and adverse outcomes in patients with coronary artery disease: the VIVA (VH-IVUS in Vulnerable Atherosclerosis) Study. *JACC Cardiovascular imaging* **4**:894-901.
- Cancer Research Institute. Queen's University at Kingston (2016) What is Flow Cytometry? .
- Carr AC and Frei B (2001) The nitric oxide congener nitrite inhibits myeloperoxidase/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup>-mediated modification of low density lipoprotein. *The Journal of biological chemistry* **276**:1822-1828.
- Carter AJ, Laird JR, Farb A, Kufs W, Wortham DC and Virmani R (1994) Morphologic characteristics of lesion formation and time course of smooth muscle cell proliferation in a porcine proliferative restenosis model. *Journal of the American College of Cardiology* **24**:1398-1405.
- Carter P, Gray LJ, Troughton J, Khunti K and Davies MJ (2010) Fruit and vegetable intake and incidence of type 2 diabetes mellitus: systematic review and meta-analysis. *BMJ (Clinical research ed)* **341**:c4229.

- Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK and Deanfield JE (1992) Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* **340**:1111-1115.
- Chan KF, Siegel MR and Lenardo JM (2000) Signaling by the TNF receptor superfamily and T cell homeostasis. *Immunity* **13**:419-422.
- Chan TYK (2011) Vegetable-borne nitrate and nitrite and the risk of methaemoglobinaemia. *Toxicol Lett* **200**:107-108.
- Channon KM (2012) Tetrahydrobiopterin: a vascular redox target to improve endothelial function. *Current vascular pharmacology* **10**:705-708.
- Charakida M, de Groot E, Loukogeorgakis SP, Khan T, Luscher T, Kastelein JJ, Gasser T and Deanfield JE (2013) Variability and reproducibility of flow-mediated dilatation in a multicentre clinical trial. *European heart journal* **34**:3501-3507.
- Chavez-Sanchez L, Espinosa-Luna JE, Chavez-Rueda K, Legorreta-Haquet MV, Montoya-Diaz E and Blanco-Favela F (2014) Innate immune system cells in atherosclerosis. *Archives of medical research* **45**:1-14.
- Chen Z, Zhang J and Stamler JS (2002) Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proceedings of the National Academy of Sciences of the United States of America* **99**:8306-8311.
- Chia S, Ludlam CA, Fox KAA and Newby DE (2003) Acute systemic inflammation enhances endothelium-dependent tissue plasminogen activator release in men. *Journal of the American College of Cardiology* **41**:333-339.

- Chiang N, Fredman G, Backhed F, Oh SF, Vickery T, Schmidt BA and Serhan CN (2012) Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature* **484**:524-528.
- Chinetti-Gbaguidi G, Colin S and Staels B (2015) Macrophage subsets in atherosclerosis. *Nat Rev Cardiol* **12**:10-17.
- Choudhury RP, Lee JM and Greaves DR (2005) Mechanisms of disease: macrophage-derived foam cells emerging as therapeutic targets in atherosclerosis. *Nature clinical practice Cardiovascular medicine* **2**:309-315.
- Chowienczyk PJ, Watts GF, Cockcroft JR and Ritter JM (1992) Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet* **340**:1430-1432.
- Christian RC, Liu PY, Harrington S, Ruan M, Miller VM and Fitzpatrick LA (2006) Intimal estrogen receptor (ER)beta, but not ERalpha expression, is correlated with coronary calcification and atherosclerosis in pre- and postmenopausal women. *The Journal of clinical endocrinology and metabolism* **91**:2713-2720.
- Cipollone F, Ferri C, Desideri G, Paloscia L, Materazzo G, Mascellanti M, Fazia M, Iezzi A, Cuccurullo C, Pini B, Bucci M, Santucci A, Cuccurullo F and Mezzetti A (2003) Preprocedural level of soluble CD40L is predictive of enhanced inflammatory response and restenosis after coronary angioplasty. *Circulation* **108**:2776-2782.
- Cipollone F, Marini M, Fazia M, Pini B, Iezzi A, Reale M, Paloscia L, Materazzo G, D'Annunzio E, Conti P, Chiarelli F, Cuccurullo F and Mezzetti A (2001) Elevated circulating levels of monocyte chemoattractant protein-1 in



- patients with restenosis after coronary angioplasty. *Arteriosclerosis, thrombosis, and vascular biology* **21**:327-334.
- Colas RA, Shinohara M, Dalli J, Chiang N and Serhan CN (2014) Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Am J Physiol Cell Physiol* **307**:C39-54.
- Colby JK, Abdulnour RE, Sham HP, Dalli J, Colas RA, Winkler JW, Hellmann J, Wong B, Cui Y, El-Chemaly S, Petasis NA, Spite M, Serhan CN and Levy BD (2016) Resolvin D3 and Aspirin-Triggered Resolvin D3 Are Protective for Injured Epithelia. *Am J Pathol* **186**:1801-1813.
- Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A and Mallat Z (2008) Combined inhibition of CCL2, CX3CR1, and CCR5 abrogates Ly6C(hi) and Ly6C(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. *Circulation* **117**:1649-1657.
- Comly H.H. (1945) Cyanosis in infants caused by nitrates in well water, . *JAMA* **129**:112-116.
- Cooper AL, Horan MA, Little RA and Rothwell NJ (1992) Metabolic and febrile responses to typhoid vaccine in humans: effect of beta-adrenergic blockade. *Journal of applied physiology (Bethesda, Md : 1985)* **72**:2322-2328.
- Cornblath M and Hartmann AF (1948) Methemoglobinemia in young infants. *The Journal of pediatrics* **33**:421-425.
- Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J and Vogel R (2002) Guidelines for the ultrasound assessment of

- endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *Journal of the American College of Cardiology* **39**:257-265.
- Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO, 3rd and Gladwin MT (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nature medicine* **9**:1498-1505.
- Couper KN, Blount DG and Riley EM (2008) IL-10: the master regulator of immunity to infection. *J Immunol* **180**:5771-5777.
- Cox DA, Vita JA, Treasure CB, Fish RD, Alexander RW, Ganz P and Selwyn AP (1989) Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation* **80**:458-465.
- Creager MA, Cooke JP, Mendelsohn ME, Gallagher SJ, Coleman SM, Loscalzo J and Dzau VJ (1990) Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *The Journal of clinical investigation* **86**:228-234.
- Cross AJ, Ferrucci LM, Risch A, Graubard BI, Ward MH, Park Y, Hollenbeck AR, Schatzkin A and Sinha R (2010) A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. *Cancer research* **70**:2406-2414.
- Cuevas AM and Germain AM (2004) Diet and endothelial function. *Biological research* **37**:225-230.

- Cunningham R, Mustoe E, Spiller L, Lewis S and Benjamin N (2014) Acidified nitrite: a host defence against colonization with *C. difficile* spores? *Journal of Hospital Infection* **86**:155-157.
- Cybalsky MI and Gimbrone MA, Jr. (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science (New York, NY)* **251**:788-791.
- Dalli J and Serhan C (2016) Macrophage Proresolving Mediators-the When and Where. *Microbiology spectrum* **4**.
- Dalli J and Serhan CN (2012) Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* **120**:e60-72.
- Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB and Gudnason V (2004) C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *The New England journal of medicine* **350**:1387-1397.
- Davies WR, Brown AJ, Watson W, McCormick LM, West NEJ, Dutka DP and Hoole SP (2013) Remote Ischemic Preconditioning Improves Outcome at 6 Years After Elective Percutaneous Coronary Intervention: The CRISP Stent Trial Long-term Follow-up. *Circulation: Cardiovascular Interventions* **6**:246-251.
- Day RM, Harbord M, Forbes A and Segal AW (2001a) Cantharidin blisters: a technique for investigating leukocyte trafficking and cytokine production at sites of inflammation in humans. *Journal of immunological methods* **257**:213-220.
- Day RM, Harbord M, Forbes A and Segal AW (2001b) Cantharidin blisters: a technique for investigating leukocyte trafficking and cytokine production

- at sites of inflammation in humans. *Journal of Immunological Methods* **257**:213-220.
- de Winter RJ, Heyde GS, Koch KT, Fischer J, van Straalen JP, Bax M, Schotborgh CE, Mulder KJ, Sanders GT, Piek JJ and Tijssen JG (2002) The prognostic value of pre-procedural plasma C-reactive protein in patients undergoing elective coronary angioplasty. *European heart journal* **23**:960-966.
- Deanfield JE, Halcox JP and Rabelink TJ (2007) Endothelial function and dysfunction: testing and clinical relevance. *Circulation* **115**:1285-1295.
- Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT and Schechter AN (2005) Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* **106**:734-739.
- Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO, 3rd, Schechter AN and Gladwin MT (2007) Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation* **116**:1821-1831.
- Dellavalle CT, Xiao Q, Yang G, Shu XO, Aschebrook-Kilfoy B, Zheng W, Lan Li H, Ji BT, Rothman N, Chow WH, Gao YT and Ward MH (2014) Dietary nitrate and nitrite intake and risk of colorectal cancer in the Shanghai Women's Health Study. *International journal of cancer Journal international du cancer* **134**:2917-2926.
- Diacovo TG, Roth SJ, Buccola JM, Bainton DF and Springer TA (1996) Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* **88**:146-157.

- Dibra A, Mehilli J, Braun S, Hadamitzky M, Baum H, Dirschinger J, Schuhlen H, Schomig A and Kastrati A (2003) Association between C-reactive protein levels and subsequent cardiac events among patients with stable angina treated with coronary artery stenting. *The American journal of medicine* **114**:715-722.
- Dibra A, Mehilli J, Braun S, Hadamitzky M, Baum H, Dirschinger J, Schuhlen H, Schomig A and Kastrati A (2005) Inflammatory response after intervention assessed by serial C-reactive protein measurements correlates with restenosis in patients treated with coronary stenting. *American heart journal* **150**:344-350.
- Dickinson HO, Nicolson DJ, Campbell F, Beyer FR and Mason J (2006) Potassium supplementation for the management of primary hypertension in adults. *Cochrane Database Syst Rev*:CD004641.
- Dietrich M, Block G, Pogoda JM, Buffler P, Hecht S and Preston-Martin S (2005) A review: dietary and endogenously formed N-nitroso compounds and risk of childhood brain tumors. *Cancer causes & control : CCC* **16**:619-635.
- Dinh PH, Corraza F, Mestdagh K, Kassengera Z, Doyen V and Michel O (2011) Validation of the cantharidin-induced skin blister as an in vivo model of inflammation. *British journal of clinical pharmacology* **72**:912-920.
- Doel JJ, Benjamin N, Hector MP, Rogers M and Allaker RP (2005) Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci* **113**:14-19.
- DOH (2003) 5 A Day Programme, (Health Do ed).

- Domingues-Montanari S, Subirana I, Tomas M, Marrugat J and Senti M (2008) Association between ESR2 genetic variants and risk of myocardial infarction. *Clinical chemistry* **54**:1183-1189.
- Dona M, Fredman G, Schwab JM, Chiang N, Arita M, Goodarzi A, Cheng G, von Andrian UH and Serhan CN (2008) Resolvin E1, an EPA-derived mediator in whole blood, selectively counterregulates leukocytes and platelets. *Blood* **112**:848-855.
- Donald AE, Charakida M, Cole TJ, Friberg P, Chowienczyk PJ, Millasseau SC, Deanfield JE and Halcox JP (2006) Non-Invasive Assessment of Endothelial Function Which Technique? *Journal of the American College of Cardiology* **48**:1846-1850.
- Doring Y, Drechsler M, Soehnlein O and Weber C (2015) Neutrophils in atherosclerosis: from mice to man. *Arteriosclerosis, thrombosis, and vascular biology* **35**:288-295.
- Doyle MP and Hoekstra JW (1981) Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *Journal of Inorganic Biochemistry* **14**:351-358.
- Doyle MP, Pickering RA, DeWeert TM, Hoekstra JW and Pater D (1981) Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. *The Journal of biological chemistry* **256**:12393-12398.
- Drexler H and Zeiher AM (1991) Endothelial function in human coronary arteries in vivo. Focus on hypercholesterolemia. *Hypertension* **18**:ii90-99.
- Druckrey H, Schildbach A, Schmaehl D, Preussmann R and Ivankovic S (1963) [QUANTITATIVE ANALYSIS OF THE CARCINOGENIC EFFECT OF DIETHYLNITROSAMINE]. *Arzneimittel-Forschung* **13**:841-851.

- Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M and Benjamin N (1995) Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nature medicine* **1**:546-551.
- Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT and Lefer DJ (2005) Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *The Journal of clinical investigation* **115**:1232-1240.
- Durante W, Johnson FK and Johnson RA (2007) Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clinical and experimental pharmacology & physiology* **34**:906-911.
- Dyckhuizen RS, Fraser A, McKenzie H, Golden M, Leifert C and Benjamin N (1998) Helicobacter pylori is killed by nitrite under acidic conditions. *Gut* **42**:334-337.
- Dzavik V (2003) New frontiers and unresolved controversies in percutaneous coronary intervention. *The American journal of cardiology* **91**:27a-33a.
- Edwards DA, Fletcher K and Rowlands EN (1954) Antagonism between perchlorate, iodide, thiocyanate, and nitrate for secretion in human saliva; analogy with the iodide trap of the thyroid. *Lancet* **266**:498-499.
- Efsa (2008) Nitrate in vegetables. Scientific Opinion of the Panel on Contaminants in the Food chain *The EFSA Journal* **689**:1-79.
- Eichholzer M and Gutzwiller F (1998) Dietary nitrates, nitrites, and N-nitroso compounds and cancer risk: a review of the epidemiologic evidence. *Nutrition reviews* **56**:95-105.

- Ernst E, Hammerschmidt DE, Bagge U, Matrai A and Dormandy JA (1987) Leukocytes and the risk of ischemic diseases. *Jama* **257**:2318-2324.
- Ernst E and Resch KL (1993) Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. *Annals of internal medicine* **118**:956-963.
- Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA and Martinez-Gonzalez MA (2013) Primary prevention of cardiovascular disease with a Mediterranean diet. *The New England journal of medicine* **368**:1279-1290.
- Evangelista O and McLaughlin MA (2009) Review of cardiovascular risk factors in women. *Gender medicine* **6 Suppl 1**:17-36.
- Evans BJ, Haskard DO, Finch JR, Hambleton IR, Landis RC and Taylor KM (2008) The inflammatory effect of cardiopulmonary bypass on leukocyte extravasation in vivo. *The Journal of Thoracic and Cardiovascular Surgery* **135**:999-1006.
- Fao/Who (2003) Nitrate (and potential endogenous formation of N-nitroso compounds), in *WHO Food Additive series 50*, World Health Organisation, Geneva.
- Farb A, Weber DK, Kolodgie FD, Burke AP and Virmani R (2002) Morphological predictors of restenosis after coronary stenting in humans. *Circulation* **105**:2974-2980.
- Fatourechi V (2009) Subclinical hypothyroidism: an update for primary care physicians. *Mayo Clinic proceedings* **84**:65-71.



- Feig JE, Parathath S, Rong JX, Mick SL, Vengrenyuk Y, Grauer L, Young SG and Fisher EA (2011) Reversal of hyperlipidemia with a genetic switch favorably affects the content and inflammatory state of macrophages in atherosclerotic plaques. *Circulation* **123**:989-998.
- Feil S, Fehrenbacher B, Lukowski R, Essmann F, Schulze-Osthoff K, Schaller M and Feil R (2014) Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. *Circulation research* **115**:662-667.
- Feldman LJ, Mazighi M, Scheuble A, Deux JF, De Benedetti E, Badier-Commander C, Brambilla E, Henin D, Steg PG and Jacob MP (2001) Differential expression of matrix metalloproteinases after stent implantation and balloon angioplasty in the hypercholesterolemic rabbit. *Circulation* **103**:3117-3122.
- Ferrucci LM, Sinha R, Ward MH, Graubard BI, Hollenbeck AR, Kilfoy BA, Schatzkin A, Michaud DS and Cross AJ (2010) Meat and components of meat and the risk of bladder cancer in the NIH-AARP Diet and Health Study. *Cancer* **116**:4345-4353.
- Fine DH, Ross R, Rounbehler DP, Silvergleid A and Song L (1977) Formation in vivo of volatile N-nitrosamines in man after ingestion of cooked bacon and spinach. *Nature* **265**:753-755.
- Fleenor BS, Sindler AL, Eng JS, Nair DP, Dodson RB and Seals DR (2012) Sodium nitrite de-stiffening of large elastic arteries with aging: role of normalization of advanced glycation end-products. *Experimental gerontology* **47**:588-594.

- Fleming RM, Kirkeeide RL, Smalling RW and Gould KL (1991) Patterns in visual interpretation of coronary arteriograms as detected by quantitative coronary arteriography. *Journal of the American College of Cardiology* **18**:945-951.
- Folco EJ, Sheikine Y, Rocha VZ, Christen T, Shvartz E, Sukhova GK, Di Carli MF and Libby P (2011) Hypoxia but not inflammation augments glucose uptake in human macrophages: Implications for imaging atherosclerosis with 18fluorine-labeled 2-deoxy-D-glucose positron emission tomography. *Journal of the American College of Cardiology* **58**:603-614.
- Follin P (1999) Skin chamber technique for study of in vivo exudated human neutrophils. *Journal of immunological methods* **232**:55-65.
- Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I and Kleinert H (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* **23**:1121-1131.
- Foster-Schubert KE, Alfano CM, Duggan CR, Xiao L, Campbell KL, Kong A, Bain CE, Wang C-Y, Blackburn GL and McTiernan A (2012) Effect of diet and exercise, alone or combined, on weight and body composition in overweight-to-obese postmenopausal women. *Obesity (Silver Spring, Md)* **20**:1628-1638.
- Fredman G, Ozcan L, Spolitu S, Hellmann J, Spite M, Backs J and Tabas I (2014) Resolvin D1 limits 5-lipoxygenase nuclear localization and leukotriene B(4) synthesis by inhibiting a calcium-activated kinase pathway. *Proceedings of the National Academy of Sciences of the United States of America* **111**:14530-14535.

- Fries DM, Paxinou E, Themistocleous M, Swanberg E, Griendling KK, Salvemini D, Slot JW, Heijnen HF, Hazen SL and Ischiropoulos H (2003) Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: role of reactive oxygen species. *The Journal of biological chemistry* **278**:22901-22907.
- Furchgott RF (1955) The pharmacology of vascular smooth muscle. *Pharmacological reviews* **7**:183-265.
- Furchgott RF (1988) Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor in nitric oxide., in *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium* (Vanhoutte PM ed) pp pp.401– 414, New York: Raven.
- Furchgott RF and Vanhoutte PM (1989) Endothelium-derived relaxing and contracting factors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **3**:2007-2018.
- Furchgott RF and Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**:373-376.
- Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB and Michelson AD (1998) Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *Journal of the American College of Cardiology* **31**:352-358.
- Furukawa Y, Matsumori A, Ohashi N, Shioi T, Ono K, Harada A, Matsushima K and Sasayama S (1999) Anti-monocyte chemoattractant protein-1/monocyte

- chemotactic and activating factor antibody inhibits neointimal hyperplasia in injured rat carotid arteries. *Circulation research* **84**:306-314.
- Fuster V, Falk E, Fallon JT, Badimon L, Chesebro JH and Badimon JJ (1995) The three processes leading to post PTCA restenosis: dependence on the lesion substrate. *Thrombosis and haemostasis* **74**:552-559.
- Gamgee A (1868) Researches on the blood.-On the action of nitrites on blood. *PhiloTransRSocLond(Biol)* **158**:589-625.
- Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A and Sessa WC (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* **392**:821-824.
- Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S, Lisanti MP and Sessa WC (1997) Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *The Journal of biological chemistry* **272**:25437-25440.
- Garcia-Garcia HM, Gogas BD, Serruys PW and Bruining N (2011) IVUS-based imaging modalities for tissue characterization: similarities and differences. *The international journal of cardiovascular imaging* **27**:215-224.
- Garcia-Santos Mdel P, Calle E and Casado J (2001) Amino acid nitrosation products as alkylating agents. *Journal of the American Chemical Society* **123**:7506-7510.
- Garg P, Cohen DJ, Gaziano T and Mauri L (2008) Balancing the risks of restenosis and stent thrombosis in bare-metal versus drug-eluting stents: results of a decision analytic model. *Journal of the American College of Cardiology* **51**:1844-1853.

- Garrone P, Biondi-Zoccai G, Salvetti I, Sina N, Sheiban I, Stella PR and Agostoni P (2009) Quantitative coronary angiography in the current era: principles and applications. *Journal of interventional cardiology* **22**:527-536.
- Gerhard M, Roddy MA, Creager SJ and Creager MA (1996) Aging progressively impairs endothelium-dependent vasodilation in forearm resistance vessels of humans. *Hypertension* **27**:849-853.
- Gharavi NM, Alva JA, Mouillesseaux KP, Lai C, Yeh M, Yeung W, Johnson J, Szeto WL, Hong L, Fishbein M, Wei L, Pfeffer LM and Berliner JA (2007) Role of the Jak/STAT pathway in the regulation of interleukin-8 transcription by oxidized phospholipids in vitro and in atherosclerosis in vivo. *The Journal of biological chemistry* **282**:31460-31468.
- Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, Khambata R, Maleki-Toyserkani S, Yousuf M, Benjamin N, Webb AJ, Caulfield MJ, Hobbs AJ and Ahluwalia A (2013) Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. *Hypertension* **61**:1091-1102.
- Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A and Benjamin N (2013) Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. *Free radical biology & medicine* **60**:89-97.
- Gilchrist M, Winyard PG, Fulford J, Anning C, Shore AC and Benjamin N (2014) Dietary nitrate supplementation improves reaction time in type 2 diabetes: Development and application of a novel nitrate-depleted beetroot juice placebo. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **40c**:67-74.

- Ginnan R, Guikema BJ, Halligan KE, Singer HA and Jourd'heuil D (2008) Regulation of smooth muscle by inducible nitric oxide synthase and NADPH oxidase in vascular proliferative diseases. *Free radical biology & medicine* **44**:1232-1245.
- Gkaliagkousi E, Ritter J and Ferro A (2007) Platelet-derived nitric oxide signaling and regulation. *Circulation research* **101**:654-662.
- Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR, Feelisch M and Lundberg JO (2005) The emerging biology of the nitrite anion. *Nat Chem Biol* **1**:308-314.
- Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, Ognibene FP and Cannon RO, 3rd (2000) Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proceedings of the National Academy of Sciences of the United States of America* **97**:11482-11487.
- Goldsmith IR, Blann AD, Patel RL and Lip GY (2000) Plasma fibrinogen, soluble P-selectin, and von Willebrand factor in aortic valve disease: evidence for abnormal haemorheology, platelet activation, and endothelial dysfunction. *Heart (British Cardiac Society)* **83**:577-578.
- Gomez-Sanchez L, García-Ortiz L, Recio-Rodríguez JI, Patino-Alonso MC, Agudo-Conde C, Rigo F, Ramos R, Martí R, Gomez-Marcos MA and for the MG (2015) Leukocyte Subtype Counts and Its Association with Vascular Structure and Function in Adults with Intermediate Cardiovascular Risk. MARK Study. *PloS one* **10**:e0119963.

- Gomma AH, Hirschfield GM, Gallimore JR, Jr., Lowe GD, Pepys MB and Fox KM (2004) Preprocedural inflammatory markers do not predict restenosis after successful coronary stenting. *American heart journal* **147**:1071-1077.
- Gonzalez FM, Shiva S, Vincent PS, Ringwood LA, Hsu LY, Hon YY, Aletras AH, Cannon RO, 3rd, Gladwin MT and Arai AE (2008) Nitrite anion provides potent cytoprotective and antiapoptotic effects as adjunctive therapy to reperfusion for acute myocardial infarction. *Circulation* **117**:2986-2994.
- Gonzalo N, Serruys PW, Okamura T, van Beusekom HM, Garcia-Garcia HM, van Soest G, van der Giessen W and Regar E (2009) Optical coherence tomography patterns of stent restenosis. *American heart journal* **158**:284-293.
- Gottsauer-Wolf M, Zasmata G, Hornykewycz S, Nikfardjam M, Stepan E, Wexberg P, Zorn G, Glogar D, Probst P, Maurer G and Huber K (2000) Plasma levels of C-reactive protein after coronary stent implantation. *European heart journal* **21**:1152-1158.
- Goulart AC, Zee RY and Rexrode KM (2009) Association of estrogen receptor 2 gene polymorphisms with obesity in women (obesity and estrogen receptor 2 gene). *Maturitas* **62**:179-183.
- Green DJ, Dawson EA, Groenewoud HMM, Jones H and Thijssen DHJ (2014) Is Flow-Mediated Dilation Nitric Oxide Mediated?: A Meta-Analysis. *Hypertension* **63**:376-382.
- Greer FR and Shannon M (2005) Infant methemoglobinemia: the role of dietary nitrate in food and water. *Pediatrics* **116**:784-786.
- Gubbels Bupp MR (2015) Sex, the aging immune system, and chronic disease. *Cellular Immunology* **294**:102-110.

- Ha T, Hua F, Liu X, Ma J, McMullen JR, Shioi T, Izumo S, Kelley J, Gao X, Browder W, Williams DL, Kao RL and Li C (2008) Lipopolysaccharide-induced myocardial protection against ischaemia/reperfusion injury is mediated through a PI3K/Akt-dependent mechanism. *Cardiovascular research* **78**:546-553.
- Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER and Barton M (2009) Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circulation research* **104**:288-291.
- Habermeyer M, Roth A, Guth S, Diel P, Engel KH, Epe B, Furst P, Heinz V, Humpf HU, Joost HG, Knorr D, de Kok T, Kulling S, Lampen A, Marko D, Rechkemmer G, Rietjens I, Stadler RH, Vieths S, Vogel R, Steinberg P and Eisenbrand G (2015) Nitrate and nitrite in the diet: how to assess their benefit and risk for human health. *Molecular nutrition & food research* **59**:106-128.
- Hafezi-Moghadam A, Thomas KL, Prorock AJ, Huo Y and Ley K (2001) L-Selectin Shedding Regulates Leukocyte Recruitment. *The Journal of experimental medicine* **193**:863-872.
- Halcox JP, Donald AE, Ellins E, Witte DR, Shipley MJ, Brunner EJ, Marmot MG and Deanfield JE (2009) Endothelial function predicts progression of carotid intima-media thickness. *Circulation* **119**:1005-1012.
- Harding SA, Din JN, Sarma J, Jessop A, Weatherall M, Fox KA and Newby DE (2007) Flow cytometric analysis of circulating platelet-monocyte aggregates in



- whole blood: methodological considerations. *Thrombosis and haemostasis* **98**:451-456.
- Haverkate F, Thompson SG, Pyke SD, Gallimore JR and Pepys MB (1997) Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet* **349**:462-466.
- He FJ, Nowson CA and MacGregor GA (2006) Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. *Lancet* **367**:320-326.
- Heidt T, Sager HB, Courties G, Dutta P, Iwamoto Y, Zaltsman A, von Zur Muhlen C, Bode C, Fricchione GL, Denninger J, Lin CP, Vinegoni C, Libby P, Swirski FK, Weissleder R and Nahrendorf M (2014) Chronic variable stress activates hematopoietic stem cells. *Nature medicine* **20**:754-758.
- Heinrich TA, da Silva RS, Miranda KM, Switzer CH, Wink DA and Fukuto JM (2013) Biological nitric oxide signalling: chemistry and terminology. *British journal of pharmacology* **169**:1417-1429.
- Heiss C, Meyer C, Totzeck M, Hendgen-Cotta UB, Heinen Y, Luedike P, Keymel S, Ayoub N, Lundberg JO, Weitzberg E, Kelm M and Rassaf T (2012) Dietary inorganic nitrate mobilizes circulating angiogenic cells. *Free Radical Biology and Medicine* **52**:1767-1772.
- Hellings WE, Peeters W, Moll FL, Piers SR, van Setten J, Van der Spek PJ, de Vries JP, Seldenrijk KA, De Bruin PC, Vink A, Velema E, de Kleijn DP and Pasterkamp G (2010) Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome: a prognostic study. *Circulation* **121**:1941-1950.

- Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT, Kelm M and Rassaf T (2008) Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *ProcNatlAcadSciUSA* **105**:10256-10261.
- Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G and Kroczek RA (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* **391**:591-594.
- Hermann M, Flammer A and Lüscher TF (2006) Nitric Oxide in Hypertension. *The Journal of Clinical Hypertension* **8**:17-29.
- Herzenberg LA and De Rosa SC (2000) Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine. *Immunology today* **21**:383-390.
- Hickson SS, Butlin M, Broad J, Avolio AP, Wilkinson IB and McEniery CM (2009) Validity and repeatability of the Vicorder apparatus: a comparison with the SphygmoCor device. *Hypertens Res* **32**:1079-1085.
- Hingorani AD, Cross J, Kharbanda RK, Mullen MJ, Bhagat K, Taylor M, Donald AE, Palacios M, Griffin GE, Deanfield JE, MacAllister RJ and Vallance P (2000a) Acute systemic inflammation impairs endothelium-dependent dilatation in humans. *Circulation* **102**:994-999.
- Hingorani AD, Cross J, Kharbanda RK, Mullen MJ, Bhagat K, Taylor M, Donald AE, Palacios M, Griffin GE, Deanfield JE, MacAllister RJ and Vallance P (2000b) Acute Systemic Inflammation Impairs Endothelium-Dependent Dilatation in Humans. *Circulation* **102**:994-999.

- Ho JE, Paultre F and Mosca L (2005) The gender gap in coronary heart disease mortality: is there a difference between blacks and whites? *Journal of women's health (2002)* **14**:117-127.
- Hodges YK, Tung L, Yan XD, Graham JD, Horwitz KB and Horwitz LD (2000) Estrogen receptors alpha and beta: prevalence of estrogen receptor beta mRNA in human vascular smooth muscle and transcriptional effects. *Circulation* **101**:1792-1798.
- Hodgson JM and Marshall JJ (1989) Direct vasoconstriction and endothelium-dependent vasodilation. Mechanisms of acetylcholine effects on coronary flow and arterial diameter in patients with nonstenotic coronary arteries. *Circulation* **79**:1043-1051.
- Honkanen RE (1993) Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. *FEBS letters* **330**:283-286.
- Hord NG, Tang Y and Bryan NS (2009a) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *The American journal of clinical nutrition* **90**:1-10.
- Hord NG, Tang Y and Bryan NS (2009b) Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *American Journal of Clinical Nutrition* **90**:1-10.
- Hulsmans M and Holvoet P (2013) MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. *Cardiovascular research* **100**:7-18.
- Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr. and Boerwinkle E (1997) Circulating adhesion molecules VCAM-1, ICAM-1, and

- E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. *Circulation* **96**:4219-4225.
- Hyde ER, Andrade F, Vaksman Z, Parthasarathy K, Jiang H, Parthasarathy DK, Torregrossa AC, Tribble G, Kaplan HB, Petrosino JF and Bryan NS (2014a) Metagenomic Analysis of Nitrate-Reducing Bacteria in the Oral Cavity: Implications for Nitric Oxide Homeostasis. *PLoS ONE* **9**:e88645.
- Hyde ER, Luk B, Cron S, Kusic L, McCue T, Bauch T, Kaplan H, Tribble G, Petrosino JF and Bryan NS (2014b) Characterization of the rat oral microbiome and the effects of dietary nitrate. *Free Radical Biology and Medicine* **77**:249-257.
- Ignarro LJ, Buga GM, Wood KS, Byrns RE and Chaudhuri G (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America* **84**:9265-9269.
- Ignarro LJ, Byrns RE and Wood KS (1988) Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radical, in *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium* pp 401-414, New York: Raven.
- Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE and Byrns RE (1993a) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci USA* **90**:8103-8107.
- Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE and Byrns RE (1993b) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison

- with enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Sciences of the United States of America* **90**:8103-8107.
- Imola F, Mallus MT, Ramazzotti V, Manzoli A, Pappalardo A, Di Giorgio A, Albertucci M and Prati F (2010) Safety and feasibility of frequency domain optical coherence tomography to guide decision making in percutaneous coronary intervention. *EuroIntervention : journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology* **6**:575-581.
- Inoue T, Sakai Y, Hoshi K, Yaguchi I, Fujito T and Morooka S (1998) Lower expression of neutrophil adhesion molecule indicates less vessel wall injury and might explain lower restenosis rate after cutting balloon angioplasty. *Circulation* **97**:2511-2518.
- International Agency for Research on Cancer, Ingested Nitrate and Nitrite and Cyanobacterial Peptide Toxins (International Agency for Research on Cancer) **Lyon, 2010**.
- Ishiwata H, Boriboon P, Harada M, Tanimura A and Ishidate M (1975) Changes of nitrite and nitrate concentration in incubated human saliva. *J Food Hyg Soc* **16**:93-98.
- Ishiwata H, Tanimura, A., Ishidate, M. (1975) Nitrite and nitrate concentrations in human saliva collected from salivary ducts. *J Food Hyg Soc Jpn* **16**:89-92.
- Janes KA and Yaffe MB (2006) Data-driven modelling of signal-transduction networks. *Nat Rev Mol Cell Biol* **7**:820-828.
- Jang IK, Bouma BE, Kang DH, Park SJ, Park SW, Seung KB, Choi KB, Shishkov M, Schlendorf K, Pomerantsev E, Houser SL, Aretz HT and Tearney GJ (2002)

- Visualization of coronary atherosclerotic plaques in patients using optical coherence tomography: comparison with intravascular ultrasound. *Journal of the American College of Cardiology* **39**:604-609.
- Jang IK, Tearney GJ, MacNeill B, Takano M, Moselewski F, Iftima N, Shishkov M, Houser S, Aretz HT, Halpern EF and Bouma BE (2005) In vivo characterization of coronary atherosclerotic plaque by use of optical coherence tomography. *Circulation* **111**:1551-1555.
- Jenner W, Motwani M, Veighey K, Newson J, Audzevich T, Nicolaou A, Murphy S, Macallister R and Gilroy DW (2014) Characterisation of leukocytes in a human skin blister model of acute inflammation and resolution. *PLoS one* **9**:e89375.
- Jeserich M, Munzel T, Just H and Drexler H (1992) Reduced plasma L-arginine in hypercholesterolaemia. *Lancet* **339**:561.
- John JH, Ziebland S, Yudkin P, Roe LS and Neil HAW (2002) Effects of fruit and vegetable consumption on plasma antioxidant concentrations and blood pressure: a randomised controlled trial. *The Lancet* **359**:1969-1974.
- Jones DA, Khambata RS, Andiapen M, Rathod KS, Mathur A and Ahluwalia A (2017) Intracoronary nitrite suppresses the inflammatory response following primary percutaneous coronary intervention. *Heart (British Cardiac Society)* **103**:508-516.
- Jones DA, Pellaton C, Velmurugan S, Rathod KS, Andiapen M, Antoniou S, van Eijl S, Webb AJ, Westwood M, Parmar M, Mathur A and Ahluwalia A (2014) Randomized Phase 2 Trial of Intra-Coronary Nitrite During Acute Myocardial Infarction. *Circulation research* **116**:437-447.

- Jones SP and Bolli R (2006) The ubiquitous role of nitric oxide in cardioprotection. *Journal of Molecular and Cellular Cardiology* **40**:16-23.
- Joris PJ and Mensink RP (2013) Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. *Atherosclerosis* **231**:78-83.
- Josefsson E, Tarkowski A and Carlsten H (1992) Anti-inflammatory properties of estrogen. I. In vivo suppression of leukocyte production in bone marrow and redistribution of peripheral blood neutrophils. *Cell Immunol* **142**:67-78.
- Joshi KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Hennekens CH, Spiegelman D and Willett WC (1999) Fruit and vegetable intake in relation to risk of ischemic stroke. *JAMA* **282**:1233-1239.
- Joshi KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D and Willett WC (2001) The effect of fruit and vegetable intake on risk for coronary heart disease. *Annals of internal medicine* **134**:1106-1114.
- Kajita M, Murata T, Horiguchi K, Iizuka M, Hori M and Ozaki H (2011) iNOS expression in vascular resident macrophages contributes to circulatory dysfunction of splanchnic vascular smooth muscle contractions in portal hypertensive rats. *American journal of physiology Heart and circulatory physiology* **300**:H1021-1031.
- Kalbfleisch SJ, McGillem MJ, Pinto IM, Kavanaugh KM, DeBoe SF and Mancini GB (1990) Comparison of automated quantitative coronary angiography with caliper measurements of percent diameter stenosis. *The American journal of cardiology* **65**:1181-1184.

- Kane MO, Anselm E, Rattmann YD, Auger C and Schini-Kerth VB (2009) Role of gender and estrogen receptors in the rat aorta endothelium-dependent relaxation to red wine polyphenols. *Vascular Pharmacology* **51**:140-146.
- Kannel WB, Castelli WP and Gordon T (1979) Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham study. *Annals of internal medicine* **90**:85-91.
- Kapil V, Khambata RS, Robertson A, Caulfield MJ and Ahluwalia A (2015) Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension* **65**:320-327.
- Kapil V KR, Robertson A, Caulfield MJ, Ahluwalia A, (2015) Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: A randomized phase 2, double-blind, placebo-controlled study. *Hypertension* **65**:320-327.
- Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ and Ahluwalia A (2010a) Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* **56**:274-281.
- Kapil V, Webb AJ and Ahluwalia A (2010b) Inorganic nitrate and the cardiovascular system. *Heart (British Cardiac Society)* **96**:1703-1709.
- Kaseta JR, Skafar DF, Ram JL, Jacober SJ and Sowers JR (1999) Cardiovascular disease in the diabetic woman. *The Journal of clinical endocrinology and metabolism* **84**:1835-1838.
- Kastrati A, Mehilli J, von Beckerath N, Dibra A, Hausleiter J, Pache J, Schühlen H, Schmitt C, Dirschinger J and Schomig A (2005) Sirolimus-eluting stent or



paclitaxel-eluting stent vs balloon angioplasty for prevention of recurrences in patients with coronary in-stent restenosis: a randomized controlled trial. *Jama* **293**:165-171.

Kasuga K, Yang R, Porter TF, Agrawal N, Petasis NA, Irimia D, Toner M and Serhan CN (2008) Rapid appearance of resolvin precursors in inflammatory exudates: novel mechanisms in resolution. *J Immunol* **181**:8677-8687.

Katz DL, Davidhi A, Ma Y, Kavak Y, Bifulco L and Njike VY Effects of walnuts on endothelial function in overweight adults with visceral obesity: a randomized, controlled, crossover trial. *J Am Coll Nutr* **31**:415-423.

Kay CD, Hooper L, Kroon PA, Rimm EB and Cassidy A Relative impact of flavonoid composition, dose and structure on vascular function: a systematic review of randomised controlled trials of flavonoid-rich food products. *Molecular nutrition & food research* **56**:1605-1616.

Kereiakes DJ, Lincoff AM, Miller DP, Tcheng JE, Cabot CF, Anderson KM, Weisman HF, Califf RM and Topol EJ (1998) Abciximab therapy and unplanned coronary stent deployment: favorable effects on stent use, clinical outcomes, and bleeding complications. EPILOG Trial Investigators. *Circulation* **97**:857-864.

Kevil CG, Kolluru GK, Pattillo CB and Giordano T (2011) Inorganic nitrite therapy: historical perspective and future directions. *Free radical biology & medicine* **51**:576-593.

Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A and Caligiuri G (2010) Macrophage plasticity in experimental atherosclerosis. *PloS one* **5**:e8852.

- Khambata RS, Ghosh SM, Rathod KS, Thevathasan T, Filomena F, Xiao Q and Ahluwalia A (2017) Antiinflammatory actions of inorganic nitrate stabilize the atherosclerotic plaque. *Proceedings of the National Academy of Sciences of the United States of America* **114**:E550-e559.
- Kibbe MR, Li J, Nie S, Watkins SC, Lizonova A, Kovesdi I, Simmons RL, Billiar TR and Tzeng E (2000) Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. *Journal of vascular surgery* **31**:1214-1228.
- Kilfoy BA, Zhang Y, Park Y, Holford TR, Schatzkin A, Hollenbeck A and Ward MH (2011) Dietary nitrate and nitrite and the risk of thyroid cancer in the NIH-AARP Diet and Health Study. *International journal of cancer Journal international du cancer* **129**:160-172.
- Kim J-K, Moore DJ, Maurer DG, Kim-Shapiro DB, Basu S, Flanagan MP, Skulas-Ray AC, Kris-Etherton P and Proctor DN (2014) Acute dietary nitrate supplementation does not augment submaximal forearm exercise hyperemia in healthy young men. *Applied Physiology, Nutrition, and Metabolism* **40**:122-128.
- Kinder LS, Carnethon MR, Palaniappan LP, King AC and Fortmann SP (2004) Depression and the metabolic syndrome in young adults: findings from the Third National Health and Nutrition Examination Survey. *Psychosomatic medicine* **66**:316-322.
- Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Godecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch

- M and Kelm M (2003) Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free RadicBiolMed* **35**:790-796.
- Kleschyov AL, Oelze M, Daiber A, Huang Y, Mollnau H, Schulz E, Sydow K, Fichtlscherer B, Mulsch A and Munzel T (2003) Does nitric oxide mediate the vasodilator activity of nitroglycerin? *Circulation research* **93**:e104-112.
- Knight CJ, Panesar M, Wright C, Clarke D, Butowski PS, Patel D, Patrinely A, Fox K and Goodall AH (1997) Altered platelet function detected by flow cytometry. Effects of coronary artery disease and age. *Arteriosclerosis, thrombosis, and vascular biology* **17**:2044-2053.
- Knowles RG and Moncada S (1992) Nitric oxide as a signal in blood vessels. *Trends in biochemical sciences* **17**:399-402.
- Ko DT, Yun L, Wijeyesundera HC, Jackevicius CA, Rao SV, Austin PC, Marquis JF and Tu JV (2010) Incidence, predictors, and prognostic implications of hospitalization for late bleeding after percutaneous coronary intervention for patients older than 65 years. *Circulation Cardiovascular interventions* **3**:140-147.
- Komatsu R, Ueda M, Naruko T, Kojima A and Becker AE (1998) Neointimal tissue response at sites of coronary stenting in humans: macroscopic, histological, and immunohistochemical analyses. *Circulation* **98**:224-233.
- Kornowski R, Hong MK, Tio FO, Bramwell O, Wu H and Leon MB (1998) In-stent restenosis: contributions of inflammatory responses and arterial injury to neointimal hyperplasia. *Journal of the American College of Cardiology* **31**:224-230.

- Kortboyer J OM, Zeilmaker MJ. (1997) The oral bioavailability of sodium nitrite investigated in healthy adult volunteers. *Bilthoven, Netherlands: National Institute of Public Health and the Environment*.
- Kosaka H, Imaizumi K, Imai K and Tyuma I (1979) Stoichiometry of the reaction of oxyhemoglobin with nitrite. *Biochimica et biophysica acta* **581**:184-188.
- Kozinski M, Krzewina-Kowalska A, Kubica J, Zbikowska-Gotz M, Dymek G, Piasecki R, Sukiennik A, Grzesk G, Bogdan M, Chojnicki M, Dziedziczko A and Sypniewska G (2005) Percutaneous coronary intervention triggers a systemic inflammatory response in patients treated for in-stent restenosis -- comparison with stable and unstable angina. *Inflammation research : official journal of the European Histamine Research Society [et al]* **54**:187-193.
- Kucharzik T, Luger N, Pauels HG, Domschke W and Stoll R (1998) IL-4, IL-10 and IL-13 down-regulate monocyte-chemoattracting protein-1 (MCP-1) production in activated intestinal epithelial cells. *Clinical and experimental immunology* **111**:152-157.
- Laakso J, Mervaala E, Himberg JJ, Teravainen TL, Karppanen H, Vapaatalo H and Lapatto R (1998) Increased kidney xanthine oxidoreductase activity in salt-induced experimental hypertension. *Hypertension* **32**:902-906.
- Lablanche JM, Grollier G, Lusson JR, Bassand JP, Drobinski G, Bertrand B, Battaglia S, Desveaux B, Juilliere Y, Juliard JM, Metzger JP, Coste P, Quiret JC, Dubois-Rande JL, Crochet PD, Letac B, Boschat J, Viroit P, Finet G, Le Breton H, Livarek B, Leclercq F, Beard T, Giraud T, Bertrand ME and et al. (1997) Effect of the direct nitric oxide donors linsidomine and molsidomine on

- angiographic restenosis after coronary balloon angioplasty. The ACCORD Study. *Angiographic Coronary Restenosis After Percutaneous Coronary Intervention With Diltiazem*. *Circulation* **95**:83-89.
- Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, Gilchrist M, Benjamin N and Jones AM (2011) Acute dietary nitrate supplementation improves cycling time trial performance. *Medicine and science in sports and exercise* **43**:1125-1131.
- Larsen FJ, Ekblom B, Sahlin K, Lundberg JO and Weitzberg E (2006) Effects of dietary nitrate on blood pressure in healthy volunteers. *The New England journal of medicine* **355**:2792-2793.
- Lauck S, Johnson JL and Ratner PA (2009) Self-care behaviour and factors associated with patient outcomes following same-day discharge percutaneous coronary intervention. *European journal of cardiovascular nursing : journal of the Working Group on Cardiovascular Nursing of the European Society of Cardiology* **8**:190-199.
- Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M and Kelm M (2001) Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci USA* **98**:12814-12819.
- Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, Pannier B, Vlachopoulos C, Wilkinson I and Struijker-Boudier H (2006) Expert consensus document on arterial stiffness: methodological issues and clinical applications. *European heart journal* **27**:2588-2605.
- Law M, Wald N and Morris J (2003) Lowering blood pressure to prevent myocardial infarction and stroke: a new preventive strategy. *Health Technol Assess* **7**:1-94.

- Lee K, Santibanez-Koref M, Polvikoski T, Birchall D, Mendelow AD and Keavney B (2013) Increased expression of fatty acid binding protein 4 and leptin in resident macrophages characterises atherosclerotic plaque rupture. *Atherosclerosis* **226**:74-81.
- Leifeld L, Fielenbach M, Dumoulin FL, Speidel N, Sauerbruch T and Spengler U (2002) Inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) expression in fulminant hepatic failure. *Journal of hepatology* **37**:613-619.
- Lemieux S, Despres JP, Moorjani S, Nadeau A, Theriault G, Prud'homme D, Tremblay A, Bouchard C and Lupien PJ (1994) Are gender differences in cardiovascular disease risk factors explained by the level of visceral adipose tissue? *Diabetologia* **37**:757-764.
- Lerner DJ and Kannel WB (1986) Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population. *American heart journal* **111**:383-390.
- Lewington S, Clarke R, Qizilbash N, Peto R and Collins R (2002) Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* **360**:1903-1913.
- Ley K, Miller YI and Hedrick CC (2011) Monocyte and macrophage dynamics during atherogenesis. *Arteriosclerosis, thrombosis, and vascular biology* **31**:1506-1516.
- Li M, Fan Y, Zhang X, Hou W and Tang Z (2014) Fruit and vegetable intake and risk of type 2 diabetes mellitus: meta-analysis of prospective cohort studies. *BMJ Open* **4**:e005497.

Libby P (2005) *The vascular biology of atherosclerosis*. , Elsevier Saunders Philadelphia

Libby P, Egan D and Skarlatos S (1997) Roles of infectious agents in atherosclerosis and restenosis: an assessment of the evidence and need for future research. *Circulation* **96**:4095-4103.

Libby P, Ridker PM and Hansson GK (2009) Inflammation in atherosclerosis: from pathophysiology to practice. *Journal of the American College of Cardiology* **54**:2129-2138.

Libby P, Simon D and Rogers C (2003) Inflammation and arterial injury, in *Textbook of interventional cardiology* (Topol EJ ed) pp 381-389, Saunders, Philadelphia.

Libby P and Simon DI (2001) Inflammation and thrombosis: the clot thickens. *Circulation* **103**:1718-1720.

Licht WR, Schultz DS, Fox JG, Tannenbaum SR and Deen WM (1986) Mechanisms for nitrite loss from the stomach. *Carcinogenesis* **7**:1681-1687.

Lilly L (2007) Pathophysiology of heart disease, Lippincott Williams and Wilkins, Baltimore.

Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, AlMazroa MA, Amann M, Anderson HR, Andrews KG, Aryee M, Atkinson C, Bacchus LJ, Bahalim AN, Balakrishnan K, Balmes J, Barker-Collo S, Baxter A, Bell ML, Blore JD, Blyth F, Bonner C, Borges G, Bourne R, Boussinesq M, Brauer M, Brooks P, Bruce NG, Brunekreef B, Bryan-Hancock C, Bucello C, Buchbinder R, Bull F, Burnett RT, Byers TE, Calabria B, Carapetis J, Carnahan E, Chafe Z, Charlson F, Chen H, Chen JS, Cheng AT-A, Child JC, Cohen A, Colson KE,

Cowie BC, Darby S, Darling S, Davis A, Degenhardt L, Dentener F, Des Jarlais DC, Devries K, Dherani M, Ding EL, Dorsey ER, Driscoll T, Edmond K, Ali SE, Engell RE, Erwin PJ, Fahimi S, Falder G, Farzadfar F, Ferrari A, Finucane MM, Flaxman S, Fowkes FGR, Freedman G, Freeman MK, Gakidou E, Ghosh S, Giovannucci E, Gmel G, Graham K, Grainger R, Grant B, Gunnell D, Gutierrez HR, Hall W, Hoek HW, Hogan A, Hosgood Iii HD, Hoy D, Hu H, Hubbell BJ, Hutchings SJ, Ibeanusi SE, Jacklyn GL, Jasrasaria R, Jonas JB, Kan H, Kanis JA, Kassebaum N, Kawakami N, Khang Y-H, Khatibzadeh S, Khoo J-P, Kok C, Laden F, Lalloo R, Lan Q, Lathlean T, Leasher JL, Leigh J, Li Y, Lin JK, Lipshultz SE, London S, Lozano R, Lu Y, Mak J, Malekzadeh R, Mallinger L, Marcenes W, March L, Marks R, Martin R, McGale P, McGrath J, Mehta S, Memish ZA, Mensah GA, Merriman TR, Micha R, Michaud C, Mishra V, Hanafiah KM, Mokdad AA, Morawska L, Mozaffarian D, Murphy T, Naghavi M, Neal B, Nelson PK, Nolla JM, Norman R, Olives C, Omer SB, Orchard J, Osborne R, Ostro B, Page A, Pandey KD, Parry CDH, Passmore E, Patra J, Pearce N, Pelizzari PM, Petzold M, Phillips MR, Pope D, Pope Iii CA, Powles J, Rao M, Razavi H, Rehfuss EA, Rehm JT, Ritz B, Rivara FP, Roberts T, Robinson C, Rodriguez-Portales JA, Romieu I, Room R, Rosenfeld LC, Roy A, Rushton L, Salomon JA, Sampson U, Sanchez-Riera L, Sanman E, Sapkota A, Seedat S, Shi P, Shield K, Shivakoti R, Singh GM, Sleet DA, Smith E, Smith KR, Stapelberg NJC, Steenland K, Stöckl H, Stovner LJ, Straif K, Straney L, Thurston GD, Tran JH, Van Dingenen R, van Donkelaar A, Veerman JL, Vijayakumar L, Weintraub R, Weissman MM, White RA, Whiteford H, Wiersma ST, Wilkinson JD, Williams HC, Williams W, Wilson N, Woolf AD, Yip P, Zielinski JM, Lopez AD, Murray CJL and Ezzati M (2012) A



comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet* **380**:2224-2260.

Lincoff AM, Kereiakes DJ, Mascelli MA, Deckelbaum LI, Barnathan ES, Patel KK, Frederick B, Nakada MT and Topol EJ (2001) Abciximab suppresses the rise in levels of circulating inflammatory markers after percutaneous coronary revascularization. *Circulation* **104**:163-167.

Lincoff AM, Topol EJ and Ellis SG (1994) Local drug delivery for the prevention of restenosis. Fact, fancy, and future. *Circulation* **90**:2070-2084.

Lindahl B, Toss H, Siegbahn A, Venge P and Wallentin L (2000) Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease. *The New England journal of medicine* **343**:1139-1147.

Lindner V, Kim SK, Karas RH, Kuiper GG, Gustafsson JA and Mendelsohn ME (1998) Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. *Circulation research* **83**:224-229.

Liu AH, Bondonno CP, Croft KD, Puddey IB, Woodman RJ, Rich L, Ward NC, Vita JA and Hodgson JM (2013a) Effects of a nitrate-rich meal on arterial stiffness and blood pressure in healthy volunteers. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **35**:123-130.

Liu AH, Bondonno CP, Croft KD, Puddey IB, Woodman RJ, Rich L, Ward NC, Vita JA and Hodgson JM (2013b) Effects of a nitrate-rich meal on arterial stiffness

and blood pressure in healthy volunteers. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **35**:123-130.

Liu PY, Christian RC, Ruan M, Miller VM and Fitzpatrick LA (2005) Correlating androgen and estrogen steroid receptor expression with coronary calcification and atherosclerosis in men without known coronary artery disease. *The Journal of clinical endocrinology and metabolism* **90**:1041-1046.

Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB and Maseri A (1994) The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *The New England journal of medicine* **331**:417-424.

Liuzzo G, Buffon A, Biasucci LM, Gallimore JR, Caligiuri G, Vitelli A, Altamura S, Ciliberto G, Rebuzzi AG, Crea F, Pepys MB and Maseri A (1998) Enhanced inflammatory response to coronary angioplasty in patients with severe unstable angina. *Circulation* **98**:2370-2376.

Loh YH, Jakszyn P, Luben RN, Mulligan AA, Mitrou PN and Khaw K-T (2011) N-nitroso compounds and cancer incidence: the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk Study. *The American journal of clinical nutrition* **93**:1053-1061.

Lorenzo C, Williams K, Hunt KJ and Haffner SM (2007) The National Cholesterol Education Program - Adult Treatment Panel III, International Diabetes Federation, and World Health Organization definitions of the metabolic syndrome as predictors of incident cardiovascular disease and diabetes. *Diabetes care* **30**:8-13.

- Lowe HC, Oesterle SN and Khachigian LM (2002) Coronary in-stent restenosis: current status and future strategies. *Journal of the American College of Cardiology* **39**:183-193.
- Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW and Ganz P (1986) Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *The New England journal of medicine* **315**:1046-1051.
- Lundberg JO, Feelisch M, Bjorne H, Jansson EA and Weitzberg E (2006) Cardioprotective effects of vegetables: is nitrate the answer? *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **15**:359-362.
- Lundberg JO and Govoni M (2004) Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free radical biology & medicine* **37**:395-400.
- Lundberg JO, Weitzberg E, Cole JA and Benjamin N (2004) Nitrate, bacteria and human health. *Nature reviews Microbiology* **2**:593-602.
- Lundberg JO, Weitzberg E, Lundberg JM and Alving K (1994) Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* **35**:1543-1546.
- Lupattelli G, Marchesi S, Lombardini R, Roscini AR, Trinca F, Gemelli F, Vaudo G and Mannarino E (2004) Artichoke juice improves endothelial function in hyperlipemia. *Life Sci* **76**:775-782.
- Maas AH and Appelman YE (2010) Gender differences in coronary heart disease. *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* **18**:598-602.

- Mach F, Schonbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS and Libby P (1997) Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**:1931-1936.
- Magee PN and Barnes JM (1956) The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. *British journal of cancer* **10**:114-122.
- Maier W, Cosentino F, Lutolf RB, Fleisch M, Seiler C, Hess OM, Meier B and Luscher TF (2000) Tetrahydrobiopterin improves endothelial function in patients with coronary artery disease. *Journal of cardiovascular pharmacology* **35**:173-178.
- Mamode N, Cobbe S and Pollock JG (1995) Infarcts after surgery. *BMJ (Clinical research ed)* **310**:1215-1216.
- Mankad R and Best PJ (2008) Cardiovascular disease in older women: a challenge in diagnosis and treatment. *Women's health (London, England)* **4**:449-464.
- Marin E and Sessa WC (2007) Role of endothelial-derived nitric oxide in hypertension and renal disease. *Current opinion in nephrology and hypertension* **16**:105-110.
- Marshall WM, C.R. (1945) The action of nitrites on blood. *Journal of Biological Chemistry* **158**:187-208.
- Martensson UE, Salehi SA, Windahl S, Gomez MF, Sward K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grande PO, Owman C, Rosen CJ, Adamo ML, Lundquist I, Rorsman P, Nilsson BO, Ohlsson C, Olde B and Leeb-Lundberg LM (2009) Deletion of the G protein-coupled receptor 30

impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice.

*Endocrinology* **150**:687-698.

Maseri A (1997) Inflammation, atherosclerosis, and ischemic events -- exploring the hidden side of the moon. *The New England journal of medicine* **336**:1014-1016.

Masoura C, Pitsavos C, Aznaouridis K, Skoumas I, Vlachopoulos C and Stefanadis C (2011) Arterial endothelial function and wall thickness in familial hypercholesterolemia and familial combined hyperlipidemia and the effect of statins. A systematic review and meta-analysis. *Atherosclerosis* **214**:129-138.

Matsumoto D, Shite J, Shinke T, Otake H, Tanino Y, Ogasawara D, Sawada T, Paredes OL, Hirata K and Yokoyama M (2007) Neointimal coverage of sirolimus-eluting stents at 6-month follow-up: evaluated by optical coherence tomography. *European heart journal* **28**:961-967.

Mauri L, Orav EJ and Kuntz RE (2005) Late loss in lumen diameter and binary restenosis for drug-eluting stent comparison. *Circulation* **111**:3435-3442.

McDonald BJ and Bennett BM (1990) Cytochrome P-450 mediated biotransformation of organic nitrates. *Canadian journal of physiology and pharmacology* **68**:1552-1557.

McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M and Benjamin N (1997) Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* **40**:211-214.

- Mehdizadeh A and Norouzpour A (2009) New insights in atherosclerosis: endothelial shear stress as promoter rather than initiator. *Medical hypotheses* **73**:989-993.
- Mehran R, Dangas G, Abizaid AS, Mintz GS, Lansky AJ, Satler LF, Pichard AD, Kent KM, Stone GW and Leon MB (1999) Angiographic patterns of in-stent restenosis: classification and implications for long-term outcome. *Circulation* **100**:1872-1878.
- Meier CR, Jick SS, Derby LE, Vasilakis C and Jick H (1998) Acute respiratory-tract infections and risk of first-time acute myocardial infarction. *Lancet* **351**:1467-1471.
- Mendelsohn ME and Karas RH (2005) Molecular and cellular basis of cardiovascular gender differences. *Science (New York, NY)* **308**:1583-1587.
- Mendy A (2018) Association of Urinary Nitrate With Lower Prevalence of Hypertension and Stroke and With Reduced Risk of Cardiovascular Mortality. *Circulation* **137**:2295-2297.
- Mercado N, Boersma E, Wijns W, Gersh BJ, Morillo CA, de Valk V, van Es GA, Grobbee DE and Serruys PW (2001) Clinical and quantitative coronary angiographic predictors of coronary restenosis: a comparative analysis from the balloon-to-stent era. *Journal of the American College of Cardiology* **38**:645-652.
- Meuwissen M, van der wal AC, de Winter RJ, Koch KT, Becker AE and Piek JJ (2002) Images in cardiovascular medicine. Stent inflammation: stent footprint in restenotic tissue retrieved by directional atherectomy. *Circulation* **106**:1176-1177.

- Michel JB, Virmani R, Arbustini E and Pasterkamp G (2011) Intraplaque haemorrhages as the trigger of plaque vulnerability. *European heart journal* **32**:1977-1985, 1985a, 1985b, 1985c.
- Michel O, Nagy AM, Schroeven M, Duchateau J, Neve J, Fondu P and Sergysels R (1997) Dose-response relationship to inhaled endotoxin in normal subjects. *American journal of respiratory and critical care medicine* **156**:1157-1164.
- Mills C, Faconti L, Govoni V, Morant S, Casagrande M-L, Gu H, Jiang B, Webb A and Cruickshank K (2016) *Reducing arterial stiffness independently of BP: Proof of concept? CAVI, PWV and cardiac data in the 6-month VaSera trial.*
- Mintz GS, Nissen SE, Anderson WD, Bailey SR, Erbel R, Fitzgerald PJ, Pinto FJ, Rosenfield K, Siegel RJ, Tuzcu EM and Yock PG (2001) American College of Cardiology Clinical Expert Consensus Document on Standards for Acquisition, Measurement and Reporting of Intravascular Ultrasound Studies (IVUS). A report of the American College of Cardiology Task Force on Clinical Expert Consensus Documents. *Journal of the American College of Cardiology* **37**:1478-1492.
- Mintz GS, Popma JJ, Pichard AD, Kent KM, Satler LF, Wong C, Hong MK, Kovach JA and Leon MB (1996) Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. *Circulation* **94**:35-43.
- Mitra AK and Agrawal DK (2006) In stent restenosis: bane of the stent era. *Journal of clinical pathology* **59**:232-239.
- Modarai B, Kapadia YK, Kerins M and Terris J (2002) Methylene blue: a treatment for severe methaemoglobinaemia secondary to misuse of amyl nitrite. *Emergency medicine journal : EMJ* **19**:270-271.

- Modin A, Bjorne H, Herulf M, Alving K, Weitzberg E and Lundberg JO (2001) Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta physiologica Scandinavica* **171**:9-16.
- Mohlke KL, Boehnke M and Abecasis GR (2008) Metabolic and cardiovascular traits: an abundance of recently identified common genetic variants. *Human molecular genetics* **17**:R102-108.
- Moncada S and Higgs A (1993) The L-arginine-nitric oxide pathway. *The New England journal of medicine* **329**:2002-2012.
- Moncada S and Higgs EA (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **9**:1319-1330.
- Montenegro MF, Sundqvist ML, Larsen FJ, Zhuge Z, Carlstrom M, Weitzberg E and Lundberg JO (2017) Blood Pressure-Lowering Effect of Orally Ingested Nitrite Is Abolished by a Proton Pump Inhibitor. *Hypertension* **69**:23-31.
- Morris T, Stables M, Colville-Nash P, Newson J, Bellingan G, de Souza PM and Gilroy DW (2010) Dichotomy in duration and severity of acute inflammatory responses in humans arising from differentially expressed proresolution pathways. *Proceedings of the National Academy of Sciences of the United States of America* **107**:8842-8847.
- Mosser DM and Zhang X (2008) Interleukin-10: new perspectives on an old cytokine. *Immunological reviews* **226**:205-218.
- Mount PF, Kemp BE and Power DA (2007) Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J Mol Cell Cardiol* **42**:271-279.



- Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, Deanfield JE and MacAllister RJ (2001) Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circulation research* **88**:145-151.
- Mullen MJ, Thorne SA, Deanfield JE and Jones CJ (1997) Non-invasive assessment of endothelial function. *Heart (British Cardiac Society)* **77**:297-298.
- Munzel T, Daiber A and Gori T (2013) More answers to the still unresolved question of nitrate tolerance. *European heart journal* **34**:2666-2673.
- Munzel T, Sayegh H, Freeman BA, Tarpey MM and Harrison DG (1995) Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *The Journal of clinical investigation* **95**:187-194.
- Napoli C, Cirino G, Del Soldato P, Sorrentino R, Sica V, Condorelli M, Pinto A and Ignarro LJ (2001) Effects of nitric oxide-releasing aspirin versus aspirin on restenosis in hypercholesterolemic mice. *Proceedings of the National Academy of Sciences of the United States of America* **98**:2860-2864.
- Napoli C, Paolisso G, Casamassimi A, Al-Omran M, Barbieri M, Sommese L, Infante T and Ignarro LJ (2013) Effects of nitric oxide on cell proliferation: novel insights. *Journal of the American College of Cardiology* **62**:89-95.
- National, Institute, for, Health, and, Care and Excellence (2011) Management of stable angina. Accessed from: <http://www.nice.org.uk/guidance/cg126/chapter/1-recommendations#information-and-support-for-people-with-stable-angina>.

- Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C and Ganz P (2005) Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. *The New England journal of medicine* **352**:29-38.
- Norling LV, Dalli J, Flower RJ, Serhan CN and Perretti M (2012) Resolvin D1 limits polymorphonuclear leukocyte recruitment to inflammatory loci: receptor-dependent actions. *Arteriosclerosis, thrombosis, and vascular biology* **32**:1970-1978.
- Nowicki A, Trawinski Z, Gambin B, Secomski W, Szubielski M, Parol M and Olszewski R (2018) 20-MHz Ultrasound for Measurements of Flow-Mediated Dilation and Shear Rate in the Radial Artery. *Ultrasound in medicine & biology* **44**:1187-1197.
- O'Rourke MF (1970) Influence of ventricular ejection on the relationship between central aortic and brachial pressure pulse in man. *Cardiovascular research* **4**:291-300.
- Oh SF, Dona M, Fredman G, Krishnamoorthy S, Irimia D and Serhan CN (2012) Resolvin E2 formation and impact in inflammation resolution. *J Immunol* **188**:4527-4534.
- Ohkawa F, Ikeda U, Kanbe T, Kawasaki K and Shimada K (1995) Effects of inflammatory cytokines on vascular tone. *Cardiovascular research* **30**:711-715.
- Okada M, Nomura S, Ikoma Y, Yamamoto E, Ito T, Mitsui T, Tamakoshi K and Mizutani S (2003) Effects of postmenopausal hormone replacement therapy on HbA(1c) levels. *Diabetes care* **26**:1088-1092.

- Okey R and Stewart D (1932) Diet and blood cholesterol in normal women. *J Biol Chem* **99**:717.
- Ott I, Neumann FJ, Gawaz M, Schmitt M and Schomig A (1996) Increased neutrophil-platelet adhesion in patients with unstable angina. *Circulation* **94**:1239-1246.
- Oyebode O, Gordon-Dseagu V, Walker A and Mindell JS (2014) Fruit and vegetable consumption and all-cause, cancer and CVD mortality: analysis of Health Survey for England data. *Journal of epidemiology and community health* **68**:856-862.
- Palmer AJ, Bulpitt CJ, Fletcher AE, Beevers DG, Coles EC, Ledingham JG, O'Riordan PW, Petrie JC, Rajagopalan BE, Webster J and et al. (1992) Relation between blood pressure and stroke mortality. *Hypertension* **20**:601-605.
- Palmer RM, Ashton DS and Moncada S (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**:664-666.
- Palmer RM, Ferrige AG and Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**:524-526.
- Panaro MA, Brandonisio O, Acquafredda A, Sisto M and Mitolo V (2003) Evidences for iNOS expression and nitric oxide production in the human macrophages. *Current drug targets Immune, endocrine and metabolic disorders* **3**:210-221.
- Pannala AS, Mani AR, Spencer JP, Skinner V, Bruckdorfer KR, Moore KP and Rice-Evans CA (2003) The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. *Free radical biology & medicine* **34**:576-584.

- Panza JA, Quyyumi AA, Brush JE, Jr. and Epstein SE (1990) Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *The New England journal of medicine* **323**:22-27.
- Parathath S, Mick SL, Feig JE, Joaquin V, Grauer L, Habel DM, Gassmann M, Gardner LB and Fisher EA (2011) Hypoxia is present in murine atherosclerotic plaques and has multiple adverse effects on macrophage lipid metabolism. *Circulation research* **109**:1141-1152.
- Park JW, Piknova B, Huang PL, Noguchi CT and Schechter AN (2013) Effect of blood nitrite and nitrate levels on murine platelet function. *PloS one* **8**:e55699.
- Park JW, Piknova B, Nghiem K, Lozier JN and Schechter AN (2014) Inhibitory effect of nitrite on coagulation processes demonstrated by thrombelastography. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **40**:45-51.
- Parker BA, Tschakovsky ME, Augeri AL, Polk DM, Thompson PD and Kiernan FJ (2011) Heterogenous vasodilator pathways underlie flow-mediated dilation in men and women. *American Journal of Physiology - Heart and Circulatory Physiology* **301**:H1118-H1126.
- Patel MR, Dehmer GJ, Hirshfeld JW, Smith PK and Spertus JA (2009) ACCF/SCAI/STS/AATS/AHA/ASNC 2009 Appropriateness Criteria for Coronary Revascularization: a report by the American College of Cardiology Foundation Appropriateness Criteria Task Force, Society for Cardiovascular Angiography and Interventions, Society of Thoracic Surgeons, American Association for Thoracic Surgery, American Heart Association, and the American Society of Nuclear Cardiology Endorsed by the American Society of Echocardiography, the Heart Failure Society of

America, and the Society of Cardiovascular Computed Tomography. *Journal of the American College of Cardiology* **53**:530-553.

Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, 3rd, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC, Jr., Taubert K, Tracy RP and Vinicor F (2003) Markers of inflammation and cardiovascular disease: application to clinical and public health practice: A statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. *Circulation* **107**:499-511.

Peretz A, Leotta DF, Sullivan JH, Trenga CA, Sands FN, Aulet MR, Paun M, Gill EA and Kaufman JD (2007) Flow mediated dilation of the brachial artery: an investigation of methods requiring further standardization. *BMC cardiovascular disorders* **7**:11.

Perez-Lopez FR, Chedraui P, Gilbert JJ and Perez-Roncero G (2009) Cardiovascular risk in menopausal women and prevalent related co-morbid conditions: facing the post-Women's Health Initiative era. *Fertility and sterility* **92**:1171-1186.

Perez Lopez F, Cuadros-Lopez J, Fernandez A, Cuadros-Celorrio A, Sabatel-Lopez R and P. C (2009) Assessing fatal cardiovascular disease with the SCORE scale in postmenopausal women 10 years after different hormonal treatment regimens., (Endocrinology. G ed).

Perfetto SP, Chattopadhyay PK and Roederer M (2004) Seventeen-colour flow cytometry: unravelling the immune system. *Nature reviews Immunology* **4**:648-655.

Pergola C, Dodt G, Rossi A, Neunhoeffler E, Lawrenz B, Northoff H, Samuelsson B, Rådmark O, Sautebin L and Werz O (2008) ERK-mediated regulation of

- leukotriene biosynthesis by androgens: A molecular basis for gender differences in inflammation and asthma. *Proceedings of the National Academy of Sciences* **105**:19881-19886.
- Phillips GB (2005) Is atherosclerotic cardiovascular disease an endocrinological disorder? The estrogen-androgen paradox. *The Journal of clinical endocrinology and metabolism* **90**:2708-2711.
- Phillips JW, Barringhaus KG, Sanders JM, Hesselbacher SE, Czarnik AC, Manka D, Vestweber D, Ley K and Sarembock IJ (2003) Single injection of P-selectin or P-selectin glycoprotein ligand-1 monoclonal antibody blocks neointima formation after arterial injury in apolipoprotein E-deficient mice. *Circulation* **107**:2244-2249.
- Pierce JM and Nielsen MS (1989) Acute acquired methaemoglobinaemia after amyl nitrite poisoning. *BMJ (Clinical research ed)* **298**:1566.
- Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers J-W, Ulfman LH, Leenen LP, Pickkers P and Koenderman L A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. *The Journal of clinical investigation* **122**:327-336.
- Pinheiro LC, Amaral JH, Ferreira GC, Portella RL, Ceron CS, Montenegro MF, Toledo JC, Jr. and Tanus-Santos JE (2015) Gastric S-nitrosothiol formation drives the antihypertensive effects of oral sodium nitrite and nitrate in a rat model of renovascular hypertension. *Free radical biology & medicine* **87**:252-262.
- Plotkin SA and Bouveret-Le Cam N (1995) A new typhoid vaccine composed of the Vi capsular polysaccharide. *Archives of internal medicine* **155**:2293-2299.
- Pluta RM, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, Devroom HL, Nahavandi M, Woo S, Figg WD and Lonser RR (2011) Safety and feasibility of long-term

- intravenous sodium nitrite infusion in healthy volunteers. *PloS one* **6**:e14504.
- Poitou C, Dalmás E, Renovato M, Benhamo V, Hajdúch F, Abdennour M, Kahn JF, Veyrie N, Rizkalla S, Fridman WH, Sautes-Fridman C, Clement K and Cremer I (2011) CD14<sup>dim</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> monocytes in obesity and during weight loss: relationships with fat mass and subclinical atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **31**:2322-2330.
- Prati F, Regar E, Mintz GS, Arbustini E, Di Mario C, Jang IK, Akasaka T, Costa M, Guagliumi G, Grube E, Ozaki Y, Pinto F and Serruys PW (2010) Expert review document on methodology, terminology, and clinical applications of optical coherence tomography: physical principles, methodology of image acquisition, and clinical application for assessment of coronary arteries and atherosclerosis. *European heart journal* **31**:401-415.
- Puig O, Yuan J, Stepaniants S, Zieba R, Zycband E, Morris M, Coulter S, Yu X, Menke J, Woods J, Chen F, Ramey DR, He X, O'Neill EA, Hailman E, Johns DG, Hubbard BK, Yee Lum P, Wright SD, Desouza MM, Plump A and Reiser V (2011) A gene expression signature that classifies human atherosclerotic plaque by relative inflammation status. *Circulation Cardiovascular genetics* **4**:595-604.
- Qin L, Liu X, Sun Q, Fan Z, Xia D, Ding G, Ong HL, Adams D, Gahl WA, Zheng C, Qi S, Jin L, Zhang C, Gu L, He J, Deng D, Ambudkar IS and Wang S (2012) Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* **109**:13434-13439.

- Quartin AA, Schein RM, Kett DH and Peduzzi PN (1997) Magnitude and duration of the effect of sepsis on survival. Department of Veterans Affairs Systemic Sepsis Cooperative Studies Group. *Jama* **277**:1058-1063.
- Quinn MJ, Bhatt DL, Zidar F, Vivekananthan D, Chew DP, Ellis SG, Plow E and Topol EJ (2004) Effect of clopidogrel pretreatment on inflammatory marker expression in patients undergoing percutaneous coronary intervention. *The American journal of cardiology* **93**:679-684.
- Radomski MW, Palmer RM and Moncada S (1987a) Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet* **2**:1057-1058.
- Radomski MW, Palmer RM and Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proceedings of the National Academy of Sciences of the United States of America* **87**:5193-5197.
- Radomski MW, Palmer RMJ and Moncada S (1987b) Comparative Pharmacology of Endothelium-Derived Relaxing Factor, Nitric-Oxide and Prostacyclin in Platelets. *British journal of pharmacology* **92**:181-187.
- Radu MD, Yamaji K, Garcia-Garcia HM, Zaugg S, Taniwaki M, Koskinas KC, Serruys PW, Windecker S, Dijkstra J and Raber L (2016) Variability in the measurement of minimum fibrous cap thickness and reproducibility of fibroatheroma classification by optical coherence tomography using manual versus semi-automatic assessment. *EuroIntervention : journal of EuroPCR in collaboration with the Working Group on Interventional Cardiology of the European Society of Cardiology* **12**:e987-e997.



- Rammos C, Hendgen-Cotta UB, Sobierajski J, Bernard A, Kelm M and Rassaf T (2014) Dietary nitrate reverses vascular dysfunction in older adults with moderately increased cardiovascular risk. *Journal of the American College of Cardiology* **63**:1584-1585.
- Raphael I, Nalawade S, Eagar TN and Forsthuber TG (2015) T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine* **74**:5-17.
- Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, Nalbone G, Castier Y, Leseche G, Lehoux S, Tedgui A and Boulanger CM (2011) Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. *Circulation research* **108**:335-343.
- Rexrode KM, Ridker PM, Hegener HH, Buring JE, Manson JE and Zee RY (2007) Polymorphisms and haplotypes of the estrogen receptor-beta gene (ESR2) and cardiovascular disease in men and women. *Clinical chemistry* **53**:1749-1756.
- Reynolds S, Waterhouse K and Miller KH (2001) Patient care after percutaneous transluminal coronary angioplasty. *Nursing management* **32**:51-54, 56.
- Richardson G, Hicks SL, O'Byrne S, Frost MT, Moore K, Benjamin N and McKnight GM (2002) The ingestion of inorganic nitrate increases gastric S-nitrosothiol levels and inhibits platelet function in humans. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **7**:24-29.
- Ridker PM, Cushman M, Stampfer MJ, Tracy RP and Hennekens CH (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *The New England journal of medicine* **336**:973-979.

Ridker PM, Cushman M, Stampfer MJ, Tracy RP and Hennekens CH (1998a) Plasma concentration of C-reactive protein and risk of developing peripheral vascular disease. *Circulation* **97**:425-428.

Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Jr., Kastelein JJ, Koenig W, Libby P, Lorenzatti AJ, MacFadyen JG, Nordestgaard BG, Shepherd J, Willerson JT and Glynn RJ (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *The New England journal of medicine* **359**:2195-2207.

Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P and Glynn RJ (2017) Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *The New England journal of medicine* **377**:1119-1131.

Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ and Allen J (1998b) Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* **351**:88-92.

Ridker PM and Morrow DA (2003) C-reactive protein, inflammation, and coronary risk. *Cardiology clinics* **21**:315-325.

Ridker PM, Rifai N, Rose L, Buring JE and Cook NR (2002) Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *The New England journal of medicine* **347**:1557-1565.

- Rioufol G, Finet G, Ginon I, Andre-Fouet X, Rossi R, Vialle E, Desjoyaux E, Convert G, Huret JF and Tabib A (2002) Multiple atherosclerotic plaque rupture in acute coronary syndrome: a three-vessel intravascular ultrasound study. *Circulation* **106**:804-808.
- Ritchie JL, Alexander HD and Rea IM (2000) Flow cytometry analysis of platelet P-selectin expression in whole blood--methodological considerations. *Clinical and laboratory haematology* **22**:359-363.
- Rittersma SZ, de Winter RJ, Koch KT, Schotborgh CE, Bax M, Heyde GS, van Straalen JP, Mulder KJ, Tijssen JG, Sanders GT and Piek JJ (2004) Preprocedural C-reactive protein is not associated with angiographic restenosis or target lesion revascularization after coronary artery stent placement. *Clinical chemistry* **50**:1589-1596.
- Robbins CS, Hilgendorf I, Weber GF, Theurl I, Iwamoto Y, Figueiredo JL, Gorbатов R, Sukhova GK, Gerhardt LM, Smyth D, Zavitz CC, Shikatani EA, Parsons M, van Rooijen N, Lin HY, Husain M, Libby P, Nahrendorf M, Weissleder R and Swirski FK (2013) Local proliferation dominates lesional macrophage accumulation in atherosclerosis. *Nature medicine* **19**:1166-1172.
- Rodriguez-Mateos A, Hezel M, Aydin H, Kelm M, Lundberg JO, Weitzberg E, Spencer JPE and Heiss C (2015) Interactions between cocoa flavanols and inorganic nitrate: Additive effects on endothelial function at achievable dietary amounts. *Free Radical Biology and Medicine* **80**:121-128.
- Roe HE (1933) Methemoglobinemia following the administration of bismuth subnitrate. Report of a fatal case. *JAMA : the journal of the American Medical Association* **101**:352-354.
- Rollins BJ (1997) Chemokines. *Blood* **90**:909-928.

- Rong JX, Shapiro M, Trogan E and Fisher EA (2003) Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading. *Proceedings of the National Academy of Sciences of the United States of America* **100**:13531-13536.
- Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**:801-809.
- Ross R (1999a) Atherosclerosis--an inflammatory disease. *The New England journal of medicine* **340**:115-126.
- Ross R (1999b) Atherosclerosis -- An Inflammatory Disease. *The New England Journal of Medicine* **340**:115-126.
- Russell KS, Haynes MP, Caulin-Glaser T, Rosneck J, Sessa WC and Bender JR (2000) Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release. *J BiolChem* **275**:5026-5030.
- Sage PR, de la Lande IS, Stafford I, Bennett CL, Phillipov G, Stubberfield J and Horowitz JD (2000) Nitroglycerin tolerance in human vessels: evidence for impaired nitroglycerin bioconversion. *Circulation* **102**:2810-2815.
- Sakshaug J, Sognen E, Hansen MA and Koppang N (1965) Dimethylnitrosamine; its hepatotoxic effect in sheep and its occurrence in toxic batches of herring meal. *Nature* **206**:1261-1262.
- Salvatore Davide Tomasello LCaARG (2011) Quantitative Coronary. Angiography in the Interventional Cardiology, Advances in the Diagnosis of Coronary Atherosclerosis, (Kirac PS ed).
- Samouilov A, Woldman YY, Zweier JL and Khramtsov VV (2007) Magnetic resonance study of the transmembrane nitrite diffusion. *Nitric oxide* :

*biology and chemistry / official journal of the Nitric Oxide Society* **16**:362-370.

Santamaria P (2006) Nitrate in vegetables: toxicity, content, intake and EC regulation. *J Sci Food Agr* **86**:10-17.

Schafer AI, Alexander RW and Handin RI (1980) Inhibition of platelet function by organic nitrate vasodilators. *Blood* **55**:649-654.

Schissel SL, Tweedie-Hardman J, Rapp JH, Graham G, Williams KJ and Tabas I (1996) Rabbit aorta and human atherosclerotic lesions hydrolyze the sphingomyelin of retained low-density lipoprotein. Proposed role for arterial-wall sphingomyelinase in subendothelial retention and aggregation of atherogenic lipoproteins. *The Journal of clinical investigation* **98**:1455-1464.

Schlitt A, Heine GH, Blankenberg S, Espinola-Klein C, Dopheide JF, Bickel C, Lackner KJ, Iz M, Meyer J, Darius H and Rupprecht HJ (2004) CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thrombosis and haemostasis* **92**:419-424.

Schmidt HH, Nau H, Wittfoht W, Gerlach J, Prescher KE, Klein MM, Niroomand F and Bohme E (1988) Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol* **154**:213-216.

Schmitt JM, Knuttel A and Bonner RF (1993) Measurement of optical properties of biological tissues by low-coherence reflectometry. *Applied optics* **32**:6032-6042.

Schmitt M, Avolio A, Qasem A, McEniery CM, Butlin M, Wilkinson IB and Cockcroft JR (2005) Basal NO locally modulates human iliac artery function in vivo. *Hypertension* **46**:227-231.

- Schreiber F, Stief P, Gieseke A, Heisterkamp IM, Verstraete W, de Beer D and Stoodley P (2010) Denitrification in human dental plaque. *BMC biology* **8**:24.
- Schuddeboom L (1993) Nitrates and nitrites in food stuffs, Council of Europe Press, Brussels.
- Schuit SC, de Jong FH, Stolk L, Koek WN, van Meurs JB, Schoofs MW, Zillikens MC, Hofman A, van Leeuwen JP, Pols HA and Uitterlinden AG (2005) Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women. *European journal of endocrinology* **153**:327-334.
- Schuit SC, Oei HH, Witteman JC, Geurts van Kessel CH, van Meurs JB, Nijhuis RL, van Leeuwen JP, de Jong FH, Zillikens MC, Hofman A, Pols HA and Uitterlinden AG (2004) Estrogen receptor alpha gene polymorphisms and risk of myocardial infarction. *Jama* **291**:2969-2977.
- Schulz E, Tsilimingas N, Rinze R, Reiter B, Wendt M, Oelze M, Woelken-Weckmuller S, Walter U, Reichenspurner H, Meinertz T and Munzel T (2002) Functional and biochemical analysis of endothelial (dys)function and NO/cGMP signaling in human blood vessels with and without nitroglycerin pretreatment. *Circulation* **105**:1170-1175.
- Segev A, Kassam S, Buller CE, Lau HK, Sparkes JD, Connelly PW, Seidelin PH, Natarajan MK, Cohen EA and Strauss BH (2004) Pre-procedural plasma levels of C-reactive protein and interleukin-6 do not predict late coronary angiographic restenosis after elective stenting. *European heart journal* **25**:1029-1035.
- Semaan HB, Gurbel PA, Anderson JL, Muhlestein JB, Carlquist JF, Horne BD and Serebruany VL (2000) Soluble VCAM-1 and E-selectin, but not ICAM-1

- discriminate endothelial injury in patients with documented coronary artery disease. *Cardiology* **93**:7-10.
- Sen NP, Smith DC and Schwinghamer L (1969) Formation of N-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Food and cosmetics toxicology* **7**:301-307.
- Serhan CN (2014) Pro-resolving lipid mediators are leads for resolution physiology. *Nature* **510**:92-101.
- Serhan CN, Dalli J, Colas RA, Winkler JW and Chiang N (2015) Protectins and maresins: New pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome. *Biochimica et biophysica acta* **1851**:397-413.
- Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P and et al. (1994) A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. *The New England journal of medicine* **331**:489-495.
- Serruys PW, Reiber JH, Wijns W, van den Brand M, Kooijman CJ, ten Katen HJ and Hugenholtz PG (1984) Assessment of percutaneous transluminal coronary angioplasty by quantitative coronary angiography: diameter versus densitometric area measurements. *The American journal of cardiology* **54**:482-488.
- Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, Swiatlowska P, Newman AA, Greene ES, Straub AC, Isakson B, Randolph GJ and Owens GK (2015) KLF4-dependent phenotypic modulation of smooth

muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nature medicine* **21**:628-637.

Sharma RV, Tan E, Fang S, Gurjar MV and Bhalla RC (1999) NOS gene transfer inhibits expression of cell cycle regulatory molecules in vascular smooth muscle cells. *The American journal of physiology* **276**:H1450-1459.

Shaul PW, Smart EJ, Robinson LJ, German Z, Yuhanna IS, Ying Y, Anderson RG and Michel T (1996) Acylation targets endothelial nitric-oxide synthase to plasmalemmal caveolae. *The Journal of biological chemistry* **271**:6518-6522.

Shearman AM, Cooper JA, Kotwinski PJ, Miller GJ, Humphries SE, Ardlie KG, Jordan B, Irenze K, Lunetta KL, Schuit SC, Uitterlinden AG, Pols HA, Demissie S, Cupples LA, Mendelsohn ME, Levy D and Housman DE (2006) Estrogen receptor alpha gene variation is associated with risk of myocardial infarction in more than seven thousand men from five cohorts. *Circulation research* **98**:590-592.

Shimizu M and Kario K (2008) Role of the augmentation index in hypertension. *Therapeutic advances in cardiovascular disease* **2**:25-35.

Shiva S, Frizzell S and Gladwin MT (2010) Nitrite and heme globins: reaction mechanisms and physiological targets, in *Nitric Oxide: Biology And Pathobiology* (Ignarro LJ ed) pp 605-626, Elsevier, Los Angeles.

Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM and Gladwin MT (2007a) Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circulation research* **100**:654-661.



- Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ and Gladwin MT (2007b) Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med* **204**:2089-2102.
- Shu X, Keller TC, Begandt D, Butcher JT, Biwer L, Keller AS, Columbus L and Isakson BE (2015) Endothelial nitric oxide synthase in the microcirculation. *Cellular and molecular life sciences : CMLS* **72**:4561-4575.
- Siddiqi N, Neil C, Bruce M, MacLennan G, Cotton S, Papadopoulou S, Feelisch M, Bunce N, Lim PO, Hildick-Smith D, Horowitz J, Madhani M, Boon N, Dawson D, Kaski JC and Frenneaux M (2014) Intravenous sodium nitrite in acute ST-elevation myocardial infarction: a randomized controlled trial (NIAMI). *European heart journal* **35**:1255-1262.
- Sidhu JS, Cowan D, Tooze JA and Kaski J-C (2004) Peroxisome proliferator-activated receptor- $\gamma$  agonist rosiglitazone reduces circulating platelet activity in patients without diabetes mellitus who have coronary artery disease. *American heart journal* **147**:1032-1037.
- Siervo M, Lara J, Chowdhury S, Ashor A, Oggioni C and Mathers JC (2014) Effects of the Dietary Approach to Stop Hypertension (DASH) diet on cardiovascular risk factors: a systematic review and meta-analysis. *The British journal of nutrition*:1-15.
- Sindler AL, Fleenor BS, Calvert JW, Marshall KD, Zigler ML, Lefer DJ and Seals DR (2011) Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. *Aging cell* **10**:429-437.

Singh M, Gersh BJ, McClelland RL, Ho KK, Willerson JT, Penny WF and Holmes DR, Jr. (2004) Clinical and angiographic predictors of restenosis after percutaneous coronary intervention: insights from the Prevention of Restenosis With Tranilast and Its Outcomes (PRESTO) trial. *Circulation* **109**:2727-2731.

Sinha R, Park Y, Graubard BI, Leitzmann MF, Hollenbeck A, Schatzkin A and Cross AJ (2009) Meat and meat-related compounds and risk of prostate cancer in a large prospective cohort study in the United States. *American journal of epidemiology* **170**:1165-1177.

Sluimer JC and Daemen MJ (2009) Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *The Journal of pathology* **218**:7-29.

Smith SC, Jr., Feldman TE, Hirshfeld JW, Jr., Jacobs AK, Kern MJ, King SB, 3rd, Morrison DA, O'Neill WW, Schaff HV, Whitlow PL, Williams DO, Antman EM, Smith SC, Jr., Adams CD, Anderson JL, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Hunt SA, Jacobs AK, Nishimura R, Ornato JP, Page RL and Riegel B (2006) ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/SCAI Writing Committee to Update the 2001 Guidelines for Percutaneous Coronary Intervention). *Journal of the American College of Cardiology* **47**:e1-121.

Sobko T, Marcus C, Govoni M and Kamiya S (2010) Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **22**:136-140.

- Sodring M, Oostindjer M, Egelanddal B and Paulsen JE (2015) Effects of hemin and nitrite on intestinal tumorigenesis in the a/j min/+ mouse model. *PLoS one* **10**:e0122880.
- Spahn M, Heer V and Freytag R (2003) [Flat-panel detectors in X-ray systems]. *Der Radiologe* **43**:340-350.
- Sparacino-Watkins CE, Tejero J, Sun B, Gauthier MC, Thomas J, Ragireddy V, Merchant BA, Wang J, Azarov I, Basu P and Gladwin MT (2014) Nitrite Reductase and Nitric-oxide Synthase Activity of the Mitochondrial Molybdopterin Enzymes mARC1 and mARC2. *Journal of Biological Chemistry* **289**:10345-10358.
- Spaulding C, Daemen J, Boersma E, Cutlip DE and Serruys PW (2007) A pooled analysis of data comparing sirolimus-eluting stents with bare-metal stents. *The New England journal of medicine* **356**:989-997.
- Speijers G and van den Brandt P (2003) Nitrate (and potential endogenous formation of N--nitrosocompounds). **World Health Organization, Geneva.**
- Spiegelhalder B, Eisenbrand G and Preussmann R (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. *Food and cosmetics toxicology* **14**:545-548.
- Spite M, Norling LV, Summers L, Yang R, Cooper D, Petasis NA, Flower RJ, Perretti M and Serhan CN (2009) Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. *Nature* **461**:1287-1291.
- Srihirun S, Sriwantana T, Unchern S, Kittikool D, Noulsri E, Pattanapanyasat K, Fucharoen S, Piknova B, Schechter AN and Sibmooh N (2012) Platelet

inhibition by nitrite is dependent on erythrocytes and deoxygenation. *PloS one* **7**:e30380.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *The New England journal of medicine* **320**:915-924.

Steinberg DH, Gaglia MA, Jr., Pinto Slottow TL, Roy P, Bonello L, De Labriolle A, Lemesle G, Torguson R, Kineshige K, Xue Z, Suddath WO, Kent KM, Satler LF, Pichard AD, Lindsay J and Waksman R (2009) Outcome differences with the use of drug-eluting stents for the treatment of in-stent restenosis of bare-metal stents versus drug-eluting stents. *The American journal of cardiology* **103**:491-495.

Steinberg HO, Bayazeed B, Hook G, Johnson A, Cronin J and Baron AD (1997) Endothelial dysfunction is associated with cholesterol levels in the high normal range in humans. *Circulation* **96**:3287-3293.

Stieglitz EJ, Palmer, A.E. (1936) Studies on the Pharmacology of the Nitrite Effect of Bismuth Subnitrate. *The Journal of Pharmacology and Experimental Therapeutics* **56**:216-222.

Stokes KY, Dugas TR, Tang Y, Garg H, Guidry E and Bryan NS (2009) Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *American journal of physiology Heart and circulatory physiology* **296**:H1281-1288.

Stone GW, Moses JW, Ellis SG, Schofer J, Dawkins KD, Morice MC, Colombo A, Schampaert E, Grube E, Kirtane AJ, Cutlip DE, Fahy M, Pocock SJ, Mehran R and Leon MB (2007) Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. *The New England journal of medicine* **356**:998-1008.

Stone GW, Witzenbichler B, Weisz G, Rinaldi MJ, Neumann FJ, Metzger DC, Henry TD, Cox DA, Duffy PL, Mazzaferri E, Gurbel PA, Xu K, Parise H, Kirtane AJ, Brodie BR, Mehran R and Stuckey TD (2013) Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. *Lancet* **382**:614-623.

Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ and O'Grady NP (1999) New insights into the biology of the acute phase response. *Journal of clinical immunology* **19**:203-214.

Suzuki H, DeLano FA, Parks DA, Jamshidi N, Granger DN, Ishii H, Suematsu M, Zweifach BW and Schmid-Schonbein GW (1998) Xanthine oxidase activity associated with arterial blood pressure in spontaneously hypertensive rats. *Proceedings of the National Academy of Sciences of the United States of America* **95**:4754-4759.

Swirski FK, Weissleder R and Pittet MJ (2009) Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology* **29**:1424-1432.

Szmitko PE, Wang CH, Weisel RD, de Almeida JR, Anderson TJ and Verma S (2003) New markers of inflammation and endothelial cell activation: Part I. *Circulation* **108**:1917-1923.

Tabas I, Williams KJ and Boren J (2007) Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* **116**:1832-1844.

Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ and Randolph GJ (2007)

Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *The Journal of clinical investigation* **117**:185-194.

Taguchi K, Matsumoto T, Kamata K and Kobayashi T (2012) Akt/eNOS pathway activation in endothelium-dependent relaxation is preserved in aortas from female, but not from male, type 2 diabetic mice. *Pharmacological Research* **65**:56-65.

Takarada S, Imanishi T, Liu Y, Ikejima H, Tsujioka H, Kuroi A, Ishibashi K, Komukai K, Tanimoto T, Ino Y, Kitabata H, Kubo T, Nakamura N, Hirata K, Tanaka A, Mizukoshi M and Akasaka T (2010) Advantage of next-generation frequency-domain optical coherence tomography compared with conventional time-domain system in the assessment of coronary lesion. *Catheterization and cardiovascular interventions : official journal of the Society for Cardiac Angiography & Interventions* **75**:202-206.

Tanaka A, Imanishi T, Kitabata H, Kubo T, Takarada S, Tanimoto T, Kuroi A, Tsujioka H, Ikejima H, Komukai K, Kataiwa H, Okouchi K, Kashiwaghi M, Ishibashi K, Matsumoto H, Takemoto K, Nakamura N, Hirata K, Mizukoshi M and Akasaka T (2009) Lipid-rich plaque and myocardial perfusion after successful stenting in patients with non-ST-segment elevation acute coronary syndrome: an optical coherence tomography study. *European heart journal* **30**:1348-1355.

Tannenbaum SR, Weisman M and Fett D (1976) The effect of nitrate intake on nitrite formation in human saliva. *Food and cosmetics toxicology* **14**:549-552.

Tearney GJ, Regar E, Akasaka T, Adriaenssens T, Barlis P, Bezerra HG, Bouma B, Bruining N, Cho JM, Chowdhary S, Costa MA, de Silva R, Dijkstra J, Di Mario C, Dudek D, Falk E, Feldman MD, Fitzgerald P, Garcia-Garcia HM, Gonzalo N, Granada JF, Guagliumi G, Holm NR, Honda Y, Ikeno F, Kawasaki M, Kochman J, Koltowski L, Kubo T, Kume T, Kyono H, Lam CC, Lamouche G, Lee DP, Leon MB, Maehara A, Manfrini O, Mintz GS, Mizuno K, Morel MA, Nadkarni S, Okura H, Otake H, Pietrasik A, Prati F, Raber L, Radu MD, Rieber J, Riga M, Rollins A, Rosenberg M, Sirbu V, Serruys PW, Shimada K, Shinke T, Shite J, Siegel E, Sonoda S, Suter M, Takarada S, Tanaka A, Terashima M, Thim T, Uemura S, Ughi GJ, van Beusekom HM, van der Steen AF, van Es GA, van Soest G, Virmani R, Waxman S, Weissman NJ and Weisz G (2012) Consensus standards for acquisition, measurement, and reporting of intravascular optical coherence tomography studies: a report from the International Working Group for Intravascular Optical Coherence Tomography Standardization and Validation. *Journal of the American College of Cardiology* **59**:1058-1072.

Teirstein PS, Massullo V, Jani S, Popma JJ, Mintz GS, Russo RJ, Schatz RA, Guarneri EM, Steuterman S, Morris NB, Leon MB and Tripuraneni P (1997) Catheter-based radiotherapy to inhibit restenosis after coronary stenting. *The New England journal of medicine* **336**:1697-1703.

Tektonidis TG, Akesson A, Gigante B, Wolk A and Larsson SC (2015) A Mediterranean diet and risk of myocardial infarction, heart failure and stroke: A population-based cohort study. *Atherosclerosis* **243**:93-98.

- Terashima M, Kaneda H and Suzuki T (2012) The role of optical coherence tomography in coronary intervention. *The Korean journal of internal medicine* **27**:1-12.
- Thom DH, Grayston JT, Siscovick DS, Wang SP, Weiss NS and Daling JR (1992) Association of prior infection with *Chlamydia pneumoniae* and angiographically demonstrated coronary artery disease. *Jama* **268**:68-72.
- Thompson D, Pepys MB and Wood SP (1999) The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure (London, England : 1993)* **7**:169-177.
- Tillett HE, Smith JW and Gooch CD (1983) Excess deaths attributable to influenza in England and Wales: age at death and certified cause. *International journal of epidemiology* **12**:344-352.
- Tomasello SD, Costanzo L and Galassi AR (2011) Quantitative Coronary Angiography in the Interventional Cardiology, in *Advances in the Diagnosis of Coronary Atherosclerosis*, (Kirac PS ed) pp 256-272, InTech.
- Tomaszewski M, Charchar FJ, Maric C, Kuzniewicz R, Gola M, Grzeszczak W, Samani NJ and Zukowska-Szczechowska E (2009) Association between lipid profile and circulating concentrations of estrogens in young men. *Atherosclerosis* **203**:257-262.
- Topol EJ and Nissen SE (1995) Our preoccupation with coronary luminology. The dissociation between clinical and angiographic findings in ischemic heart disease. *Circulation* **92**:2333-2342.
- Topol EJ and Serruys PW (1998) Frontiers in interventional cardiology. *Circulation* **98**:1802-1820.



- Toutouzas K, Colombo A and Stefanadis C (2004) Inflammation and restenosis after percutaneous coronary interventions. *European heart journal* **25**:1679-1687.
- Trevisi P, Casini L, Nisi I, Messori S and Bosi P (2011) Effect of high oral doses of nitrate on salivary recirculation of nitrates and nitrites and on bacterial diversity in the saliva of young pigs. *Journal of animal physiology and animal nutrition* **95**:206-213.
- Uemura S, Ishigami K, Soeda T, Okayama S, Sung JH, Nakagawa H, Somekawa S, Takeda Y, Kawata H, Horii M and Saito Y (2012) Thin-cap fibroatheroma and microchannel findings in optical coherence tomography correlate with subsequent progression of coronary atheromatous plaques. *European heart journal* **33**:78-85.
- Ugocsai P, Hohenstatt A, Paragh G, Liebisch G, Langmann T, Wolf Z, Weiss T, Groitl P, Dobner T, Kasprzak P, Gobolos L, Falkert A, Seelbach-Goebel B, Gellhaus A, Winterhager E, Schmidt M, Semenza GL and Schmitz G (2010) HIF-1beta determines ABCA1 expression under hypoxia in human macrophages. *The international journal of biochemistry & cell biology* **42**:241-252.
- Valtonen V, Kuikka A and Syrjanen J (1993) Thrombo-embolic complications in bacteraemic infections. *European heart journal* **14 Suppl K**:20-23.
- van der Zwet PM and Reiber JH (1994) A new approach for the quantification of complex lesion morphology: the gradient field transform; basic principles and validation results. *Journal of the American College of Cardiology* **24**:216-224.
- van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-

- Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J and Gladwin MT (2009) Nitrite as regulator of hypoxic signaling in mammalian physiology. *Medicinal research reviews* **29**:683-741.
- Van Herck PL, Gavit L, Gorissen P, Wuyts FL, Claeys MJ, Bosmans JM, Benali K and Vrints CJ (2004) Quantitative coronary arteriography on digital flat-panel system. *Catheterization and cardiovascular interventions : official journal of the Society for Cardiac Angiography & Interventions* **63**:192-200.
- van Leeuwen M, Gijbels MJ, Duijvestijn A, Smook M, van de Gaar MJ, Heeringa P, de Winther MP and Tervaert JW (2008) Accumulation of myeloperoxidase-positive neutrophils in atherosclerotic lesions in LDLR<sup>-/-</sup> mice. *Arteriosclerosis, thrombosis, and vascular biology* **28**:84-89.
- van Velzen AG, Sips AJ, Schothorst RC, Lambers AC and Meulenbelt J (2008) The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol Lett* **181**:177-181.
- Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, Benjamin N, Winyard PG and Jones AM (2010) Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *American journal of physiology Regulatory, integrative and comparative physiology* **299**:R1121-1131.
- Vanhoutte PM (1996) Endothelial dysfunction in hypertension. *Journal of hypertension Supplement : official journal of the International Society of Hypertension* **14**:S83-93.
- Vavra AK, Havelka GE, Martinez J, Lee VR, Fu B, Jiang Q, Keefer LK and Kibbe MR (2011) Insights into the effect of nitric oxide and its metabolites nitrite and

nitrate at inhibiting neointimal hyperplasia. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **25**:22-30.

Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, Van Eijl S, Sagi-Kiss V, Chowdhury TA, Curtis M, Kuhnle GG, Wade WG and Ahluwalia A (2016) Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *The American journal of clinical nutrition* **103**:25-38.

Velmurugan S, Kapil V, Ghosh SM, Davies S, McKnight A, Aboud Z, Khambata RS, Webb AJ, Poole A and Ahluwalia A (2013) Antiplatelet effects of dietary nitrate in healthy volunteers: Involvement of cGMP and influence of sex. *Free Radical Biology and Medicine* **65**:1521-1532.

Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, Cassella CP, Moore KJ, Ramsey SA, Miano JM and Fisher EA (2015) Cholesterol loading reprograms the microRNA-143/145-myocardin axis to convert aortic smooth muscle cells to a dysfunctional macrophage-like phenotype. *Arteriosclerosis, thrombosis, and vascular biology* **35**:535-546.

Verma S and Anderson TJ (2002) Fundamentals of endothelial function for the clinical cardiologist. *Circulation* **105**:546-549.

Vermeer KA, Mo J, Weda JJ, Lemij HG and de Boer JF (2013) Depth-resolved model-based reconstruction of attenuation coefficients in optical coherence tomography. *Biomedical optics express* **5**:322-337.

Versaci F, Gaspardone A, Tomai F, Ribichini F, Russo P, Proietti I, Ghini AS, Ferrero V, Chiariello L, Gioffre PA, Romeo F and Crea F (2002) Immunosuppressive Therapy for the Prevention of Restenosis after Coronary Artery Stent

- Implantation (IMPRESS Study). *Journal of the American College of Cardiology* **40**:1935-1942.
- Villar IC, Scotland RS, Khambata RS, Chan M, Duchene J, Sampaio AL, Perretti M, Hobbs AJ and Ahluwalia A (2011) Suppression of endothelial P-selectin expression contributes to reduced cell trafficking in females: an effect independent of NO and prostacyclin. *Arteriosclerosis, thrombosis, and vascular biology* **31**:1075-1083.
- Vink A, Schoneveld AH, Lamers D, Houben AJ, van der Groep P, van Diest PJ and Pasterkamp G (2007) HIF-1 alpha expression is associated with an atheromatous inflammatory plaque phenotype and upregulated in activated macrophages. *Atherosclerosis* **195**:e69-75.
- Viola A and Luster AD (2008) Chemokines and Their Receptors: Drug Targets in Immunity and Inflammation. *Annual Review of Pharmacology and Toxicology* **48**:171-197.
- Virmani R and Farb A (1999) Pathology of in-stent restenosis. *Current opinion in lipidology* **10**:499-506.
- Virmani R, Kolodgie FD, Farb A and Lafont A (2003) Drug eluting stents: are human and animal studies comparable? *Heart (British Cardiac Society)* **89**:133-138.
- Vishnevetsky D, Kiyani VA and Gandhi PJ (2004) CD40 ligand: a novel target in the fight against cardiovascular disease. *The Annals of pharmacotherapy* **38**:1500-1508.
- Vitale C, Mendelsohn ME and Rosano GMC (2009) Gender differences in the cardiovascular effect of sex hormones. *Nat Rev Cardiol* **6**:532-542.

- Vivekananthan DP, Bhatt DL, Chew DP, Zidar FJ, Chan AW, Moliterno DJ, Ellis SG and Topol EJ (2004) Effect of clopidogrel pretreatment on periprocedural rise in C-reactive protein after percutaneous coronary intervention. *The American journal of cardiology* **94**:358-360.
- Vlachopoulos C, Aznaouridis K and Stefanadis C (2010) Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *Journal of the American College of Cardiology* **55**:1318-1327.
- Vogt F, Zerneck A, Beckner M, Krott N, Bosserhoff AK, Hoffmann R, Zandvoort MA, Jahnke T, Kelm M, Weber C and Blindt R (2008) Blockade of angio-associated migratory cell protein inhibits smooth muscle cell migration and neointima formation in accelerated atherosclerosis. *Journal of the American College of Cardiology* **52**:302-311.
- Wagner DA, Schultz DS, Deen WM, Young VR and Tannenbaum SR (1983) Metabolic fate of an oral dose of 15N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer research* **43**:1921-1925.
- Waksman R, Ajani AE, Gruberg L, Pinnow E, GebreEyesus A, Deible R, Satler LF, Pichard AD, Torguson R, Kent KM, Suddath W and Lindsay J (2004) Use of IIb/IIIa inhibitors in patients with in-stent restenosis treated with intracoronary gamma-radiation. Integrilin WRIST. *Catheterization and cardiovascular interventions : official journal of the Society for Cardiac Angiography & Interventions* **62**:162-166.
- Waksman R, Lipinski MJ, Acampado E, Cheng Q, Adams L, Torii S, Gai J, Torguson R, Hellenga DM, Westman PC, Joner M, Zumstein P, Kolodgie FD and Virmani R (2017) Comparison of Acute Thrombogenicity for Metallic and Polymeric

- Bioabsorbable Scaffolds: Magmaris Versus Absorb in a Porcine Arteriovenous Shunt Model. *Circulation Cardiovascular interventions* **10**.
- Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA, Freij A and Thorsen M (2009) Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *The New England journal of medicine* **361**:1045-1057.
- Walter DH, Fichtlscherer S, Sellwig M, Auch-Schwelk W, Schachinger V and Zeiher AM (2001) Preprocedural C-reactive protein levels and cardiovascular events after coronary stent implantation. *Journal of the American College of Cardiology* **37**:839-846.
- Walton G (1951) Survey of literature relating to infant methemoglobinemia due to nitrate-contaminated water. *American journal of public health and the nation's health* **41**:986-996.
- Wang X, Ouyang Y, Liu J, Zhu M, Zhao G, Bao W and Hu FB (2014) Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *BMJ (Clinical research ed)* **349**:g4490.
- Wang X OY, Liu J, Zhu M, Zhao G, Bao W, Hu FB. (2014) Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *BMJ (Clinical research ed)* **349**.
- Webb A, Bond R, McLean P, Uppal R, Benjamin N and Ahluwalia A (2004a) Reduction of nitrite to nitric oxide during ischemia protects against

- myocardial ischemia-reperfusion damage. *Proceedings of the National Academy of Sciences of the United States of America* **101**:13683-13688.
- Webb A, Bond R, McLean P, Uppal R, Benjamin N and Ahluwalia A (2004b) Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *ProcNatlAcadSciUSA* **101**:13683-13688.
- Webb AJ and Ahluwalia A (2010) *Nitrite Reduction in Ischaemia in the Cardiovascular System*, Elsevier, Los Angeles.
- Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ and Ahluwalia A (2008) Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* **51**:784-790.
- Weintraub WS (2007) The pathophysiology and burden of restenosis. *The American journal of cardiology* **100**:3k-9k.
- Weisz G, Leon MB, Holmes DR, Jr., Kereiakes DJ, Popma JJ, Teirstein PS, Cohen SA, Wang H, Cutlip DE and Moses JW (2009) Five-year follow-up after sirolimus-eluting stent implantation results of the SIRIUS (Sirolimus-Eluting Stent in De-Novo Native Coronary Lesions) Trial. *Journal of the American College of Cardiology* **53**:1488-1497.
- Wennmalm A, Benthin G and Petersson AS (1992) Dependence of the metabolism of nitric oxide (NO) in healthy human whole blood on the oxygenation of its red cell haemoglobin. *British journal of pharmacology* **106**:507-508.
- Werns SW, Walton JA, Hsia HH, Nabel EG, Sanz ML and Pitt B (1989) Evidence of endothelial dysfunction in angiographically normal coronary arteries of patients with coronary artery disease. *Circulation* **79**:287-291.

- Whelton PK, He J, Cutler JA, Brancati FL, Appel LJ, Follmann D and Klag MJ (1997) Effects of oral potassium on blood pressure. Meta-analysis of randomized controlled clinical trials. *JAMA* **277**:1624-1632.
- Wilkinson IB, Qasem A, McEniery CM, Webb DJ, Avolio AP and Cockcroft JR (2002) Nitric oxide regulates local arterial distensibility in vivo. *Circulation* **105**:213-217.
- Willerson JT and Ridker PM (2004) Inflammation as a cardiovascular risk factor. *Circulation* **109**:12-10.
- Williams B, Poulter NR, Brown MJ, Davis M, McNnes GT, Potter JF, Sever PS and Thom SM (2004) British Hypertension Society guidelines for hypertension management 2004 (BHS-IV): summary. *BMJ (Clinical research ed)* **328**:634-640.
- Williams DO, Holubkov R, Yeh W, Bourassa MG, Al-Bassam M, Block PC, Coady P, Cohen H, Cowley M, Dorros G, Faxon D, Holmes DR, Jacobs A, Kelsey SF, King SB, 3rd, Myler R, Slater J, Stanek V, Vlachos HA and Detre KM (2000) Percutaneous coronary intervention in the current era compared with 1985-1986: the National Heart, Lung, and Blood Institute Registries. *Circulation* **102**:2945-2951.
- Windecker S, Neumann FJ, Juni P, Sousa-Uva M and Falk V (2018) Considerations for the choice between coronary artery bypass grafting and percutaneous coronary intervention as revascularization strategies in major categories of patients with stable multivessel coronary artery disease: an accompanying article of the task force of the 2018 ESC/EACTS guidelines on myocardial revascularization. *European heart journal*.



- Windecker S, Remondino A, Eberli FR, Juni P, Raber L, Wenaweser P, Togni M, Billinger M, Tuller D, Seiler C, Roffi M, Corti R, Sutsch G, Maier W, Luscher T, Hess OM, Egger M and Meier B (2005) Sirolimus-eluting and paclitaxel-eluting stents for coronary revascularization. *The New England journal of medicine* **353**:653-662.
- Winter JW, Paterson S, Scobie G, Wirz A, Preston T and McColl KE (2007) N-nitrosamine generation from ingested nitrate via nitric oxide in subjects with and without gastroesophageal reflux. *Gastroenterology* **133**:164-174.
- Witkowska AM and Borawska MH (2004) Soluble intercellular adhesion molecule-1 (sICAM-1): an overview. *European cytokine network* **15**:91-98.
- Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, De Servi S, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM and Antman EM (2007) Prasugrel versus clopidogrel in patients with acute coronary syndromes. *The New England journal of medicine* **357**:2001-2015.
- Wohrle J, Hoher M, Nusser T, Hombach V and Kochs M (2003) No effect of highly dosed nitric oxide donor molsidomine on the angiographic restenosis rate after percutaneous coronary angioplasty: a randomized, placebo controlled, double-blind trial. *The Canadian journal of cardiology* **19**:495-500.
- Xia DS, Liu Y, Zhang CM, Yang SH and Wang SL (2006) Antimicrobial effect of acidified nitrate and nitrite on six common oral pathogens in vitro. *Chinese medical journal* **119**:1904-1909.

- Xu H, Manivannan A, Crane I, Dawson R and Liversidge J (2008) Critical but divergent roles for CD62L and CD44 in directing blood monocyte trafficking in vivo during inflammation. *Blood* **112**:1166-1174.
- Yabushita H, Bouma BE, Houser SL, Aretz HT, Jang IK, Schlendorf KH, Kauffman CR, Shishkov M, Kang DH, Halpern EF and Tearney GJ (2002) Characterization of human atherosclerosis by optical coherence tomography. *Circulation* **106**:1640-1645.
- Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR and Herrington DM (2009) Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation* **120**:502-509.
- Yeo EL, Sheppard JA and Feuerstein IA (1994) Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* **83**:2498-2507.
- Yonetsu T, Kim JS, Kato K, Kim SJ, Xing L, Yeh RW, Sakhuja R, McNulty I, Lee H, Zhang S, Uemura S, Yu B, Kakuta T and Jang IK (2012) Comparison of incidence and time course of neoatherosclerosis between bare metal stents and drug-eluting stents using optical coherence tomography. *The American journal of cardiology* **110**:933-939.
- Yu X, Hirono KI, Ichida F, Uese K, Rui C, Watanabe S, Watanabe K, Hashimoto I, Kumada T, Okada E, Terai M, Suzuki A and Miyawaki T (2004) Enhanced iNOS expression in leukocytes and circulating endothelial cells is associated with the progression of coronary artery lesions in acute Kawasaki disease. *Pediatric research* **55**:688-694.

- Zakynthinos E and Pappa N (2009) Inflammatory biomarkers in coronary artery disease. *Journal of cardiology* **53**:317-333.
- Zand J, Lanza F, Garg HK and Bryan NS (2011) All-natural nitrite and nitrate containing dietary supplement promotes nitric oxide production and reduces triglycerides in humans. *Nutrition Research* **31**:262-269.
- Zernecke A, Bot I, Djalali-Talab Y, Shagdarsuren E, Bidzhekov K, Meiler S, Krohn R, Schober A, Sperandio M, Soehnlein O, Bornemann J, Tacke F, Biessen EA and Weber C (2008) Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circulation research* **102**:209-217.
- Zipes D, Libby P, Bonow R and Braunwald E (2005) *The vascular biology of atherosclerosis*. , Elsevier Saunders, Philadelphia.
- Zweier JL, Wang P, Samouilov A and Kuppusamy P (1995) Enzyme-independent formation of nitric oxide in biological tissues. *Nature medicine* **1**:804-809.

## Appendix 1: Patient Information Leaflet for the NITRATE-OCT Study



### Patient Information Sheet

## **NITRATE-OCT: A randomised, double-blind, placebo-controlled study investigating the effects of dietary nitrate on vascular function, platelet reactivity and restenosis in stable angina**

### **Part 1**

#### **Invitation paragraph**

You are being invited to take part in a research study. Before deciding to take part it is important for you to understand why this research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- **Part 1 tells you the purpose of this study and what will happen if you take part**
- **Part 2 gives you more detailed information about the conduct of the study**

One of our research team will go through the information with you to make sure that you understand. Ask us if there is anything that is unclear or if you would like more information or more time.

Queen Mary University of London (QMUL) is the Sponsor of this research.

### **What is the purpose of this study?**

Nitric oxide is a gas normally produced by healthy blood vessels that plays an important role in preventing blood vessels from hardening and blocking up. This blocking up of blood vessels results in block of blood flow that ultimately causes heart attacks and strokes. Research shows that in people who have hardening of blood vessels, that their blood vessels have a reduced ability to make nitric oxide. Diets rich in fruits and vegetables reduce the risk of heart attack and strokes. Many vegetables, particularly the green leafy ones and beetroot, are rich sources of dietary nitrate. Our recent research in healthy volunteers shows that consuming dietary nitrate results in more nitric oxide being produced in the body. In this study we want to find out whether consuming nitrate in the form of beetroot juice (since beetroot juice has a high concentration of inorganic nitrate) might improve blood levels of nitrate and therefore nitric oxide in individuals with hardening of blood vessels and blockages of vessels.

## **What is the substance that is being tested?**

The test substance is inorganic nitrate. Inorganic nitrate is present in our food (especially in vegetables) and we also produce it ourselves in our body. We are able to transform inorganic nitrate, in our mouths, to inorganic nitrite, which can then be further converted to nitric oxide within our blood vessels. Inorganic nitrate is found in high concentrations within vegetables such as beetroot but also lettuce and cabbage. After you have your angioplasty and stent insertion, there is a risk of re-narrowing (known as restenosis) and sudden complete blockage (called stent thrombosis) which can result in a heart attack. Although the new drug coating on the stents are thought to cause less re-narrowing than previous generations of drug-coated stents, there is a small but significant risk that complications will still occur. We believe that the inorganic nitrate found in beetroot can reduce the amount of re-narrowing and reduce the risk of stent thrombosis by improving the degree of re-growth of coronary artery (the blood vessels providing blood to the heart) lining cells (endothelium) over the stent as well as by reducing the stickiness of blood. The degree of re-growth of coronary artery lining cells (endothelium) over the stent is a major determinant of both restenosis and stent thrombosis, and we will assess the degree of re-growth of endothelium with a technique called optical coherence tomography (OCT). OCT is a tiny device that is passed into the coronary artery and inside the stent. OCT is emerging as the new best test for imaging of stents and endothelium. It provides extremely detailed pictures of the coronary arteries and stent and in this way a clear measure of re-narrowing. We will perform OCT 6 months after your surgery.. Previous studies suggest that this is the optimal time to perform OCT evaluation of these new drug coated stents. Performing OCT means that you will need to undergo a second angiogram procedure with OCT assessment 6 months after the stent is implanted.

## **Why have I been chosen?**

You have been chosen because you have been diagnosed with stable angina and are due to undergo **planned** coronary angioplasty and stent insertion.

We are inviting potential participants by means of posters displayed in hospital departments within Barts Health NHS Trust including general outpatients, Accident and Emergency Reception in The Royal London Hospital and in King George Hospital. A general advertisement has also been placed in a London based newspaper.

This study will aim to recruit 246 patients like you who are having coronary stents inserted within Barts Health NHS trust within the next 3 years.

## **Do I have to take part?**

NO. It is up to you to decide whether or not to take part. The purpose of this information sheet is to help you make a decision. The study will also be explained to you by a member of the research team. If you decide to take part you will be asked to sign a consent form. You are free to withdraw from the study at any time without giving a reason. A decision to participate, not participate or withdraw from the study will not affect your medical treatment or planned stent insertion.

## **What will happen to me if I take part?**

This study will take place at The William Harvey Clinical Trials Centre in Barts & The London Medical School, Queen Mary University of London in Charterhouse Square and at The Barts Heart Centre, St. Bartholomew's Hospital (Barts Health NHS Trust)

and will be overseen by the Imperial Clinical Trials Unit. When you come for your first visit (Screening visit) we will explain the study to you, obtain your written consent, take a brief medical history, take some blood, collect a urine sample and do a basic physical examination. If you pass screening and you provide full consent you will then be entered for our study.

On the day of each study visit you will need to arrive in the morning after an overnight fast, so you will have to miss breakfast. You will also be asked to avoid caffeine or high nitrate-containing food or drink (such as green leafy or root vegetables or beetroot juice) for 12 hours before each study visit, and avoid taking strenuous exercise for 24 hours before the study. We will give you advice on high nitrate containing foods but this includes green leafy vegetables and processed meats such as bacon and sausages.

This study is a randomised placebo-controlled, double blind, clinical trial. This means that you will be randomly selected to receive either nitrate rich beetroot juice or a special nitrate-deplete juice as placebo. The studies are classified as “blinded” as neither you or the investigators know which type of juice you are taking. This is kept confidential until the whole study is complete. The aim of doing a study in this way is to prevent bias and ensure the results we get are as accurate as possible.

After the screening you will need to visit the Clinical Trials Unit four times in total and once to the Barts Heart Centre, St.Bartholomew’s Hospital. What will happen to you on each of these visits is detailed below:

### ***Study Visit 2***

Visit 2 will take place at a convenient time for you but not less than 1 month after successful screening and successful consent has been completed. On visit 2 you will have to fast from midnight the night before.

After you have arrived we will collect a urine and saliva sample from you, which we will freeze, for measurement at a later time of the levels of nitric oxide..

After this we will perform special techniques called pulse wave velocity and pulse wave analysis to get a measure of how stiff your arteries are. You will be lying on a couch and special cuffs placed on your brachial (arm) artery, your carotid (neck) artery and femoral (groin) artery. We will use a measuring tape to measure distances from your neck to your collar bone and from your collar bone to your belly button and from your belly button to your groin artery. You won’t be exposed as you will not be asked to undress - so don’t worry. The whole process takes around 20 minutes and doesn’t involve any needles. These cuffs will only inflate slightly and you should not feel any tightness around your neck. However, if you feel any discomfort then the cuffs will be removed immediately.

After this we will take 40 ml of blood from your arm vein (this is approximately 3 tablespoons). This shouldn’t be too uncomfortable and is not dissimilar to a routine blood test. Taking 40 ml of blood will not harm your blood count. We will use this blood to study your blood’s stickiness and measure your blood levels of nitric oxide. We will also measure the levels of a substance called thromboxane A2, and two proteins called P-selectin and CD40L which tell us about your platelets (the cells that make clots). The samples will be processed and disposed within 3 years of collection.

After this you will be free to leave but we will also offer you a light lunch of a sandwich and a drink.

You will then take the juice at home (either nitrate rich or nitrate deplete beetroot juice) and continue this every day for 6 months in total. The juice will have been couriered to your preferred address. We will provide you with a diary for you to keep a daily record of taking the juice over the 6 month period.

You will then have your scheduled balloon angioplasty and stent implantation at The Barts Heart Centre, St. Bartholomew's Hospital as planned. The trial will not affect any routine care given around the time of your coronary stent procedure. The planned angioplasty and stenting procedure will be performed as usual.

### ***Study Visit 3***

6 months  $\pm$  2 weeks after your procedure, you will return to The William Harvey Clinical Trials Unit for your third visit. During this visit we will repeat all of the things that you did on the second study visit although we won't expect you to take any further juice. This will enable us to see if the nitrate ingestion has had a beneficial effect on the stickiness of your blood and whether there has been an increase in nitrate levels in your blood.

### ***Study Visit 4***

Study visit 4 will take place at The Barts Heart Centre, St. Bartholomew's Hospital. Here, you will undergo another angiogram procedure 6 months ( $\pm$  1 month) after the stent was implanted. We will also study the stent with OCT during this procedure. We will ask you to come to the Barts Heart Centre, St. Bartholomew's Hospital for one day for this procedure to be performed. You should not need to stay overnight. The angiogram will be performed as usual with a small tube being inserted from either the wrist or top of the leg. The OCT requires a tiny wire to be advanced down the coronary artery. Over this wire the OCT probe can be advanced giving extremely detailed pictures of the stent and coronary artery. The procedure should last between 30 minutes and 1 hour. All the tubes and wires are removed at the end of the procedure. You may find the tubes being put into the wrist or leg a little uncomfortable but once the tubes are in the body you should not feel any pain.

On this visit, one of the research team will discuss with you how you have been over the six month period since the stent was implanted. This consultation should take no longer than 30 minutes.

We do not plan to use the OCT images to make any changes to your health care. However, if you have new concerning symptoms, or significant changes with your angiogram, we may organise additional cardiac tests and cardiology follow up as necessary.

## ***Study Visits 5***

At 12 months after your initial procedure, you will return to The Clinical Trials Unit for your final visit. During this visit we will repeat the urine, saliva and blood collection and the blood pressure measurements that we did on the second study visit. This will again enable us to see if the nitrate ingestion has had a beneficial effect on the stickiness of your blood, your blood vessel function and whether there has been an increase in nitrate levels in your blood.

In total, you will visit the Clinical Trials Unit four times and once to The Barts Heart Centre, St.Bartholomew's Hospital as part of the study.

Following this, you will be contacted by telephone after 1 year (after your last visit) to ask about your health.

The whole duration of the study will be 2 years in total.

### **What are the alternatives for treatment?**

If you were not taking part in the study your angioplasty and stenting procedure will proceed as normal with your Consultant Cardiologist deciding further management during your procedure as planned. Any changes to your health care will be fully discussed with you prior to anything being organised.

By agreeing to tissue storage, you will only be giving us permission to store your blood for future research of the type described above. Any other use that we may want to make of your sample in the future will require approval by a Research Ethics Committee, which is an independent panel of experts who assess all research projects for safety, ethical acceptability and who protect volunteers' interests. None of the work that we are proposing will have any direct implications for your personal health. You do not have to agree to the storage of your tissue.

### **What are the possible risks and side effects of any treatment received when taking part?**

For the blood sampling, inserting a needle in a vein causes a stinging sensation, after which blood sampling is usually painless, although there may be some mild aching from the needle. If this becomes uncomfortable the needle will be removed. Also, we will be placing a cuff around your neck, thigh and arm, although these will inflate slightly, it should not cause too much discomfort. If you feel any discomfort at any point, then these will be removed immediately.

The trial also involves a repeat coronary angiogram and OCT at 6 months, which is associated with a small risk of adverse events. This procedure requires a catheter to be introduced into the coronary artery via a small cut in the artery at the top of the leg or in the wrist. Rarely there may be injury to the artery of the leg or wrist. This occurs in < 1 in 100 procedures. Once the catheter is in the coronary artery a tiny wire is advanced down the coronary artery, over which the OCT catheter is passed. There is a risk of a serious complication (including heart attack, stroke and need for urgent heart bypass



surgery) in <1 in 200 of patients. These procedures will be undertaken by trained senior medical staff in the angiography department, where this is considered a standard procedure. During this study you will receive approximately 44% more exposure to radiation from X-rays than if you did not participate and this will be at 6 months for the OCT. It is estimated that the total dose of radiation gives an additional risk of cancer of approximately 1 in 2000. The risk from the dose additional to standard care (i.e. from the OCT at 6 months) is approximately 1 in 4000. The total dose is equivalent to about 5 years natural background radiation dose. The regulatory body (PHE Radiation Protection Division) describes this risk as low risk.

### **What do I have to do?**

We need to know whether you are taking any medication or if you have had any reactions to drugs in the past as this may exclude you from the study.

The day before your study visits, we would ask you to keep to a low nitrate diet (avoid green vegetables, beetroot and processed meats – advice will be given), avoid alcohol and caffeine, and fast after your evening meal (except water) until you are finished with us the following morning. We will provide tea/ coffee and toast at the end of each visit, or a light lunch, if you require after each study visit. You should avoid strenuous exercise on the day before the study. If you feel unwell (e.g. have a cold, fever, sore throat, headache) you should inform the researcher. If you are a blood donor you should refrain from giving blood from one week before you start the study until one week after you have finished the study.

We also ask that you avoid paracetamol and any non steroidal anti inflammatories eg. ibuprofen and diclofenac for 1 week prior to study visits. This is because these tablets can affect the way your blood cells respond. We know this can be difficult so if once you are enrolled in the study you happen to take a paracetamol tablet for example for a headache, please let us know. For example if you are due for a study visit we would simply delay the visit for a further 1 week to ensure the effect of the tablet on your body would have worn off. Please note: If you take regular aspirin or clopidogrel to reduce the stickiness of your blood that is ok.

We also need to know whether you are pregnant. Women of childbearing age in whom the possibility of being pregnant cannot be otherwise excluded must have a negative pregnancy test before their participation begins and this will be done by the investigator. Women who are not pregnant must use an effective form of barrier contraception or be on the oral contraceptive pill. If you suspect that you may have become pregnant during the study in which you are taking part, you must immediately inform the investigator who will advise and help you. If necessary, you will be referred to other clinicians and counsellors for specialist care.

You will be given either nitrate rich beetroot juice or a placebo juice (which is nitrate-deplete beetroot juice), to take during the study. You will be chosen to receive whichever juice by a random process and both you and the investigators won't know which juice you will have been consuming at each stage until the study has ended and all results analysed. This makes for a fairer and more scientific study. The ingestion of either nitrate rich or deplete (i.e. placebo) beetroot juice will take place for 6 months in total.

### **What are the benefits of taking part?**

It is possible that your blood vessel stiffness and blood stickiness may improve after you have taken the nitrate, however it is also possible that the study may be no benefit to you at all. It is hoped that the results of this and similar studies will help treat patients in the future.

Although we do not plan to use the OCT data to make changes to your health care, the repeat angiogram may give us use useful information about changes in your heart arteries over the 6 month period from the stent implantation. We may make changes to medications or organise further cardiac testing and/or cardiology follow up based upon the results of the angiogram.

### **Expenses and Payments**

A payment of up to £80 will be organised for travel expenses for visits within Greater London, on provision of receipts. Payment is by cheque (payable from Barts and the London School of Medicine and Dentistry): this may take 2-3 weeks; or may be made in cash on the day, depending on the arrangements at the time.

### **What if new information becomes available?**

Sometimes new information becomes available during the course of a research project that is directly relevant to participants within the research. If significant new information came to light that had a bearing on the study and your participation, then this information would be considered by a committee who oversee the conduct of the trial and you would be informed.

### **What happens when the research study stops?**

When the study is completed you will not be required to continue with any of the study procedures and your care will continue as usual.

### **What if there is a problem?**

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in part 2 of the patient information sheet. If you have a complaint please contact the following in the first instance: Dr Krishnaraj Rathod or Professor Amrita Ahluwalia.

### **Who will have access to the research notes?**

Only the researchers, representative of the Sponsor and regulatory bodies and a representative of the Research Ethics Committee will have access to the data collected during the study. Personal data about you will only be stored in the (paper) clinical study notes and (paper) case report form. Personal data will not be stored or transmitted electronically, and will not be used in any presentations or publications about this research, so it will not be possible for you to be identified as a result of having taken part in the study. The findings of this study will be published in a scientific journal,

which is freely available, and discussed at international scientific meetings, but again it will not be possible to identify you from these sources.

### **Will my taking part in this study be kept confidential?**

All the information that is collected about you during the course of the study will be kept strictly confidential and will be kept in a locked room and stored on a password-protected computer. Only the investigators will have access to the data. Any information about you that leaves the centre will have your name and address removed so that you cannot be recognised from it.

Your GP will be notified of any important results, namely blood pressure readings, in the study, if you agree.

#### **Contact Details:**

**If you require any further information please contact**

Dr Krishnaraj S Rathod,  
Clinical Research Fellow  
Clinical Pharmacology,  
Charterhouse Square,  
London,  
EC1M 6BQ  
*Tel:* 020 7882 5720 / 8931  
*Email:* [k.s.rathod@qmul.ac.uk](mailto:k.s.rathod@qmul.ac.uk)

Professor Amrita Ahluwalia,  
Professor of Vascular Pharmacology  
Clinical Pharmacology,  
Charterhouse Square,  
London,  
EC1M 6BQ  
*Tel:* 020 7882 8377;  
*Email:* [a.ahluwalia@qmul.ac.uk](mailto:a.ahluwalia@qmul.ac.uk)

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering taking part, please continue to read the additional information in Part 2 before making any decision.

## **Part 2**

### **What will happen if I don't want to carry on in the study?**

You are free to withdraw from the study at any time although we would be happy to assist you to stay and complete the study with us. If you become ill for any reason during the study your health would obviously come first and we may advise you to withdraw from the study. We must stress that beetroot juice or placebo juice are not known to have made anyone seriously ill.

### **What if something goes wrong?**

The chances of any harm befalling you in this study are very small. However, Queen Mary University London (QMUL) have provided indemnity/insurance against negligent harm and if you are harmed through your participation in this study, you will be entitled to compensation.

If you are harmed by taking part in this study and it is not the fault of the Investigators, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you

have been approached or treated during the course of this study, then you should first of all discuss this with Dr Rathod, Prof Mathur or Prof Ahluwalia. If you would rather discuss any problems with individuals independent of the study at QMUL / Barts Health NHS Trust you can contact Professor Mark Caulfield on 020 7882 3402.

### **Who has reviewed the study?**

This study has been reviewed by the NRES Committee London - City Road & Hampstead, South West REC Centre Research Ethics Committee.

### **Complaints**

You will always be able to contact an investigator to discuss your concerns and/or to get help:

Dr Krishnaraj S Rathod, Clinical Pharmacology, Charterhouse Square, London EC1M 6BQ

Telephone number: 020 7882 5720 / 8931; Email: [k.s.rathod@qmul.ac.uk](mailto:k.s.rathod@qmul.ac.uk)

or

Professor Amrita Ahluwalia, Clinical Pharmacology, Charterhouse Square, London EC1M 6BQ, Telephone number: 020 7882 8377; Email: [a.ahluwalia@qmul.ac.uk](mailto:a.ahluwalia@qmul.ac.uk)

or

Professor Anthony Mathur, Clinical Pharmacology, Charterhouse Square, London EC1M 6BQ, Telephone number: 020 7882 5906; Email: [a.mathur@qmul.ac.uk](mailto:a.mathur@qmul.ac.uk)

or

Imperial Clinical Trials Unit, Professor Neil Poulter, Professor of Preventative Cardiovascular Medicine, International Centre for Circula, 59/61 North Wharf Road, St Mary's Campus Telephone number: 020 7594 3446; Email: [n.poulter@imperial.ac.uk](mailto:n.poulter@imperial.ac.uk)

Alternatively, you may wish to speak someone who is independent of the study (not actively involved), but knows about the study and the techniques involved. We suggest:

The INVOLVE organisation. This is a national advisory group that supports greater public involvement in NHS, public health and social care research. INVOLVE shares knowledge and learning on public involvement in research. They can be approached via their website:

<http://www.invo.org.uk/>

### **What happens to the results of the research study?**

The results from each volunteer will be pooled to obtain average results, so that no individual or their results will be identifiable in any form of publication. The results from the research will be presented as a talk (or poster) at a local meeting within the University of London, or a national meeting, or possibly a European or international meeting. The results will also be written up as a full paper and be submitted for publication in an international journal. These events will most likely occur 2-3 years after your participation in the study. If you would like a copy of the paper you can contact the researcher to send you one when it becomes available.

**Who is organising and funding the research?**

The research is being organised by the Centre of Clinical Pharmacology of Queen Mary University London and Sponsored by Queen Mary University of London. It is being performed by doctors at the Barts Health NHS Trust who have time allocated to undertake research at The William Harvey Research Institute which is part of Queen Mary University of London. The study is funded by the National Institute for Health Research.

None of the doctors will be paid for including you in this study.

Thank you for taking the time to read this patient information sheet. If you decide to take part we will ask you to sign a consent form. You will be given a copy of the signed consent form and the patient information sheet.

**Thank you for taking the time to read this. Please ask if there are any questions.**