

Biomarkers of COX-2 Inhibition and NSAID Cardiovascular Side Effects

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Declaration

I, Dr Ricky Vaja declare that the work presented in this thesis is my own. Information obtained from other sources and work conducted in collaboration has been appropriately acknowledged or cited.

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Dedication

I dedicate this thesis to my family, who have been integral to my success from the beginning.

First and foremost my parents Geeta and Karata Vaja who have made sacrifices that have enabled me to persevere, for that I am forever grateful. Thank you.

My Grandparents; Ishwarlal, Shantaben and Charlie Vaja, Babubhai and Ratanben Sudra and my uncle Kishore Vaja who have been pillars of support throughout my life.

Finally my siblings Nikita, Milan, Rishi and Riva Vaja who have had to put up with me and my harmonica over the years.

Abstract

Non-steroidal anti-inflammatory drugs, which work by blocking cyclooxygenase-2 (COX-2) can increase a person's risk of having a heart attack or stroke by around 30% after just 1-2 weeks of use. After more than 20 years of research in this field and despite being one the most commonly used medications world-wide, we still do not fully understand how COX-2 protects the cardiovascular system. In this thesis, I performed a multi-omic analysis of serum samples from healthy volunteers taking the COX-2 inhibitor celecoxib and linked these to cardiovascular and renal physiological endpoints measured in parallel. I also performed a similar multi-omic analysis of samples from patients having had cardiovascular events versus matched case controls taken from the SCOT (Standard Care versus Celecoxib Outcome Trial) biobank.

The key novel findings within this thesis include:

- I. In a double blind, placebo controlled, randomised trial in healthy volunteers, 7 days of NSAID (celecoxib) use was associated with a significant increase in augmentation index (surrogate for vascular stiffness). This was linked to changes in serum protein markers relating to the extracellular matrix (ECM) as well a reduction in arginine/ADMA ratio.
- II. Similar changes in ECM proteins were also seen in serum samples from real world NSAID users who go onto have a cardiovascular event within 1 year.
- III. GDF-15 was identified as a candidate biomarker to predict early NSAID cardiovascular events. What makes this a remarkable discovery is that GDF-15 is induced by NSAIDs and provides a novel mechanism for NSAID cardiovascular events.

Using a multi-omics approach has provided novel mechanistic insight into how COX-2 protects the cardiovascular system and has highlighted several key areas for future research in this field.

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Contents

Biomarkers of COX-2 Inhibition and NSAID Cardiovascular Side Effects.....	1
Declaration	2
Copyright Declaration	2
Dedication	3
Abstract	4
Acknowledgements	5
Contents	6
List of Figures	12
Chapter 1: Introduction	19
1.1 Prostaglandins	20
1.2 Cyclooxygenase.....	20
1.3 Prostacyclin and thromboxane.....	22
1.4 Non-steroidal anti-inflammatory drugs	23
1.4.1 Cardiovascular Safety of Celecoxib, Naproxen or Ibuprofen for Arthritis (PRECISION trial).....	24
1.4.2 Standard Care vs Celecoxib Outcome Trial (SCOT)	25
1.4.3 NSAIDs as a chemo-preventative agent.....	26
1.4.4 Meta-analysis.....	26
1.4.5 Scale of the problem	27
1.4.6 Variation in practice	28
1.5 How does COX-2 protect the cardiovascular system?	28
1.6 Biomarkers in cardiovascular disease.....	30
1.7 Promising biomarkers of NSAID cardiovascular events	30
1.7.1 Methylarginines.....	31
1.8 ‘Omic biomarker technology and application in NSAID use	33
1.8.1 Genome wide association study (GWAS)	33
1.8.2 Transcriptomics	34
1.8.3 Proteomics.....	35
1.8.4 Metabolomics.....	38
1.9 The endothelium	40

1.10 Endothelial dependant dilatation	42
1.10.1 Intra-vascular infusion.....	42
1.10.2 Post-occlusive reactive hyperaemia; flow mediated dilatation	42
1.10.3 Peripheral arterial tonometry (PAT)- EndoPAT 2000 ®	42
1.10.4 Other techniques	43
1.11 NSAIDs and Endothelial Function	43
1.12 Summary.....	51
1.13 Hypothesis and Aims	51
Chapter 2: General Methods	52
2.1 Animal Studies	53
2.1.1 Ethics	53
2.1.2 Cyclooxygenase-2 (COX-2) wild type and knockout (KO) mice.....	53
2.1.3 Prostacyclin synthase (PGIS) wild type and knockout mice	53
2.1.4 Proprotein convertase subtilisin/kexin type 9 (PCSK9) mice	53
2.2 Clinical Studies	53
2.2.1 ASCENT	53
2.2.2 SCOT Samples	53
2.3 Cell Culture	54
2.3.1 COX-2 inhibition Bioassay	54
2.3.2 Bioassay- Kidney Organ Culture.....	55
2.4 ELISAs	55
2.4.1 ADMA/ L-Arginine	56
2.4.2 IL-6	57
2.4.3 Endothelin-1.....	58
2.4.4 MCP-1.....	59
2.4.5 Cystatin C	60
2.4.6 Urea	60
2.4.7 PGE ₂	61
2.4.8 PGI ₂	61
2.4.9 MultiPlex assays	61
2.5 Transcriptomics.....	64
2.5.1 RNA isolation and purification.....	64
2.5.2 RNAseq and data analysis.....	64
2.6 Proteomics	66

2.6.1 Sequential window acquisition of all theoretical mass spectra (SWATH-MS)	66
2.6.2 Olink	66
2.6.1 Metabolomics- amine and methylarginine quantification using UHPLC/MS/MS	67
2.7 Data analysis	68
Chapter 3: Effect of Global Genetic Loss of COX-2 Pathways on Biomarker Identification and Mechanistic Insights in Mouse and Human Models	69
3.1 Rationale	70
3.1.1 Hypothesis	71
3.1.2 Declaration	71
3.2 Methods	72
3.3 Results	72
3.3.1 Global COX-2 KO mice- Amines	72
3.3.2 PGIS KO Mice- Amines	75
3.3.3 Link with renal function	76
3.4 Summary	81
3.4.1 Limitations	81
3.4.2 Future Studies	81
Chapter 4: The Effects of Celecoxib on Cardiovascular Biomarkers in Healthy Volunteers; A Double Blind, Placebo Controlled Randomised Trial	83
4.3 Rationale	84
4.3.1 Hypothesis	85
4.4 Methods	85
4.4.1 Study team and roles	85
4.4.2 Population	86
4.4.3 Interventions	88
4.4.4 Comparator- Placebo	89
4.4.5 Outcomes	89
4.4.6 Study Design	89
4.4.7 Sample size calculation and interim analysis	91
4.4.8 Randomisation, allocation concealment and blinding-	91
4.5 Results	92
4.5.1 Interim analysis- primary outcome (endothelial function)	95

4.5.2 Secondary outcomes	98
4.5.3 Compliance.....	104
4.5.4 Reproducibility of EndoPAT readings.	105
4.5.5 Correlation of reactive hyperaemia and augmentation Index.....	106
4.6 Summary.....	106
4.6.1 Limitations.....	107
4.6.2 Future work.....	109
Chapter 5: COX-2 Inhibition in Healthy Volunteers and ‘Omic’ Biomarkers	110
5.3 Rationale.....	111
5.3.1 Hypothesis.....	111
5.4 Methods	112
5.5 Results	113
5.5.1 Methylarginines and amines measured using mass spectrometry.....	113
5.5.2 Endothelial, Inflammatory and Thrombosis Markers.....	116
5.5.3 Whole Blood Transcriptome Using RNASeq.....	118
5.5.4 Targeted Proteomic analysis using Olink.....	124
5.6 Summary.....	132
5.6.1 Limitations.....	132
5.6.2 Future Work	133
Chapter 6: Clinical Drivers of Cardiovascular Events Amongst NSAID users	134
6.3 Rationale.....	135
6.3.1 Hypothesis.....	136
6.4 Methods	136
6.4.1 Exploratory analysis using the SCOT dataset.....	136
6.5 Results	140
6.5.1 Exploratory analysis using the SCOT dataset.....	140
6.5.2 Matching SCOT samples	151
6.6 Summary.....	156
6.6.1 Limitations.....	157
6.6.2 Future Work	157
Chapter 7: Cardiovascular Biomarker Identification in the SCOT Trial Biobank (SCOT-1).....	159
7.3 Rationale.....	160

7.3.1 Hypothesis	160
7.4 Methods	161
7.5 Results	161
7.5.1 Amines and methylarginines assessed using Mass spectrometry	161
7.5.2 Renal and Thrombosis Markers Measured Using Multiplex.....	168
7.5.3 Analytes measured using ELISA	170
7.5.4 Proteomics using MS SWATH	174
7.5.5 Subgroup Analysis of Early Events.....	180
7.6 Summary.....	183
7.6.1 Limitations.....	184
7.6.2 Future Work.....	185
Chapter 8: Biomarkers of Early Events in the SCOT Cohort (SCOT-2)	186
8.1 Rationale.....	187
8.1.1 Hypothesis	187
8.2 Methods	188
8.2.1 Designing the new study (SCOT-2)	188
8.2.2 Planning experiments and aliquoting	190
8.3 Results	192
8.3.1 Amines and methylarginines assessed using Mass spectrometry.	192
8.3.2 Proteomics measured using O link	196
8.3.3 Biomarkers of Cardiovascular Events.....	208
8.4 Summary.....	215
8.4.1 Limitations.....	215
8.4.2 Future Work	216
Chapter 9: Discussion	217
9.1 Discussion.....	218
9.1.1 COX-2 Methylarginine Axis and Renal Function in Extreme Models	219
9.1.2 Effects of Celecoxib on Endothelial Function in Healthy Volunteers.....	219
9.1.3 Effects of Celecoxib on Cardiovascular Biomarkers in Healthy Volunteers	220
9.1.4 Clinical Predictors of Cardiovascular Events Amongst NSAID Users.	222
9.1.5 Biomarkers of NSAID Cardiovascular Events	225
9.2 Priority Areas for Future Research.....	227
9.3 General Conclusions.....	227

References	229
Appendix 1- Example Standard Curves Multiplex Assay.....	247
Appendix 2- List of Proteins Measured using Olink.....	249
Appendix 3- Example Standard Curves for ADMA and SDMA Measured using UHPLC/MS/MS.....	252
Appendix 4- SAMURAI Study Protocol	254
Appendix 5- Raw Concentrations for Analytes Measured in Chapter 3	269
Appendix 6- Raw Concentrations for Analytes measured in Chapter 7	273
Appendix 7- Raw Concentrations for Analytes Measured in Chapter 8 (1-year events)	276
Appendix 8- Raw Concentrations for Analytes Measured in Chapter 8 (6-month subgroup).....	279

List of Figures

Figure 1.1 Downstream Pathways of Cyclooxygenase Activity.	22
Figure 1.2 Endothelial Nitric Oxide Pathway Endothelial nitric oxide synthase (eNOS) acts on the pre-cursor L-arginine to produce nitric oxide and L-citrulline.....	31
Figure 1.3. Publications using Proteomics & NSAIDs..	38
Figure 1.4. Publications using Metabolomics & NSAIDs..	40
Figure 1.5. Studies that demonstrate NSAIDs reduce endothelial function.	48
Figure 1.6. Studies that demonstrate NSAIDs either reduce or have no effect on endothelial function.	50
Figure 2.1 Standard curves for the L-arginine and ADMA ELISA.....	57
Figure 2.2 Standard curve for the IL-6 ELISA.	58
Figure 2.3 Standard curve for the ET-1 ELISA.....	59
Figure 2.4 Standard curve for the MCP-1 ELISA.	60
Figure 2.5 Principles of MultiPlex assay.....	62
Figure 2.6 Table of analytes and top standard concentrations.....	63
Figure 2.7 Table showing the RNA concentration (ng/ml) and the quality of the RNA (ratio of the absorbance at 260/280) following RNA extraction from whole blood from ASCENT volunteers.	65
Figure 2.8 Table summarising the interpretation of Cohen's D.	68
Figure 3.1 PCA plot demonstrating batch effect.	73
Figure 3.2 Quantification of amines and methylarginines in COX-2 knockout (KO) mice using UHPLC/MS/MS.	74
Figure 3.3 Figure 3.3 Quantification of amines and methylarginines in prostacyclin synthase (PGIS) knockout (KO) mice using UHPLC/MS/MS.	76
Figure 3.4 Correlation between renal function and plasma methylarginines in COX-2 and PGIS knockout mice.....	77
Figure 3.5 Correlation between renal function (urea) and plasma methylarginines in COX-2 and PGIS knockout mice.	78

Figure 3.6 Correlation between renal function (cystatin c) and plasma methylarginines in PLA ₂ deficient patient before and after renal transplant.	78
Figure 3.7 Correlation between renal function (urea) and plasma methylarginines in a PLA ₂ deficient patient before and after renal transplant.	79
Figure 3.8 ADMA (a) and prostacyclin (b) release by organotypic kidney slices from wild type (WT) and COX-2 Knockout (KO) mice.	80
Figure 4.1 Inclusion & exclusion criteria for the ASCENT trial.....	86
Figure 4.2 ASCENT trial flow diagram.....	90
Figure 4.3 Label used for dispensing the study medication.....	92
Figure 4.4 ASCENT Trial CONSORT Flow Diagram.....	93
Figure 4.5 Monthly Recruitment Figures.	94
Figure 4.6 Baseline Characteristics Table.....	94
Figure 4.7 Scatter Plot showing the difference in endothelial readouts between celecoxib and placebo group.	95
Figure 4.8 Post hoc power calculations and estimated effect size.	96
Figure 4.9. Scatterplot showing the difference in blood pressure readouts between visit 2 and visit 1.....	98
Figure 4.10 Graph showing the difference in blood pressure readout over time.	99
Figure 4.11. Scatterplot showing the difference in heart rate between visit 2 and visit 1.....	100
Figure 4.12 Scatterplot showing the difference in serum biochemistry readings between visit 2 and visit 1.	101
Figure 4.13 Scatterplot showing the difference in serum haematology readings between visit 2 and visit 1.	102
Figure 4.14 Scatterplot showing the difference in fasting serum lipids between visit 2 and visit 1.....	103
Figure 4.15 Scatterplot showing the difference in clotting parameters between visit 2 and visit 1.....	103
Figure 4.16 Scatterplot showing the difference in Prostaglandin E ₂ (PGE ₂) production from J774 cultures in plasma from participants.....	104

Figure 4.17 . Scatterplot showing the correlation of EndoPAT readouts between visit 0 (screening) and visit 1.	105
Figure 4.18 Cochrane Risk of Bias summary for the ASCENT Trial.....	108
Figure 5.1 Principal Component Analysis Plot (PCA).....	113
Figure 5.2 Scatter Plot of Nitric Oxide Amines.	114
Figure 5.3 Heatmap of analytes with corresponding P-values.	115
Figure 5.4 Scatter Plot of 4-hydroxyproline.	116
Figure 5.5 Scatter Plot of endothelin-1 (ET-1) and monocyte chemoattractant protein-1 (MCP-1).....	116
Figure 5.6. Scatter Plot of thrombosis markers measured using Multiplex assay.	117
Figure 5.7 Principal Component Analysis Plot (PCA) of whole blood transcriptome measured using RNASeq.....	118
Figure 5.8 (A) Scatter Plot of top 9 genes at a threshold of $p < 0.05$ and fold change more than ± 1.5 and (B) corresponding table summarising the P-value, Q Value and fold change.....	121
Figure 5.9 Volcano plots showing transcripts that are significantly different between celecoxib visit 1 and visit 2.	122
Figure 5.10 (A) Enrichment plot and table summarising the top 10 gene sets (B).	124
Figure 5.11 Principal Component Analysis Plot (PCA).....	124
Figure 5.12 (A) Volcano plot showing proteins that are significantly different between celecoxib and placebo group and (b) table showing the Q values and fold change for the 21 proteins.	125
Figure 5.13 . Scatter Plot of 21 proteins that were significantly different between celecoxib and placebo group.....	127
Figure 5.14 Top 10 pathways identified using Over-representation analysis in REACTOME.....	128
Figure 5.15 Heatmaps of top 21 proteins that were significantly different between celecoxib and placebo.....	128
Figure 5.16 (a) Table summarising the correlations between delta protein and delta augmentation index with Scatterplots for the 3 significant correlations (b).	131
Figure 6.1 Directed Acyclic Graph assessing causal and biasing relationships between clinical predictors of cardiovascular events within the SCOT dataset.	139

Figure 6.2 Baseline characteristics table comparing cases (cardiovascular event) and controls (no event).....	142
Figure 6.3 Factors associated with a cardiovascular event using binary logistic regression.	144
Figure 6.4 Kaplan-Meier survival curves showing the impact of clinical variables on the occurrence of a cardiovascular event along with a number at risk table.	150
Figure 6.5 Factors associated with a cardiovascular event Cox regression.	151
Figure 6.6 Baseline characteristics table comparing cases (cardiovascular event) and controls (no event).....	154
Figure 6.7 Table of prioritisation of experiments with the volume of sample required.	155
Figure 6.8 Example plate layout for case control study.	156
Figure 6.9 Table of prioritisation of experiments with the volume and number of samples per experiment.	156
Figure 7.1 Principal Component Analysis Plot (PCA).....	162
Figure 7.2 Principal Component Analysis Plot (PCA).....	163
Figure 7.3 Loadings Plot.	163
Figure 7.4 Scatter Plot of Nitric Oxide Amines.	164
Figure 7.5 Table Summarising the difference in amines between the event and control group.	165
Figure 7.6 Correlation Matrix of Amines.	166
Figure 7.7 Amines associated with an increased odds of a cardiovascular event using binary logistic regression.	167
Figure 7.8 Scatter plot showing differences in renal markers between event and control group.	168
Figure 7.9 Scatter plot showing differences in thrombosis markers between event and control group.	169
Figure 7.10 Differences in ADMA and Arginine between groups assessed using ELISA.	170
Figure 7.11 11 Differences in (a) ET-1, (b) IL-6 and (c) MCP-1 between groups assessed using ELISA..	171

Figure 7.12 . Scatterplot of differences in cytokine levels between parecoxib treated and control mice in an atherosclerotic model.	173
Figure 7.13 Principal Component Analysis plot.	174
Figure 7.14 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups.	175
Figure 7.15 Principal Component Analysis plot.	175
Figure 7.16 Principal Component Analysis plot.	176
Figure 7.17 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after removing the Alfie instrument.	176
Figure 7.18 Principal Component Analysis plot after removing the Alfie and SadCafe samples.	177
Figure 7.19 Figure 7.19. Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after removing the Alfie and SadCafe instruments.	177
Figure 7.20 Principal Component Analysis plot after correcting for batch effect. ...	178
Figure 7.21 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after correcting for batch effect.	178
Figure 7.22 Principal Component Analysis plot after correcting for batch effect and removing the SadCafe instrument.	179
Figure 7.23 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after correcting for batch effect & removing SadCafe instrument.	179
Figure 7.24 Subgroup analysis assessing differences in amines in earlier events vs controls.	181
Figure 7.25 Graph showing ADMA levels as non-cumulative events over time using Mass spectrometry (a) and ELISA (b).	181
Figure 7.26 Subgroup analysis assessing differences in analytes in earlier events vs controls.	182
Figure 7.27 Principal Component Analysis plot.	182
Figure 7.28 Volcano plot (a) and table (b) showing the top proteins that are significantly different between events at 6 months vs controls.	183
Figure 8.1 . Baseline characteristics table comparing cases (cardiovascular event) and controls (no event).	190

Figure 8.2 Example randomised plate layout and aliquots for experiments.	191
Figure 8.3 Principal Component Analysis Plot (PCA).	192
Figure 8.4 Scatter Plot of Nitric Oxide Amines.	193
Figure 8.5 QQ plots for ADMA and SDMA.	194
Figure 8.6 Principal Component Analysis Plot (PCA).	194
Figure 8.7 Scatter Plot of Nitric Oxide Amines.	195
Figure 8.8 Principal Component Analysis Plot (PCA).	196
Figure 8.9 Volcano plots showing proteins that are significantly different between event and non-event groups before and after false discovery rate (FDR) correction.	197
Figure 8.10 Table showing proteins that are different between 12-month events and matched controls with fold change.	198
Figure 8.11 Scatter plot of top 20 significantly changed proteins between events at 12 months and matched controls.	200
Figure 8.12 Top 10 pathways identified in 1 year event group using Over-representation analysis in REACTOME.	200
Figure 8.13 Heatmaps of top 36 proteins in events at 1 year grouped according to pathways highlighted in REACTOME.	201
Figure 8.14 Venn diagram showing overlap in significant proteins and methylarginines between ASCENT and SCOT-2 study.	203
Figure 8.15 Principal Component Analysis Plot (PCA).	204
Figure 8.16 Volcano plots showing proteins that are significantly different between 6-month event and non-event groups before and after false discovery rate (FDR) correction.	205
Figure 8.17 Table showing proteins that are different between 6-month events and matched controls with fold change.	206
Figure 8.18 Top 10 pathways identified in 6-month event group using Over-representation analysis in REACTOME.	207
Figure 8.19 Heatmaps of top 28 proteins in events at 6 Months year grouped according to pathways highlighted in REACTOME.	207
Figure 8.20 Venn diagram showing overlap in significant proteins at 6 months and 1 year.	208

Figure 8.21 Receiver Operating Characteristic (ROC) Curves (A.) and table of Area Under Curve (AUC) analysis (B.) for 10 proteins used to predict cardiovascular events within 1 year.....	210
Figure 8.22 Proteins associated with a cardiovascular event within 1 year using binary logistic regression.....	211
Figure 8.23 Receiver Operating Characteristic (ROC) Curves and table of Area Under Curve (AUC) analysis for GDF15 +SSC4D score to predict events within 1 year.	211
Figure 8.24 Receiver Operating Characteristic (ROC) Curves and table of Area Under Curve (AUC) analysis for GDF15 + SSC4D score to predict cardiovascular events within 6 months.	212
Figure 8.25 Factors associated with a cardiovascular event using binary logistic regression.	213
Figure 8.26 Receiver Operating Characteristic (ROC) Curve and table of Area Under Curve (AUC) analysis for clinical risk score to predict cardiovascular events in NSAID users.	214
Figure 8.27 Receiver Operating Characteristic (ROC) Curve and table of Area Under Curve (AUC) analysis for clinical risk score to predict cardiovascular events in NSAID users.	214

Chapter 1: Introduction

1.1 Prostaglandins

Prostaglandins are a group of fatty acids that exert a diverse range of physiological effects and are found in almost all human and animal tissues. They were first discovered in 1935 by Ulf von Euler who isolated them from ram seminal fluid. [1]. In early experiments using a more purified form of prostaglandins and in vitro smooth muscle bioassay systems, dramatic effects were observed including contraction of uterine, placental and intestine preparations and vasodilation of systemic and pulmonary vessels [2]. It was initially thought that prostaglandins were only produced by the seminal vesicles, however, it is now known that prostaglandins are produced by most tissues including blood vessels[3]. In the early 1960's, work by Bergstrom and Van Dorp identified prostaglandin precursors such as arachidonic acid and dihomo- γ -linolenic acid (DGLA) which are unsaturated fats[2]. We now know that prostaglandins, thromboxanes and prostacyclins form a subclass of physiologically active compounds called prostanoids. These belong to the eicosanoid family and are catalysed by cyclooxygenase (COX).

Arachidonic acid is itself highly reactive and extremely sensitive to oxidation and is therefore not normally free in cells. Instead it is stored in phospholipid membranes and is released by the actions of phospholipase A₂ (PLA₂)[3]. Although PLA₂ has multiple isoforms, cPLA₂ α is the isoform that is mainly responsible for arachidonic acid release in blood vessels, immune cells and platelets [4].

Arachidonic acid is a pre-cursor for two main pathways

- 1) The COX pathway which is responsible for the formation of prostanoids
- 2) The lipoxygenase pathway which is responsible for the formation of leukotrienes

The focus of this thesis will be on the COX pathway.

1.2 Cyclooxygenase

COX, also known as prostaglandin-endoperoxide synthase (PTGS), is present in two isoforms, both of which are involved in the formation of prostanoids. COX was first isolated from sheep vesicular glands in 1977 by Hemler & Lands and identified as a haem protein which we now know as COX-1 [5]. This isoform was subsequently

sequenced by DeWitt & Smith in 1988 [6] which ultimately laid the foundations for the discovery of COX-2 a year later[7-9]. Early experiments demonstrated that an inflammatory stimulus was required to induce the COX-2 isoform in tracheal epithelial cells, mouse macrophages and chick fibroblasts [2]. Sequencing of COX-2 revealed transcription pathways including cAMP response element-binding protein (CREB) and NF- κ B, nuclear factor of activated T cells (NFAT) [3]. This led to the hypothesis that COX-1 is constitutively expressed, and COX-2 is an inducible form found at the site of inflammation. We now understand that COX-2 is also constitutively expressed in a variety of organs including the kidneys, lung, brain and thymus[10].

Both COX-1 and COX-2 convert arachidonic acid into prostaglandin H_2 (PGH₂). Although the COX isoenzymes play a role in prostanoid synthesis, the prostanoid produced by a cell is influenced by downstream enzymes[11] **Figure 1.1**. These include PGF synthase (PGFS), microsomal PGE synthases (mPGES)-1 and -2, cytosolic PGE synthase (cPGES), prostacyclin synthase (PGIS), Thromboxane synthase (TXAS), lipocalin-type PGD synthase (LPGDS) and haematopoietic-type PGD synthase (HPGDS)[3]. Whilst other prostanoids exert cardiovascular effects [12], as discussed below, prostacyclin and thromboxane are considered to be central to cardiovascular health and disease.

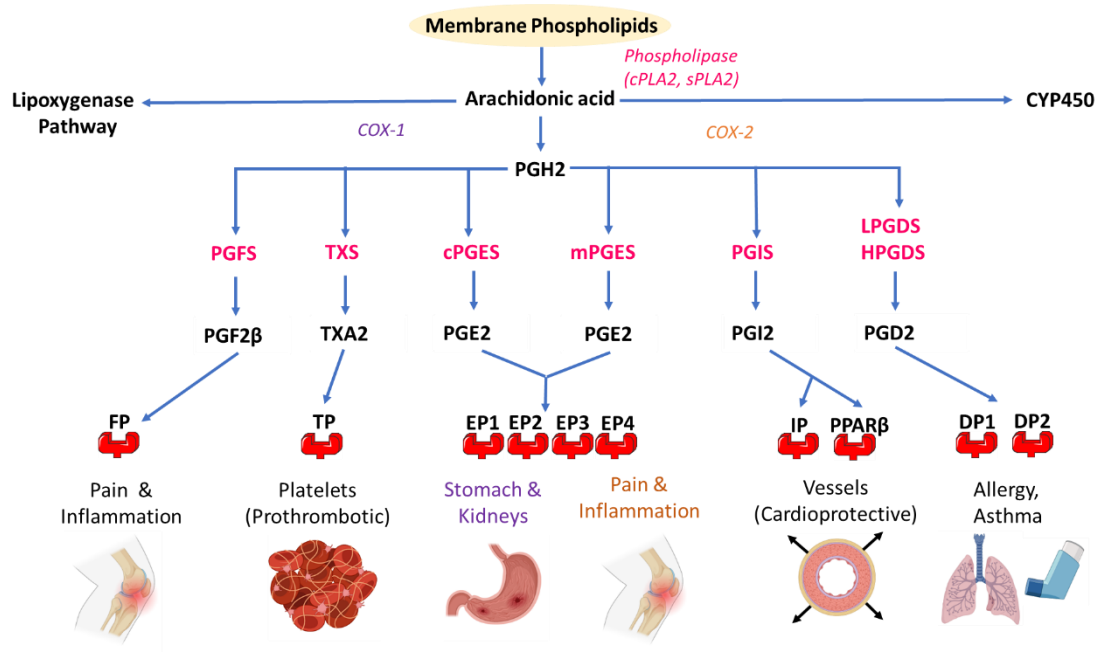


Figure 1.1 Downstream Pathways of Cyclooxygenase Activity. Phospholipase acts on membrane phospholipids to release arachidonic acid which is a precursor for the cyclooxygenase pathway. Both COX-1 & 2 convert arachidonic acid into prostaglandin H₂ (PGH₂). Prostanoids produced by cells are influenced by downstream enzymes. These include PGF synthase (PGFS), microsomal PGE synthases (mPGES)-1 and -2, cytosolic PGE synthase (cPGES), prostacyclin synthase (PGIS), Thromboxane synthase (TXAS), lipocalin-type PGD synthase (LPGDS) and haematopoietic-type PGD synthase (HPGDS). Created with Biorender.com

1.3 Prostacyclin and thromboxane

Prostacyclin was discovered by John Vane which together with his elucidation of the mechanism of action of NSAIDs was the basis of his Nobel prize in 1982[3]. Prostacyclin is now recognised as a cardioprotective hormone exerting effects on vasodilation and inhibition of platelet aggregation as well as limiting lipid uptake[13] and providing cytoprotection[14]. Blood vessels contain large amounts of prostacyclin synthase (PGIS) and COX so it is unsurprising that prostacyclin is predominantly released[3]. The importance of PGIS in cardiovascular protection can be demonstrated in PGIS knockout mice that develop renal dysfunction, hypertension and vascular hypertrophy[15]. This is further supported by evidence from patients who have polymorphisms and mutations in PGIS who have an increased risk of heart attacks and strokes as well as higher blood pressure[16].

Prostacyclin acts on the cell surface GPCR IP receptors and the cytosolic nuclear receptor PPAR β to exert both acute signalling events as well as alterations in gene

expression [17]. IP receptor activation results in downstream conversion of ATP to cAMP through activation of membrane bound adenylate cyclase. cAMP activates PKA resulting in reduction of intracellular calcium, increased levels of which is required for both vasoconstriction and inhibition of platelet aggregation. Activation of PPAR β can also explain some of the acute effects of prostacyclin seen in blood vessels and platelets[18-20].

In addition to the acute effects, activation of both IP (cAMP response element binding protein; CREB) and PPAR β (via well described signalling pathways[3]) receptors can also regulate gene expression.

Thromboxane (TX) is synthesised in platelets by the actions of COX-1 and thromboxane synthase and is prothrombotic, proangiogenic and a vasoconstrictor. It exerts its effects by binding to the thromboxane receptor (TP). TP receptors are membrane bound G protein-coupled receptors (GPCR) and are expressed on numerous cell types including macrophages and vascular endothelial cells[21]. Following activation, TP is no longer able to further stimulate cell function or mobilise G protein targets. This is due to a process called homologous desensitisation in addition TP, can undergo heterologous desensitisation by activation of IP receptor[22]. These mechanisms help limit the potentially harmful cardiovascular effects of thromboxane.

Since prostacyclin and thromboxane have opposing effects, they are considered to be in balance within a healthy cardiovascular system. The key role of thromboxane in thrombosis can be highlighted by the effects of low dose aspirin which exerts its effects by selectively inhibiting COX-1 derived thromboxane[23], thereby tipping the balance in favour of prostacyclin.

1.4 Non-steroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) have both analgesic and anti-inflammatory properties and inhibit both COX-1 and COX-2. It was initially believed that the therapeutic benefits of NSAIDs were due to COX-2 inhibition and the primary side effects of concern at the time (of COX-2 discovery); gastrointestinal toxicity, were due to inhibition of COX-1. This led to the hypothesis that COX-2 could be a potential therapeutic target for a new class of drugs [24, 25]. Selective COX-2 inhibitors (Coxibs)

such as celecoxib, rofecoxib and etoricoxib were subsequently developed and introduced to the clinic. However, it soon became apparent that their use was associated with an increase in cardiovascular events and in 2004 Rofecoxib was withdrawn following the release of data confirming increased cardiovascular events seen in a randomised placebo-controlled trial[26]. Valdecoxib was withdrawn in 2005 due to serious concerns of increased cardiovascular events seen in a study in patients following cardiac surgery[27, 28]. Lumiracoxib was withdrawn in 2007 by the European Medicines Agency (EMA) due to liver toxicity, after which the drug was not able to gain FDA approval.

It was initially thought that the cardiovascular side effects were limited to selective COX-2 inhibitors. However, as a result of subsequent epidemiology analyses[29, 30] and the publication of two large clinical cardiovascular outcome studies, SCOT[31] and PRECISION [32], it is now clear that traditional NSAIDs including naproxen and ibuprofen may carry at least as great a risk of cardiovascular and renal events as selective COX-2 inhibitors.

1.4.1 Cardiovascular Safety of Celecoxib, Naproxen or Ibuprofen for Arthritis (PRECISION trial)

The Cardiovascular Safety of Celecoxib, Naproxen, or Ibuprofen for Arthritis (PRECISION)[32] trial was a landmark trial comparing the cardiovascular safety of celecoxib versus non-selective NSAIDs. This was a multi-centre, double blind, non-inferiority RCT in adult patients with either rheumatoid or osteoarthritis requiring daily NSAID use. A key inclusion criterion was patients who were at an increased cardiovascular risk. Patients were randomly allocated to receive either celecoxib 100mg BD, Ibuprofen 600mg TDS, Naproxen 375 BD with matching placebo. The clinicians also had the option of increasing the doses to; celecoxib 200mg BD (in patients with rheumatoid arthritis only), ibuprofen 800mg TDS and Naproxen 500mg BD. The primary composite outcome was a composite of cardiovascular death, myocardial infarction and stroke. There were 24,081 participants randomised with a mean follow up of 34+-13.4 months. In both the intention to treat and on-treatment analysis, celecoxib was found to be non-inferior to non-selective NSAIDs in relation to cardiovascular events. There were also fewer gastrointestinal and renal events with celecoxib. Whilst this was a landmark paper, there was a lot of criticism and

controversy. The majority of patients had osteoarthritis and could be deemed low cardiovascular risk as the event rate was low at ~1% per year and only ~50% were on aspirin or statin therapy. Due to the low event rate, the upper bound for non-inferiority was changed during the study from 1.3 to 1.4 with a power of ~80% whilst previous studies including patient level meta-analysis of RCTs showed a relative risk of a cardiovascular event with celecoxib was 1.37. The second major concern was compliance and dropout as approximately 60% of participants had stopped taking their allocated drug by the end of the study and ~30% were lost to follow up. The final and perhaps most important criticism relates to the dosing and whether adequate levels were obtained. Due to prescribing restrictions, dose escalation was only possible in patients with rheumatoid arthritis and the average dose of celecoxib was ~209mg/day which could be deemed to be in the lower therapeutic range. In previous RCTs at this dose, the relative risk is ~0.95 which is comparable to placebo. In contrast, higher doses of 400 and 800mg/day were associated with relative risks of 1.29 and 2.96 respectively [33].

1.4.2 Standard Care vs Celecoxib Outcome Trial (SCOT)

SCOT was a prospective, multi-centre, randomized, open-label trial comparing the gastrointestinal and cardiovascular safety of prescribed NSAIDs with switching to celecoxib in patients with rheumatoid or osteoarthritis. In contrast to PRECISION, the population of interest was those with a low cardiovascular risk. Similar to PRECISION, the primary outcome was a composite of cardiovascular death, myocardial infarction and stroke. In the original power calculation, 13,682 patients needed to be followed up for 2 years and with an anticipated event rate ~2%/year, 611 patients who reached the primary endpoint were required. Within the study there were 7,297 patients enrolled, of which, 249 patients had a cardiovascular event which was lower than expected; observed: 0.9% vs expected 2%. The follow up period was therefore increased to account for the low event rate. Furthermore, the dosing data was only available for Scottish patients and the mean dose of NSAIDs per day were 170mg for celecoxib, 676 for ibuprofen, 581 for naproxen and 79mg for diclofenac. At the time of screening, participants had serum samples taken and bio-banked for future research. Within this thesis I have used the SCOT serum biobank to investigate biomarkers that could potentially 'predict' cardiovascular events in NSAID users.

1.4.3 NSAIDs as a chemo-preventative agent

In addition to their anti-inflammatory properties, NSAIDs have also demonstrated a chemo-preventative action against a range of malignant conditions and in particular colorectal cancer. Colorectal cancer is the 4th most common type of cancer in the UK and across a lifetime will occur in approximately 1 in every 15-18 people[34]. It has now become apparent that COX-2 is inducible in tumorigenesis and plays a key role in cancer formation and metastasis. The use of NSAIDs as chemo preventative agents has therefore been explored for over 20 years and there have since been 8 double blind randomised controlled trials performed in colorectal cancer alone[35]. In 2005, the Cardiovascular Events Associated with Rofecoxib in a Colorectal Adenoma Chemoprevention Trial (APPROVe)[26] reported that in 2586 patients that were randomised to either rofecoxib or placebo, rofecoxib was associated with an increased risk of thrombotic events; relative risk 1.92 (95% CI 1.19 to 3.11). These findings were confirmed in the Cardiovascular Risk Associated with Celecoxib in a Clinical Trial for Colorectal Adenoma Prevention (APC)[36] which was stopped early due to the dose dependant increases in cardiovascular risk seen in patients receiving either 200mg BD (HR: 2.3; 95% CI 0.9 to 5.5) or 400mgBD (HR:3.4; 95% CI 1.4 to 7.8) versus placebo. Due to concerns around the side-effect – efficacy balance, including increased cardiovascular risk, celecoxib is no longer approved for use in colon cancer in Europe[37]. This has had the knock-on effect of virtual arrest in the clinical use of NSAIDs in cancer prevention. This is despite studies such as the PreSAP [38] trial looking at the prevention of colorectal adenomatous polyps, where celecoxib use was associated with an ~50% reduction in the development of malignant polyps in young patients with adenomatous disease and so the potential therapeutic benefit is huge. Understanding the mechanisms behind NSAID cardiovascular events could unlock a huge therapeutic benefit for patients with malignancy.

1.4.4 Meta-analysis

The first landmark meta-analysis of individual patient data from 280 trials (124,513 participants and 68,342 patient years) conducted by the Coxib and traditional NSAID trialists' (CNT) collaboration in 2013 found that coxibs increase cardiovascular risk by approximately 30% (RR 1.37, 95% CI 1.12 to 1.66; p:0.0009)[39]. More recently in 2017, Bally conducted a Bayesian meta-analysis of individual patient level data using

datasets from Canada, UK and Finland consisting of a cohort of 446,763 individuals of which 61,460 had a myocardial infarction during the study period [40]. The meta-analysis found that the current use of an NSAID (celecoxib, diclofenac, ibuprofen, naproxen and rofecoxib) was associated with a significant increased risk of myocardial infarction. This supports the growing body of evidence suggesting that traditional NSAIDs carry the at least the same cardiovascular risk as selective COX-2 inhibitors. These findings are consistent with other epidemiological studies.[30, 41, 42]

It is clear from clinical trials, population-based studies and meta-analysis that there is an association between NSAID use and risk of a cardiovascular event. This still leaves the unanswered questions of

- 1) What are the mechanisms of COX-2 inhibition and cardiovascular events?
- 2) Are there any biomarkers to predict cardiovascular events amongst NSAID users?

1.4.5 Scale of the problem

Ibuprofen is readily available as an over-the-counter drug and is one of the most commonly used medications (used by approximately 20-30% of the population)[43]. Given that it can increase the risk of cardiovascular complications by 30% [41, 44], we could speculate that NSAID use can be thought of as independent risk factor and potentially accounts for up to 50,000 of the 456,000 heart attacks and strokes in the UK each year[39, 45]. Moreover, we now know that this risk emerges with just 1-2 weeks of use[40].

Although NSAIDs are associated with cardiovascular, renal and gastrointestinal (GI) complications, they remain extremely effective analgesic and anti-inflammatory drugs and alternatives such as opioids carry problems of tolerance and addiction. As discussed, NSAIDs are also effective chemo-preventative therapies, however, due to the risk of cardiovascular and GI side effects, they are no longer used and have been withdrawn from the European market[37]. Clearly the potential for NSAIDs to reduce cancer further adds to the importance of understanding the cardiovascular side effects of NSAIDs.

1.4.6 Variation in practice

Despite the evidence linking NSAID use with increased cardiovascular risk, there is variation in NSAID use and restrictions across the globe. In the UK, whilst some NSAIDs including celecoxib and naproxen are prescription only, ibuprofen is readily available over the counter at supermarkets at a recommended dose of up to 1.2g daily despite the warning of increased cardiovascular risk in the British National Formulary[46] although the greatest risk is seen in prescription doses of ibuprofen. In France, since 2020, rules are stricter with both ibuprofen and paracetamol only available behind the counter at pharmacists, and patients are advised of the risks before receiving them[47]. In 2015, the New Delhi government in India banned over the counter NSAIDs without a prescription[48] . In the USA, all NSAIDs now come with a black-box FDA warning of cardiovascular and gastrointestinal risks[49].

1.5 How does COX-2 protect the cardiovascular system?

Vane et al[50] first hypothesised in the early 1980s that an imbalance in thromboxane and prostacyclin maybe critical for thrombus formation. It is now generally agreed that prostacyclin is the prostanoid involved in the cardio protective effects of COX-2 and while an inducible enzyme at the site of inflammation, we now know it is the *constitutively* expressed COX-2 that has a cardioprotective role[51, 52], this is because the increased risk of cardiovascular events is seen even in individuals without ongoing inflammation. There is however, controversy regarding the relative homeostatic role of COX-2 in main two locations: (i) the kidney and/or (ii) vascular endothelium[3].

The first hypothesis is that COX-2 in the vascular endothelium, driven by shear stress, is the primary cardioprotective site. This is based on experiments using urinary metabolites of both thromboxane and prostacyclin (Tx-M and PGI-M) that show selective reduction of PGI-M in healthy volunteers taking celecoxib or rofecoxib and in COX-2 knockout mice[3]. There is debate as to whether urinary markers of prostacyclin reflect systemic levels[3] and moreover, evidence now suggests that PGI-M is a renal metabolite and therefore represents local levels in the kidney and not

systemic levels [53]. In addition, there is now a strong body of evidence showing the importance of constitutive COX-1 in vascular prostacyclin production. This has been demonstrated by experiments showing minimal release of prostacyclin in vessels from COX-1 knockout mice and near normal levels in COX-2 knockout mice[54]. Furthermore, experiments using tissue specific knockout mice have demonstrated that endothelial COX-1 accounts for all of the endothelial produced prostacyclin in major vessels (i.e. aorta)[3]. If the endothelium is not the ubiquitous site for COX-2 derived prostacyclin production, there must be another mechanism by which COX-2 protects the cardiovascular system.

The second hypothesis is that renal COX-2 plays a role in cardiovascular protection. Studies have shown that the kidney, and specifically the renal medulla is a major site for constitutively expressed COX-2[55] and its expression is driven in the absence of an inflammatory stimulus by the transcription factor NFAT[52]. We also know that COX-2 plays a key role in reno-vascular homeostasis through regulation of blood pressure and the renin/angiotensin system[55]. Finally, knocking out COX-2 in mice has significant impact on renal transcription whilst sparing the heart and aorta causing significant renal compromise [51]. We also know that NSAID use in humans can cause renal dysfunction and increased blood pressure [56]. Although it is far from definitive, COX-2 in the kidney may provide an explanation (at least in part) for NSAID induced cardiovascular side effects.

Finally, COX-2 is also constitutively expressed in the gut, lung, brain and thymus[10]. Whilst this thesis will focus on the cardiorenal system, these other organs could also play a role in NSAID cardiovascular effects.

Despite more than 15 years of research into this area, we still do not fully understand how COX-2 protects the cardiovascular system. A vital area of unmet need is the identification of biomarkers of NSAID induced cardiovascular adverse events. This may not only help to identify patients at risk of heart attacks and strokes but could also provide key mechanistic insights into how COX-2 protects the cardiovascular system revealing mitigating therapeutic strategies.

1.6 Biomarkers in cardiovascular disease

A biomarker can be defined as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [57]. Biomarkers have transformed clinical care in cardiovascular disease[58]. The first biomarker identified for the diagnosis of acute myocardial infarction was Aspartate Transaminase (AST) in 1954 [59] however this is not specific for cardiac damage. In the 1970s, Lactate dehydrogenase (LDH) and creatinine kinase (CK) were identified and in the late 1980’s myoglobin became a biomarker for myocardial infarction[60]. Through advances in electrophoresis, cardiac specific iso-enzymes such as CK-MB were identified[61] and although it was included in the World Health Organisation (WHO) diagnostic criteria for acute myocardial infarction[62], the low specificity and high rates of false positive results limited its use. Troponins, which are a contractile component of cardiac myocytes have since been discovered as biomarkers and remain in clinical practice today; specifically newer 5th generation troponins are in use which are highly sensitive and specific for myocardial damage[58]. Of course, troponins are not the only success story, there are a wide range of biomarkers used in clinical practice for cardiovascular disease/dysfunction, including B-type natriuretic peptide (BNP) as a biomarker of heart failure status and low-density lipoprotein (LDL) cholesterol a biomarker of cardiovascular disease[58].

1.7 Promising biomarkers of NSAID cardiovascular events

Although there are currently no biomarkers used in clinical practice to predict NSAID induced cardiovascular adverse events, there have been a few promising leads.

A link between COX-2 inhibition and the endothelial NOS (eNOS) pathway has now been established in studies by Ahmetaj-Shala et al[51] and Yu et al [63]. eNOS is cardioprotective and is expressed throughout the vasculature. Inhibition/genetic deletion of COX-2 was shown to be associated with reduced eNOS gene expression or increases in levels of the naturally occurring methylarginine inhibitors, ADMA and

L-NMMA which prevent access of the substrate, L-arginine, to the eNOS active site[64].

1.7.1 Methylarginines

Methylarginines (ADMA, LNMMA & SDMA) are methylated forms of arginine that are derived with the involvement of the enzyme's protein methyltransferase type 1 and type 2 (PRMT1 & PRMT2). The formation of ADMA and LNMMA is catalysed by PRMT-1 whilst PRMT-2 is involved in the methylation of proteins to release SDMA and LNMMA.

Vallance and colleagues first reported in 1992 that ADMA is a natural endogenous inhibitor of eNOS which reduces NO production[65]. Both ADMA and LNMMA, which have asymmetrically methylated residues on guaidino nitrogens of 'arginine', are competitive eNOS inhibitors that prevents access of the substrate, L-arginine, to the eNOS active site[66] **Figure 1.2**. Conversely the symmetrically methylated arginine (SDMA) does not inhibit eNOS. As discussed earlier, NO is a key cardioprotective player and it is therefore unsurprising that ADMA is a recognised biomarker of cardiovascular disease, all-cause mortality and end-stage renal failure[67].

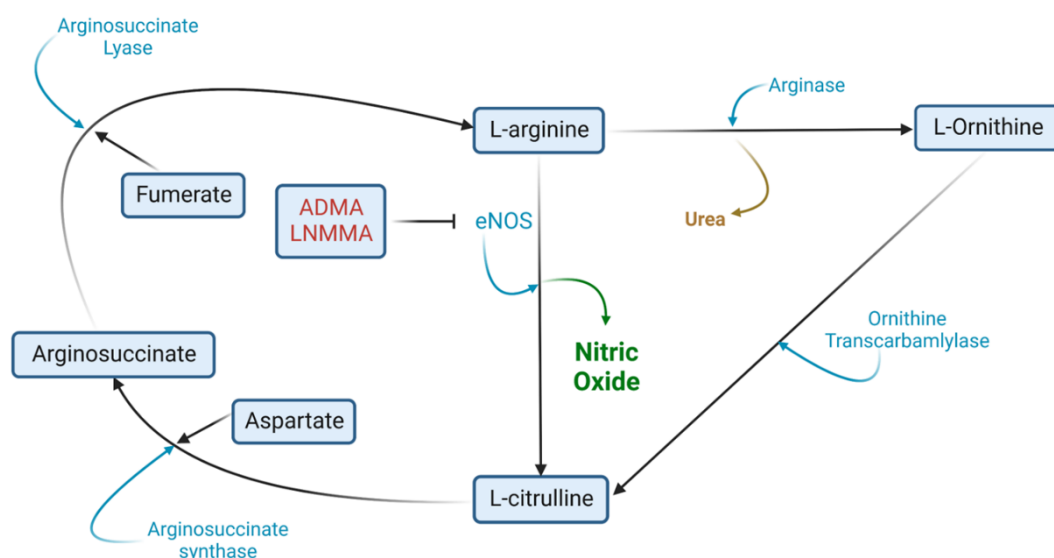


Figure 1.2 Endothelial Nitric Oxide Pathway Endothelial nitric oxide synthase (eNOS) acts on the pre-cursor L-arginine to produce nitric oxide and L-citrulline. The methylarginines ADMA and LNMMA competitive eNOS inhibitors that prevents access of the substrate, L-arginine, to the eNOS active site and therefore inhibit NO formation.

ADMA was first isolated in significant amounts in the urine by Kakimoto in 1970 which led to the initial assumption that renal excretion was the sole route for removal of free methylarginines[68]. However, a subsequent study found that in rabbits, urinary excretion of SDMA was 30 times higher than both LNMMA or ADMA suggesting alternative pathways for its removal[69]. We now know that NG dimethylarginine dimethylaminohydrolase (DDAH) is a key hydrolytic enzyme involved in the metabolism of ADMA and LNMMA but not SDMA. DDAH has two main isoforms and it's primarily DDAH-2 is predominantly found in tissues containing eNOS[70]. DDAH works by converting ADMA/ LNMMA to L-citrulline and either di or mono- methylamine respectively.

DDAH activity has been found in various regions including the kidneys, aorta, pancreas and brain. Pharmacological inhibition of DDAH increases ADMA and subsequently reduces NO synthesis[71]. To the contrary, transgenic overexpression of DDAH reduces ADMA and increases NO[72] and furthermore, DDAH overexpression appears to be cardioprotective in animal models of endothelial repair following vascular injury and in models of myocardial reperfusion injury[72].

Further studies have demonstrated that ADMA levels are increased in plasma from prostacyclin synthase (PGIS) knockout mice but not in PGE₂ synthase knockout mice [73]. This suggests that the COX-2 end-product that limits ADMA levels is prostacyclin. Prostacyclin and nitric oxide (NO) synergistically inhibit platelet function and act additively as vasodilators [3]. Therefore, this is one potential mechanism by which NSAIDs predispose users to cardiovascular side effects with ADMA as a potential biomarker.

It should be noted that changes in ADMA levels alone do not necessarily reflect potential changes in NOS activity. To resolve this, this group has previously developed a cell-based bioassay that can be used to test the overall ability of human plasma 'ex vivo' to 'support NOS activity'; this assay can be used as a supportive biomarker in parallel with measuring ADMA[74].

1.8 ‘Omic biomarker technology and application in NSAID use

Although conventional hypothesis testing experiments have furthered our understanding in this field, we now need to couple this with hypothesis generating approaches utilising newer ‘omic technologies. In the next section I will briefly describe these approaches and their application to biomarker discovery in general.

1.8.1 Genome wide association study (GWAS)

GWAS is an observational study approach used to identify associations between single-nucleotide polymorphisms (SNPs) and traits such as disease or efficacy/side effects to drugs. The most common method used to do this is via case-control studies. Typically, two large groups are required; a group affected by the condition (case group) and a “healthy” control group. Individuals are genotyped for SNPs and differences in allele frequency between the groups are investigated. Since the first successful GWAS in 2005 that compared 96 individuals with macular degeneration with 50 controls[75], there has been a trend towards larger sample sizes with the introduction of large biobanks. There have now been several studies that have included over 1 million participants[76, 77]. There have been a number of successful clinical applications for GWAS. One such example found a genetic variant associated with anti-hepatitis C virus treatment which showed that SNPs near the IL28B gene that encodes lambda 3 was associated with significant differences in treatment response [78]. These findings have allowed physicians to develop personalised medicine targeting treatment to the patients’ genotype.

GWAS has also been applied to patients taking NSAIDs. Braenne et al [79] identified 47 genes that are affected by Coxibs from a Drug Gene interaction database. They compared this with genes associated with coronary artery disease (CAD) from 85,000 patients and 200,000 controls. They found SNP’s relating to MMP9 and BCAR1 reaching significance highlighting common genes affected by CAD and COX inhibition. This now needs to be further investigated in patients who had cardiovascular events

whilst on NSAIDs. Whilst it is outside the scope of my thesis, GWAS should be further explored in this field.

1.8.2 Transcriptomics

Transcriptomics is a quantitative assessment of gene expression in a given biological state[80]. Typically, a comparison between “diseased” and “non-diseased” (control) tissues is made which provides a profile of altered gene expression. There are essentially three methods used:

- 1) Quantitative real-time polymerase chain reaction (qRT-PCR, or qPCR)
- 2) Microarray
- 3) Next-Generation Sequencing (RNAseq)

For the purpose of my thesis, I will focus on RNAseq. This technology isolates and sequences the complete complement of RNAs from a sample which then identifies the abundance of each transcript [80]. Transcriptomics has been successfully used to identify a range of biomarkers in cardiovascular medicine. Weinberg et al [81] found that when cardiac myocytes were stretched in vitro, there was a significant increase in transcription of the ST2 receptor. ST2 is a soluble protein that can be measured in peripheral blood and follow up studies have demonstrated that ST2 plays a cardioprotective role in cardiac fibroblast and myocyte signalling. Not only has this led to a novel cardiac biomarker but has also opened the door for a new therapeutic target for heart failure[82].

Despite its growing popularity, transcriptomics has not yet been extensively applied to find potential biomarkers for NSAID induced side effects. A PubMed search with the terms ‘Transcriptomics’ and ‘NSAIDs’ revealed just four papers that reported gene expression profiles in relation to hypersensitivity and hepatotoxicity induced by NSAIDs and chemoprevention and progression of Alzheimer’s disease [83-86], but no reports are available for similar studies looking at cardiovascular side effects. However, prior to my work, our group performed a basic, pilot transcriptomic analysis using Illumina microarray technology of blood from healthy volunteers taking celecoxib for 7 days. The analysis revealed altered genes mapping to key pathways of direct relevance to cardiovascular homeostasis, namely, (i) cholesterol synthesis, (ii)

interferon and (iii) vasoconstriction. My research will involve rigorous replication and validation of these findings using newly generated samples and RNA sequencing technology.

1.8.3 Proteomics

A potential limitation of both genomic and transcriptomic approaches is they can be difficult to associate observed changes to protein expression and fail to capture any post-translational modifications[87]. There are several techniques that can be used to study proteins of which the two broad categories are immunoassays and mass spectrometry. This report will focus on mass spectrometry (MS) and immunoassays. In essence, MS precisely measures the fragmentation and mass spectra of peptides that have been derived from digestion of proteins[88]. In theory, MS can analyse the whole proteome thus providing an unbiased and hypothesis generating approach. One of the early success stories of proteomics was the development of OVA1 for the management of pelvic malignancies which was the first FDA-approved in vitro diagnostic multivariate index assay[89]. It was found that using a combination of biomarkers (CA 125, apolipoprotein A1, truncated form of transthyretin & inter- α -trypsin inhibitor heavy chain H4) as a multivariate index increased both the specificity and sensitivity for the detection of early-stage ovarian cancer.

A PubMed search (August 2022) identified 15 papers using the term 'proteomics' and 'NSAIDs' (**Figure 1.3**). This technology has not only been used to further our understanding of COX and cancer but has also been used to identify biomarkers. An unpublished pilot study from our group using plasma from healthy volunteers taking using LC-MS/MS identified ≈ 30 proteins being altered at a discovery threshold of $p < 0.07$. Pathway analysis revealed altered proteins mapped to changes in acute inflammatory response and acute-phase response networks, of particular note was increases of up to 3-fold in COL11, LPS binding protein (LBP) and vascular cell adhesion protein 1 (VCAM-1). LBP and COL11 facilitate binding of agents that activate interferon pathways and VCAM-1 is an interferon response gene. Thus, the pilot data showed proteomic and transcriptomic readouts converge and overlap at the level of interferon/innate immunity pathways identified in volunteers taking celecoxib. My

research will involve replication and validation of these findings using newly generated samples and RNA sequencing technology.

More recently, Proximity Extension Assay (PEA) technology which essentially enables high-throughput multiplex immunoassays for a large number of targeted proteins and is rapidly becoming a popular alternative to mass spectrometry with over 900 publications since 2015 with almost 180 of these from January to August 2022 alone.

There is potential to now use these technologies to identify biomarkers of cardiovascular side effects associated with NSAID use.

Title	Year
Plasma proteomic changes in response to surgical trauma and a novel transdermal analgesic treatment in dogs[90].	2022
Identification and drug-induced reversion of molecular signatures of Alzheimer's disease onset and progression in AppNL-G-F, AppNL-F, and 3xTg-AD mouse models [83]	2021
Aspirin inhibits prostaglandins to prevents colon tumor formation via down-regulating Wnt production[91]	2020
The influence of hypoxia on the prostate cancer proteome[92].	2020
Serum protein expression in Equine Glandular Gastric Disease (EGGD) induced by phenylbutazone[93]	2019
Inhibition of mPGES-1 or COX-2 Results in Different Proteomic and Lipidomic Profiles in A549 Lung Cancer Cells[94]	2019
A Precision Medicine Model for Targeted NSAID Therapy in Alzheimer's Disease[95]	2018
Proteomic Analysis Shows Constitutive Secretion of MIF and p53-associated Activity of COX-2 ^{-/-} Lung Fibroblasts[96]	2017
Effects of delayed NSAID administration after experimental eccentric contraction injury - A cellular and proteomics study.[97]	2017
Prodrugs Bioactivated to Quinones Target NF-κB and Multiple Protein Networks: Identification of the Quinonome.[98]	2016
Inhibition of Cyclooxygenase-2 Prevents Chronic and Recurrent Cystitis.[99]	2014
Major involvement of Na(+) -dependent multivitamin transporter (SLC5A6/SMVT) in uptake of biotin and pantothenic acid by human brain capillary endothelial cells.[100]	2015
The influence of chronic ibuprofen treatment on proteins expressed in the mouse hippocampus [101]	2015
Sulindac modulates secreted protein expression from LIM1215 colon carcinoma cells prior to apoptosis[102]	2013
Non-steroidal anti-inflammatory drugs target the pro-tumorigenic extracellular matrix of the postpartum mammary gland [103]	2011
Secretome-based proteomics reveals sulindac-modulated proteins released from colon cancer cells [104]	2009
Proteomic Profiling Identifies Cyclooxygenase-2-Independent Global Proteomic Changes by Celecoxib in Colorectal Cancer Cells [105]	2006

Protein biomarkers of nephrotoxicity; a review and findings with cyclosporin A, a signal transduction kinase inhibitor and N-phenylanthranilic acid [106]	2005
High-throughput proteomics and protein biomarker discovery in an experimental model of inflammatory hyperalgesia: effects of nimesulide [107]	2003

Figure 1.3. Publications using Proteomics & NSAIDs. A table summarising the publications found on PubMed with the search terms “proteomics” AND “NSAIDs”.

1.8.4 Metabolomics

In recent years, metabolomics has become a complimentary technique to genomics, transcriptomics and proteomics [108]. It identifies the levels of smaller molecules (<10 kDa) that are not captured by the other mentioned techniques. It provides a snapshot of the metabolic profile of a system and how it changes in response to stress, disease or drugs. An example of how metabolomics can be used to predict cardiovascular disease can be seen in a study by Tang et al[109]. Using liquid chromatography and tandem MS, they found that increased plasma levels of trimethylamine-N-oxide (TMAO) (the production of which is dependent on intestinal microbes) were significantly associated with an increase in adverse cardiovascular events (Hazard ratio 2.54; 95% confidence interval, 1.96 to 3.28; $P < 0.001$).

A PubMed search (September 2022) identified 8 papers using ‘metabolomics’ and ‘NSAIDS’ (**Figure 1.4**) suggesting that this technology could identify potential biomarkers of cardiovascular events in NSAID users. Our team have developed a novel ultra-high performance liquid chromatography with electrospray ionisation (ESI) coupled to tandem mass spectrometry (UHPLC-MS/MS) platform for the quantification of amino acids and methylarginines which was applied to samples from patients undergoing coronary artery bypass surgery [74]. As previously discussed, COX-2 inhibition is linked to increased ADMA in healthy volunteers and in mice [51]. As part of my PhD thesis, I have explored the impact of COX-2 inhibition on methylarginines and amino acids.

Distinct Transcriptomic and Metabolomic Profiles Characterize NSAID-Induced Urticaria/Angioedema Patients Undergoing Aspirin Desensitization[110]	2022
Long-term exposure to environmental diclofenac concentrations impairs growth and induces molecular changes in <i>Lymnaea stagnalis</i> freshwater snails[111].	2022
Longer-Term Omega-3 LCPUFA More Effective Adjunct Therapy for Tuberculosis Than Ibuprofen in a C3HeB/FeJ Tuberculosis Mouse Model[112]	2021
Inflammatory macrophage memory in nonsteroidal anti-inflammatory drug-exacerbated respiratory disease[113].	2021
Pharmacometabolomics with a combination of PLS-DA and random forest algorithm analyses reveal meloxicam alters feline plasma metabolite profiles [114].	2020
Impact of Pre-blood Collection Factors on Plasma Metabolomic Profiles[115]	2020
Progress in understanding hypersensitivity reactions to nonsteroidal anti-inflammatory drugs[84]	2019
Development of a novel UHPLC-MS/MS-based platform to quantify amines, amino acids and methylarginines for applications in human disease phenotyping [74]	2018
Stereoselective effects of ibuprofen in adult zebrafish (<i>Danio rerio</i>) using UPLC-TOF/MS-based metabolomics[116]	2018

Genomic, Lipidomic and Metabolomic Analysis of Cyclooxygenase-null Cells: Eicosanoid Storm, Cross Talk, and Compensation by COX-1[117]	2016
Metabolomics assessment of the effects of diclofenac exposure on <i>Mytilus galloprovincialis</i> : Potential effects on osmoregulation and reproduction[118]	2016
H-Nuclear magnetic resonance-based metabolic profiling of nonsteroidal anti-inflammatory drug-induced adverse effects in rats [119]	2014
Hydroxyproline, a serum biomarker candidate for gastric ulcer in rats: a comparison study of metabolic analysis of gastric ulcer models induced by ethanol, stress, and aspirin [120]	2012
Genetically based impairment in CYP2C8- and CYP2C9-dependent NSAID metabolism as a risk factor for gastrointestinal bleeding: is a combination of pharmacogenomics and metabolomics required to improve personalized medicine? [121]	2009

Figure 1.4. Publications using Metabolomics & NSAIDs. A table summarising the publications found on PubMed with the search terms “proteomics” AND “NSAIDs”.

1.9 The endothelium

The endothelium has metabolic and endocrine functions supporting its role as a critical regulator of vascular homeostasis in processes including regulation of vascular tone, smooth muscle cell proliferation, cellular adhesion and vascular inflammation[122]. The importance of the endothelium in regulating vascular tone was firmly established by the seminal work of Furchgott and Zawadzki's published in 1980[123]. In this landmark study, the authors described the 'obligatory role' of the endothelium in relaxing vessels to acetylcholine, the nature of the mediator could not be identified at that time and so they named the entity as 'endothelial derived relaxing factor' or EDRF. We now know that EDRF is the bioactive gaseous mediator nitric oxide (NO). Nitric Oxide synthases (NOS) are a family of enzymes that catalyse the production of NO. There are 3 isoforms of the enzyme including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[124]. The focus in the thesis will be on pathways

and interactions relating to eNOS although it should be noted that nNOS and iNOS can mediate vascular responses in some settings.

NO is produced from L-arginine by the actions of eNOS and citrulline plus H₂O is formed as a by-product [125]. As discussed earlier, prostacyclin (derived from the actions of COX plus PGIS) is also produced by endothelial cells and works both independently and additively with NO to suppress platelet aggregation and cellular adhesion. NO derived from the endothelium is a powerful vasodilator and together with the sympathetic nervous system, helps to regulate vascular tone. As a reactive gas, NO has a short half-life and is rapidly oxidised to nitrite and then nitrate in neutral aqueous solutions and the presence of oxygenated haemoglobin[126].

Vascular endothelium is also responsible for making Endothelin-1 which is an extremely potent vasoconstrictor that was first isolated in 1988 [127]. These are just some examples of the diverse roles the endothelium has, and it is therefore unsurprising that the endothelium plays a key role in the pathogenesis of vascular diseases such as atherosclerosis. Similarly the underlying smooth muscle cells can express cyclooxygenase and NOS (iNOS) in inflammation and atherosclerosis where cells switch from a contractile phenotype to an “active” type that is geared towards a host defence response. In inflammation the normal protective functions of the endothelium can be overwhelmed resulting in a switch towards activation of redox signalling which results in the generation of reactive oxygen species (ROS)[128]. Whilst this “activated” endothelium plays a role in normal host defence and is not necessarily proatherogenic in isolation, the addition of clinical risk factors such as dyslipidaemia, hypertension and diabetes could tip the balance to drive atheroma formation. “Activated” endothelium, which will be referred to as endothelial dysfunction hereafter, is therefore considered to be an independent cardiovascular risk factor in its own right and there are a number of clinical methods that have been developed to assess endothelial function. In essence, the principal of most of these tests is to assess endothelial dependant dilation in vivo using a physiological/pharmacological stimulation to release endothelial NO and other endothelial derived vasoactive compounds.

1.10 Endothelial dependant dilatation

1.10.1 Intra-vascular infusion

The first clinical studies measuring endothelial function were conducted using coronary angiography and under normal physiological conditions, acetylcholine stimulates the release of NO in vessels but in patients with endothelial dysfunction (e.g. patients with coronary artery disease), it causes vasoconstriction[129]. Intra-brachial infusion of acetylcholine with ultrasound measurement of dilation is typically performed although alternative NO agonists such as bradykinin and methacholine can be used[130]. Whilst these techniques are relatively safe, they are still invasive compared to other methods.

1.10.2 Post-occlusive reactive hyperaemia; flow mediated dilatation

In addition to chemical stimuli, the normal physiological hyperaemic response of the endothelium to ischemia can also be utilised to assess endothelial dependant dilatation. When assessing larger vessels, flow mediated dilatation (FMD) is commonly used and involves inducing ischaemia by inflating a pressure cuff around the arm to supra-systolic pressures for a period of around 5 minutes. Imaging (typically using ultrasound) is used to measure brachial artery dilation following rapid release of the pressure cuff (reactive hyperaemia). An FMD index is calculated using the maximal arterial diameter pre and post occlusion.[131] Whilst this is a non-invasive technique, due to the use of ultra-sound, it is operator dependant which could affect reproducibility. Alternative imaging techniques such as Magnetic resonance imaging (MRI) have been used to improve reproducibility, however this is expensive and requires highly skilled personnel[132].

1.10.3 Peripheral arterial tonometry (PAT)- EndoPAT 2000 ®

The assessment of reactive hyperaemia in the microvasculature was first introduced in 2003 by Kuvin and Colleagues who found that pulse wave analysis (PWA) using peripheral artery tonometry (PAT) was comparable to FMD[133]. Disposable plethysmography finger probes are used to measure beat-to-beat pulse waveform and a reactive hyperaemia index (RHI) is calculated using pre and post occlusion readings. Subsequent metanalysis[134] have shown that both FMD and reactive hyperaemia

PAT (RH-PAT) predict future cardiovascular events and in fact the EndoPAT® 2000 device which utilises this technology is the first FDA approved non-invasive test for endothelial dysfunction[135]. In addition to providing RHI, the EndoPAT® device also provides augmentation index measured using PWA which is a surrogate for vascular stiffness. Whilst changes in vascular stiffness can occur independently of the endothelium due to influences of sympathetic tone and/or changes to the structural integrity of a vessel, endothelial dysfunction can also increase vascular stiffness. Vascular stiffness and augmentation index will be discussed further in **Chapter 9 (Discussion)**. The EndoPAT® has been used in a range of clinical studies with a PubMed search (September 2022) of EndoPAT returning over 200 publications. The most notable publications using the EndoPAT device and cardiovascular disease were in the Framingham Heart study cohorts [136, 137] and in a comparative study of 5000 participants in the Gutenberg Heart Study[138] which found an association between reduced RHI (endothelial dysfunction) and cardiovascular risk.

As previously discussed, one of the major limitations of FMD using ultra-sound is its operator dependency and reproducibility. There have been 4 publications using EndoPAT demonstrating day to day reproducibility and reliability [139-142] which suggests it is operator independent. However, the device is probably not suitable for repeated measurements within the same day [142]. For the aforementioned reasons, I have used EndoPAT® to assess the effects of NSAIDs on endothelial function. The technique is described in depth in the **Chapter 2 (Methods)**.

1.10.4 Other techniques

Similar techniques to those described above can also be employed in the venous system (e.g. venous occlusion plethysmography [143]) and skin microvasculature using laser doppler[144].

1.11 NSAIDs and Endothelial Function

Assessing how NSAIDs effect endothelial function is not a novel concept. For example, a scoping review (August 2022) of the literature identified 33 human mechanistic studies assessing NSAIDs and endothelial function.

There were 21 studies that reported a reduction in endothelial function with NSAID use **Figure 1.5**, 9 studies that reported no difference **Figure 1.6** and 3 studies that reported an improvement in endothelial function **Figure 1.6**. Given the heterogeneity due to differences in 1) study population, 2) NSAIDs used including dosage and duration and 3) modalities used to assess endothelial function, meta-analysis is not meaningful and so was not performed. Furthermore, the techniques used in these studies are operator dependant which may explain some of the conflicting findings. This highlights that the effect of NSAIDs on endothelial function remains an area of uncertainty despite over 30 years of research. Within this thesis, I will try and address these uncertainties by measuring endothelial function using the FDA approved EndoPAT® device which is non-operator dependant.

Study	Study Design	Outcome	Drug, dose, Duration	Method of Detection	Population
Kellogg 2005 [145] <i>Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin</i>	Mechanistic study	Ketorolac reduced Ach induced increase in Skin blood flow (SkBF) 55 +/- 7% maximal CVC	Ketorolac, 10 mM via intradermal micro dialysis.	SkBF by laser-Doppler flowmetry	11 healthy subjects (5 men and 6 women)
Khan 1997 [146] <i>Cutaneous vascular responses to acetylcholine are mediated by a prostanoid-dependent mechanism in man</i>	Mechanistic study	Aspirin reduced Ach induced blood flow Reduction: 54% (13.6 ± 3.0 to 6.3 ± 1.7 AuC, p = 0.06, t-test)	Aspirin 600 mg daily for 3 days	SkBF by laser-Doppler flowmetry	25 Healthy male volunteers
Noon 1998 [147] <i>Studies with iontophoretic administration of drugs to human dermal vessels in vivo: cholinergic vasodilation is mediated by dilator prostanoids rather than nitric oxide</i>	Mechanistic study	Aspirin reduced Ach blood flow (approx. 53% reduction)	Aspirin 600 mg injected IV over 5 minutes	SkBF by laser-Doppler flowmetry	6 healthy male volunteers aged 23–36 years

Carlsson 1986 [148] <i>The role of myogenic relaxation, adenosine and prostaglandins in human forearm reactive hyperaemia.</i>	Mechanistic study	Ibuprofen reduced reactive hyperaemia	1200mg orally 1 hour before experiment	(Reactive hyperaemia) Forearm blood flow (Monograph)	16 healthy male and female volunteers
Kilbom 1976 [149] <i>Endogenous prostaglandins as local regulators of blood flow in man: effect of indomethacin on reactive and functional hyperaemia.</i>	Mechanistic study	Indomethacin reduced reactive hyperaemia (approx. 25%)	Indomethacin 1-25 mg/kg body weight rectally 1 hour before blood flow protocol	Forearm blood flow by venous occlusion plethysmography	10 healthy volunteers, 5 men and 5 women
Duffy 1998 [150] <i>Continuous release of vasodilator prostanoids contributes to regulation of resting forearm blood flow in humans.</i>	Mechanistic study	Aspirin inhibits resting blood flow but not increased blood flow by ACh	Aspirin infused via the brachial artery in three incremental doses of 1, 3, and 10 mg/min, acute	Forearm blood flow by venous occlusion plethysmography	10 healthy subjects
Wilson 1993 [151] <i>Contribution of prostaglandins to exercise-induced vasodilation in humans.</i>	Mechanistic study	Indomethacin reduced basal blood flow and blood flow after exercise.	intra-arterial infusion of indomethacin (0.3 mg/100 ml forearm vol)	forearm blood flow by plethysmography – basal & after exercise	12 normal subjects
Nowak 1979 [152] <i>A study on the role of endogenous prostaglandins in the development of exercise-induced and post-occlusive hyperemia in human limbs. Abstract only available</i>	Mechanistic study	Indomethacin significantly reduced the magnitude of the reactive hyperaemia both in the forearm and in the calf, decreasing both the peak value and the duration of the vasodilation	Abstract only available	Forearm blood flow	Abstract only
Friedman 1981 [153] <i>Coronary vasoconstrictor effect of indomethacin in patients with coronary-artery disease</i>	Mechanistic study	Coronary sinus blood flow decreased after indomethacin coronary blood flow fell significantly, from 181 ± 29 to 111 ± 14 ml per minute ($P < 0.05$).	before and 20 minutes after an intravenous dose of indomethacin (0.5 mg per kilogram of body weight)	Coronary blood flow using thermodilution.	Nine Patients with coronary artery disease.

Carlsson 1983 [154] <i>Effect of different prostaglandin synthesis inhibitors on post-occlusive blood flow in human forearm;</i>	Mechanistic study	Reduction in forearm blood flow in: Aspirin (56%), indomethacin(67 %), diclofenac(56%), ibuprofen(71%), piroxicam(56%), naproxen(34%).	The highest recommended 24 hrs dose of each drug was divided and given in 2 portions, at times chosen to result in peak plasma concentrations at the time of the study.	Forearm blood flow (monograph)	Ten healthy volunteers, 5 men and 5 women
Cherney 2010 [155] <i>Renal Hyperfiltration Is a Determinant of Endothelial Function Responses to Cyclooxygenase 2 Inhibition in Type 1 Diabetes.</i>	Mechanistic study Mechanistic study	COX2 inhibition suppressed FMD in normofiltering (10.2+-5.3% to 5.8+-3.4%, P_0.006) versus hyperfiltering subjects (ANOVA interaction, P_0.003).	200mg celecoxib/day orally for 14 days.	Flow mediated dilatation measured using ultrasound.	13 participants with type 1 diabetes.
Engelke 2019 [156] <i>Contribution of nitric oxide and prostaglandins to reactive hyperemia in the human forearm.</i>	Mechanistic study	Reduction in peak vasodilation by 22% but no difference in total flow.	1200mg oral ibuprofen.	forearm blood flow by plesmography	7 healthy volunteers
Hojs 2009 [157] <i>The effect of glibenclamide on acetylcholine and sodium nitroprusside induced vasodilatation in human cutaneous microcirculation.</i>	Mechanistic study	Diclofenac reduced blood flow at rest and in Ach induced blood flow.	10 µl of 31.4mM solution microinjection.	SkBF by laser-Doppler flowmetry	14 young male volunteers.
Crilly 2011 [158] <i>Non-steroidal anti-inflammatory drug (NSAID) related inhibition of aldosterone glucuronidation and arterial dysfunction in patients with rheumatoid arthritis: a cross-sectional clinical study</i>	Cross sectional study.	NSAID users with a higher aldosterone glucuronidation inhibition had a higher augmentation index and lower reflected wave transit time.	NSAID users with at least 3 months consecutive use included. NSAIDs: naproxen, celecoxib, metoricoxib, ibuprofen, meloxicam, indomethacin, other.	Radial pulse wave analysis.	Participants with rheumatoid arthritis. 60 NSAID users and 25 non-users.
Carter 2014 [159] <i>The role of prostaglandin and antioxidant availability in recovery from forearm ischemia</i>	Mechanistic study	Ibuprofen reduced FMD	1200mg Ibuprofen orally 1 hour before procedure.	Flow mediated dilatation measured using ultrasound.	12 healthy participants (5 women and 7 men).

<i>reperfusion injury in humans.</i>					
Dzeka 2000 [160] <i>Venous responsiveness to norepinephrine in healthy subjects: Effects of single doses of 325 mg aspirin.</i>	Mechanistic study	Aspirin increased venoconstriction to norepinephrine	325mg Aspirin at least 90 minutes before test.	Dorsal hand vein distension using linear variable differential transformer technique.	12 healthy participants (7 women and 5men).
Lee 2017 [161] <i>Electroacupuncture prevents endothelial dysfunction induced by ischemia-reperfusion injury via a cyclooxygenase-2-dependent mechanism: A randomized controlled crossover trial.</i>	Mechanistic Study. Crossover RCT.	No difference in baseline blood flow. Celecoxib reduced FMD in patients receiving electrical acupuncture and ischaemia/reperfusion (Pre-IR: 11.05 $\pm$ 3.27%; Post-IR: 4.20 $\pm$ 1.68%, p = 0.001)	400mg celecoxib/day for 5 days.	Brachial FMD measured using ultrasound.	
Kwong 2015 [162] <i>Cyclooxygenase inhibition and rosuvastatin-induced vascular protection in the setting of ischemia-reperfusion: A human in vivo study.</i>	Mechanistic study	FMD remained unchanged in the placebo group (8.1 $\pm$ 1.0 vs 7.2 $\pm$ 0.8%; P=NS) ASA 81 mg group, there was no change in FMD following IR (6.7 $\pm$ 0.6 vs 6.1 $\pm$ 0.7%; P=NS). significant decrease in FMD in the ASA 325 mg group (7.2 $\pm$ 0.8 vs 3.3 $\pm$ 0.7%, P b 0.001), the ibuprofen group (6.8 $\pm$ 0.7 vs 2.6 $\pm$ 0.8%; P = 0.001), as well as the celecoxib group (7.3 $\pm$ 1.5 vs 2.6 $\pm$ 1.5%, P b 0.01).	Patients received : Aspirin 81 mg OD Aspirin 325mg OD Celecoxib 200mg BD Ibuprofen 400mg QDS. Or Placebo for 5 days. Each participant also then received 40mg of rosuvastatin on day 5. Test performed on day 6	Radial artery FMD measured using ultrasound.	Placebo n:2 Aspirin 81mg n:15 Aspirin 325mg n:15 Celecoxib n:12 Ibuprofen n:12
Liuni 2010 [163] <i>Rosuvastatin Prevents Conduit Artery Endothelial Dysfunction Induced by Ischemia and Reperfusion by a Cyclooxygenase-2-Dependent Mechanism.</i>	Mechanistic study	Celecoxib blunted FMD post IR (pre-IR: 8.1 $\pm$ 1.9%; post-IR: 2.2 $\pm$ 2.8%, p <0.001)	Celecoxib 200mg BD for 5 days. On day 4 participants also received 1 dose of 40mg rosuvastatin or placebo.	Radial artery FMD measured using ultrasound.	18 healthy volunteers

Moppett 2003 [164] <i>non-selective and cyclo-oxygenase-2-specific non-steroidal anti-inflammatory drugs impair the hyperaemic response of skin to brief axillary artery occlusion.</i>	Mechanistic study	Hyperaemic response ratio was reduced in both ibuprofen; from 2.92 (2.38±3.86) to 2.09 (1.45±3.54); rofecoxib, from 3.36 (2.06±5.16) to 2.67 (1.15 ±3.84); P<0.01).	Single dose of either 800mg ibuprofen or rofecoxib 2-3 hours before test.	SkBF by laser-Doppler flowmetry	10 subjects
Liu 2015 [165] <i>Remote ischemic precondition prevents radial artery endothelial dysfunction induced by ischemia and reperfusion based on a cyclooxygenase-2-dependent mechanism</i>	Prospective Mechanistic study, RCT. Unblinded.	Celecoxib reduced FMD post IR injury even after 24 hours of ischaemic preconditioning.	Celecoxib 200mg BD for 5 days.	Radial artery FMD using ultrasound,	Twenty healthy volunteers

Figure 1.5. Studies that demonstrate NSAIDs reduce endothelial function. Table summarising studies that demonstrate a reduction in endothelial function with NSAID use.

Study	Study Design	Outcome	Drug, dose, duration	Method Of detection	Population
Chenevard 2003 [166]. <i>Selective COX-2 Inhibition improves endothelial function in coronary artery disease</i>	Double Blind, placebo controlled cross-over trial	<i>Celecoxib improved FMD Compared to placebo (3.3±0.4% vs 2±0.5%, p:0.026)</i>	Celecoxib 200 mg BD for 2 weeks or placebo	Brachial artery FMD using ultrasound.	Fourteen male patients with severe coronary artery disease (all on statin and aspirin)
Title 2003 [167] <i>Effect of Cyclooxygenase-2 Inhibition With Rofecoxib on Endothelial Dysfunction and Inflammatory Markers in Patients With Coronary Artery Disease</i>	Prospective Mechanistic study, RCT, placebo controlled double blind.	No difference between FMD between rofecoxib and placebo group: 4.0 ± 3.0% to 4.0 ± 3.8% vs. 2.7 ± 2.7% to 3.1 ± 2.7%, respectively	Rofecoxib 25mg/day for 8 weeks or placebo.	Brachial artery FMD using ultrasound.	Patients with coronary artery disease on low dose aspirin. 30 allocated to placebo, 30 to rofecoxib.
Cavka 2015 [168] <i>The role of cyclo-oxygenase-1 in high-salt diet-induced microvascular dysfunction in humans</i>	Prospective Mechanistic study, RCT, placebo controlled, single blind. Data presented is a subgroup analysis.	Indomethacin improved reactive hyperaemia _R-O HS diet 1.0 ± 0.2 vs. HS diet + indomethacin 1.3 ± 0.5, P < 0.05. No difference in reactive hyperaemia with	Participants received a high salt diet for 7 days plus either 100mg indomethacin OD or celecoxib 200mg	SkBF by laser-Doppler flowmetry	Healthy female medical students. Indomethacin n:13 Celecoxib n:10

		celecoxib (R-OHS diet 1.1 ± 0.4 vs. HS diet $\pm$ celecoxib 1.1 ± 0.3 , $P = 0.552$) compared to baseline.			
Luca 2013 [169] <i>Daily Ischemic Preconditioning Provides Sustained Protection From Ischemia-Reperfusion Induced Endothelial Dysfunction: A Human Study.</i>	Prospective Mechanistic study. Crossover RCT, double blinded.	No difference in FMD or reactive hyperaemia between celecoxib or placebo with or without ischaemic preconditioning.	Celecoxib 200mg BD for 7 days or placebo.	Radial artery FMD using ultrasound.	30 young male volunteers.
Verma 2001 [170] <i>Cyclooxygenase-2 Blockade Does Not Impair Endothelial Vasodilator Function in Healthy Volunteers Randomized Evaluation of Rofecoxib Versus Naproxen on Endothelium-Dependent Vasodilatation</i>	Prospective Mechanistic study. RCT. Outcome assessor blinded.	Naproxen or celecoxib had no change in Ach or sodium nitroprusside induced blood flow	7 days treatment with either rofecoxib 25mg/day or naproxen 750mg/day.	Forearm plethysmography.	Naproxen n:17 Rofecoxib n:17
Widlansky 2003 [171] <i>Short- and Long-Term COX-2 Inhibition Reverses Endothelial Dysfunction In patients With Hypertension.</i>	Prospective Mechanistic study. Placebo controlled RCT. Double blinded.	Treatment with celecoxib improved FMD 3 hours and 1 week after the first dose; Baseline $7.9 \pm 4.5\%$ to $9.9 \pm 5.1\%$ ($P=0.005$) 3 hours after the first dose and to $10.1 \pm 6.1\%$ 1 weeks after ($P=0.006$).	Celecoxib 200mg BD for 7 days or placebo.	Brachial FMD using ultrasound.	Placebo n:15 Celecoxib n:14
Solmaz 2012 [172] <i>The effect of non-steroidal anti-inflammatory drugs on the endothelial function of patients with osteoarthritis in short term.</i>	Prospective Mechanistic study. Placebo controlled RCT. Unblinded.	Diclofenac or naproxen had no effect on FMD or on nitroglycerin-induced vasodilation.	Diclofenac 75mg BD or naproxen 500mg BD for 7 days.	Brachial FMD using ultrasound.	Participants with osteoarthritis. Diclofenac n:20 Naproxen n:20
Florez 2009 [173] <i>Selective cyclooxygenase-2 inhibition reduces endothelial dysfunction and</i>	Prospective Mechanistic study. Placebo controlled RCT. Unblinded.	There was an increase in FMD both 3 hours ; ($3.33\% [4.11\%]$ vs $6.97\% [3.27\%]$; P	Celecoxib 200mg BD for 7 days.	Brachial FMD using ultrasound.	Patients with intermittent claudication.

<i>improves inflammatory status in patients with intermittent claudication.</i>		=.008); and 1 week after treatment; (3.33% [4.11%] vs 7.09% [4.4%]; P=.001.			
Calkin 2002 [174] <i>Rapid Potentiation of Endothelium-Dependent Vasodilation by Estradiol in Postmenopausal Women Is Mediated via Cyclooxygenase 2</i>	Prospective Mechanistic study. Placebo controlled RCT. Double blinded.	Vasodilator response to Ach after oestrogen administration was abolished. There was no difference in response with aspirin, diclofenac or placebo.	6-week treatment of either: celecoxib 200mg BD Diclofenac 50mg BD Aspirin 300mg BD Placebo	SkBF by laser-Doppler flowmetry	32 post-menopausal women.
Markwald 2011 [175] <i>Combined inhibition of nitric oxide and vasodilating prostaglandins abolishes forearm vasodilatation to systemic hypoxia in healthy humans</i>	Prospective Mechanistic study.	COX inhibition with ketorolac and NOS inhibition with L-NAME together reduced forearm vascular conductance. COX-2 inhibition alone had no effect.	Ketorolac 600ug/min for 5 minutes followed by maintenance doses of 150ug/min.	Brachial FMD using ultrasound.	group contained 8 men and 2 women,
Shoemaker 1996 [176] <i>Failure of prostaglandins to modulate the time course of blood flow during dynamic forearm exercise in humans.</i>	Prospective Mechanistic study. Placebo controlled RCT. Double blinded.	Ibuprofen had no effect on FMD following 5 mins of dynamic hand grip exercise	Placebo or 1.2g/day ibuprofen for 2 days	Brachial mean blood velocity using ultrasound.	8 healthy male volunteers
Bogaty 2004 [177] <i>Impact of Prolonged Cyclooxygenase-2 Inhibition on Inflammatory Markers and Endothelial Function in Patients With Ischemic Heart Disease and Raised C-Reactive Protein</i>	Placebo controlled, double blind RCT.	Rofecoxib had no effect on FMD. compared to placebo.	Rofecoxib 25mg OD or Placebo for 6 months	Brachial FMD using ultrasound.	35 patients with ≥2 previous acute coronary events and CRP>2mg/L.

Figure 1.6. Studies that demonstrate NSAIDs either reduce or have no effect on endothelial function.
Table summarising studies that demonstrate an increase or a reduction in endothelial function with NSAID use.

1.12 Summary

COX-2 has a central role in protecting the cardiovascular system. The use of NSAIDs, which work by blocking COX-2, is globally prolific and associated with increased risk of cardiovascular events. The concern associated with NSAIDs has led to a virtual arrest of drug development in this area and the removal of celecoxib as a chemo preventative agent. Whilst it is clear that prostacyclin is a key mediator in how COX-2 protects the cardiovascular system, a granular picture of the up and downstream mechanisms remain incompletely understood. Thus, it is timely that a comprehensive physiological and Omic approach be taken in order to advance our understanding of this important area.

1.13 Hypothesis and Aims

Hypothesis: A multi-omics approach will allow the identification of mechanism-based biomarkers that predict cardiovascular events in those taking NSAIDs and will reveal novel pathways by which COX-2 and prostacyclin protect the cardiovascular system

Objective: The overall aim of my thesis is to use an 'omics' approach to identify biomarkers that predict cardiovascular risk in individuals taking NSAIDs and understand mechanistic pathways through which COX-2 protects the cardiovascular system.

To achieve this I addressed the following aims:

1. Perform multi-omic (transcriptomic, proteomic, & amino acid profiling) analysis of serum samples from healthy volunteers taking the COX-2 inhibitor celecoxib and link these to cardiovascular (including blood pressure and endothelial function) and renal physiological endpoints measured in parallel
2. Perform a similar multi-omic analysis of samples from patients having had cardiovascular events versus identically matched case controls taken from the SCOT (Standard Care versus Celecoxib Outcome Trial) bio-bank[31]
3. Perform pathway analysis on omics readouts from human samples in aims 1 and 2 in order to identify biomarkers and predictive pathways for further analysis.

Chapter 2: General Methods

2.1 Animal Studies

2.1.1 Ethics

All animal studies were conducted in accordance with the UK Animals (Scientific procedures) Act 1986 Amendment (2013) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and under the jurisdiction of the UK home Office (Licence no 70/8422).

2.1.2 Cyclooxygenase-2 (COX-2) wild type and knockout (KO) mice

Samples were donated by Prof Rolf Nusing (Goethe University, Frankfurt, Germany) and after approval by Animal Welfare Committee of the State Agency Darmstadt. Details have previously been published [64].

2.1.3 Prostacyclin synthase (PGIS) wild type and knockout mice

Samples were donated by Prof Bin Liu and Prof Yingbi Zhou (Shantou Medical University, China) and collected under ethics number SUMC2019-336 after approval by the Shantou University Institutional Animal Research and Use Committee. Details have previously been published [178].

2.1.4 Proprotein convertase subtilisin/kexin type 9 (PCSK9) mice

Samples were donated by Prof Jacob Bentzon (Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain) and local ethical approval was obtained. Details of the model have previously been published [179].

2.2 Clinical Studies

2.2.1 ASCENT

Further description of the study design has also been provided in **Chapter 4**. Ethical approval was obtained; REC reference 20/PR/0378. The trial was registered on clinicaltrials.gov before commencement (NCT04765644).

2.2.2 SCOT Samples

Further details on samples and study design can be found in **Chapter 6**.

2.3 Cell Culture

Materials for cell culture and associated chemicals were obtained from Sigma Aldrich (UK). L-glutamine and 100x non-essential amino acids were obtained from (Thermo Fisher Scientific™; 25030081) and 100x nonessential amino acids (Invitrogen™, UK; 11140-035). Cell culture work was performed under sterile conditions. Incubation of cells took place in an incubator with a humidified atmosphere of 5% CO₂ with a temperature of 37°C. The media was replaced as required.

J774 mouse macrophages were obtained from American Type Culture Collection (USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; D6546). This was supplemented with 100U/ml penicillin, 0.1g/ml streptomycin, 10% filtered foetal bovine serum (FCS), 1x non-essential amino acids and 2mM L-glutamine. Cells were grown in T75 flasks and once they'd reached >70% confluence, they were scraped and collected and centrifuged at 400 RCF using maximum acceleration and medium brake settings for 5 minutes. The pellet was reconstituted using DMEM.

2.3.1 COX-2 inhibition Bioassay

Day 1

J774 cells were plated in a 96 well plate (10,000 cells per well). Cell count was obtained using a haemocytometer and cells were cultured in DMEM+10% FCS for 24 hours.

Day 2

The media was then discarded and 200ul of LPS media (DMEM+10% FCS + 1μM LPS) was added to each well and cultured in the incubator overnight for 18 hours to induce COX-2 in the cells.

Day 3

The next day, the plate was centrifuged at 500g for 5 mins and the media was removed. Next 90ul of plasma from the volunteers was added to each well and incubated for 30 minutes. Next 10ul of 0.01uM of A23187 was added to each well to stimulate prostaglandin release. After a further 30 minutes of incubation, 20ul of

diclofenac stop solution was added to each well and the plate was centrifuged at 500g for 5 minutes. The media was removed, and a prostaglandin E2 (PGE2) Enzyme-linked immunosorbent assay (ELISA) was performed.

2.3.2 Bioassay- Kidney Organ Culture

Tissue collection was performed by Professor Jane Mitchell and Dr Nicholas Kirkby. Briefly, mice were euthanised with CO₂ and kidneys were removed in tubes with sterile PBS. Harvested kidneys were then sliced and placed onto a 48 well plate along with Dulbecco's Modified Eagle Medium (DMEM; D6546) (Sigma Aldrich (UK) supplemented with 100U/ml penicillin, 0.1g/ml streptomycin, 1x non-essential amino acids and 2mM L-glutamine. Kidney slices were cultured for 24 and 72 hours in an incubator with a humidified atmosphere of 5% CO₂ with a temperature of 37°C. Media was collected at 24 and 72 hours and Asymmetric dimethylarginine (ADMA) and Prostacyclin (PGI₂) was measured using ELISA by Matthew Rickman and analysed by me.

2.4 ELISAs

ELISA is an analytical biochemistry test used to detect the presence of a ligand using antibodies. There are four distinct types of ELISA which include direct, indirect, sandwich and competitive. The basic principles behind each type of ELISA are broadly similar and requires an antigen to be first attached to the surface of a plate. A matching enzyme-linked antibody is then applied which binds the antigen and unbound antibodies are subsequently removed. Finally the substrate is added, and any binding produces a colour change that is detected using a spectrophotometer. This is compared to a standard curve which allows estimation of the concentration of substrate in each well.

All ELISA kits were used according to manufacturer's instructions. A summary of each is provided below.

2.4.1 ADMA/ L-Arginine

Human ADMA and L-arginine concentrations were detected using a competitive ELISA kit (EA207 / 192; DLD Diagnostika, Germany). As both L-arginine and ADMA lack immunogenicity, the process first requires an acylation step with an epitope that is recognised by the antibodies. Serum or plasma samples from either human or mouse were thawed from -80°C storage and 20µl was added to a 96 well plate. Next 25µl of acylation buffer and 200µl of equalising agent was added, and the plate was mixed for 10 seconds using an orbital shaker. Next 50µl of acylation reagent was added to each well and the plates were incubated for 20 minutes at room temperature on an orbital shaker at medium speed. Separate volumes of 10µl and 25 µl for L-arginine and ADMA respectively were transferred from each well onto the relevant ADMA and ELISA plates. The kit provided standards ranging from 5-300µM for L-arginine and 0.2- 3µM for ADMA which were also added to the respective plates. Next 50µl of antiserum was added to each plate which was then covered in foil and incubated on an orbital shaker for 90 minutes at room temperature. Plates were subsequently washed four times using 300µl of wash buffer in each well. Next 100µl of enzyme conjugate was added to both L-arginine and ADMA plates and incubated for 30 mins at room temperature on an orbital shaker. Each well was then washed four times using 300µl of wash buffer. Then 100µl of substrate was added to each well and the plates were incubated for 25 mins at room temperature on an orbital shaker. Finally 100µl of stop solution was added to each well and the absorbance was measured at 450nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9). **Figure 2.1.**

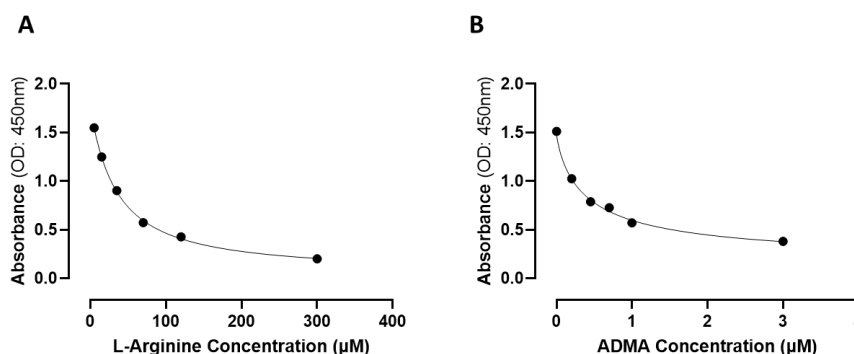


Figure 2.1 Standard curves for the L-arginine and ADMA ELISA. An increase in L-arginine (Figure A) and ADMA (Figure B) concentrations is associated with a reduction in the number of antibodies that bind to the solid phase which translates to a reduction in optical density (OD)

2.4.2 IL-6

Human Interleukin-6 (IL-6) concentrations were detected using a sandwich ELISA kit (430504, BioLegend, California, USA). A human specific rat monoclonal antibody provided by the manufacturer was first coated onto a 96 well plate and incubated overnight for 16 hours between 2°C and 6°C. The following day, the plate was washed four times using 300ul of wash buffer per well. Next, 200μl of assay diluent was added to each well and left to incubate for 1 hour on an orbital shaker (500rpm) at room temperature to block non-specific binding. Each well was then again washed four times using 300μl of wash buffer. Next 100μl of standards ranging from 7.8 to 500 pg/ml were added in duplicate. As the expected concentration of IL-6 in healthy participants would fall in an around the lower limit of detection, two further standards at 3.9 and 1.95pg/ml were added to the plate for both SCOT and ASCENT trial samples. As seen in **Figure 2.2**, samples that fall on the lower range of detection on the standard curve may not be reliably interpreted. Next 100μl of neat serum sample was added to each well, the plate was sealed and incubated at room temperature for 2 hours. Each well was then washed four times using 300ul of wash buffer and then 100μl of a detection antibody was added to each well and incubated for 1 hour at room temperature. Each well was then washed five times using 300μl of wash buffer. A 100μl of diluted Avidin-HRP solution was added to each well and incubated at room temperature for 30 minutes. Each well was then washed 5 times using 300μl of wash buffer. Finally 100μl of TMB substrate solution was added to each well and

after 20 minutes of incubation in the dark, 100µl of stop solution was added to each well and the absorbance was measured at 450nm with a wavelength correction of 570nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9) **Figure 2.2.**

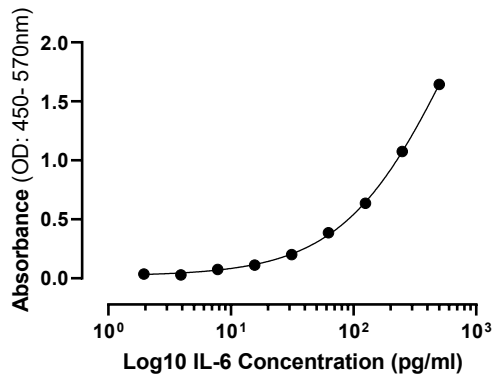


Figure 2.2 Standard curve for the IL-6 ELISA. An increase in IL-6 concentration is associated with an increase in the number of antibodies that bind to the solid phase which translates to an increase in optical density (OD).

2.4.3 Endothelin-1

Human Endothelin-1 (ET-1) concentrations were detected using a sandwich ELISA kit (DET100, R&D Systems, Minneapolis, USA). A monoclonal antibody specific for ET-1 was pre-coated onto a 96 well plate provided by the manufacturer. Serum or plasma samples from either human or mouse were thawed from -80°C storage and 50µl was added to a 96 well plate. The kit standards and QCs were also added. Next 175µl of Assay diluent was added to each well and the plate was covered and incubated for 1 hour at room temperature on an orbital shaker at 500rpm. Each plate well was then washed four times using 400µl of wash buffer. Next 200µl of ET-1 conjugate was added to each well, the plate was covered and incubated for 3 hours at room temperature on an orbital shaker. Each well was again washed four times using 400µl of wash buffer. Next 200µl of substrate solution was added to each well, the plate covered and incubated for 30 minutes at room temperature. Finally 50µl of stop solution was added to each well and the absorbance was measured at 450nm with a wavelength correction of 570nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9). **Figure 2.3.**

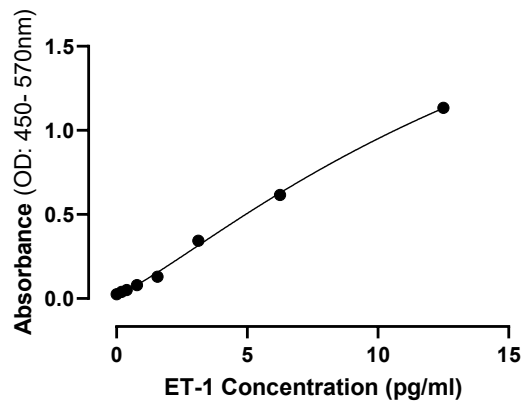


Figure 2.3 Standard curve for the ET-1 ELISA. An increase in ET-1 concentration is associated with an increase in the number of antibodies that bind to the solid phase which translates to an increase in optical density (OD).

2.4.4 MCP-1

Human MCP-1 concentrations were detected using a sandwich ELISA kit (438804, BioLegend, California, USA). A human MCP-1 specific mouse monoclonal antibody was diluted in equal volumes with a coating buffer and 100µl was added to a 96 well plate and was incubated overnight for 16 hours between 2°C and 6°C. The following day, the plate was washed four times using 300µl of wash buffer per well. Next, 200µl of assay diluent was added to each well and left to incubate for 1 hour on an orbital shaker (500rpm) at room temperature to block non-specific binding. Each well was then again washed four times using 300µl of wash buffer. Next 100µl of standards ranging from 7.8 to 500 pg/ml were added in duplicate. In order to determine the optimal range of detection for human serum samples from SCOT, a test run whereby quadruplicates of 100%, 50% and 25% of serum sample was performed **Figure 2.4**. A 50% dilution was used for both SCOT and ASCENT trial samples and 100µl of sample was added to each well, the plate was sealed and incubated at room temperature for 2 hours. Each well was then washed four times using 300µl of wash buffer and then 100µl of a biotinylated Armenian hamster monoclonal antibody is added to each well and incubated for 1 hour at room temperature. Each well was then washed five times using 300µl of wash buffer. Finally 100µl of TMB substrate solution was added to each well and after 20 minutes

of incubation in the dark, 100 μ l of stop solution was added to each well and the absorbance was measured at 450nm with a wavelength correction of 570nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9).

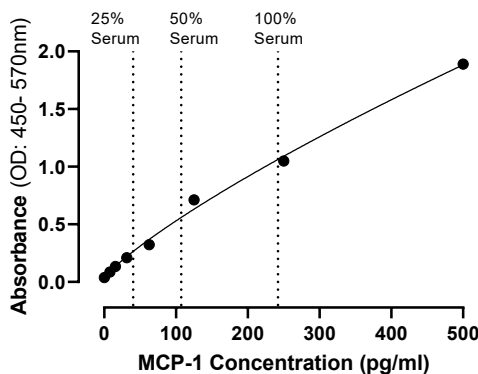


Figure 2.4 Standard curve for the MCP-1 ELISA. Human serum samples from the SCOT cohort were first tested in quadruplicate using 100%, 50% and 25% of serum diluted using assay diluent to determine the optimum dilution. The mean concentrations of MCP-1 at each dilution is indicated by the dotted lines.

2.4.5 Cystatin C

Mouse cystatin C concentrations were detected using an ELIS kit (DY1238, R&D systems, Minneapolis, USA). Briefly, a 96 well plate was coated with 100 μ l capture antibody and incubated overnight. Wells were then washed 3 times using 400 μ l of wash buffer. Next 100 μ l of sample or standard were added to the wells and incubated for 2 hours at room temperature. Wells were again washed 3 times and then 100 μ l of streptavidin-HRP was added to each well and incubated for 20 minutes. Wells were again washed 3 times and 100 μ l of substrate solution was added incubated for a further 20 mins at room temperature. Finally 50 μ l of stop solution was added to each well and the absorbance was measured at 450nm with a wavelength correction of 570nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9).

2.4.6 Urea

Human and mouse urea nitrogen concentrations were detected using a colorimetric assay kit (EIABUN, ThermoFischer Scientific, MD, USA). Briefly, plasma samples were diluted 1:20 in deionized water. Next 50 μ l of sample or standard was added to a 96 well plate. Next 75 μ l of reagent A and 75 μ l of reagent B were added to each

well. Following 30 minutes of incubation at room temperature, absorbance was measured at 450nm with a wavelength correction of 570nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9).

2.4.7 PGE₂

Human PGE₂ concentrations were detected using an ELISA kit (514010, Cayman Chemical, MI, USA). Briefly, 100µl of ELISA buffer was added to each well with 50µl of either sample or standard followed by 50µl of PGE₂ acetylcholinesterase (AChE) tracer and 50µl of PGE₂ monoclonal antibody. Wells were then washed 5 times with wash buffer and 200µl of Ellman's reagent was added to each well. The plate was then incubated on an orbital shaker for 60-90 minutes and absorbance was measured at 420nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9).

2.4.8 PGI₂

PGI₂ levels were approximated by measuring the stable PGI₂ breakdown product 6-keto-PGF1α using an ELISA kit (515211, Cayman Chemical, MI, USA). Briefly, 100µl of ELISA buffer was added to each well with 50µl of either sample or standard followed by 50µl of 6-keto-PGF1α AChE tracer and 50µl of 6-keto-PGF1α anti-serum. Wells were then washed 5 times with wash buffer and 200ul of Ellman's reagent was added to each well. The plate was then incubated on an orbital shaker for 90-120 minutes and absorbance was measured at 420nm using a spectrophotometer (Infinite F50; Tecan, Switzerland). Concentrations were interpolated using nonlinear regression (GraphPad Prism, V9).

2.4.9 MultiPlex assays

A panel of kidney function and vascular inflammation markers were measured using LEGENDplex™. The assays follow the same principles as sandwich immunoassays whereby beads which are differentiated by fluorescence intensity and size are used to measure a panel of analytes simultaneously.

Details of the assay principles are provided in **Figure 2.5**.

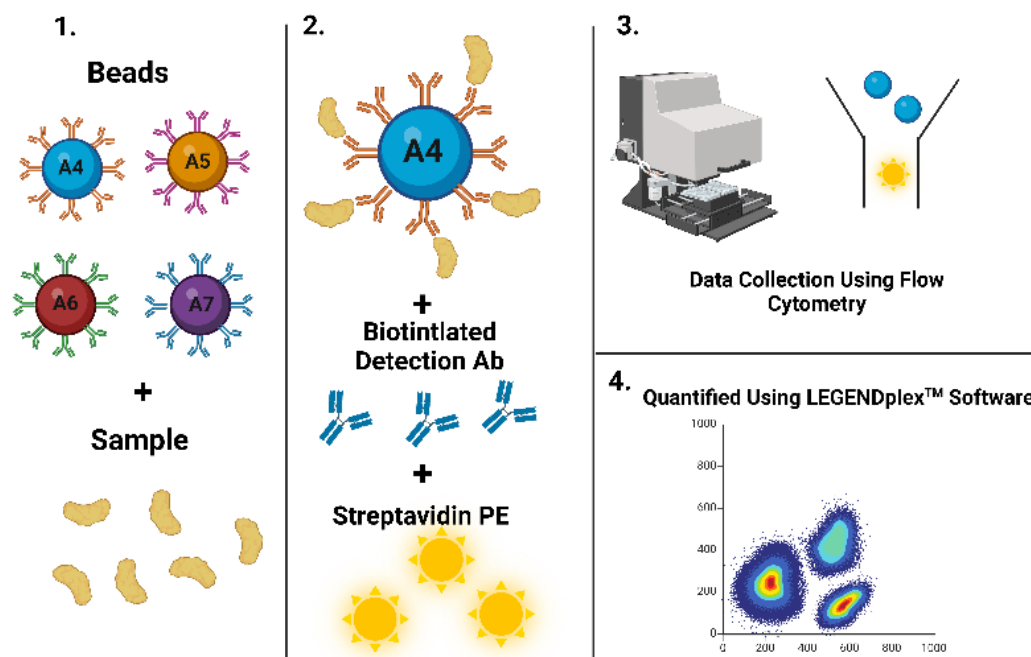


Figure 2.5 Principles of MultiPlex assay. (1) Each set of beads is conjugated with an antibody and when these are mixed and incubated with a sample, the targeted analyte binds to the specific capture beads. (2) Washing is then followed by the addition of a biotinylated antibodies forming capture bead analyte detection antibody sandwiches. Finally streptavidin-phycoerythrin is added which bind the detection antibodies and provides fluorescent signal intensities. (3) Beads are then differentiated by size and fluorescent intensity using a flow cytometer and concentrations are determined from the standard curves.

Three separate panels of analytes were measured:

LEGENDplex™ Human Kidney Function Panel 2 (740583)

LEGENDplex™ Human Thrombosis Panel (7-plex) (740905)

LEGENDplex™ Mouse Inflammation Panel (13-plex) (740446)

The general steps for each of the assays is the same and is described below:

A pre prepared mixture of standards provided in the kit was first reconstituted using 250µl of reconstitution buffer. The top standards for each of the analytes is provided in the **Figure 2.6** and serial ¼ dilutions were made using assay buffer. Next serum samples were diluted 1/50 using 4µl of sample and 196µl of assay buffer. Next 25µl of assay buffer was added to each well followed by 25µl of standards or diluted serum sample and 25µl of beads (for thrombosis panel and inflammation panel, 25µl of Matrix C is also added to each well). The plates were sealed and placed onto an orbital shaker at 500 RPM for 2hours at room temperature. Next plates were washed

by adding 200µl of wash buffer which was then removed using vacuum filtration. This was done twice. Next 25µl of detection antibody was added to each well and the plate was sealed and placed on an orbital shaker at 500 RPM for 1 hour at room temperature. Then 25µl of streptavidin-phycoerythrin is added which binds the detection antibodies and provides fluorescent signal intensities. The plate was sealed and incubated for 30 minutes on an orbital shaker at 500 RPM at room temperature. Wells were then washed twice using wash buffer and vacuum filtration. Finally 150µl of wash buffer was added to resuspend the beads. Samples were then read on a flow cytometer BD (LSR Fortessa II TM). Concentrations were calculated using the generated standard curves, examples of which are provided in **Appendix 1**. Quality control was performed and any samples with less than 100 bead counts per analyte were excluded from the analysis.

<u>LEGENDplex TM Human Kidney Function Panel 2 (740583)</u>		<u>LEGENDplex TM Human Thrombosis Panel (7-plex) (740905)</u>		<u>LEGENDplex TM Mouse Inflammation Panel (13-plex) (740446)</u>	
Analyte	Top Standard (ng/ml)	Analyte	Top Standard (ng/ml)	Analyte	Top Standard (ng/ml)
IGFBP7	100	P-Selectin	400	IL-23	50
NGAL	100	D-Dimer	100	IL-1α	10
Cystatin C	800	PSGL-1	50	IFN-γ	10
TIMP-2	400	tPA	100	TNF-α	10
OPN	10	SCD40L	50	MCP-1	10
TFF3	10	PAI-1	100	IL-12p70	10
Uromodulin	250	Factor IX	2500	IL1-β	10
				IL-10	10
				IL-6	10
				IL-27	50
				IL-17A	10
				IFN-β	150
				GM-CSF	10

Figure 2.6 Table of analytes and top standard concentrations. In total 27 analytes across 3 assays was performed. The table details the analytes in each assay alongside the top concentration of the highest standard.

2.5 Transcriptomics

2.5.1 RNA isolation and purification

Whole blood collected in Tempus™ blood RNA tubes was immediately stored at -80°C. When extracting RNA, the collection tubes were thawed at room temperature. The Tempus™ spin RNA isolation kit was used for extraction and purification. Contents (9ml) were transferred to a 50ml conical tube and 3ml of PBS (CA²⁺/Mg²⁺ free) was added to bring the total volume to 12ml. The mixture was vigorously vortexed for 30 seconds. Next the Conical tubes were centrifuged at 4 °C at 3,000xg for 30 minutes. The supernatant was carefully discarded, and the RNA pellet was resuspended using 400µl of RNA purification resuspension solution and vortexed. The collected RNA was then purified. An RNA purification filter was inserted into a waste collection tube and the membrane was pre-wetted using 100µl of RNA purification wash solution 1. Next µl of the resuspended RNA was added to the filter and centrifuged for 30 seconds at 16,000xg. The purification filter was removed, liquid discarded, and filter re-inserted into the tube. Next 500µl of purification wash solution 1 was added and centrifuged for 30 seconds at 16,000xg. The purification filter was removed, liquid discarded and filter re-inserted into the tube. Next 500µl of purification wash solution 2 was added and centrifuged for 30 seconds at 16,000xg. The purification filter was removed, liquid discarded and filter re-inserted into the tube. This step was performed twice. The empty tube and filter was centrifuged for 30 seconds at 16,000xg to dry the membrane. The purification filter was transferred to a new collection tube and 100µl of nucleic acid purification solution elution solution was added to the filter, incubated for 2 minutes at 70 °C and centrifuged for 30 seconds at 16,000xg. The 100µl of collected RNA eluate was added back to the filter and centrifuged for 2 minutes at 16,000xg. 80µl of the RNA eluate was transferred to a new labelled collection Tube and immediately stored at -80 °C. The remaining 10µl was used to perform quality control checks using the Nanodrop device **Figure 2.7**.

2.5.2 RNAseq and data analysis

The purified RNA was transported to the Imperial College Genomics Facility who performed sample analysis using RNAseq. Aligned and normalised count data was

provided to me in a txt.file and PARTEK® Flow ® was used for analysis. Further description of the analysis performed is provided in **Chapter 6** which includes PCA, differential expression analysis and pathway analysis.

	Visit 1		Visit 2	
Study ID	Concentration (ng/μl)	260/280 ratio	Concentration (ng/μl)	260/280 ratio
9732	77	1.91	72.3	1.93
5414	80	1.9	83.7	2.05
1780	75.8	1.93	79.5	1.95
2034	82.2	1.94	63.5	2.03
1229	80	1.91	64	1.92
4454	98.4	1.79	58.3	2
7182	100.7	1.93	64.7	2.03
9564	80.3	1.9	97.4	1.93
7653	70.9	2.29	67	1.98
8406	162.5	2.07	111.5	2.02
8800	171.2	2.4	79.5	2.07
6513	103	1.92	126	2.12
4634	73.2	2	78.5	2
3915	76.5	1.94	59.1	1.84
5245	119.7	1.71	105.5	1.91
4093	33.2	1.98	70.3	1.98
4738	63.6	2.07	82.9	1.98
4355	93.2	1.81	71.4	2.03
4105	68.2	1.95	79	2.01
5816	75.7	1.99	85.7	1.83
4786	49.8	1.96	72	2.02
9596	78.3	2.01	69.6	1.98
8940	112	2	78.2	2.11
7994	81.5	1.92	65.6	2.04
6692	99	1.85	64.2	2.07
8632	64.3	1.86	51.1	2.01
1210	78.9	2.06	67.9	2.07
5836	73.5	2.09	66.6	2.07
4902	49.2	2.08	58.1	1.94
2014	56.3	2.04	56.4	2.11
2534	90.1	2	96.8	1.9
8020	65.5	1.99	78	1.91
9481	112.2	2.02	106.5	2.06
6142	57.2	2.03	74.8	1.84
2940	78.1	1.92	65.8	1.86
4029	88.1	1.84	57.7	1.98
6230	99.7	1.73	107.2	1.73
6748	107	1.91	82.9	2.01
8025	104.1	1.92	78.2	1.85
5838	109.8	1.9	118.6	1.92

Figure 2.7 Table showing the RNA concentration (ng/ml) and the quality of the RNA (ratio of the absorbance at 260/280) following RNA extraction from whole blood from ASCENT volunteers. The average concentration of RNA extracted was 81.9 +/- 23.1ng/uL. The average 260/280 ratio was 1.97 +/- 0.1. A ratio of ~2 indicates a higher purity for RNA.

2.6 Proteomics

2.6.1 Sequential window acquisition of all theoretical mass spectra (SWATH-MS)

SWATH proteomic analysis was performed by the Stoller Biomarker discovery centre for the SCOT-1 samples (**Chapter 7**). Details on how the samples were plated including randomisation is provided in **Chapter 6**. In brief, samples were randomised and plated by me and Dr Ferreira and shipped to the Biomarker discovery centre for analysis. Samples were immunodepleted using top 14 abundant protein depletion resin (ThermoScientific UK) and total protein concentration determined. Samples were then reduced, alkylated and digested using S-trap columns prior to lyophilisation. Sample analysis was performed by SWATH-MS using a microflow LC-MS system coupled to a SCIEX 6600 triple-TOF mass spectrometer. Analysis was then performed against the stoller library. Differential expression analysis was performed by the stoller bioinformatics team with the R package Limma using an empirical bayes approach. All the analysis including correction for batch effect was performed by the Stoller bioinformatics team. A report was provided including PCAs and differential expression data.

2.6.2 Olink

Targeted proteomic analysis of 369 proteins was performed by Olink (**Appendix 2**). In brief, Olink utilises Proximity Extension Assay (PEA) technology enables the high throughput analysis of a large number of proteins from <10µl of serum sample. This was used in both the ASCENT samples (**Chapter 5**) and SCOT-2 (**Chapter 8**). Detailed description about sample preparation and randomised plate layouts is provided in **Chapters 5 and 8** accordingly. Olink performed the assays and generated raw data. They were blinded to the groups and so raw data was provided to me in the form of Normalised Protein eXpression (NPX) scores for each protein and each sample in an excel sheet. NPX is a unit derived by Olink and is calculated from the Ct values. Normalisation is performed to minimise inter and intra-assay variation. NPX is in Log2 scale so a NPX difference of 1 means a doubling of protein concentration. It should be noted that NPX should only be used for relative quantification. For ASCENT, delta was calculated between visits for celecoxib and placebo group.

PCA analysis for both ASCENT and SCOT was performed using SIMCA 13.0.3 (Sartorius Stedim biotech, France) and differential expression was performed in IBM SPSS. Graphs for individual analytes were generated using GraphPad Prism.

2.6.1 Metabolomics- amine and methylarginine quantification using UHPLC/MS/MS

I utilised an UHPLC-MS/MS platform for quantification of amino acids and methylarginines previously developed and published by my group[74]. This original method will be referred to as Method A. This was further optimised by my colleague Dr Plinio Ferreira and will be referred to as Method B.

Sample preparation for data generated in **Chapter 3 (Method A)** was performed by myself. Sample preparation for data generated in **Chapters 5,7 & 8 (Method B)** was performed by Dr Plinio Ferreira and analysis was performed by me.

Briefly, in method A, 10µl of serum sample was used and diluted 1:1 using Optima™ LC-MS grade water (Fisher Scientific; Leicester, UK) and 20µl of standard was used. The remaining steps for both sample and standard were the same. In the final analysis, differences in concentrations for standard and sample were adjusted accordingly. For Method B, 20µl of serum sample was used with no dilution together with 20µl of standard. No post-hoc adjustment for differences in concentration was therefore required. The remaining steps were the same between the methods. To this 5µl of internal standard (contains 16 stable-isotope-labelled internal standards & is used a quality check for extraction) was added. Protein was then precipitated using 40µl of 0.1% formic acid and stored at -20 °C for 20 minutes and then centrifuged for 10 minutes at 10,000g. Next 10µl of the supernatant was transferred into another Eppendorf and 70µl of borate buffer was added, and samples were centrifuged at 2000g for 2 minutes. Next sample derivatisation was performed using 20µl of 6-aminoquinolyl-N-hydroxysuccinimide carbamate (AccQTag™ kit - Waters, Wilmslow, UK). This maximises detection of amino acids and amines. Samples were heat sealed at 55°C for 10 minutes. Finally the sample was diluted 1/10 using Optima™ LC-MS grade water (Fisher Scientific; Leicester, UK). Samples were then loaded onto the UHPLC/MS/MS. Data acquired was then quantified using MassLynx V4.1 software (Waters, Milford, MA, USA). PCA analysis was performed using SIMCA 13.0.3

(Sartorius Stedim biotech, France). Example standard curves are provided in **Appendix 3**.

2.7 Data analysis

Statistical analysis (unless otherwise stated) and generation of graphs was performed using IBM SPSS and GraphPad Prism V9. Data in figures is presented as mean $\pm$ standard error of the mean. For continuous variables, normality was not assumed and was assessed using the Shapiro Wilk test and Kolmogorov-Smirnov tests. Visual distribution of the data was also assessed using histograms and QQ plots when data appeared to be normally distributed but did not pass the tests. This has been clearly stated in the Results chapters when performed. Normally distributed data was analysed using parametric tests and non-normally distributed data was analysed using non-parametric tests. Cohens D was used to estimate effect size. When interpreting effect size, the following table was used as a guide

Figure 2.8 [180].

Value for Cohen's d	Interpretation
<0.20	Small effect size
0.2-0.49	Small to medium effect size
0.5	Medium effect size
0.5-0.79	Medium to large effect size
>0.8	Large effect Size

Figure 2.8 Table summarising the interpretation of Cohen's D. These definitions have been used throughout the manuscript when describing effect size.

Chapter 3: Effect of Global Genetic Loss of COX-2 Pathways on Biomarker Identification and Mechanistic Insights in Mouse and Human Models

3.1 Rationale

Through inhibition of cyclooxygenase-2 (COX-2), non-steroidal anti-inflammatory drugs (NSAIDs) increase the risk of cardiovascular events. As discussed previously, while reduction in COX-2 driven prostacyclin (PGI₂) is a likely link in the chain, the up and downstream mechanisms associated with the increased risk remains incompletely understood and, as such, there are currently no biomarkers used in clinical practice to predict NSAID induced cardiovascular adverse events. To address this, the main aim of my thesis was to establish biomarkers and mechanisms associated with COX-2, NSAIDs and the cardiovascular system. The kidney is a well-recognised site where COX-2 is expressed constitutively and as such blocking COX-2 with NSAIDs or genetic deletion of COX-2 is associated with renal dysfunction. As renal dysfunction is associated with increased risk of cardiovascular disease [181], in this chapter I explore links between COX-2, renal function and a range of amines focusing on the methylarginines and associated amino acids.

This approach came about because of the link between COX-2 inhibition and the endothelial NOS (eNOS) pathway [51] [63]. eNOS is cardioprotective and is expressed throughout the vasculature. Inhibition/genetic deletion of COX-2 has been shown to reduce endothelial dependent vasodilation [51, 63, 64] accompanied by reduce eNOS gene expression or increases in levels of the naturally occurring methylarginines. Methylarginines asymmetric dimethylarginine (ADMA) and N(G)-monomethyl-L-arginine (L-NMMA) which prevent access of the substrate, L-arginine, to the eNOS active site are natural inhibitors of endothelial dependent vasodilation [64]. ADMA (which is regulated in the kidney) is a recognised biomarker of end-stage renal failure, cardiovascular disease and all-cause mortality[67]. ADMA is also increased in plasma from prostacyclin synthase (PGIS) knockout mice but not in prostaglandin E₂ (PGE₂) synthase knockout mice[73]. This suggests that the COX-2 end-product that limits ADMA levels is PGI₂. PGI₂ and NO synergistically inhibit platelet function and act additively as vasodilators[3]. Therefore, ADMA is a potential mechanism and/or biomarker of NSAID induced cardiovascular side effects. It should be noted that not all studies find increased ADMA in models where COX-2 is deleted or inhibited. Specifically, Ricciotti and co-workers found, in models where renal function was not

compromised, ADMA was not increased in samples from mice where COX-2 was knocked out or inhibited or in samples from patients taking NSAIDs[182].

Here I have used samples from extreme models of loss of COX-2/prostacyclin including germline global COX-2 and PGIS knock out (KO) mice and a human subject with a rare homozygous mutation in PLA₂ before and after transplant with a PLA₂ replete kidney. As PLA₂ activity is essential in this subject for prostanoid production, their mutation mimics a global COX-2 KO phenotype while, after transplant the patient could be considered as having a kidney specific COX/prostanoid knock-in phenotype.

Finally, since the impact of methylarginines on eNOS function is inherently influenced by levels of L-arginine and associated amino acids, I have utilised a metabolomic approach focused on amine analytes.

3.1.1 Hypothesis

“Renal dysfunction underpins changes in ADMA and associated amino acids in extreme human and mouse models of COX-2 deletion”.

The aims of this chapter were to:

- Quantify and compare metabolites including ADMA in plasma samples from the human patient deficient in prostanoids, due to a mutation in PLA₂, before and after renal transplant and from global germline COX-2 and PGIS KO mice.
- Measure renal function markers and make correlations with plasma methylarginines.

3.1.2 Declaration

It should be noted that the measurements of levels of methylarginines and amino acids in the patient carrying the PLA₂ mutation were performed by a previous student in the group (Yu He), which followed a pilot study (n=2) performed by an earlier master's student, Esther Ajayi, Yu He also performed the methylarginine and amino acid measurements on n=5 (batch 1) of the COX-2 KO mouse samples. I measured methylarginines and amino acids in samples from an additional n=4 (batch 2) COX-2

KO mice and in samples from the PGIS KO mice. Blood urea and cystatin C were not previously analysed.

3.2 Methods

A detailed description of the methods used for UHPLC/MS/MS, ADMA/Arginine, cystatin C ELISA, urea and the kidney organ culture are discussed in **Chapter 2 Methods**. The raw concentrations for analytes measured in this chapter are provided in **Appendix 5**.

3.3 Results

3.3.1 Global COX-2 KO mice- Amines

The data for the global COX-2 KO mice were generated from two separate batches. Analysis of Batch 1 was performed in 2018 by a previous member of the group and included n=5 wild type and n=5 COX-2 KO mice. Batch 2 was performed by me and included n=4 wild type and n=4 COX-2 KO mice. Only common amines/methylarginines quantified between the batches were included in the analysis.

Using UHPLC/MS/MS, it was possible to quantify 23 common amines and the methylarginine ADMA. To provide a validation step, for COX-2 KO mice, levels of ADMA were additionally measured by ELISA.

First, an unsupervised analysis using principal component analysis (PCA) was performed using the pooled samples. When combining data from the two batches, it became evident that there were differences in the actual quantified concentrations of amines and methylarginines due to a batch effect which is demonstrated by grouping on PCA **Figure 3.1A**.

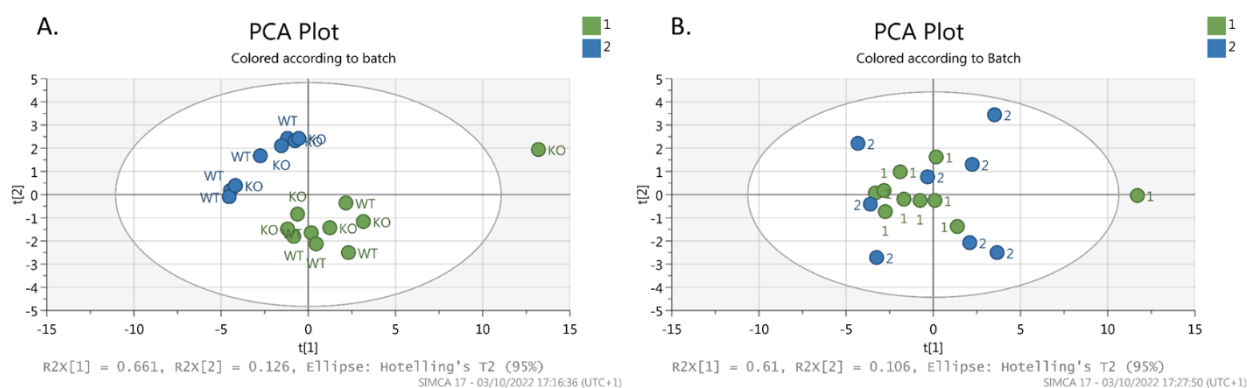


Figure 3.1 PCA plot demonstrating batch effect. (A) PCA analysis shows separation of samples according to batch (1: green, 2: blue). (B) PCA analysis shows samples no longer group according to batch following standardisation using Z-scores.

To overcome this, I standardised data from the separate batches by calculating the Z-score for each amine/ methylarginine. The Z-score or standard score is the number of standard deviations by which an individual data point is above or below the mean value of that population. In this instance, the population was a pool of COX-2 KO and wild type readouts per amine per batch (n:18). It was calculated for each amine by subtracting the population mean concentration from the raw concentration and dividing by the population standard deviation. The z-scores from each batch were then pooled together so that the overall distribution will have a mean of 0 and a standard deviation of 1. This method of standardisation is better suited for subsequent PCA analysis than just dividing by the mean of the population as values that have a high baseline concentration are likely to have more variance which can influence PCA thereby skewing the data. The pooled Z-scores were then analysed using PCA as an unsupervised approach. No additional pre-processing steps (such as scaling or transformation) were required as standardisation had already been performed. Following standardisation, samples no longer grouped according to batch **Figure 3.1b** but there was separation of the COX-2 and wild type mice **Figure 3.2a**

A loading plot was then constructed to assess the influence of each amine on the principal component. **Figure 3.2b**. Most amines clustered away from the centre suggesting that global COX-2 deletion has a profound effect on a range of amines. Next, a supervised analysis was performed by first calculating fold change between levels of analytes in wild type versus COX-2 KO mice, which is presented in a heatmap

in **Figure 3.2.c** with the associated p values. Amines with more than 1.5-fold increase and $p < 0.05$ included ADMA, arginine and citrulline.

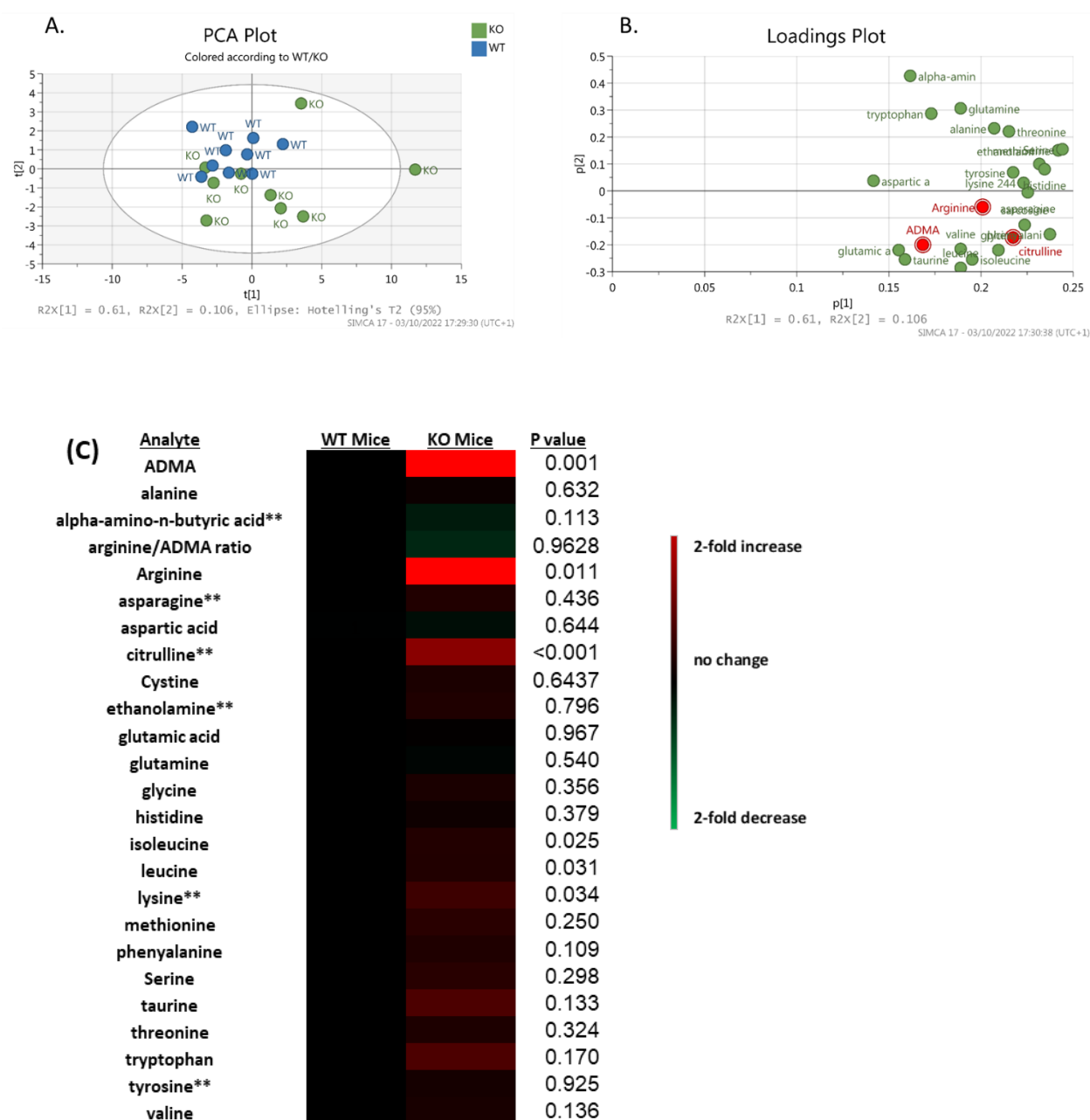


Figure 3.2 Quantification of amines and methylarginines in COX-2 knockout (KO) mice using UHPLC/MS/MS. (A) PCA analysis shows wild type (WT) mice (blue) separate from COX-2 knockout mice (green). (B) Loadings plot demonstrating the influence of each amine and methylarginine (Red) on the principal component. (C) Heat map and associated p values. **Analysed using Mann Whitney U test

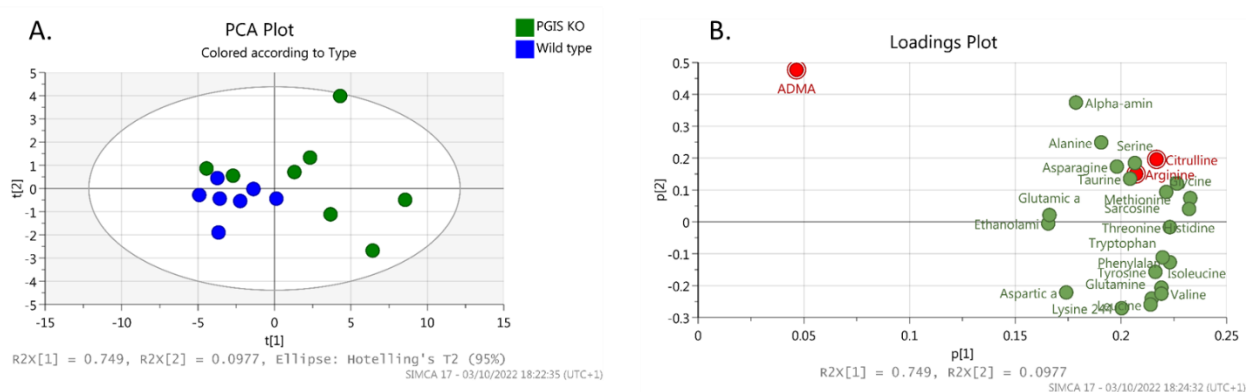
3.3.2 PGIS KO Mice- Amines

Data from PGIS KO mice was generated from n:8 PGIS KO and n:7 wild type mice. In total, 23 amines and the methylarginine ADMA were included in the analysis. These samples were analysed using UHPLC/MS/MS as one batch but to allow for consistency in data quantification, Z-scores were calculated. In regard to validation by ELISA, it should be noted that ADMA in the PGIS KO samples that I have used have previously been measured by ELISA, and were increased in KO compared to wild type controls[64].

First, an unsupervised analysis using PCA was performed which demonstrated separation of PGIS knockout and wild type mice (**Figure 3.3a**). A loading plot (**Figure 3.3b**) was constructed which showed that the majority of amines clustered away from the centre suggesting that PGIS deletion, like global COX-2 deletion has a profound effect on amines.

Next a supervised analysis was performed by calculating fold change between levels of analytes in wild type versus PGIS KO mice which is presented in **Figure 3.3c** in the form of a heat map with associated p values. 11 analytes had a fold change of more than 1.5 and $p < 0.05$ (alpha-amino-n-butyric acid, carnosine, citrulline, ethanolamine, glycine, histidine, methionine, phenylalanine, sarcosine, taurine, threonine). Cystine was the only analyte that had significantly reduced.

Next, I identified common amines that were significantly altered in both the COX-2 and PGIS KO mice. These included ADMA, arginine and citrulline. As discussed in **Chapter 1**, ADMA, arginine and citrulline are key metabolites involved in the NO pathway.



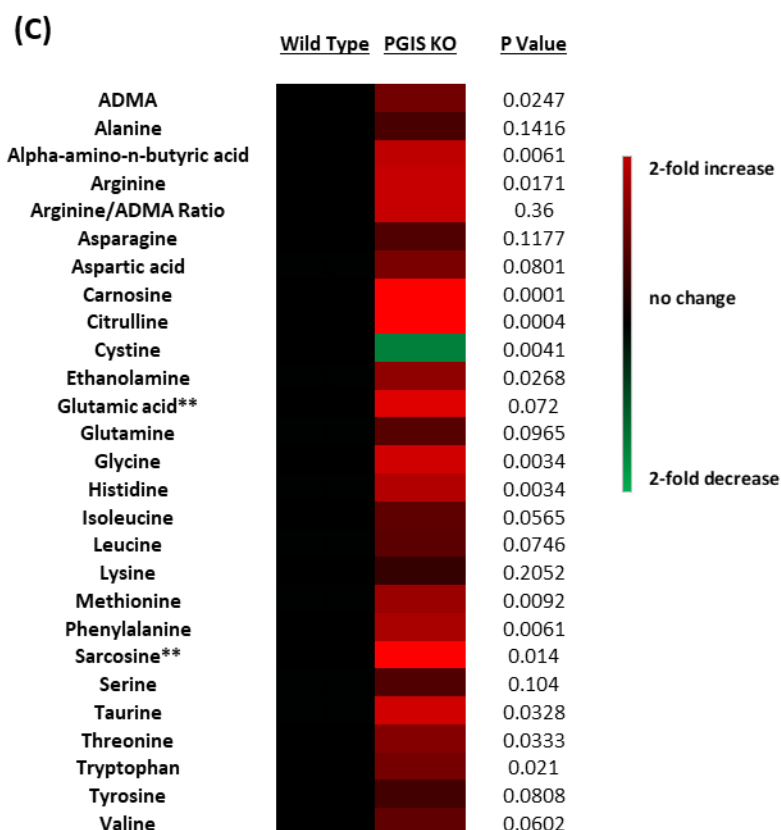


Figure 3.3 Figure 3.3 Quantification of amines and methylarginines in prostacyclin synthase (PGIS) knockout (KO) mice using UHPLC/MS/MS. (A) PCA analysis shows wild type mice (blue) separate from PGIS knockout mice (green). (B) Loadings plot demonstrating the influence of each amine and methylarginine (blue) on the principal component. (C) Heat map and associated p values. **Analysed using Mann Whitney U test.

3.3.3 Link with renal function

ADMA is a recognised marker of renal dysfunction and it has been suggested that the changes in plasma methylarginines observed in prostanoid models are due to concomitant renal dysfunction[182] which is a feature in some models of both COX-2[183] and PGIS KO [15]. To further explore this cystatin C (which is a sensitive renal dysfunction biomarker) was measured using ELISA in both sets of mice and were correlated with ADMA. Individual scatterplots for ADMA and cystatin C for wild type, COX-2 and PGIS KO in provided in **Figures 3.4abc**. ADMA (measured using ELISA and mass spectrometry) had a strong positive correlation with cystatin C **Figure 3.4de**.

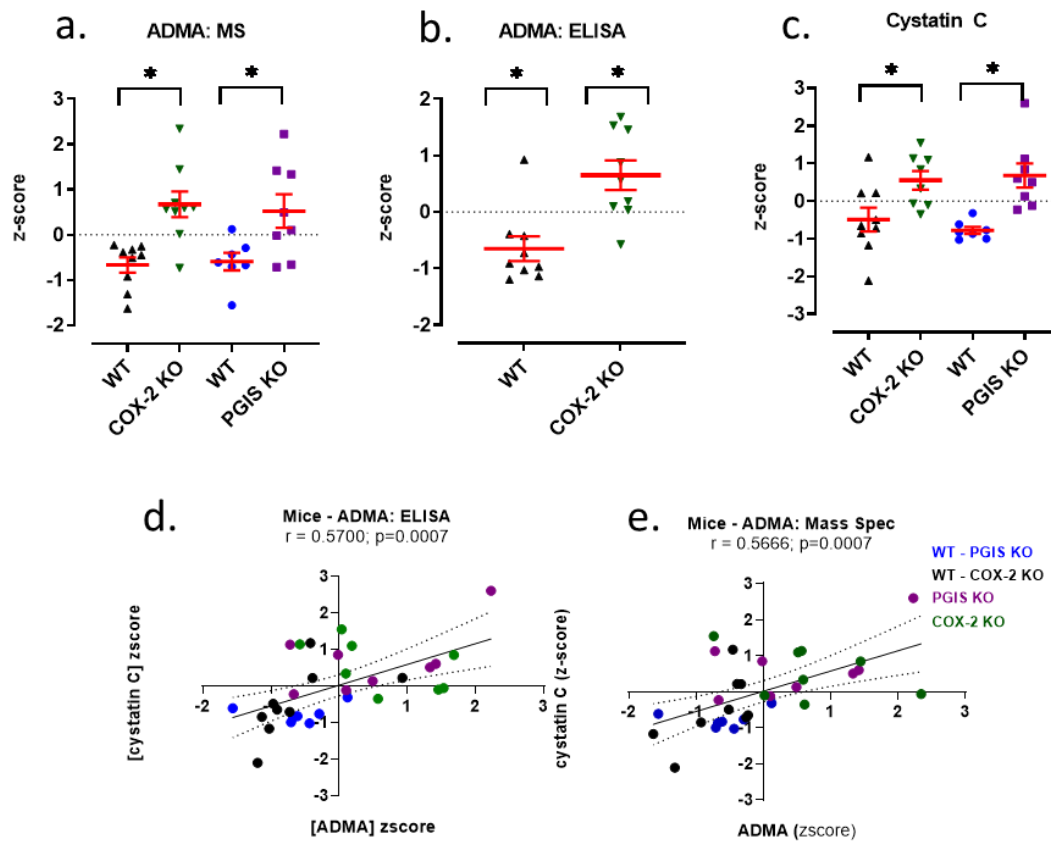


Figure 3.4 Correlation between renal function and plasma methylarginines in COX-2 and PGIS knockout mice. The Figures represent z-scores with mean $\pm$ -SEM (red error bars) for (a) ADMA measured using UHPLC-MS/MS (MS) (b) ADMA measured using ELISA, (c) cystatin C measured using ELISA. Correlations between cystatin C and ADMA by ELISA (d) and UHPLC/MS/MS (e). Data was analysed using unpaired t-tests (a-d) and Pearson's R (e-g). * $p < 0.05$.

To validate these findings, urea, which is another marker of renal function was measured using ELISA which in agreement was significantly higher in both COX-2 and PGIS KO mice **Figure 3.5a**. ADMA measured using UHPLC/MS/MS again had strong positive correlation with urea **Figure 3.5b**.

Next cystatin C was measured using the renal multiplex assay in the patient carrying the PLA₂ mutation. Individual scatterplots for ADMA and cystatin C in samples from the patient before and after renal transplant as well as healthy volunteers is provided in **Figures 3.6abc**. ADMA (measured using ELISA and UHPLC/MS/MS) had a strong positive correlation with cystatin C **Figure 3.6efg**.

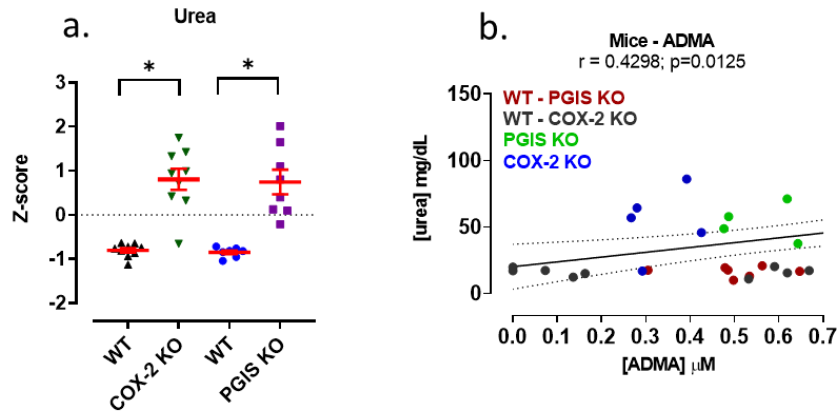


Figure 3.5 Correlation between renal function (urea) and plasma methylarginines in COX-2 and PGIS knockout mice. The Figures represent z-scores with mean+SEM (red error bars) for (a) urea measured using ELISA. Correlation between urea and ADMA by MS. Data was analysed using unpaired t-tests (a-d) and Pearson's R (e-g). * $p < 0.05$

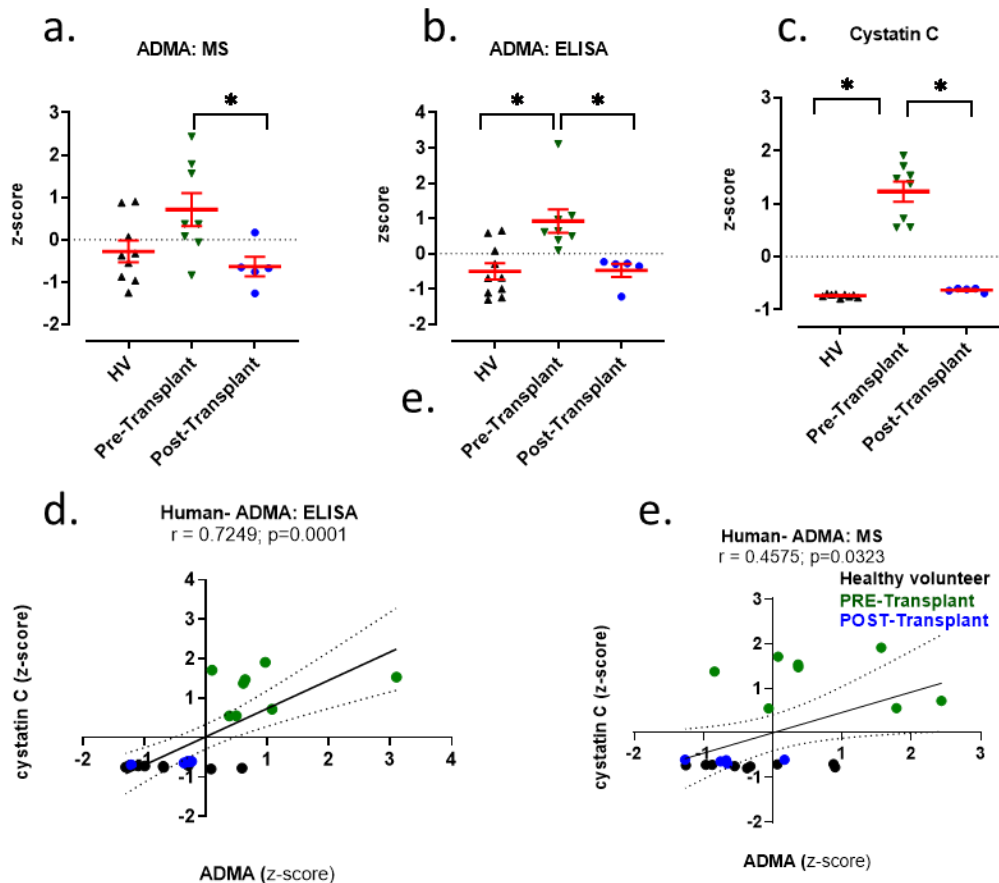


Figure 3.6 Correlation between renal function (cystatin c) and plasma methylarginines in PLA₂ deficient patient before and after renal transplant. The Figures represent z-scores with mean+SEM (red error bars) for (a) ADMA measured using UHPLC-MS/MS (MS) (b) ADMA measured using ELISA, (c) cystatin C measured using MultiPlex. Correlations between cystatin C and ADMA by ELISA (d), ADMA by MS (e). Data was analysed using one-way ANOVA followed by Dunnett's multiple comparison test(a-d) and Pearson's R (e-g). * $p < 0.05$

Finally urea was measured in samples from the patient carrying the PLA₂ mutation using ELISA which again confirmed renal dysfunction pre-transplant with return to near normal levels post-transplant **Figure 3.7a** and a strong positive correlation with ADMA **Figure 3.7b**.

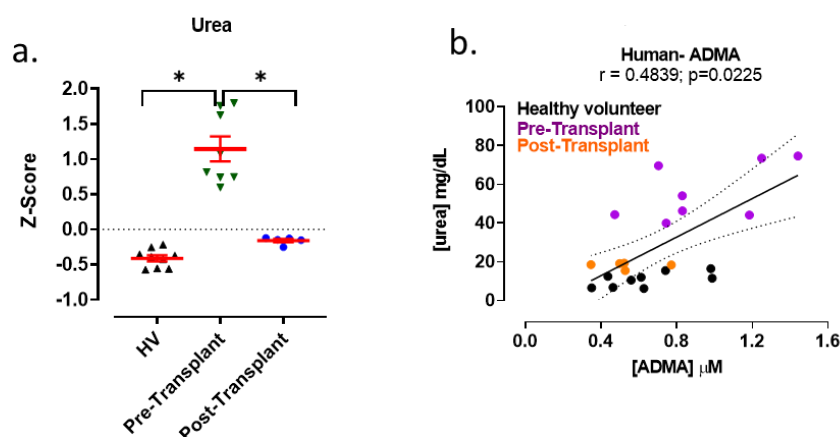


Figure 3.7 Correlation between renal function (urea) and plasma methylarginines in a PLA₂ deficient patient before and after renal transplant. The Figures represent z-scores with mean+SEM (red error bars) for (a) urea measured using ELISA. Correlations between urea and ADMA by MS (b). Data was analysed using one-way ANOVA followed by Dunnett's multiple comparison test(a-d) and Pearson's R (e-g). * $p < 0.05$

These results confirm that ADMA is a renal biomarker and deletion of COX-2[178, 184] or PGIS[15] in mice or a functional prostanoid capacity (i.e. with loss of PLA₂) in humans, is associated with altered renal morphology and renal dysfunction. What is particularly important when considering the results of samples from the human study, is that levels of ADMA decline to normal levels after the patient received a genetically normal kidney and rescued renal function. Work from our group confirms that, for this patient, renal prostanoid production was restored while whole body prostanoid capacity remained essentially absent, after the transplant[53]. While the kidney is an important location for metabolism and excretion of ADMA, other cells and tissues, including endothelial cells[185] and the liver (for ADMA) [186] are thought to contribute to the dynamic levels of ADMA in vivo. Work in this chapter utilising samples from the patient carrying the PLA₂ mutation, where increased ADMA was fully normalised after receiving a PLA₂-replete donor kidney, suggests that, where prostanoids are involved, the kidney is the only meaningful driver of circulating methylarginine levels.

As discussed in the introduction, ADMA is primarily metabolised by Dimethylarginine Dimethylaminohydrolase (DDAH) with a contribution to metabolism by alanine: glyoxylate aminotransferase 2 (AGXT2[187]), prior to renal excretion. In COX-2 knockout mice, DDAH and AGXT2 levels are reduced [51] but these findings are not seen in conditional knockout models [182]. Finally in chronic kidney disease, DDAH levels are reduced secondary to a loss in renal mass [188] which means that circulating SDMA levels are primarily driven by renal filtration whereas ADMA levels are also influenced by additional metabolic factors. What was unclear was that if the increase in ADMA seen in the extreme human and mouse models used in this chapter was due to a reduction in renal filtration or due to the influence of metabolic factors. To further understand this, we performed ex-vivo analysis using organotypic kidney slices in culture (**See Chapter 2. Methods**).

We found that conditioned media from kidney slices from wild type and COX-2 KO mice released ADMA and PGI₂ at 24 hours which significantly increased at 72 hours. However, there was no difference in the release of any of the analytes between wild type and KO mice at 24- or 72-hours **Figure 3.8**.

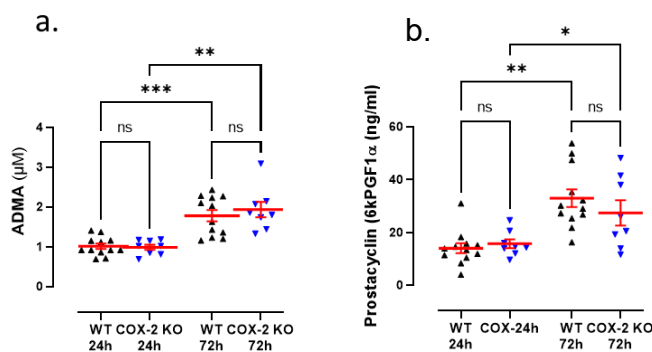


Figure 3.8 ADMA (a) and prostacyclin (b) release by organotypic kidney slices from wild type (WT) and COX-2 Knockout (KO) mice. Prostacyclin production was indirectly assessed by measuring the stable prostacyclin breakdown product 6-keto-PGF1 α . Individual data points are presented with mean $\pm$ SEM (Red bars). Data was analysed using one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$.

3.4 Summary

The results in this chapter suggests that COX-2 and PGI₂ are important regulators of renal function and the increases in ADMA directly correlate with renal function. ADMA is an inhibitor of the cardioprotective eNOS and is itself an independent predictor of both cardiovascular events and all-cause mortality. Methylarginines especially ADMA could be mechanistically important when trying to unpick NSAID cardiovascular side effects and will form a component in the remainder of this thesis.

If we accept that ADMA is a mechanistic biomarker of cardiovascular disease, then these findings are in agreement with the hypothesis that renal COX-2 is cardioprotective.

3.4.1 Limitations

Whilst these models provide insight into the importance of COX-2 and renal function, they are extreme models and may not necessarily reflect the effects COX-2 inhibition in people using NSAIDs with clinically defined, normal renal function.

Data for SDMA and LNMMA were not available for all samples and so the influence of COX-2 /PGIS deletion on other methylarginines was not explored.

The use of the ex vivo kidney slice culture model provides a good snapshot of overall function as it contains all renal cell types. As discussed in the introduction, unlike COX-1, which is expressed throughout, COX-2 expression, especially in the kidneys is located in highly localised regions which could result in the loss of signalling compartmentalisation. This could explain why we did not see a reduction in prostacyclin production in COX-2 KO kidney slices. Furthermore, the culture process itself could influence biological processes.

3.4.2 Future Studies

Repeat experiments using regional kidney slices (e.g. separate for cortex and medulla) may overcome the compartmentalisation limitation. Furthermore, measuring tissue levels of amines/methylarginines from these regions would provide better insight into the metabolic effects of COX-2 deletion on methylarginines.

As discussed above, a major limitation of this work is the translatability due to the extreme nature of the models. Instead, the results in this chapter provide an important

proof of principle such that renal function and/or methylarginine levels in the very large numbers of people taking NSAIDs may be mechanistically important in predicting and/or explaining the small but significant risk of increased cardiovascular events. In the following chapter I set out to further this idea by studying the effects of blocking COX-2 with celecoxib on cardiovascular and renal function in healthy volunteers using a placebo controlled randomised trial and later in **Chapters 6-8** [31], in real-world patients taking NSAIDs in the '*Standard care vs. Celecoxib Outcome Trial (SCOT)*' [31] respectively.

Chapter 4: The Effects of Celecoxib on Cardiovascular Biomarkers in Healthy Volunteers; A Double Blind, Placebo Controlled Randomised Trial

Acknowledgements (Chapter 4)

I would like to thank the research team; Professor Jane Mitchell, Professor Peter Collins, Dr Nicholas Kirkby, Matthew Rickman and would especially like to thank Dr Plinio Ferreira who helped to deliver this trial. I would also like to thank and acknowledge the Imperial Clinical Research Facility for providing infrastructure support. Finally I would like to thank the participants who without the study would not be possible.

4.3 Rationale

As discussed in the introduction, prostacyclin formed by the actions of either cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) is a vasodilator and anti-thrombotic agent. Similarly, nitric oxide (NO), formed by endothelial nitric oxide synthase (eNOS), expressed throughout the vasculature, protects the entire cardiovascular system against atherosclerosis, thrombosis and hypertension. The power of eNOS and COX-2 together in protecting the cardiovascular system is biologically enhanced since NO and prostacyclin (PGI₂) work additively to maintain vascular function and in a synergistic manner against thrombosis [189]. Moreover, loss of COX-2 has been associated with reduced eNOS expression increases in eNOS inhibitors and reduced endothelial function ex vivo [51, 63, 64]. Thus, this pathway, which links COX-2 and prostacyclin with eNOS, provides a plausible contributory mechanism that explains how non-steroidal anti-inflammatory drugs (NSAIDs) precipitate cardiovascular side effects. This is supported by previously published findings showing that the loss of eNOS function in blood vessels ex vivo was rescued by supplying the eNOS substrate L-arginine in vitro[51].

However, as detailed in the introduction, studies investigating the effect of COX-2 inhibition with NSAIDs in vivo on endothelial function in man have delivered varying and sometimes conflicting results confounded by (i) the type, dose and duration of NSAID used, (ii) the characteristics of the human subjects (age, sex, health status), (iii) the study design (placebo controlled, blinding stagey) and (iii) the method used to measure endothelial function. Moreover, where others have investigated the effect of NSAIDs on endothelial function, previous studies have been limited to physiological

measurements only or (at best) together with a restricted set of associated biomarkers. Thus, there is an unmet need to fully understand the effect of blocking COX-2 on endothelial function in the context of a complete assessment of multi-omic readouts to determine associations with unappreciated signalling pathways involved in vascular protection of COX-2. In my thesis I have addressed this knowledge gap by conducting a clinical study in healthy male volunteers to determine the effects of COX-2 inhibition using celecoxib on cardiovascular endpoints and coupled this with a multi-omic approach. This chapter details the effect of celecoxib on the physiological and basic clinical cardiovascular and renal biomarkers in healthy volunteers. Based on the wealth of evidence suggesting that the eNOS substrate, L-arginine can improve endothelial function in a variety of settings[190], the study was designed to allow for a second arm where volunteers would receive L-arginine together with drug or placebo if the primary endpoint was achieved.

4.3.1 Hypothesis

“Inhibition of COX-2 using celecoxib will alter cardiovascular biomarkers in healthy volunteers”.

The aims of this chapter are to:

- Assess the effects of COX-2 inhibition on endothelial function and augmentation index (AI), blood pressure and cardiovascular & renal function in healthy volunteers in a randomised controlled trial.

4.4 Methods

4.4.1 Study team and roles

While I am grateful for and acknowledge the support of my study team members, the final design and majority of the execution of the study was led and performed by me.

Study Co-ordinator: Dr Ricky Vaja

Chief Investigator: Prof Jane Mitchell

Principle Investigator: Professor Peter Collins

Co-investigators: Dr Ricky Vaja, Dr Nick Kirkby, Dr Plinio Ferreira

My roles in the study included: fine tuning the study design; creation of protocol and associated study documents; ethical application and other regulatory approvals; procurement of blood pressure machines, celecoxib and placebo; designing the REDCAP data capture tool and centralised randomisation feature; participant recruitment; trial co-ordination and delivery (screening, consent, blood/urine sample collection, EndoPAT operator, trial documentation); data analysis and interpretation.

The first challenge was to design a clinical trial that could answer the research question. This was done using the PICO framework and I will now discuss the rationale for the study design.

4.4.2 Population

As endothelial function is dynamic and can be influenced by a range of factors, it was important to carefully consider the inclusion/exclusion criteria **Figure 4.1**.

Inclusion Criteria	Exclusion Criteria
- Male, 18-40 years old - No abnormal findings on medical history, screening, physical examination, haematology, biochemistry, urinalysis and vital signs (sitting blood pressure, resting pulse rate, respiratory rate and body temperature) - Normal fasting lipid profile (Soverson et al, 1994) - Non-smoking - Clear venous access in upper limbs - BMI 18-30 - No history or signs of drug abuse - No other medication 4 weeks before or during study - No informed written consent	- Any history of allergy to NSAIDs or arginine - Significant medical conditions - Pulse rate <50 beats/minute - Sitting systolic blood pressure <80 or >160 mmHg - Sitting diastolic blood pressure <60 or >100 mmHg - Baseline endothelial dysfunction (as defined by EndoPAT; LnRHI <0.51) - Participation in other clinical study 8 weeks before or during study - Donation of blood 8 weeks before or during study - COVID-19 vaccination within 4 weeks

Figure 4.1 Inclusion & exclusion criteria for the ASCENT trial. Abbreviations: BMI: body mass index, NSAID: non-steroidal anti-inflammatory drug, LnRHI: log reactive hyperaemia index.

To minimise the risk of confounding. I have provided a summary and the rationale behind some of the inclusion/exclusion criteria below:

Include healthy 18–40-year-olds

Aging is a well-established independent risk factor for cardiovascular disease and is associated with age related endothelial dysfunction. There are various proposed mechanisms which include a) reduction in NO synthesis and therefore NO bioavailability b) increased age related oxidative stress and c) the development of a

low grade pro-inflammatory state [191]. Including only a younger and healthy population should therefore alleviate some of these confounding issues.

Include only Male participants

This is because in females, the endogenous hormones produced during the various phases of the menstrual cycle have been shown to have variable effects on endothelial function [192]. Pragmatically, this would be extremely difficult to control for.

Include only Non-smokers

Cigarette smoke induces oxidative stress which activates the endothelium to release inflammatory cytokines [193]. This reduces the NO availability within cells resulting in loss of function of smooth muscle cells with the vessel wall. Clinical trials have also shown that in young adults, cigarette smoking is associated with impairment of endothelial-dependant arterial dilatation [194].

Exclude participants who have had a COVID-19 vaccination within the last 4 weeks

During the course of the study, we had to adapt to the uncertainties surrounding COVID-19. It had become increasingly apparent that the cardiovascular sequelae of COVID-19 infection had effects on the endothelium and endothelial function [195, 196] and so a decision was made to screen for active COVID-19 infection between visits. Furthermore, during the active recruitment phase, the government had rolled out the national COVID-19 vaccination programme in our target population during the study. As this was a novel vaccine, the effects on endothelial function were unknown at the time. Previous work had shown that influenza vaccination can affect endothelial function for up to 2 weeks [197]. Following discussion with the study team, we reasoned that a 4 week “washout” period between the vaccination and the active phase of the study should be sufficient to minimise the influence of vaccination on our primary endpoint. This required me to make an amendment to our ethics.

In our final cohort of participants, one participant was unvaccinated, 2 participants had received a single dose of the vaccine, 35 participants had received 2 doses and 2 participants had received a booster. All participants had at least a 4-week washout period between last vaccine dose and the first study visit.

4.4.3 Interventions

Celecoxib

As discussed in the Introduction, the cardiovascular side effects of NSAIDs are due to inhibition of COX-2 as opposed to COX-1. Therefore, a natural choice of test drug was celecoxib which is a selective COX-2 inhibitor that is actively used in clinical practice. It was important to balance the risk of any potential adverse events in our population whilst ensuring participants had enough COX-2 inhibition to detect any changes in vascular function and cardio-renal biomarkers should they exist. In the British National Formulary, the maximum recommended daily dose is 400mg in divided doses and therefore a decision was made to use 200mg twice a day. This dosing regime is also in line with earlier studies using celecoxib [198]. As detailed in the introduction previous studies have demonstrated that celecoxib carries the same cardiovascular risk as other NSAIDs such as ibuprofen, which is readily available over the counter, although cardiovascular risk with ibuprofen is dose dependent with the highest doses restricted to prescription only. Celecoxib has a half-life of approximately 12 hours and steady state plasma concentrations are normally reached within 5 days of treatment [199]. However, as the increased cardiovascular risk can occur following one (8 days) to two weeks [40] of use, we opted for a treatment duration of 7 days to allow enough time to detect systemic effects whilst minimising the chance of major adverse events.

L-arginine

As discussed in the introduction, L-arginine has been shown to improve endothelial function in a range of cardiovascular conditions. The rationale that L-arginine may prevent any endothelial dysfunction attributed to NSAIDs is additionally strengthened since our group has reported an imbalance between arginine and ADMA in healthy volunteers taking celecoxib and in mice where COX-2 is inhibited or deleted[51]. The study design included two phases. Phase 1 to assess the effect of celecoxib on endothelial function and if the primary endpoint was achieved, progress to phase 2 where drug or placebo would be taken with a dose of L-arginine. A decision was made to administer a total of 10g per day in two divided doses as previous work has suggested this dose is safe and could increase plasma arginine levels by approximately 40% [200] .

4.4.4 Comparator- Placebo

It was important to use a placebo group as a comparator to see if any differences seen were indeed due to celecoxib use. When assessing physiological parameters such as blood pressure and endothelial function, there is a high risk of introducing performance bias and so a decision was made to blind participants, personnel and outcome assessor. This would also reduce the risk of selection and detection bias. It was important that the placebo was physiologically inactive and was visually the same as the celecoxib group. Over encapsulation was chosen as the preferred method of blinding as this was the most cost-effective.

4.4.5 Outcomes

The primary outcome was U.S Food and Drug Administration (FDA) approved endothelial function measured using the EndoPAT device. EndoPAT measures the reactive hyperaemia index and, in addition, AI. For these two parameters reactive hyperaemia index, but not augmentation index, is approved by the FDA.

The secondary outcomes included blood pressure, cardiovascular biomarkers, eicosanoid and methylarginine/amine levels, proteome and blood transcriptome.

4.4.6 Study Design

A schematic is provided in **Figure 4.2**.

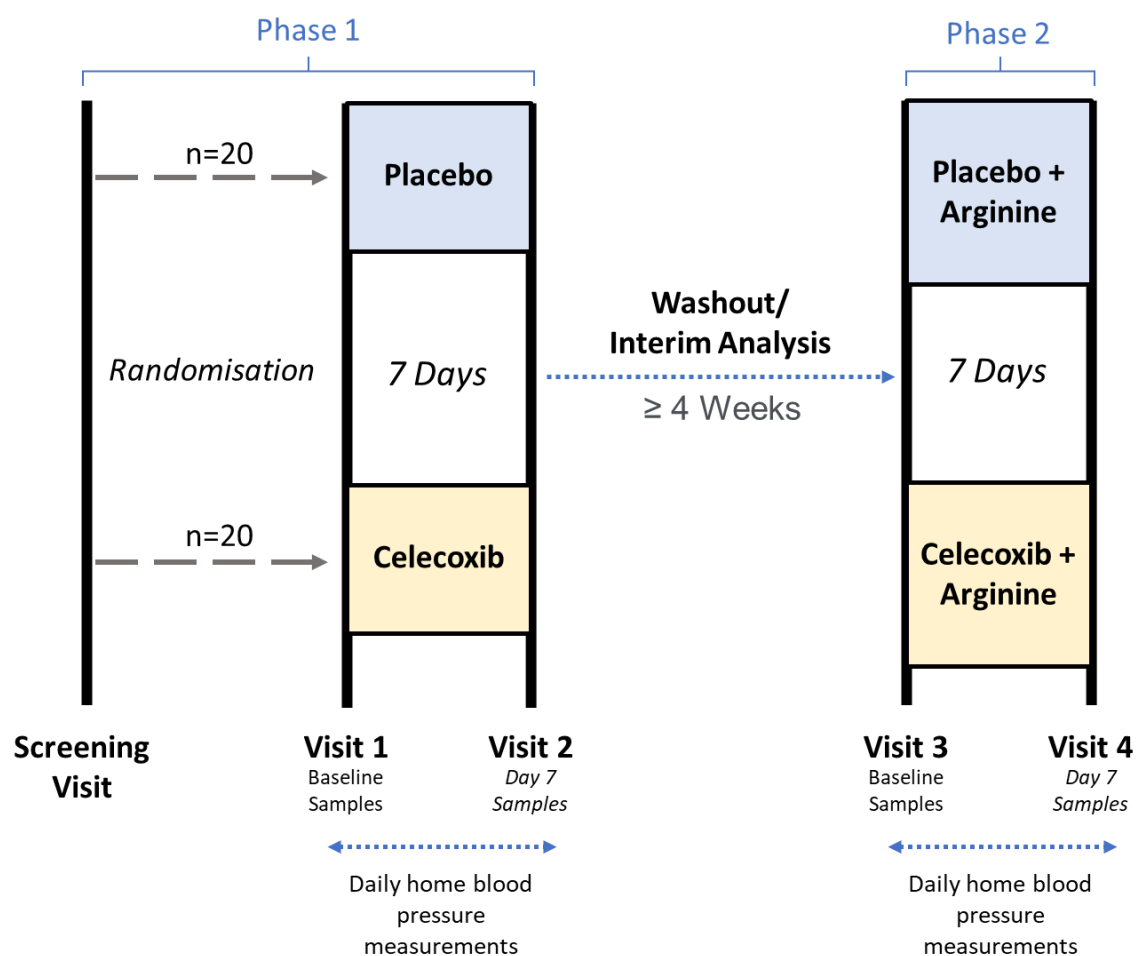


Figure 4.2 ASCENT trial flow diagram.

Phase 1

This was a prospective, single centre, participant, personnel and outcome assessor blinded, placebo controlled randomised trial designed to evaluate the effects of how COX-2 inhibition using celecoxib affects vascular function and cardiovascular 'Omic biomarkers in healthy male volunteers.

Phase 2

The same participants would remain blinded to the intervention and receive either celecoxib or placebo and arginine supplementation to see if L-arginine supplementation can reverse any endothelial dysfunction caused by celecoxib.

Ethical approval was obtained; IRAS Project ID: 280354. Written informed consent was obtained from all participants prior to enrolment. The study and protocol were

registered on clinicaltrials.gov (NCT04765644) prior to commencement to minimise the risk of reporting bias.

4.4.7 Sample size calculation and interim analysis

This was a novel mechanistic study where the primary endpoint was the effect of celecoxib on endothelial function measured using the EndoPAT device. It was anticipated that we would require approximately $n=20-40$ in each study arm to detect a difference based on previous work using similar drugs and alternative measurement approaches. For example Carlsson and Wennmalm (1983) [154] showed a reduction in endothelial function with ibuprofen (effect size: 0.80 standard deviations) and diclofenac (effect size: 1.06 standard deviations). This equates to a power of 72-92% at $n=20$ and 95-100% at $n=40$ to detect a difference between groups at a nominal significance threshold of $p<0.05$. As a different drug and an alternative measurement platform were used, power could only be estimated broadly. We therefore planned to perform an interim analysis of data after studying $n=20$ volunteers per group.

At this point, actual effect size and variance could be determined and experimental power re-calculated. If the primary endpoint was not achieved, we reserved the option to recruit up to an additional $n=20$ (to a maximum of 40) volunteers per group. This would also serve as a feasibility stop-go checkpoint for continuation of the L-arginine arm of the study to minimise any unnecessary exposure to participants.

4.4.8 Randomisation, allocation concealment and blinding-

Randomisation was performed centrally using REDCap. Dr Nick Kirkby produced a computer-generated randomisation list so that I would remain blinded throughout the study as I was the outcome assessor. A block size of 40 (included 20 celecoxib and 20 placebo) was used to ensure even allocation between groups. Using a large block size also reduces the risk of selection bias by ensuring allocation concealment. Once participants had been consented into the study by me, Prof Peter Collins enrolled the participants and Dr Kirkby centrally randomised them. The allocation was then be emailed by Dr Kirkby to pharmacy who dispensed the drug to me. The packaging and label **Figure 4.3.** tablet size and colour between the placebo and celecoxib were identical to ensure blinding of both participants, Dr Ferreira and myself throughout. I

remained blinded until the end of the study and until the relevant enzyme-linked immunosorbent assay (ELISA)s had been performed.

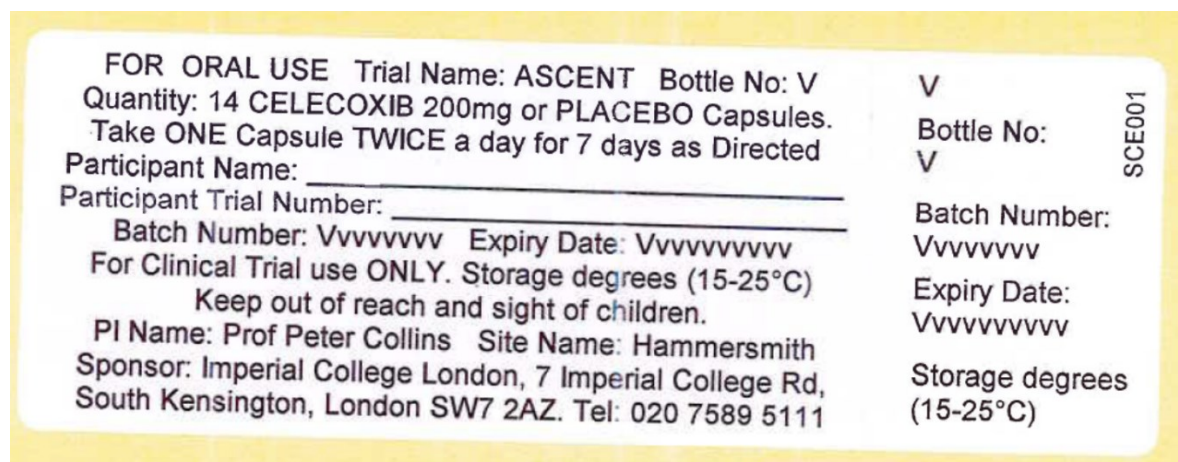


Figure 4.3 Label used for dispensing the study medication. The labels were identical between the groups to ensure blinding.

4.5 Results

The first participant attended screening on June 10th, 2021, and the final participant completed the trial on January 24th, 2022. In total 49 participants attended the screening visit, and 44 participants were randomised. A summary is provided in the CONSORT[201] flow diagram **Figure 4.4**.

There was no drug related adverse events during the study. In total 4 participants withdrew from the study (2 from each group). In those allocated to celecoxib, one withdrew prior to receiving the drug. The second participant could not attend the second screening visit due to personal reasons. They were re-enrolled as a new participant after ~6 months. In the placebo group one participant developed viral gastroenteritis and was advised to discontinue the study medication and was withdrawn from the study. The second participant developed COVID infection and again was advised to discontinue the study medication. They were re-enrolled as a new participant after a 4-week washout period

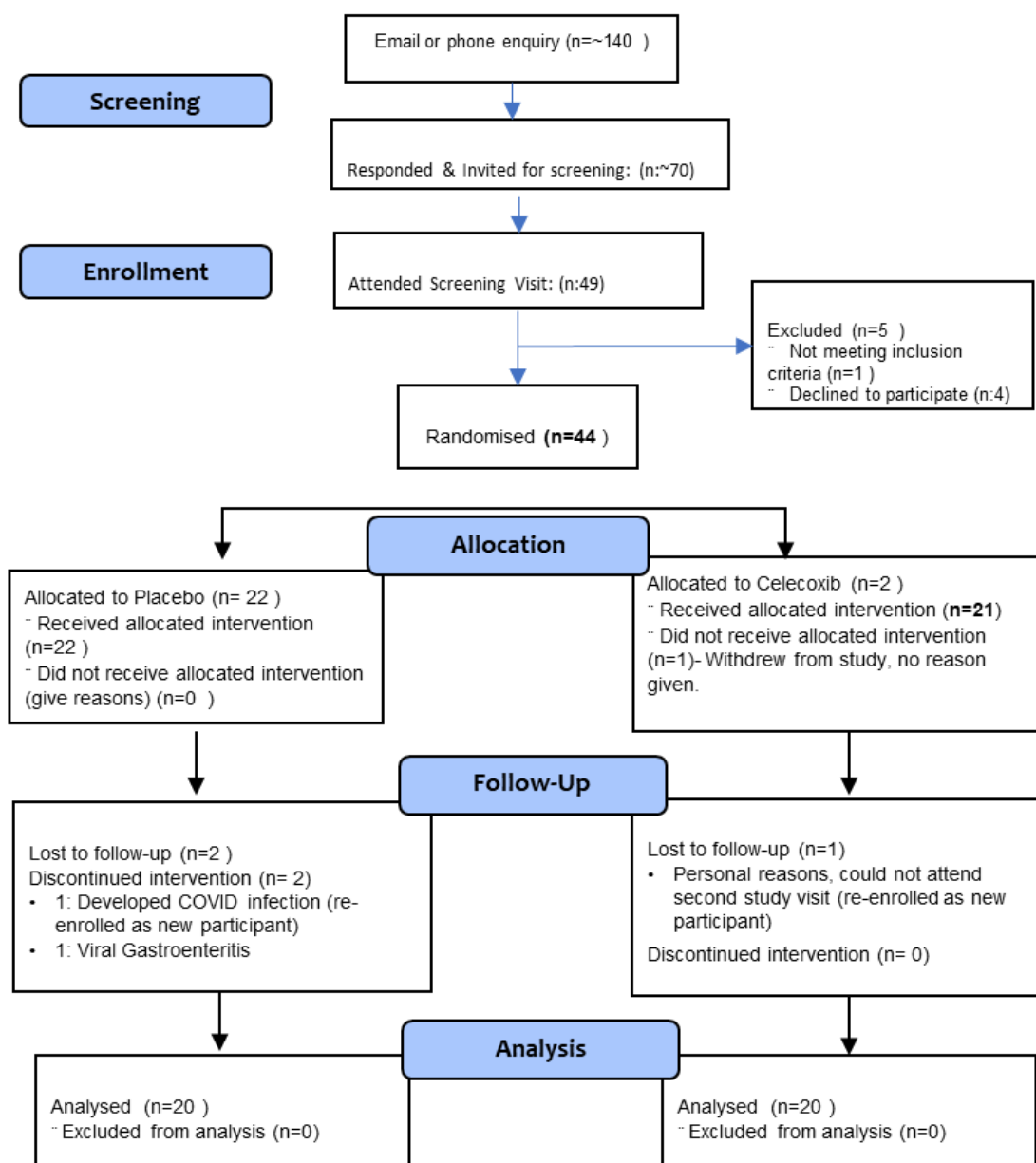


Figure 4.4 ASCENT Trial CONSORT Flow Diagram. A total of 44 participants were randomised of which 40 (20 in each group) completed follow up and were included in the final analysis.

All the analysis discussed is taken from the number of people who completed the second study visit (n:20 in each group). A summary of the recruitment trends each month is provided in **Figure 4.5**. Overall the baseline characteristics between the groups was similar **Figure 4.6** suggesting that the randomisation was adequate.

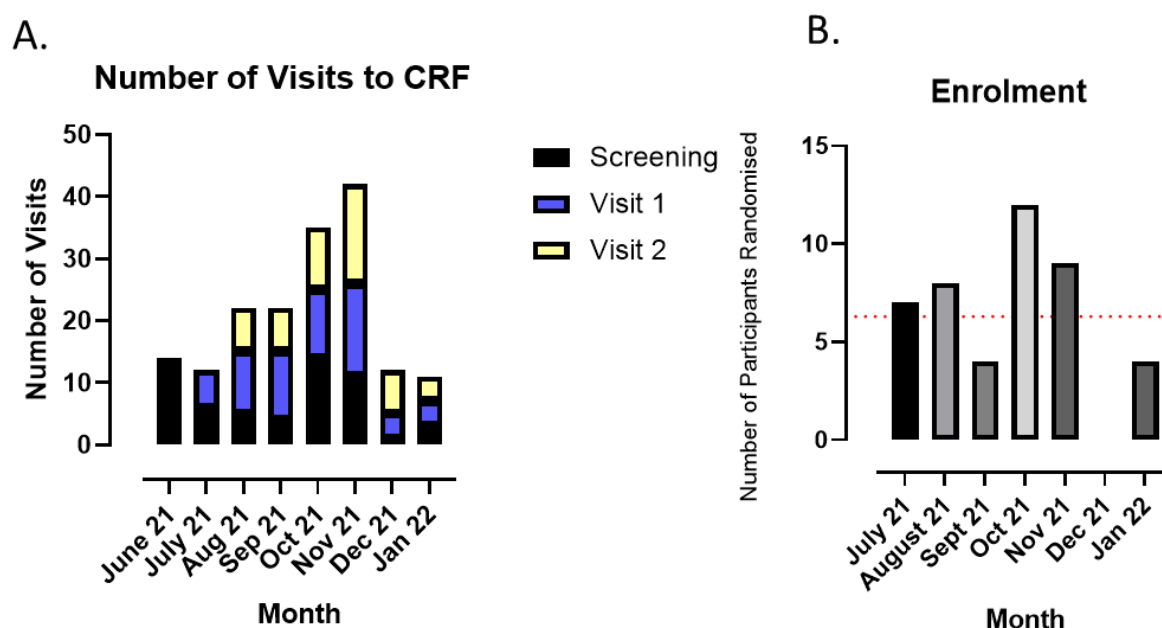


Figure 4.5 Monthly Recruitment Figures. Figure A shows the number of visits to the Clinical Research Facility (CRF) each month and Figure B shows the number of participants enrolled into the trial each month. On average there were 6.3 participants enrolled each month (red dotted line).

Variable	Celecoxib (n:20)	Placebo (n:20)
	Mean $\pm$ SD or %	Mean $\pm$ SD or %
Age	25.5 $\pm$ 6	27.1 $\pm$ 6
BMI	22.6 $\pm$ 2.3	23.6 $\pm$ 2.3
Ethnicity		
White	50%	50%
Asian	20%	35%
Oriental	20%	5%
Mixed	5%	0%
Other	5%	10%
Systolic BP (mmHg)	116.2 $\pm$ 12.1	115.9 $\pm$ 12
Diastolic BP (mmHg)	67.5 $\pm$ 7.4	62 $\pm$ 7.9
Heart Rate	68.7 $\pm$ 10.6	64 $\pm$ 10.2

Figure 4.6 Baseline Characteristics Table. This table shows the baseline characteristics are similar between the celecoxib and placebo group. Abbreviations; BMI: body mass index, BP: blood pressure.

4.5.1 Interim analysis- primary outcome (endothelial function)

The delta (Δ) in readouts between visit 2 and visit 1 was calculated for each group and then compared according to the distribution of the data unless otherwise stated.

There was no difference in Δ reactive hyperaemia index (RHI) or Δ LogRHI between the celecoxib and placebo group. There was however a significant increase in Δ AI in celecoxib versus placebo; 3.7 vs -2.5, $p:0.048$. There was also a trend towards an increase in AI corrected for a heart rate at 75 beats per minute, but this did not reach statistical significance; 3.1 vs -1.6, $p:0.13$. **Figure 4.7.**

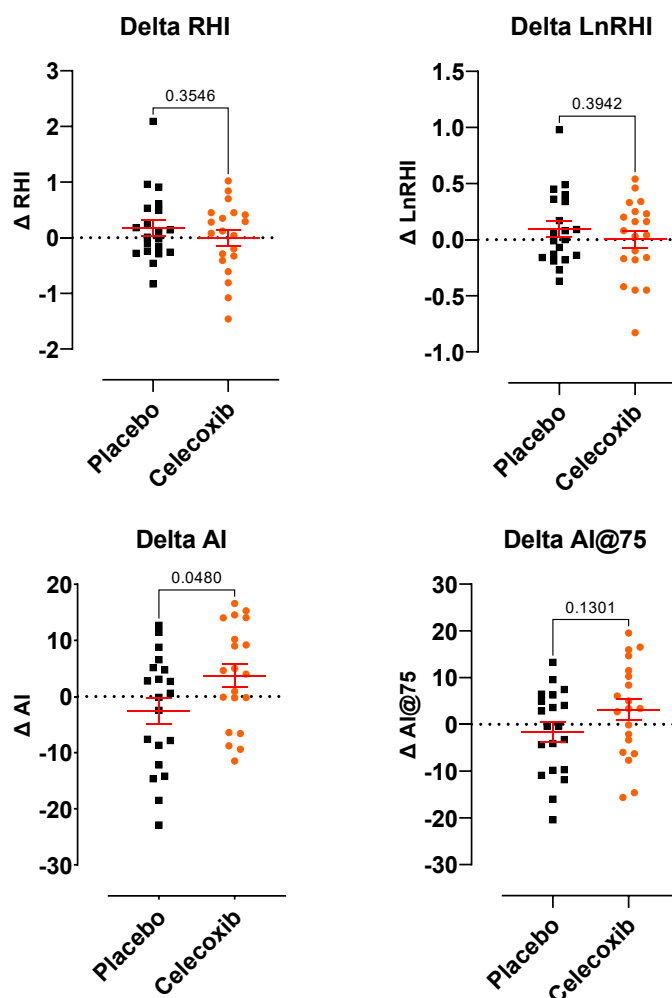


Figure 4.7 Scatter Plot showing the difference in endothelial readouts between celecoxib and placebo group. Delta represents the difference between visit 2 and visit 1 readings. There was no significant difference in Δ Reactive hyperaemia Index (RHI) Figure A. or LogRHI (LnRHI) Figure B. between the groups. Δ Augmentation index (AI) was significantly higher in the celecoxib group Figure C. There was a trend towards higher AI corrected to a heart rate of 75 beats per minute (AI@75), but this was not statistically significant Figure D.

At this point we had a decision to make as to whether to proceed to the second phase. Whilst there was no difference in reactive hyperaemia index, there was a significant increase in AI which is a marker of both endothelial function and vascular stiffness in the celecoxib group. Importantly, AI is captured prior to the reactive hyperaemia protocol. The first considerations were 1) post hoc power and 2) effect size. **Figure 4.8.**

All power calculations were estimated using an online sample size calculator (<https://sample-size.net/sample-size-means/>) that utilises the t-statistic to calculate sample size. A power of 80% and alpha of 5% were used for all calculations. The effect size (E) was calculated as the difference in means between the groups and the standard deviation of the placebo group was used. An enrolment ratio of 1:1 was used. Cohens D was calculated by determining the mean difference between the groups and dividing by the pooled standard deviation. For the key, FDA approved, readout of endothelial function (reactive hyperaemia index) we would need ~ 200 participants in each group to reach 80% power.

Parameter	Effect Size (Cohens D)	Post Hoc Power (Alpha 0.05)	Sample size to reach 80% Power (Per group)
RHI	0.3	0.15	179
LnRHI	0.27	0.13	212
AI	0.64	0.49	44
AI@75	0.49	0.31	73
SBP	0.09	0.05	2113
DBP	0.22	0.09	382

Figure 4.8 Post hoc power calculations and estimated effect size. The post hoc power analysis suggests that for Reactive hyperaemia index, the trial would need an additional ~200 participants in each arm to reach 80% power. For augmentation index, the trial is powered at ~50% and would need an additional 23 participants in each group to reach 80% power. The Cohen's d suggests the effect size of celecoxib on augmentation index is moderate to large.

For AI we would need to recruit an additional 24 people in each group (more than double our current sample size) to reach 80% power. The Cohen's d of 0.65 suggests the effect size of celecoxib on AI is moderate to large.

At this point we had 3 possible options to consider:

1) Recruit more participants

As pre-defined in the protocol, we had the option to recruit up to an additional 20 people per group. This number was wholly inadequate for endothelial function as defined by reactive hyperaemic index. We considered the validity of continuing the trial based on our positive endpoint of increased AI. However, this function of the EndoPat device is not FDA approved[202]. Furthermore, there is considerable debate within the cardiac physiology fraternity in the interpretation of peripheral pulse wave forms regarding AI and/or arterial stiffness [203-205].

Moreover, a further 20 recruits would fall short of the 24 required to achieve 80% power. Based on our initial study design and ethical approvals, which defined our use of EndoPat as 'FDA approved' we were obliged to not progress to the second phase of the study.

2) Proceed to Phase 2 with the current number of participants.

As the primary outcome, had not been met, we could not justify progress on to the second phase of the study.

3) Do not proceed to Phase 2 and end the study.

As discussed above, there was no justification to proceed to the second phase. The downside of terminating the study at phase 1 was that it would still leave the question of can arginine supplementation reverse the endothelial effects of NSAIDs based on AI, unanswered. However, as mentioned above, AI is a surrogate for both endothelial function and vascular stiffness and the EndoPAT device gives us an indication of peripheral augmentation but may not necessarily reflect central waveforms and pressures. Therefore we would need to design a new study and using EndoPAT together with additional modalities such as magnetic resonance imaging and echocardiography to firmly establish effects of celecoxib on pulse waves, arterial stiffness and AI and if successful progress to a (similar) second arm where celecoxib is administered along with L-arginine in order to provide a complete answer. For the

aforementioned reasons, it was decided that the best course of action was to end the study and not proceed to phase 2.

4.5.2 Secondary outcomes

4.5.2.1 Blood pressure

There was a trend towards a reduction in Δ systolic and an increase in Δ diastolic BP in the celecoxib group however this did not reach statistical significance. There was no difference in mean arterial pressure between the two groups. The celecoxib group had a significant reduction in Δ pulse pressure between visit 1 and 2; -2.45 vs 5.7, $p:0.04$. **Figure 4.9.**

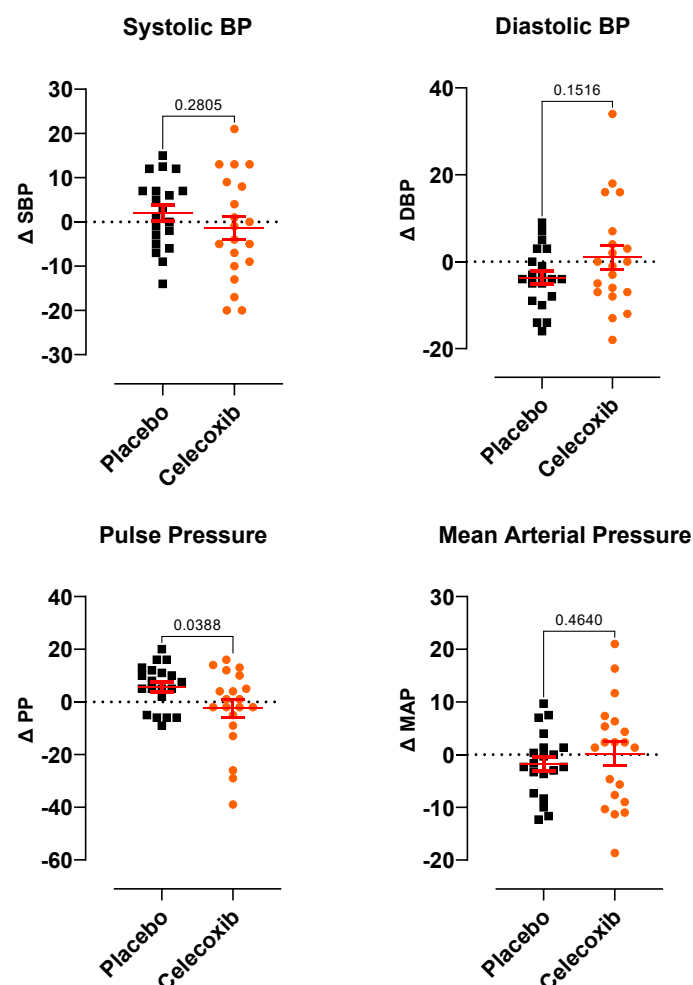


Figure 4.9. Scatterplot showing the difference in blood pressure readouts between visit 2 and visit 1. There was no difference in Δ systolic (SBP), diastolic (DBP) or mean arterial pressure (MAP) between the groups. There was a significant reduction in Δ pulse pressure (PP) in the celecoxib group.

As participants were also given a blood pressure diary for 3 readings per day, a mixed effects model was used to look at the impact of repeated measurements. The average of the 3 daily reading was taken.

Using a traditional repeated measures ANOVA was not possible as there was some missing BP diary data. As the missing data could be assumed to be random, I analysed the data by using a mixed models approach in GraphPad Prism v 8.0. This method uses Restricted Maximum Likelihood (REML) whereby the results can be interpreted in a similar fashion to repeated measures ANOVA. In the model, I defined the fixed effects as time and treatment group and the subjects were treated as a random effect. Sphericity was not assumed and so the Geisser-Greenhouse correction was used to take this into account.

Over time, there was no difference in systolic, diastolic, mean arterial or pulse pressure between the groups. **Figure 4.10.**

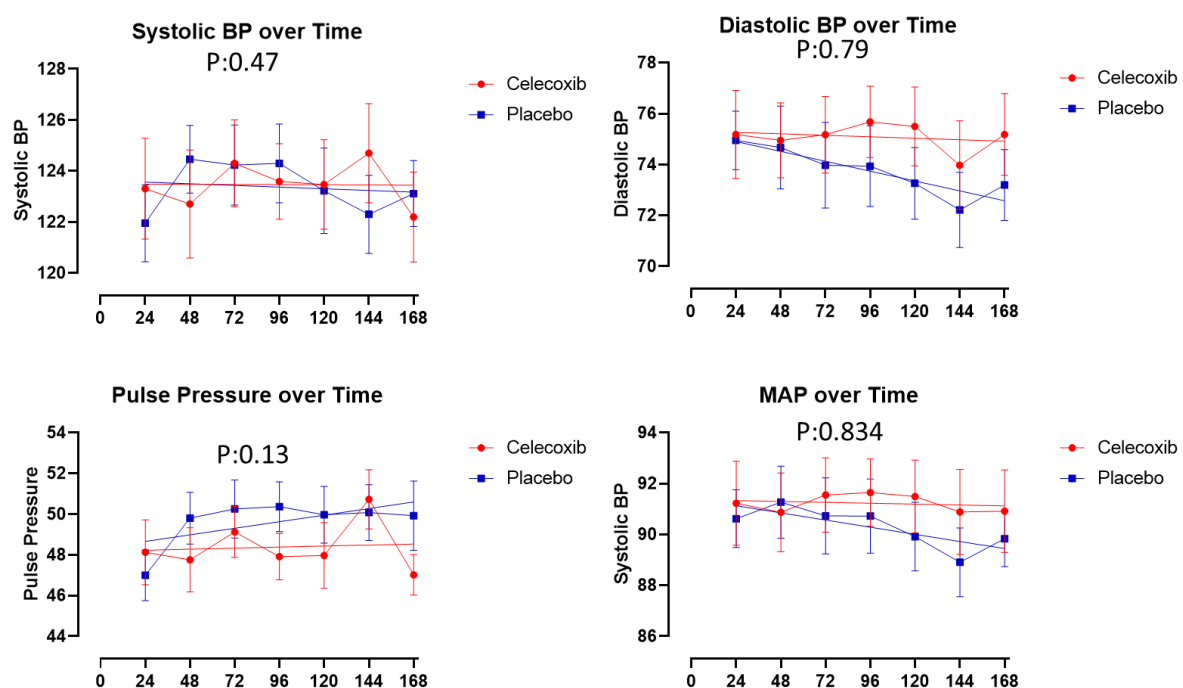


Figure 4.10 Graph showing the difference in blood pressure readout over time. The Average of 3 daily reading was taken, and the mean values are plotted along with the SEM. A mixed models approach was used to assess the effects of the treatment group over time. There was no difference in systolic, diastolic, mean arterial or pulse pressure over time between the groups.

4.5.2.2 Heart rate

There was no difference in heart rate between the groups. **Figure 4.11.**

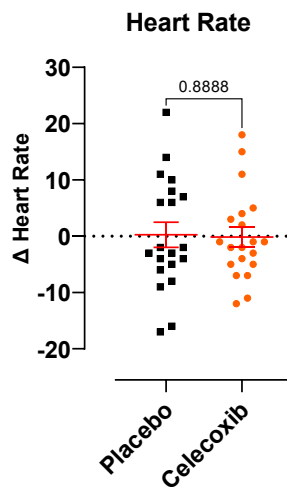


Figure 4.11. Scatterplot showing the difference in heart rate between visit 2 and visit 1. There was no difference in Δ Heart rate between the groups.

4.5.2.3 Biochemical results

Please see methods for a detailed description as to how the samples were taken and processed. All measurements were performed in either serum or plasma as stated, and data is presented as Δ between visit 2 and visit 1.

4.5.2.4 Biochemistry- Serum

There was a significant reduction in albumin concentration in the celecoxib group. There was a trend towards a reduction in sodium in the celecoxib group, but this did not reach statistical significance. There was no difference in renal markers including creatinine, urea or glomerular filtration rate (GFR) between the groups. There was no difference in any of the other analytes measured **Figure 4.12.**

4.5.2.5 Haematology- Plasma

There was an increase in mean cell volume in the celecoxib group. There was a trend towards a reduction in haemoglobin in the celecoxib group, but this did not reach statistical significance. There were no differences in any of the other analytes. **Figure 4.13.**

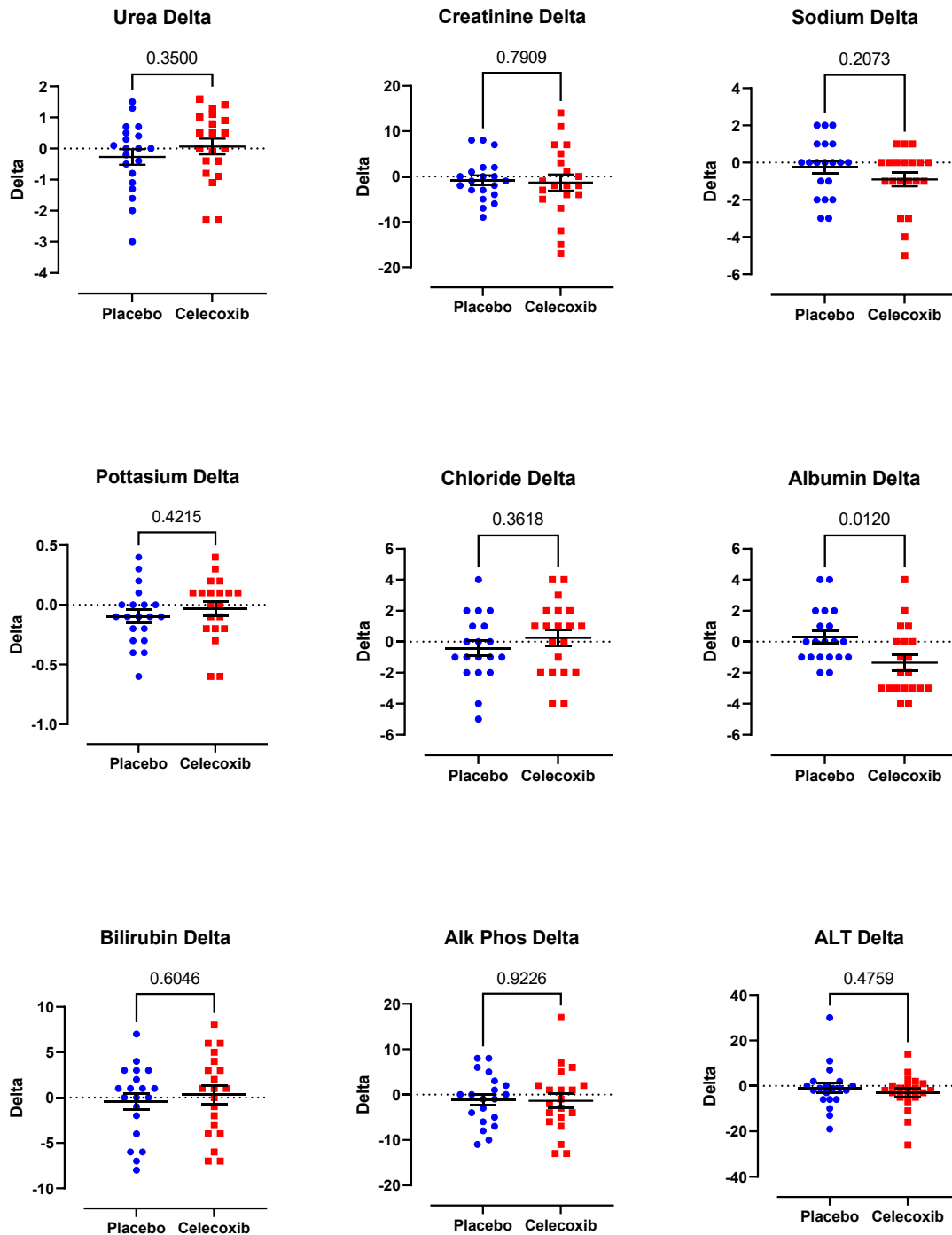


Figure 4.12 Scatterplot showing the difference in serum biochemistry readings between visit 2 and visit 1. Data is presented as mean (Horizontal bar) $\pm$ Standard error of the mean. There is no difference in renal markers including urea or creatinine. There is a significant reduction in Albumin in the Celecoxib group. Abbreviations: Alk phos: Alkaline phosphatase, ALT: Alanine transaminase.

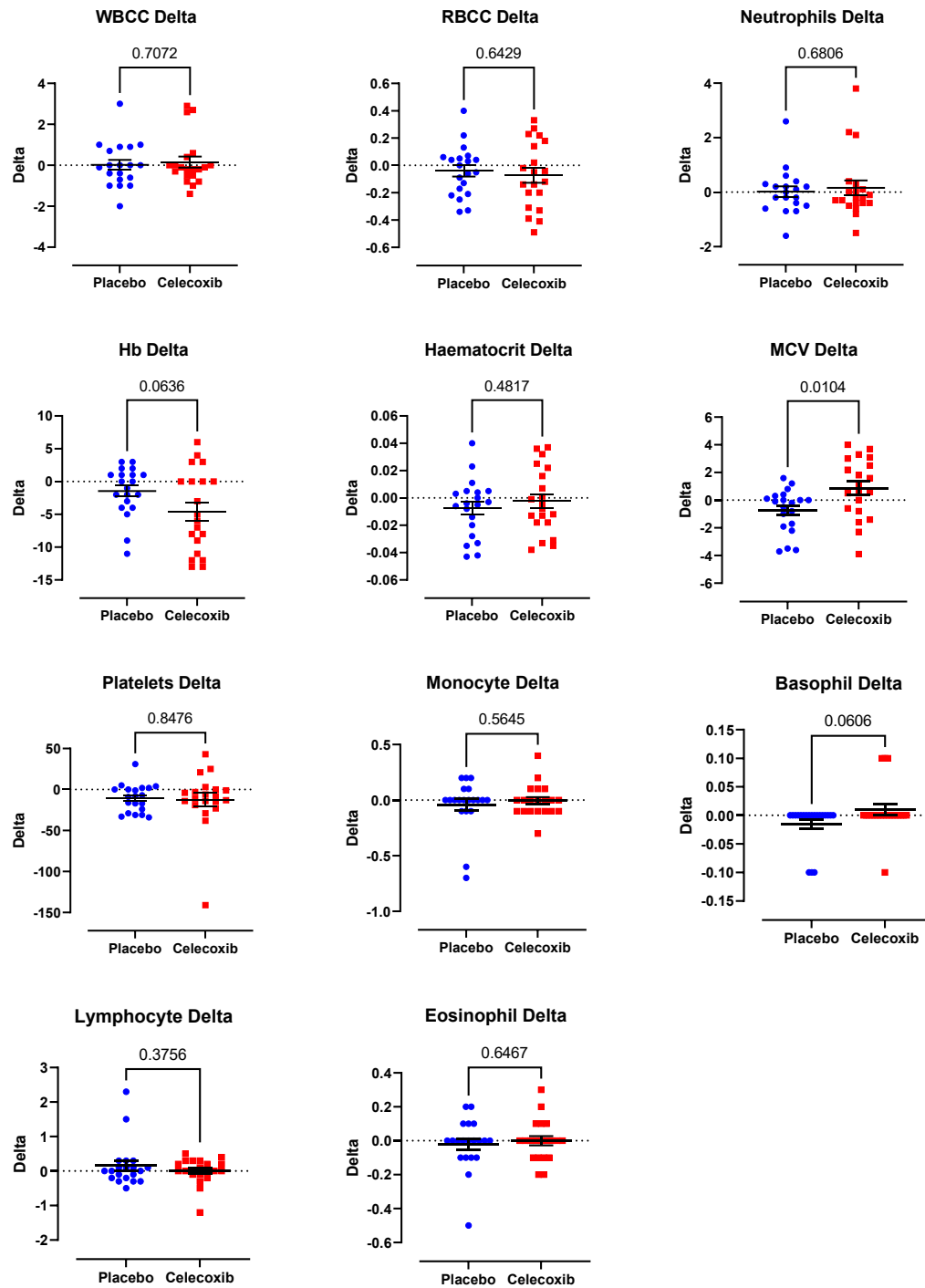


Figure 4.13 Scatterplot showing the difference in serum haematology readings between visit 2 and visit 1. Data is presented as mean (Horizontal bar) $\pm$ Standard error of the mean. There is a reduction mean cell volume (MCV) in the Celecoxib group. There is a trend towards a reduction in Haemoglobin (Hb) in the celecoxib group, but this was not significant. Abbreviations: WBCC: white blood cell count, RBCC: red blood cell count, Hb: haemoglobin, MCV: mean cell volume.

4.5.2.6 Fasting Serum Lipids

There was a significant reduction in LDL levels in the celecoxib group. There was no difference in any of the other analytes measured. **Figure 4.14.**

4.5.2.7 Clotting Factors

There was no difference in any clotting factors between the groups. **Figure 4.15.**

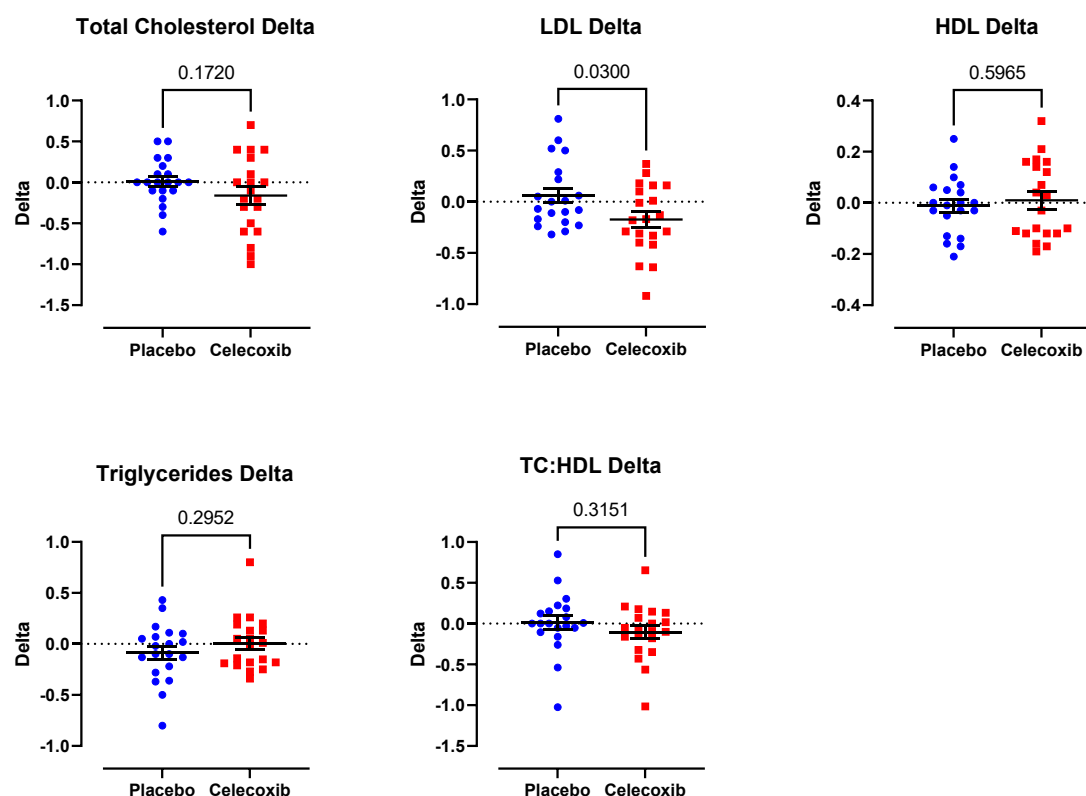


Figure 4.14 Scatterplot showing the difference in fasting serum lipids between visit 2 and visit 1. Data is presented as mean (Horizontal bar) $\pm$ Standard error of the mean. There is a significant reduction in LDL in the Celecoxib group. Abbreviations; LDL: low density lipoprotein, HDL: high density lipoprotein.

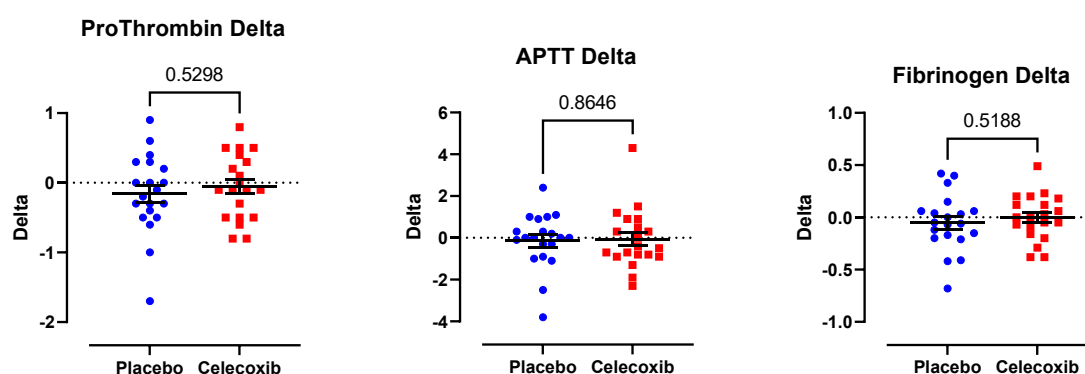


Figure 4.15 Scatterplot showing the difference in clotting parameters between visit 2 and visit 1. Data is presented as mean (Horizontal bar) $\pm$ Standard error of the mean. There is no difference in clotting function between the groups. Abbreviations; APTT: activated partial thromboplastic clotting time.

4.5.2.8 False Discovery Correction (FDR)

In this study a total of 35 readouts were calculated, these divide into 7 physiological readouts and 28 biochemical readouts. These were 'planned comparisons' and performed using several platforms and as such FDR was not applied. In the following chapters where I report omic-based data the requirement for FDR and its impact on my results is discussed in detail.

4.5.3 Compliance

Overall 37/40 (92.5%) participants reported 100% compliance to the allocated medication. One participant from the placebo group reported missing a single dose. Two participants from the celecoxib group reported missing 1 dose each (4th dose and 6th dose out of 14). Given the pharmacokinetics of celecoxib[199] and with only a single dose missed, both participants should still have a steady state plasma concentration by the final visit. To further validate compliance the plasma from participants was bio-assayed on a cell-based COX-2 system. A detailed description is provided in **Chapter 2 Methods**.

There was a 73% reduction in prostaglandin E₂ (PGE₂) production in cells cultured in plasma from the celecoxib group vs the placebo group. **Figure 4.16A**. Overall PGE₂ levels were reduced in 19/20 participants in the celecoxib group after the second visit and levels remained similar in the remaining participant **Figure 4.16B**. There was no difference in PGE₂ production between visits in the placebo group.

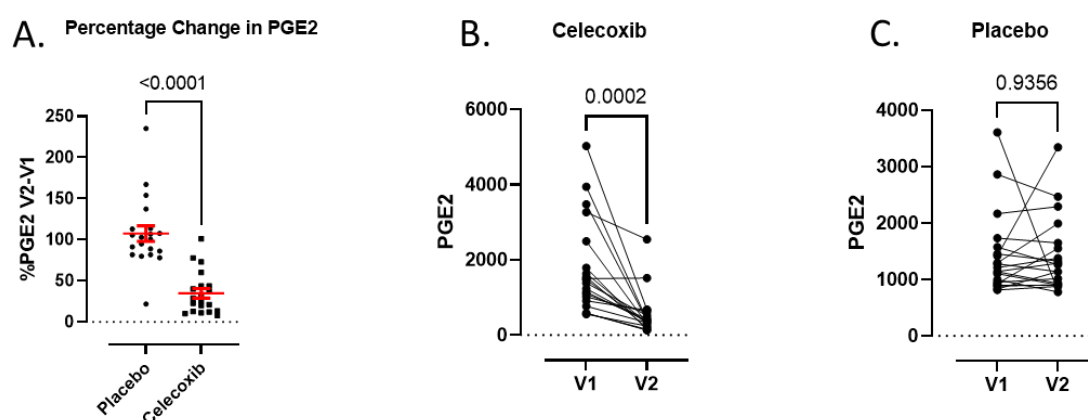


Figure 4.16 Scatterplot showing the difference in Prostaglandin E₂ (PGE₂) production from J774 cultures in plasma from participants. There was a 73% reduction in PGE₂ production in cells cultured in plasma from the celecoxib group vs the placebo group **Figure A**. There was a significant reduction in PGE₂ between visit 2 (V2) and visit 1 (V1) in the celecoxib group **Figure B**. There was no difference in PGE₂ levels between visits in the placebo group **Figure C**.

4.5.4 Reproducibility of EndoPAT readings.

As discussed in the introduction, there are several limitations of using the EndoPAT machine to assess endothelial function. The protocol for EndoPAT measurements helped to minimise environmental influence and the use of a single blinded operator helped to reduce performance bias, however, I wanted to assess how reproducible the EndoPAT readings were. I therefore decided to compare the readings from the screening visit and Visit 1 as the participants had not received any intervention. There was no correlation in LnRHI between screening and visit 1; $r:0.15$, $p:0.34$. There was a good correlation in AI and AI@75 between screening (visit 0) and visit 1. **Figure 4.17ABC.**

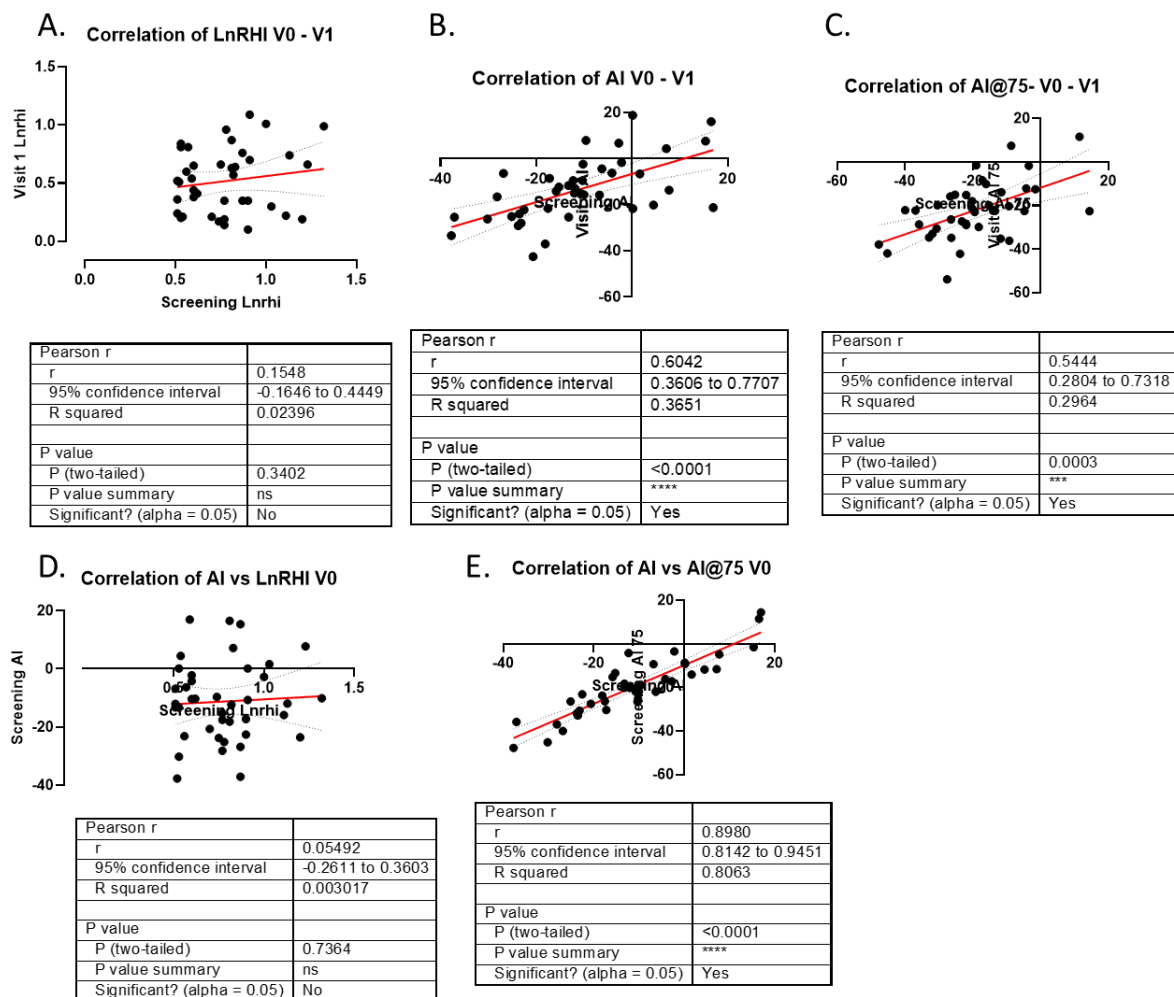


Figure 4.17 . Scatterplot showing the correlation of EndoPAT readouts between visit 0 (screening) and visit 1. Correlations of screening and visit 1 for: (A) Log Reactive Hyperaemia index (LnRHI), (B) augmentation index (AI), (C) AI corrected to heart rate of 75 beats per minute. (D) AI vs LnRHI at screening and (E) AI vs AI@75 at screening.

4.5.5 Correlation of reactive hyperaemia and augmentation Index.

Whilst reactive hyperaemia and AI are both markers of endothelial function, I wanted to see if they were correlated. **Figure 4.17DE**. There was no correlation between AI and LnRHI at the screening visit $r:0.05$, $p:0.74$. As expected, there was a strong correlation between AI and AI@75; $r: 0.9$, $p<0.0001$.

4.6 Summary

Inhibition of COX-2 with drugs including celecoxib are associated with increased risk of cardiovascular events thought to be mediated by blockade of COX-2-driven prostacyclin release from endothelial cells and/or the kidney. As prostacyclin is a vasodilator the idea that NSAIDs would impact negatively on endothelial function has been tested in a number of settings over the years using experimental in vivo and in vitro approaches. In addition, there have been several clinical studies assessing the effects of NSAIDs on endothelial function and/or cardiovascular and renal biomarkers. As documented in the introduction section of my thesis, the results of such studies are variable and inconsistent confounded by differences in study design, drug, dose, duration, patient cohort and method used to assess endothelial function. While there are a limited number of studies assessing the effects of the selective COX-2 inhibitor celecoxib on endothelial function in disease cohorts, some of which show a paradoxical improvement[166, 173], to my knowledge there are very limited studies adopting a double blind, placebo-controlled format to test the effects of celecoxib in healthy volunteers [163, 206], with just one study adopting a 7-day dosing regimen [206]. In this case the study was designed (and powered; $n=10$) to assess the effects of ischemic preconditioning on FMD in the radial artery and reactive hyperaemia but baseline data in placebo and celecoxib arms were collected and reported to be unaffected, although a trend was seen for reduced blood flow and reactive hyperaemic response in the celecoxib group. Moreover, there are no studies designed to include endothelial function as a readout in the setting of a large and comprehensive assessment of both hypothesis testing and hypothesis generating biomarkers.

A key aim of my thesis was to address this knowledge gap by conduct a fully controlled, celecoxib versus placebo healthy volunteer study where endothelial

function was captured along with carefully curated blood samples. In this chapter I reported the physiological and basic clinical biomarkers measured in the study. The key summary points are listed below:

- I found that endothelial function measured as RHI was not affected by celecoxib, but that AI was increased by celecoxib.
- Other cardiovascular physiological measurements were unchanged.
- Biochemical and clinical markers were unaffected by celecoxib except for plasma albumin and LDL which decreased by celecoxib and mean cell volume which was increased by celecoxib. This is further discussed in **Chapter 9 Discussion**.

The majority of measurements were unaffected by celecoxib. However, to my knowledge, this is the first placebo controlled randomised trial that has shown any NSAID to increase AI and therefore increase vascular stiffness. What is really striking is that these changes are seen in young, healthy volunteers following just 7 days of use and it is well established that increased AI is a risk factor for cardiovascular events.

4.6.1 Limitations

This study was a placebo controlled; participant, operator and outcome assessor blinded randomised trial and therefore represents level 1 / 2 of evidence. Furthermore, using the Cochrane risk of bias tool, the study overall has a low risk of bias **Figure 4.18**.

- | | |
|--|--|
| <ul style="list-style-type: none"> ⊕ (A) Random sequence generation (selection bias) ⊕ (B) Allocation concealment (selection bias) ⊕ (C) Blinding of participants and personnel (performance bias) ⊕ (D) Blinding of outcome assessment (detection bias) ⊕ (E) Incomplete outcome data (attrition bias) ⊕ (F) Selective reporting (reporting bias) | <p>Risk of Bias Key</p> <ul style="list-style-type: none"> ⊕ Low Risk ⊖ High Risk ⊛ Unclear Risk |
|--|--|

Support for Judgement

- A)- Computer generated randomisation
- B)- Computer generated central allocation
- C)- Participants, research staff interacting with patients blinded to the intervention. Placebo and drug were over-encapsulated and labelling on bottles was identical.
- D)- Outcome assessors were blinded to the intervention
- E)- Low attrition rate
- F) - Outcomes prespecified and protocol published prior to study start.

Figure 4.18 Cochrane Risk of Bias summary for the ASCENT Trial. I would judge the study to overall have a low risk of bias. The support for judgment for each parameter is provided.

Despite this there are some important limitations that need to be acknowledged. Whilst there was a significant increase in AI in the celecoxib group, at the current sample size the study has ~50% power so there is a small probability that the observed effects could be due to a type 1 error, however, as I will discuss later, other non-randomised studies have also demonstrated an association between NSAID use and increased AI.

The study population was limited to young healthy male volunteers primarily from Caucasian and Asian ethnic groups and so results may not be translatable to the general population.

The primary endpoint was reactive hyperaemia index measured by EndoPAT. The correlation data in **Figure 4.17** shows no association in RHI score between screening and visit 1. The reason for this discrepancy is unclear. Studies have shown that RHI is reproducible between days, although not necessary within days [139-142]. Within our study, participants were fasted for at least 8 hours, but we did not restrict diet during the study period which could be a factor. Furthermore, the trial took place during the COVID-19 pandemic. Whilst we tried to mitigate the effects of COVID-19 infection (lateral flow tests at each study visit) and vaccination (minimum 4-week washout period), this could also have impacted on the discrepancies between visits. Within our study, these discrepancies suggest that RHI was not an effective tool to determine endothelial function in this study design. AI however appears to be a far more reproducible outcome variable and itself is a recognised biomarker of cardiovascular disease.

4.6.2 Future work

Whilst it is outside the scope of this thesis, a follow up clinical trial would be the best way to validate these findings. Implementing what I have learnt from this study, I would want to capture additional measures of augmentation and cardiovascular function using echocardiography. Based on the findings in this chapter, I would want to see if arginine and/or statins can reverse the changes in AI following NSAID use. The most efficient way to perform this would be to design a multi-arm multi-stage trial. I would also want to increase the follow up duration to see how long the effects on AI persist after NSAID discontinuation. Ideally, this study would be performed on NSAID users with arthritis and potentially other cardiovascular co-morbidities.

In the next chapter I will explore how COX-2 inhibition by celecoxib affects 'omic biomarkers in this cohort.

Chapter 5: COX-2 Inhibition in Healthy Volunteers and ‘Omic’ Biomarkers

5.3 Rationale

The clinical utility of blocking cyclooxygenase-2 (COX-2) with non-steroidal anti-inflammatory drugs (NSAIDs) is firmly established and the primary mechanism of action of this class of drugs is inhibition of prostaglandins. Prostanoids promote leak (swelling) and sensitize pain responses providing a simple and well accepted, explanation of their therapeutic mechanism of action. As mentioned throughout my thesis, in terms of cardiovascular side effects, the picture is less clear with loss of prostacyclin generated from constitutively expressed COX-2 uniting the debate in this area. However, other than blockade of prostanoids driven by COX-2, a full picture of what happens to global pathways in healthy humans taking a COX-2 inhibitor is incompletely understood. This is true for pathways modulated by prostanoids as well non-prostanoid pathways affected directly by NSAIDs. Indeed, the literature suggests that NSAIDs, including aspirin and celecoxib, can have important off target effects. For example, high concentrations of aspirin have been shown to directly inhibit NFkB [207] while in preclinical models, celecoxib has a wide range of off target effects[208], including direct binding to oncogene targets and ion channels on vascular smooth muscle as well as activating AMP kinase pathways [209].

Thus, as I concluded in the previous chapter, in order to further understand the effect of celecoxib at therapeutic doses across biological pathways and to interrogate any changes in regard to cardiovascular function, a complete assessment of multi-omic readouts is required. Such a multi-omic approach has not been performed before in healthy volunteers, or indeed in any patient group but is essential to determine associations with unappreciated signalling pathways involved in vascular protection of COX-2.

In this chapter I will present my findings of a multi-omics analysis of samples generated from the ASCENT trial.

5.3.1 Hypothesis

This chapter will test two primary hypotheses. The first is a direct testable hypothesis and the second is hypothesis generating.

Firstly: 'Serum ADMA levels are different in healthy volunteers who take 7 days of celecoxib compared to placebo'

Secondly: 'Using a multi-omic approach will identify novel mechanisms associated with therapeutic doses of celecoxib and provide insight into how COX-2 protects the cardiovascular system'

Aims of this chapter:

Compared to placebo, what are the effects of 7 days of celecoxib (200mg b.d.) on:

- 1) Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), NG-Methyl-L-arginine (LNMMMA) and/or other amines measured using mass spectrometry.
- 2) Endothelial, inflammatory and thrombosis markers measured using single analyte ELISAs and multiplex assays.
- 3) Whole blood transcriptome using RNA seq.
- 4) A panel of targeted serum cardiovascular proteins measured using the Olink proximity extension assay multiplex platform.

5.4 Methods

A detailed description of the methods is provided in the **Methods** section and in **Chapter 4**.

5.5 Results

5.5.1 Methylarginines and amines measured using mass spectrometry

Using UHPLC-MS/MS, in total 31 amines and 3 methylarginines were quantified in 80 serum samples (20 before and after celecoxib vs 20 before and after placebo). Principal component analysis (PCA) was performed as an unsupervised approach and samples were labelled according to group (celecoxib or placebo) and visit (1 or 2). There was no separation between the groups **Figure 5.1**.

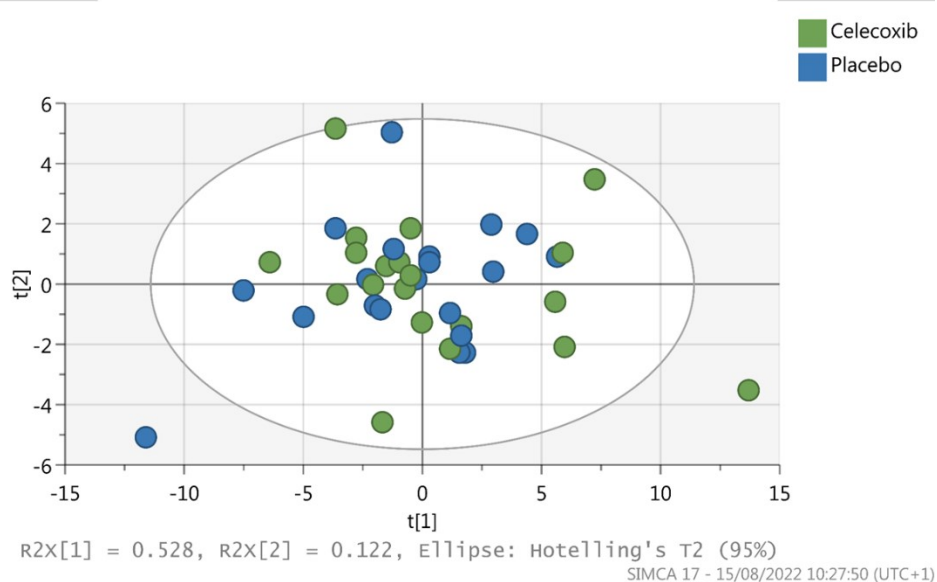


Figure 5.1 Principal Component Analysis Plot (PCA). Samples were labelled according to group (celecoxib or placebo). There was no separation between the groups.

Next the nitric oxide (NO) amines and ratios were analysed using a supervised approach. While there was no statistically significant increase in ADMA levels (in contrast to our previous clinical study, which was not placebo controlled), there was a trend for increase. Furthermore, and in line with the idea that changes in ADMA signalling could mechanistically be involved individuals taking celecoxib, the arginine/ADMA ratio was lower in the celecoxib group.

There was no significant difference in any of the other NO amines **Figure 5.2**. This is in line with previously published work [51]

A heatmap of all analytes measured and p-values are provided in **Figure 5.3**.

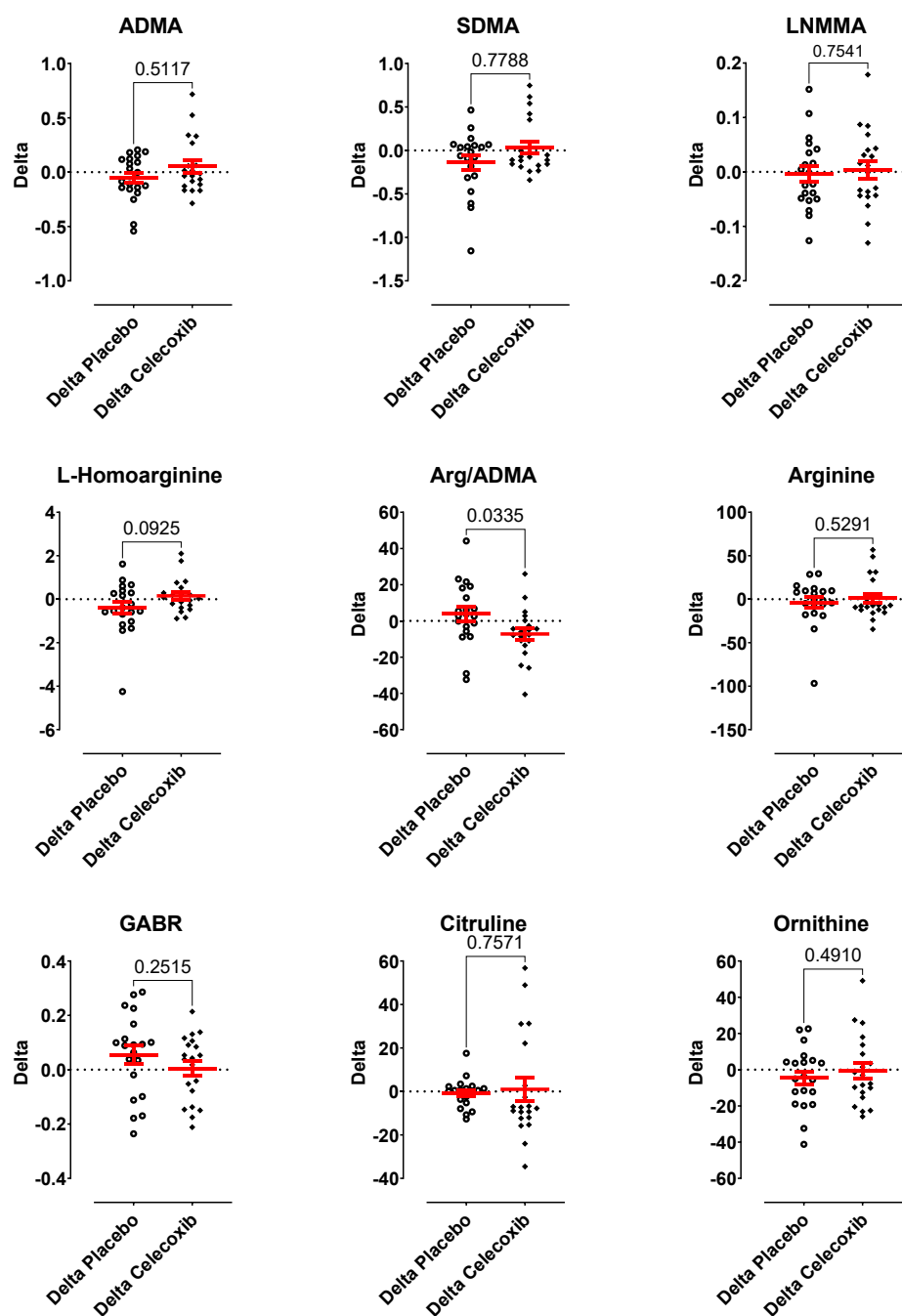


Figure 5.2 Scatter Plot of Nitric Oxide Amines. There was lower Arginine/ADMA ratio in the event group. There was no significant difference in any of the other analytes. ADMA, SDMA, Arginine and 4-hydroxyproline were analysed using Mann Whitney U Test.

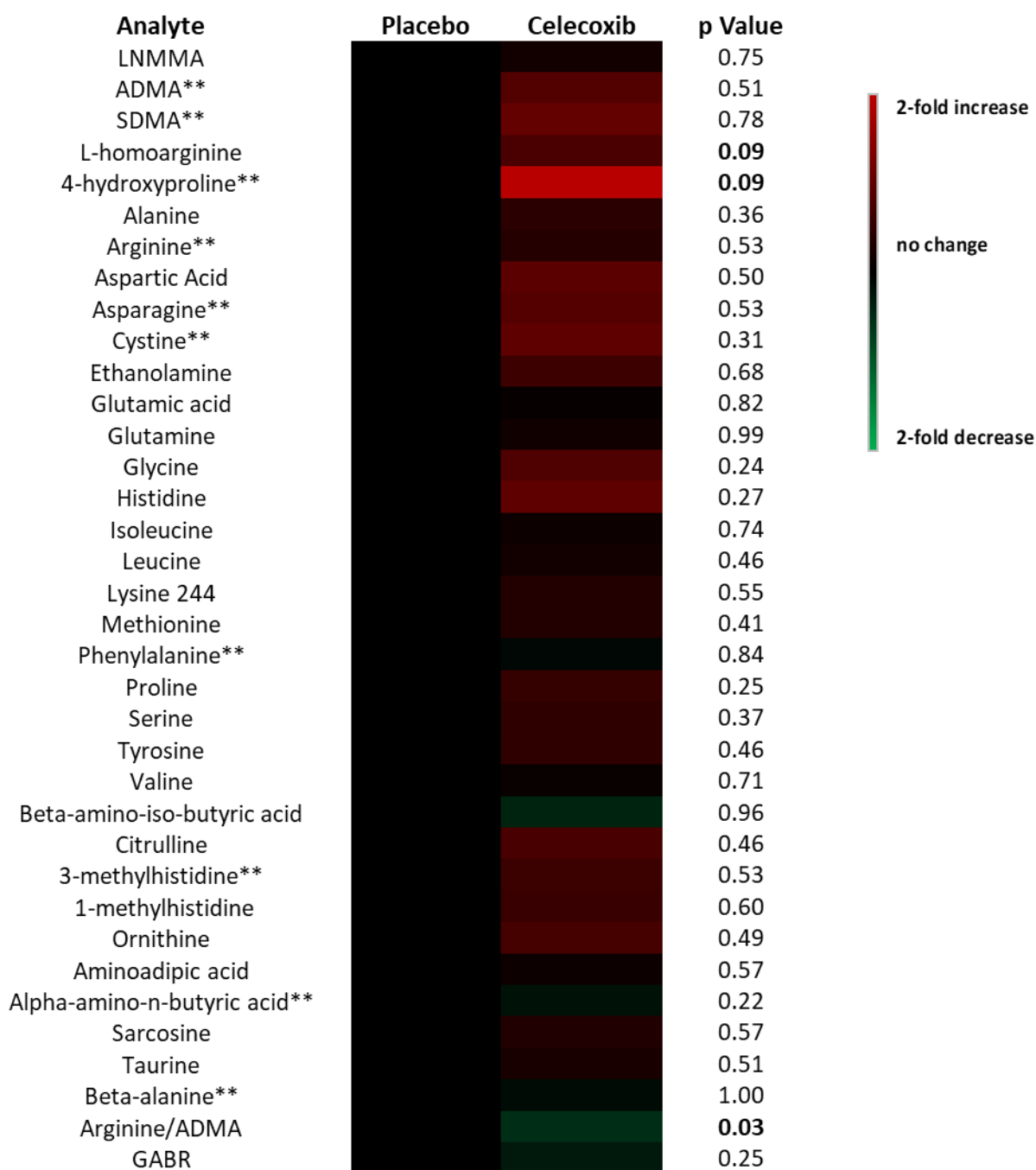
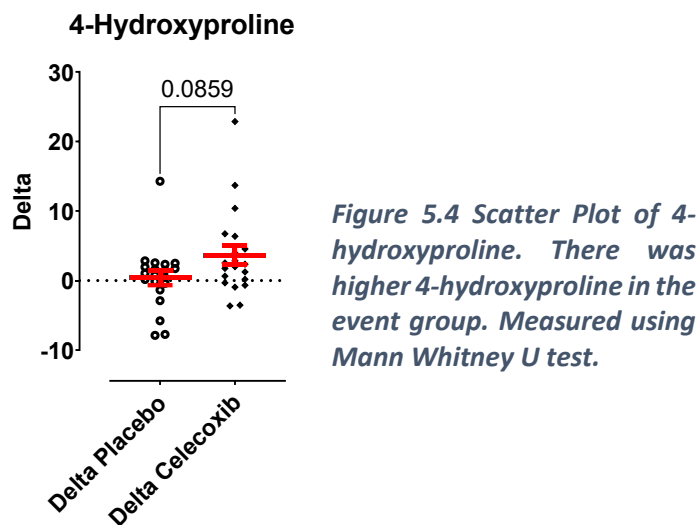


Figure 5.3 Heatmap of analytes with corresponding P-values. There was significantly lower arginine/ADMA and higher L-homoarginine and 4-hydroxyproline in the celecoxib group.

****Analysed using Mann Whitney U test.**

Interestingly, there were higher levels 4-hydroxyproline in the celecoxib group ($p:0.086$) although this did not reach statistical significance (**Figure 5.4**). While not statistically significant this trend may be important in light of results shown in Chapter 5 where AI was increased in the celecoxib arm. This is because 4-hydroxyproline is a major component of collagen and is found in very few other proteins apart from elastin [210] and is therefore used as a surrogate marker for vascular collagen turnover [211] [212] .



5.5.2 Endothelial, Inflammatory and Thrombosis Markers

There was no significant difference in serum endothelin-1 or monocyte chemoattractant protein-1 (MCP-1) levels between the groups **Figure 5.5**.

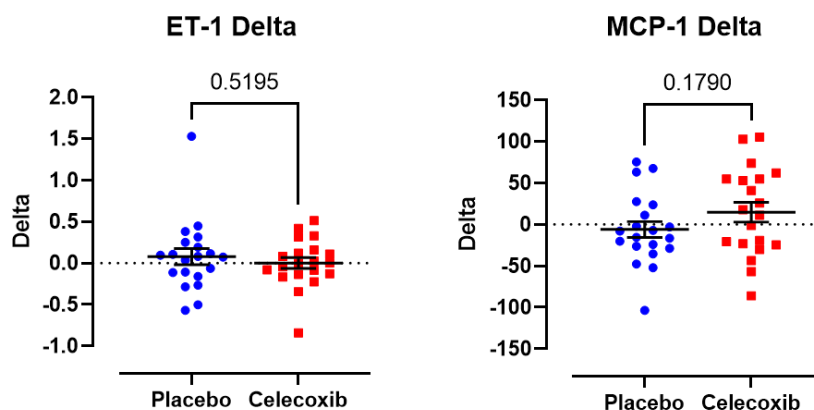


Figure 5.5 Scatter Plot of endothelin-1 (ET-1) and monocyte chemoattractant protein-1 (MCP-1). There was no significant difference between the groups.

Seven thrombosis markers were quantified in plasma samples using Multiplex assay (P-selectin, D-Dimer, PSGL-1, tPA, sCD40L, PAI-1 & Factor IX). There was no significant difference in any of the analytes between the groups **Figure 5.6**.

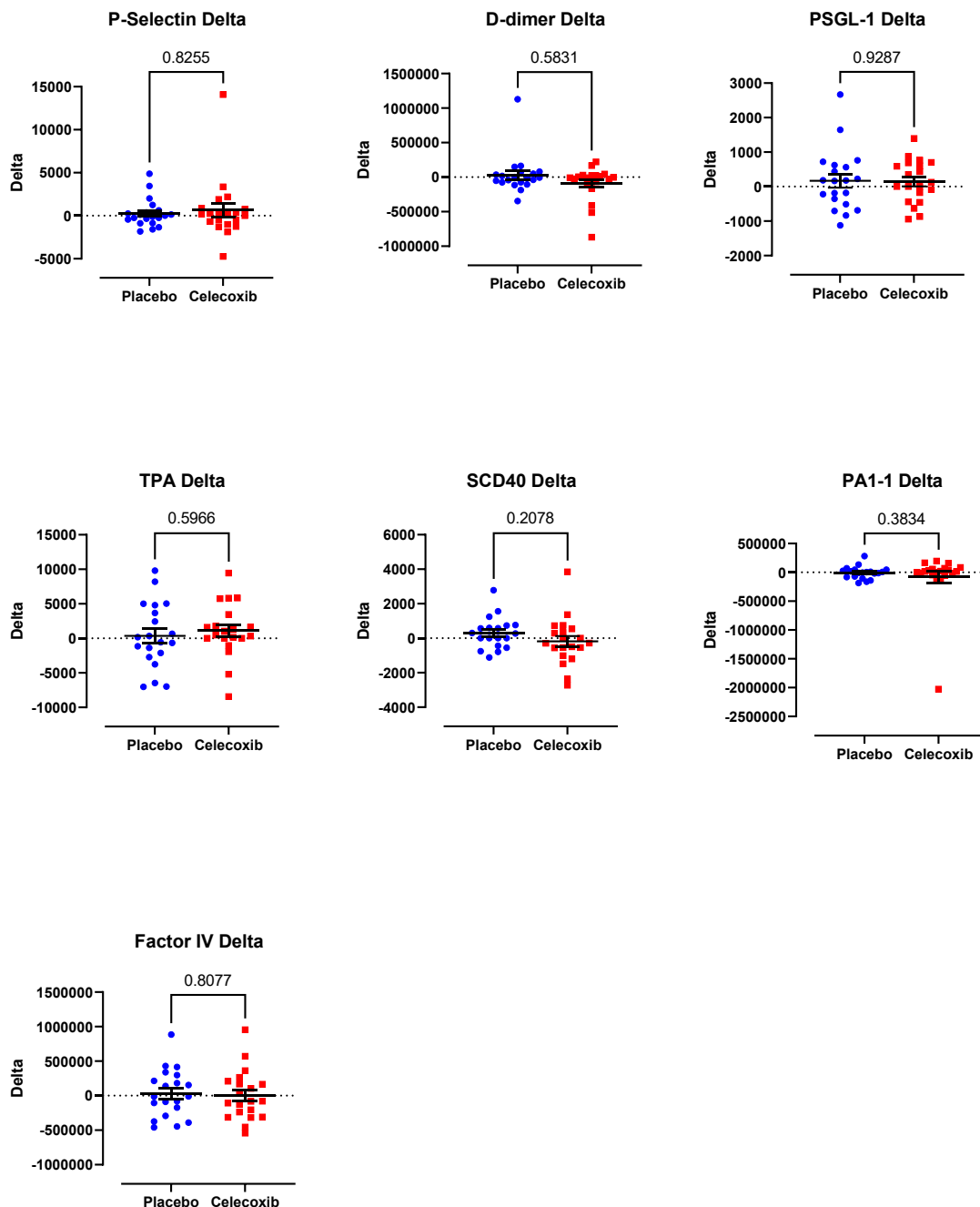


Figure 5.6. Scatter Plot of thrombosis markers measured using Multiplex assay. There was no significant difference between the groups. P selectin, D-dimer and PAI-1 analysed using Mann Whitney U test, all remaining other analytes analysed using T-test.

5.5.3 Whole Blood Transcriptome Using RNASeq

After filtering for noise reduction, 15,078 transcripts were included in the final analysis. Samples were labelled according to the condition (celecoxib visit 1, celecoxib visit 2, placebo visit 1 and placebo visit 2). PCA showed no separation between the groups **Figure 5.7**.

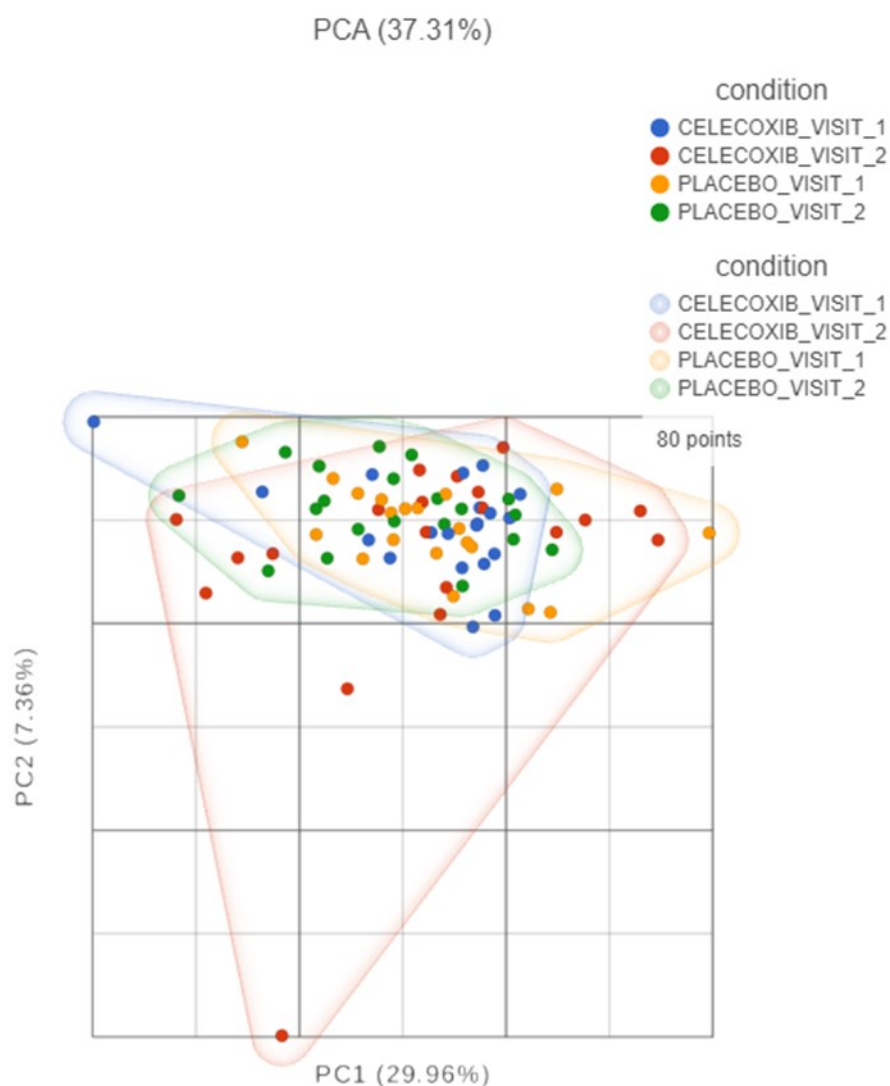


Figure 5.7 Principal Component Analysis Plot (PCA) of whole blood transcriptome measured using RNASeq. Samples were labelled according to group and visit number (celecoxib/placebo and visit 1/2). There was no separation between the groups.

Next differential analysis was performed using gene specific analysis (GSA) comparing the celecoxib visit 2 to celecoxib visit 1 group. There were no genes that were significantly different following false discovery rate (FDR correction). As a discovery approach, using an uncorrected p-value cut off $p \leq 0.05$, 1835 transcripts

that were significantly different were identified. A summary of the number of significant transcripts at different fold change cut offs can be found in the table below.

P Value cut off	Fold Change	Number of Transcripts
<0.05	+/-1.1	1583
<0.05	+/-1.2	205
<0.05	+/-1.3	74
<0.05	+/-1.4	40
<0.05	+/-1.5	16

5.5.3.1 Top altered genes:

There were 16 statistically significant transcripts with a fold change +/-1.5. Of these, 7 were noncoding regions and were excluded. The top 9 remaining transcripts are presented in **Figure 5.8** with corresponding scatterplots. None of these genes were altered in the placebo group **Figure 5.8**.

Below is a brief summary of the notoriety (based on standard PubMed searches) and function of the top nine (by fold) altered genes.

ZDHHC19 (Zinc Finger DHHC-Type Palmitoyltransferase 19): ZDHHC19 enables protein-cysteine S-palmitoyltransferase activity, involved in peptidyl-L-cysteine S-palmitoylation[213]. It is located in Golgi membrane; endoplasmic reticulum; and perinucleolar compartment. Little is known about the function of this gene with just 11 viable publications on PubMed. However, a role in driving role in proliferation and cancer has been suggested [214-216].

Septin 4: Septin 4 is a nucleotide (GTP) binding protein, originally described in yeast as cell division cycle regulatory proteins. It is highly expressed in brain and heart, reported regulate cytoskeletal organization[217]. There were 80 papers returned in a PubMed search of septin4 including publications relating to cancer and the cardiovascular system. Septin 4 was suggested to play a role in atherosclerosis, cardiac myocyte apoptosis, vascular smooth muscle cell proliferation oxidative stress in endothelial cells and a signalling molecule in platelets [218-221].

NELFCD (negative elongation factor complex member C/D): NELFCD is a member of the NELF complex of proteins which interact with DSIF proteins to repress

transcriptional elongation by RNA polymerase II[222]. There are just 7 publications returned on a PubMed search for NELFCD which include associations in cancer [223-225].

TUBA3D (Tubulin Alpha 3d): TUBA3D a member of the alpha tubulin family which are major component of microtubules in the cytoskeleton. Microtubules maintain cellular structure, function in intracellular transport, and play a role in spindle formation during mitosis.[226] Again, little is known about this gene with just four publications returning in a PubMed search which include papers associating this gene with cardiac abnormalities and arrhythmia.

C2orf74 (Chromosome 2 Open Reading Frame 74): C2orf74 is a protein encoding gene located on chromosome 2[227]. Again, little is known about the function of this gene with just six papers identified in a PubMed search. These studies suggest associations with autoimmune diseases and cancer [228, 229].

ITGA9 (Integrin alpha-9/beta-1): The protein encoded by ITGA9 is a receptor for VCAM1, cytotoxin and osteopontin[230]. More is known about ITGA9 than the other five genes described above with 100 publications returning on a PubMed search including associations with hypertension [231] and heart failure [232] with one study showing reduced ITGA6 in hypertensive high-salt fed rats[233] .

ADM (Adrenomedullin): Adrenomedullin is a well-established bioactive peptide with over 3,900 papers returning on a PubMed search. Adrenomedullin is implicated in cancer but is also an established biomarker in cardiovascular disease where it acts as a vasodilator[234].

IL27: IL27 is a member of the IL-12 cytokine family expressed by antigen presenting cells [235]. IL27 is a well-studied over 1,800 papers returning on a PubMed search. In immunology IL27 induces differentiation of the different populations of T cells and upregulates IL-10. However, IL27 is increasingly recognised as a potential modulator of atherosclerosis with increased plasma levels seen in those with coronary syndrome and myocardial infarction[236].

SPSB3 (Spla/Ryanodine Receptor Domain And SOCS Box Containing 3): [237] Again, little is known about SPSB3 with just seven papers returning on a PubMed search including studies associating SPSB3 with cancer. Interestingly it was shown that

SPSB3 positively associates with the severity grade in colorectal cancer[238]. As we found SPSB3 to be down regulated celecoxib, which is an effective preventative treatment for colorectal cancer, this observation may be important in the therapeutic utility of celecoxib in cancer.

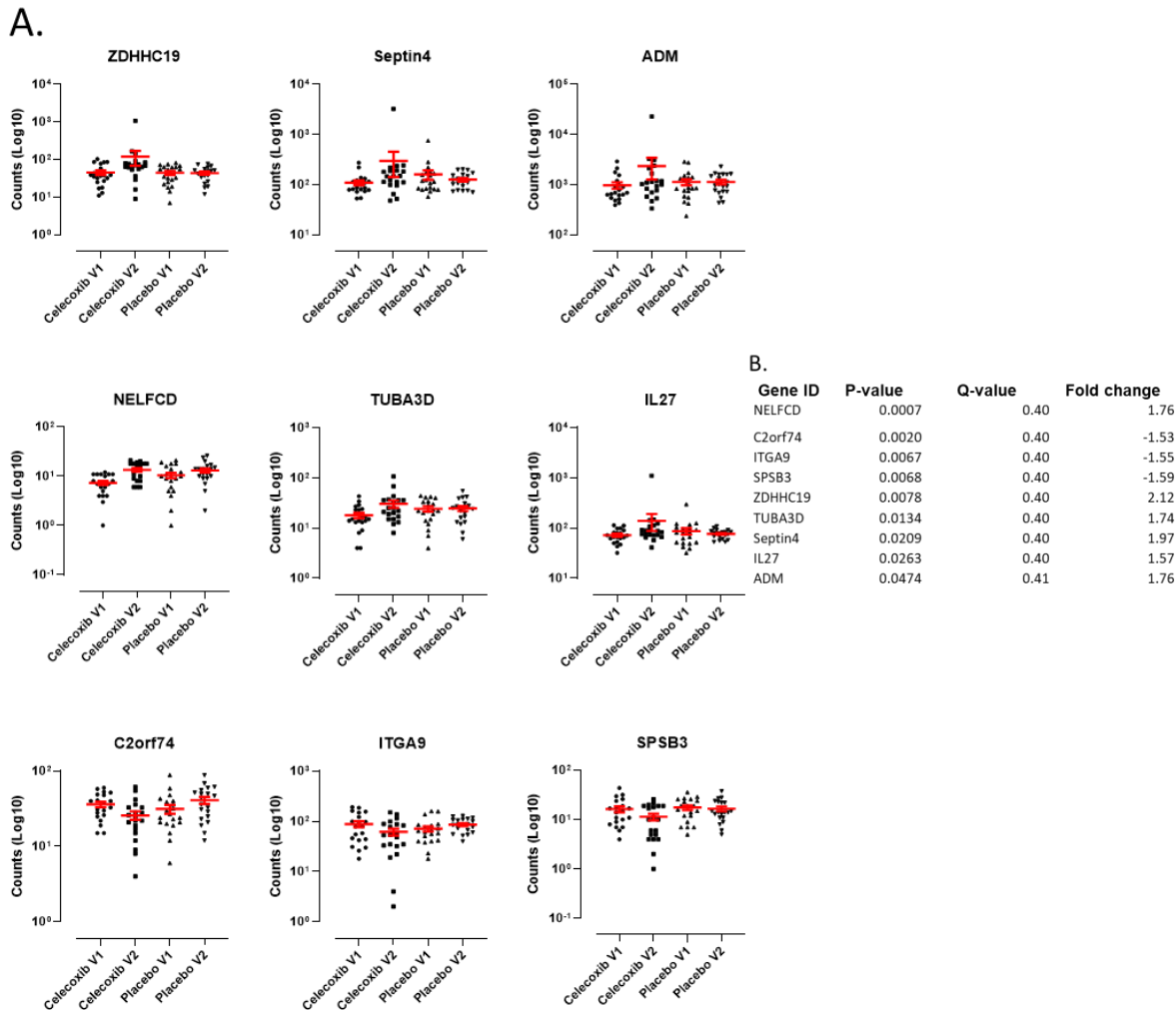


Figure 5.8 (A) Scatter Plot of top 9 genes at a threshold of $p < 0.05$ and fold change more than ± 1.5 and (B) corresponding table summarising the P-value, Q Value and fold change. Counts were log10 transformed. Abbreviations: ZDHHC19 (Zinc Finger DHHC-Type Palmitoyltransferase 19), Septin4 (Septin-4), ADM (adrenomedullin), NELFCD (Negative Elongation Factor Complex Member C/D), TUBA3D (Tubulin Alpha 3d), IL27 (interleukin 27), C2orf74 (Chromosome 2 Open Reading Frame 74), ITGA9 (Integrin Subunit Alpha 9), SPSB3 (SplA/Ryanodine Receptor Domain and SOCS Box Containing 3).

5.5.3.2 Pathway analysis:

In order to understand the pathways affected by celecoxib I then looked at genes altered before and after celecoxib at a cut off of 1.2-fold change. 205 transcripts were significantly altered in samples before versus after celecoxib (55 up and 150 downregulated; **Figure 5.9**). Next pathway enrichment analysis was performed using these 205 transcripts which were filtered and entered into the pathway analysis software. A summary of the top 10 gene sets alongside the enrichment plot is provided in **Figure 5.10**. The impact of the pathways identified on our understanding of the effects of celecoxib will be discussed in the general discussion (**Chapter 9**) but considering changes seen in the arginine: ADMA ratio shown in **Figure 5.2** it was interesting to note that biosynthesis of amino acids and arginine metabolism featured in the top 15 pathways. Similarly, in light of increases in AI in the celecoxib group shown in chapter 5, it was interesting to note that extracellular matrix (ECM) receptor interaction featured as one of the top 10 enriched gene sets.

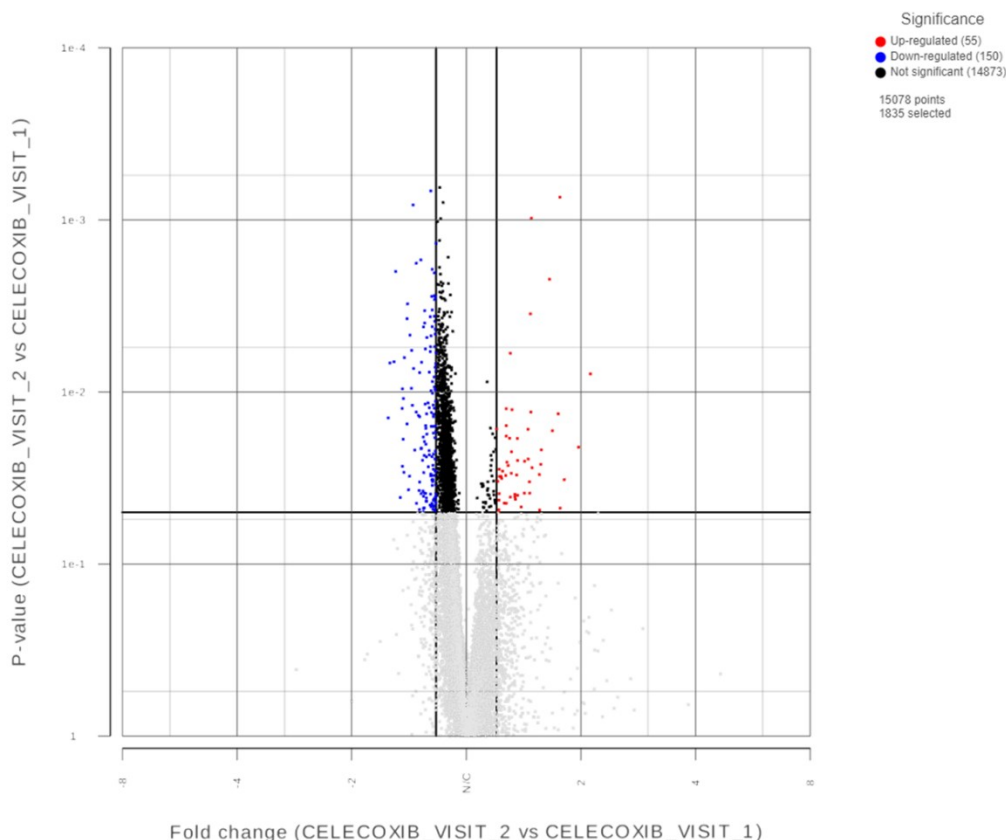
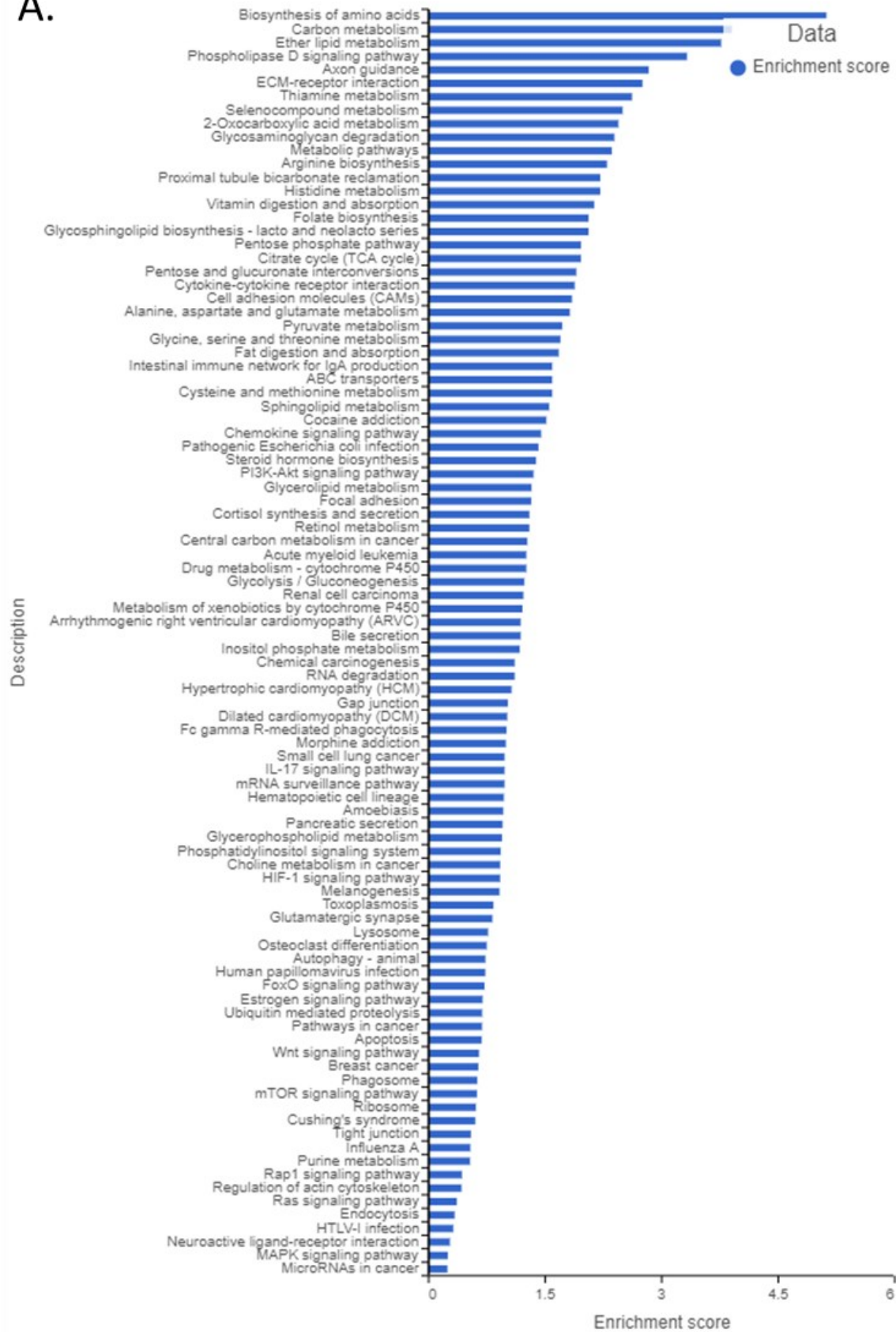


Figure 5.9 Volcano plots showing transcripts that are significantly different between celecoxib visit 1 and visit 2. At a p -value cut off of <0.05 (horizontal line) and fold change of ± 1.2 (vertical lines), 205 transcripts were significantly different including 150 down (blue) and 55 up regulated (red).

A.



B.

Gene Set	Description	Enrichment Score	P value	Number of Genes in List	Genes in List	Genes not in List
path:hsa01230	Biosynthesis of amino acids	5.12	5.98E-03	3	CTH GPT RPE	71
path:hsa01200	Carbon metabolism	3.9	0.02	3	DLAT GPT RPE	113
path:hsa00565	Ether lipid metabolism	3.76	0.02	2	ENPP2 PLPP3	45
path:hsa04072	Phospholipase D signaling pathway	3.32	0.04	3	GRM2 KIT PLPP3	142
path:hsa04360	Axon guidance	2.83	0.06	3	EPHA4 L1CAM SRGAP3	174
path:hsa04512	Extracellular matrix receptor interaction	2.75	0.06	2	ITGA9 LAMB3	80
path:hsa00730	Thiamine metabolism	2.62	0.07	1	LAMB3	14
path:hsa00450	Selenocompound metabolism	2.5	0.08	1	CTH	16
path:hsa01210	2-Oxocarboxylic acid metabolism	2.44	0.09	1	GPT	17
path:hsa00531	Glycosaminoglycan degradation	2.39	0.09	1	HYAL2	18

Figure 5.10 (A) Enrichment plot and table summarising the top 10 gene sets (B). Pathways relating to metabolism and Extracellular matrix were significantly enriched.

5.5.4 Targeted Proteomic analysis using Olink

In total 369 proteins were quantified. The full list can be found in **Appendix 2**. The difference between the visit 2 and visit 1 Log₂NPX score was calculated which equates to log₂ fold change.

PCA was performed, samples were labelled according to the allocated group. There was no separation between the groups **Figure 5.11**.

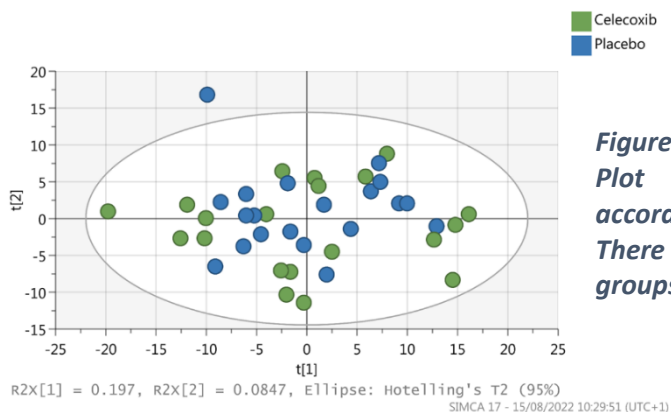


Figure 5.11 Principal Component Analysis Plot (PCA). Samples were labelled according to group (celecoxib or placebo). There was no separation between the groups.

Next a supervised analysis was performed by screening analytes using a T-test. In total there were 20 proteins that were significantly different (including 7 upregulated and 13 downregulated) between the placebo and celecoxib group **Figure 5.12a**. No proteins remained significant after FDR correction **Figure 5.12b**. CA3 was also included in the table as it was close to statistical significance (p=0.053) and had a fold change of 1.49 which could biologically be significant. Individual scatterplots for these 21 proteins is provided in **Figure 5.13**.

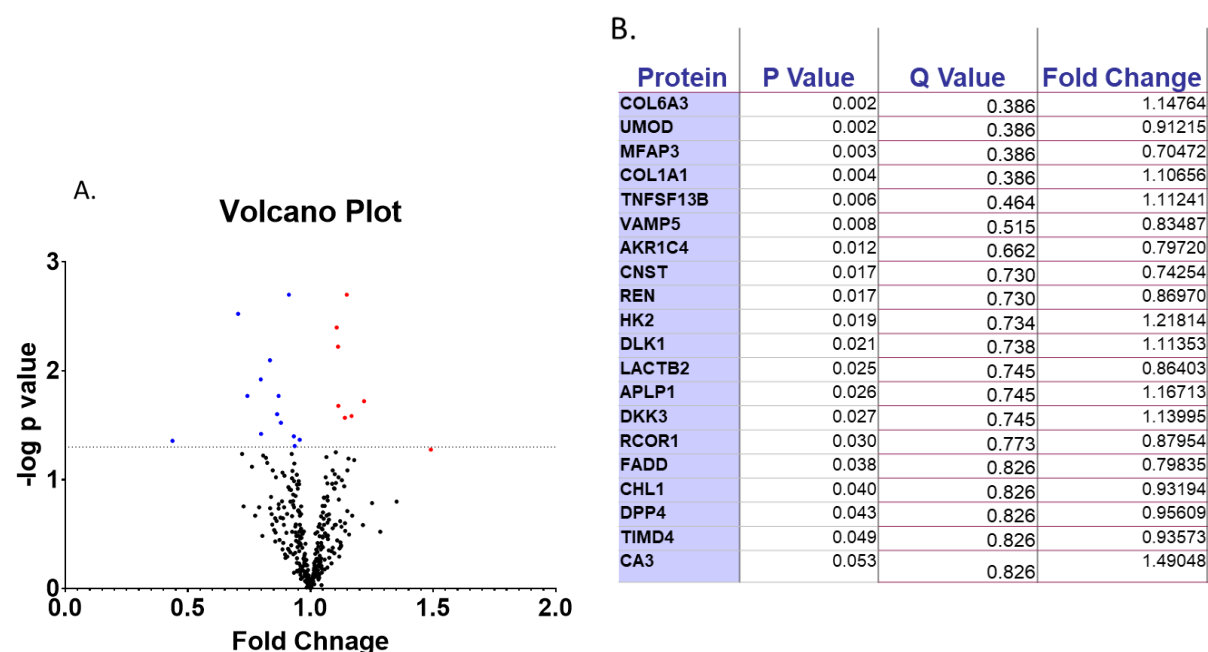
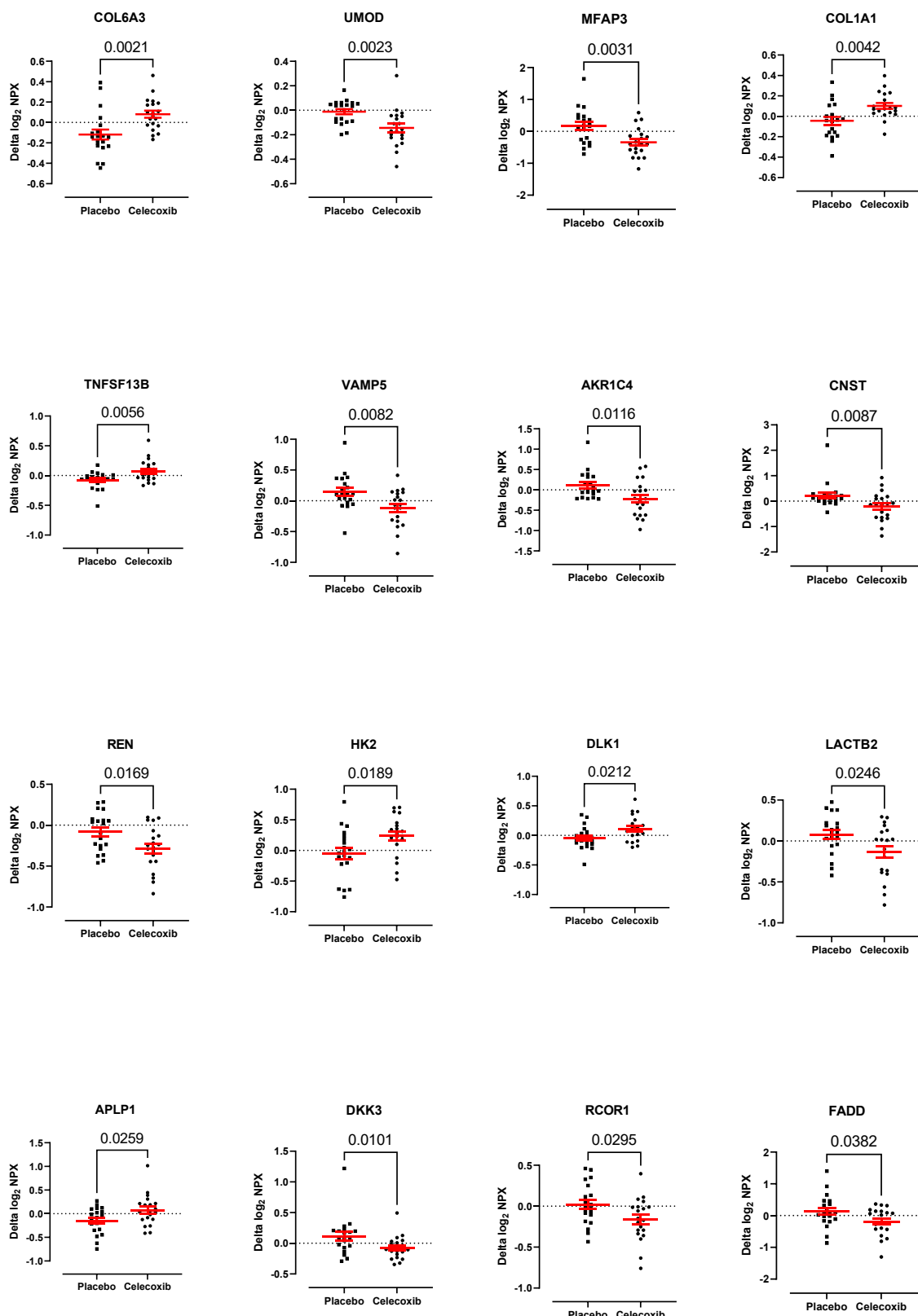


Figure 5.12 (A) Volcano plot showing proteins that are significantly different between celecoxib and placebo group and **(b)** table showing the Q values and fold change for the 21 proteins. **(a)** At a p-value cut off of <0.05 (dotted line), 20 proteins were significantly different including 13 down (blue) and 7 up regulated (red). **(b)** There were no proteins with Q<0.05 after correction for false discovery using the Benjamini, Krieger and Yekutieli method.



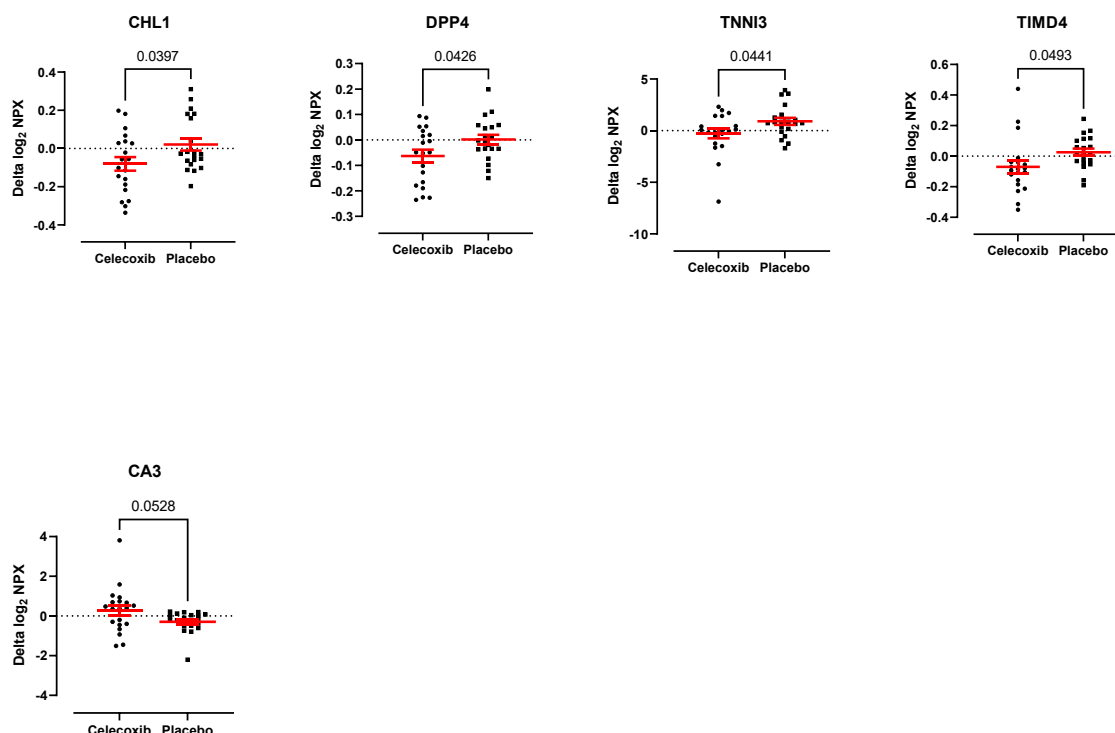


Figure 5.13 . Scatter Plot of 21 proteins that were significantly different between celecoxib and placebo group. Data is presented as mean (Horizontal bar) $\pm$ Standard error of the mean. Groups were compared using T-test for all analytes except CNST which was analysed using Mann Whitney U test.

Next pathway analysis was performed using Reactome which is a free web-based tool which supports pathway analysis of proteins using over-representation. Whilst FDR correction is useful in identifying biomarkers for discovery, it can sometimes be too strict and could lead to us missing some important pathways, therefore a decision to use p-values as a reference for 'significant proteins' for pathway analysis was made. The top 21 proteins were used for the pathway analysis. The top 10 pathways are presented in **Figure 5.14**.

Next, proteins were characterised into broad groups based on where they featured in the Reactome pathway database **Figure 5.15**.

It is important to note that some of the proteins in the 'other' or 'not found group' may be involved in ECM and inflammatory pathways when looking in the literature but for the sake of simplicity, they were grouped based on where they feature in the Reactome pathway database.

Pathway name	Entities				Reactions	
	found	ratio	P-Value	Entities FDR	Found	Reactions ratio
Reversible hydration of carbon dioxide	2/17	0.001123	3.01E-04	0.11049	2/8	5.74E-04
Collagen chain trimerization	2/44	0.002906	0.001967	0.359992	2/28	0.00201
Assembly of collagen fibrils and other multimeric structures	2/67	0.004425	0.00614	0.374175	16/26	0.001866
Collagen degradation	2/69	0.004557	0.00614	0.374175	6/34	0.00244
Defective RIPK1-mediated regulated necrosis	1/5	3.30E-04	0.00736	0.374175	1/1	7.18E-05
Glycolysis	2/110	0.007265	0.011772	0.374175	1/24	0.001723
ECM proteoglycans	2/79	0.005217	0.015639	0.374175	6/23	0.001651
CASP8 activity is inhibited	1/14	9.25E-04	0.021924	0.374175	2/3	2.15E-04
Anchoring fibril formation	1/15	9.91E-04	0.021924	0.374175	1/4	2.87E-04
Glucose metabolism	2/140	0.009246	0.022313	0.374175	1/50	0.003589

Figure 5.14 Top 10 pathways identified using Over-representation analysis in REACTOME. Broad characterisation identifies key pathway themes relating to extra-cellular matrix organisation.

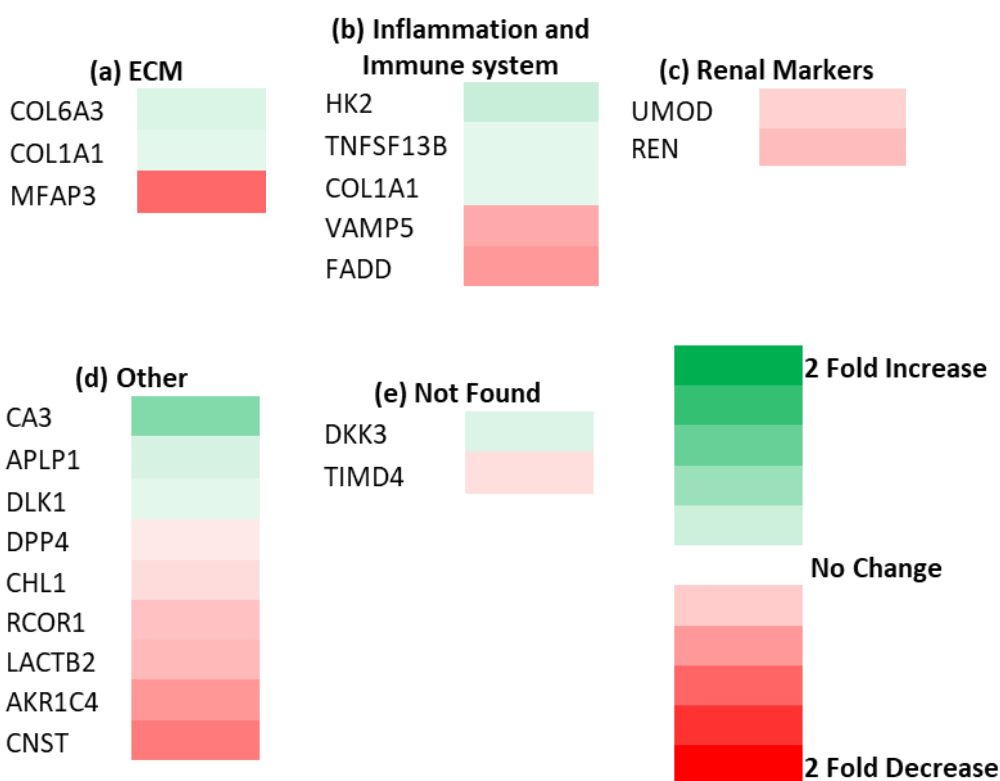


Figure 5.15 Heatmaps of top 21 proteins that were significantly different between celecoxib and placebo. Proteins were grouped according to pathways highlighted in REACTOME. Dark green indicates a 2-fold increase and dark red indicates a 2-fold decrease.

5.5.4.1 Pathways relating to ECM Organisation

Considering that celecoxib increased AI it was interesting to find that pathways relating to extracellular matrix organisation dominated the top 9 pathways, specifically these were (i) Collagen chain trimerization, (ii) Assembly of collagen fibrils and other multimeric structures, (iii) Collagen degradation, (iv) ECM proteoglycans and (v) Anchoring fibril formation (Figure 5.14). In total 3 proteins were identified as directly relevant to the extracellular matrix (**Figure 5.15a**) including higher COL1A1 and COL6A3 and lower MFAP3. What is particularly interesting is that the ECM remodelling is a key driver of arterial stiffness and is central in the pathogenesis of a range of vascular diseases including hypertension.

Increased COL1A1 and COL6A3 is also supported by the increase in 4-hydroxyproline shown earlier which is a surrogate for vascular collagen turnover. Furthermore microfibrillar-associated proteins (MFAPs) play a key role in elastin fibre formation. Whilst these are circulating serum levels which may not reflect what is happening locally at the vasculature, simplistically, this data suggests there may be an increase in collagen and a reduction in elastin formation, which are hallmark features seen with increased vascular stiffness. As mentioned above, these biological changes would also fit with the clinical finding in **Chapter 4** of increased AI (surrogate for vascular stiffness) following celecoxib treatment.

5.5.4.2 Pathways relating to inflammation and the immune system

Two pathways were identified relating to inflammation and the immune system, specifically these were (i) Defective RIPK1-mediated regulated necrosis and (ii) CASP8 activity is inhibited (Figure 5.14). In total, 5 proteins were identified **Figure 5.15b**. The protein with highest fold change was HK2 and lowest fold change was FADD.

5.5.4.3 Renal markers

Two renal markers were identified **Figure 5.15c**; uromodulin and renin which were both lower in the celecoxib group. The reduction in renin following COX-2 inhibition is already well established and provides some confidence in our other findings [239]. Uromodulin is exclusively synthesised by the kidneys and is the most abundant urinary protein in physiological conditions and lower levels are indicative of renal dysfunction [240].

5.5.4.4 Other Pathways

Nine 'other' proteins were identified including 3 up and 6 downregulated **Figure 5.15d**. The protein with the highest fold change was CA3 and lowest fold change was CNST. One of the limitations of pathway analysis is that whilst it can be useful for identifying proteins within well-established pathways, it can be more limited when trying to assess interactions with lesser-known pathways or pathways that do not feature within Reactome at that time. For example, when searching through GeneCards.org, amyloid beta precursor like protein 1 (APLP1), dipeptide peptidase 4 (DPP4) and cell adhesion molecule L1 like (CHL1) are intricately linked to ECM organisation. It should also be noted that two pathways; glycolysis and glucose metabolism were also identified and disturbances in these metabolic pathways as with diabetes mellitus, can increase cardiovascular risk.

5.5.4.5 Not Found in Reactome

There were 2 proteins found **Figure 5.15e**; dickkopf WNT signalling pathway inhibitor 3 (DKK3) was increased and T cell immunoglobulin and mucin domain containing 4 (TIMD4) was reduced.

5.5.4.6 Correlations with Augmentation Index

Next, I wanted to see how changes in serum proteins correlates with change in AI. The delta (difference between visit 2 and visit 1) for the top 21 proteins was plotted against delta AI and Pearson's r was used to assess correlation **Figure 5.16a**. MFAP3, VAMP5 and RCOR1 significantly correlated with AI **Figure 5.16b**. As discussed earlier, MFAP3 plays a key role in elastin fibre formation and so the negative correlation seen would fit a biological picture of reduced elastin = increased vascular stiffness.

a.

Protein	Pearson's r	95% Confidence Interval	P value
RCOR1	-0.3829	-0.6204 to -0.08106	0.0147
MFAP3	-0.3489	-0.5957 to -0.04200	0.0273
VAMP5	-0.3349	-0.5854 to -0.02614	0.0346
AKR1C4	-0.2635	-0.5314 to 0.05232	0.1004
UMOD	-0.2429	-0.5154 to 0.07419	0.1309
DKK3	-0.2121	-0.4911 to 0.1065	0.1889
DLK1	-0.1732	-0.4599 to 0.1462	0.2851
CNST	-0.1492	-0.4403 to 0.1702	0.3581
FADD	-0.1465	-0.4380 to 0.1729	0.367
COL1A1	0.1347	-0.1845 to 0.4282	0.4073
LACTB2	-0.1174	-0.4138 to 0.2015	0.4707
CA3	0.1164	-0.2025 to 0.4129	0.4745
TNFSF13B	0.1133	-0.2054 to 0.4104	0.4862
DPP4	0.09556	-0.2226 to 0.3953	0.5575
CHL1	-0.0885	-0.3893 to 0.2293	0.5871
COL6A3	0.08681	-0.2309 to 0.3878	0.5943
TIMD4	0.04443	-0.2708 to 0.3511	0.7854
APLP1	0.04228	-0.2728 to 0.3492	0.7956
TNNI3	0.02621	-0.2876 to 0.3350	0.8724
REN	0.01599	-0.2970 to 0.3259	0.922
HK2	0.003632	-0.3082 to 0.3148	0.9823

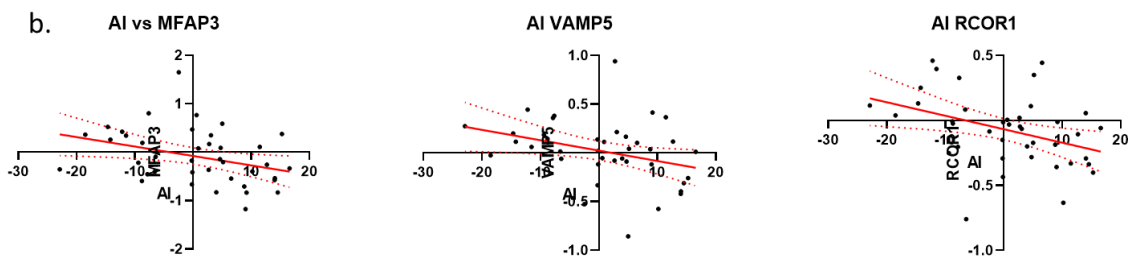


Figure 5.16 (a) Table summarising the correlations between delta protein and delta augmentation index with Scatterplots for the 3 significant correlations (b). Changes in MFAP3, VAMP5 and RCOR1 significantly correlated with changes in augmentation index (AI).

5.6 Summary

The aims of this chapter were to assess serum ADMA and other multi-omic analytes in healthy male volunteers who have taken 7 days of celecoxib and compare this to placebo. We found a significant reduction in arginine/ADMA ratio and a reduction in uromodulin with celecoxib use which fits with the hypothesis that renal COX-2 is cardioprotective and could be driving cardiovascular events amongst NSAID users.

From the proteomic analysis we identified differences in proteins relating to extracellular matrix organisation and in particular increase in serum collagen markers (COL1A1, COL6A3 and 4-hydroxyproline) and a reduction in MFAP3 which is involved in elastin formation. This is an exciting finding as it fits with the clinical finding of increased AI (vascular stiffness) with celecoxib use in this cohort and provides a novel mechanism for NSAID cardiovascular events.

5.6.1 Limitations

The samples that were analysed in this chapter were taken as part of a double blinded, placebo-controlled trial that was deemed to have a low risk of bias. None the less, there are several limitations that need to be taken into account.

Whilst the study design minimises the risk of bias, as with all studies, there is still a small chance that unknown confounders have not been taken into consideration. As the study design was limited to healthy, young male volunteers the results may not be applicable to the wider population and in particular chronic NSAID users. Participants in the study were treated with 7 days of celecoxib and this may not reflect what happens with longer term NSAID use.

Our Proteomic analysis using Olink was semi targeted towards 369 known cardiometabolic proteins. This could potentially bias any downstream pathway analysis and so should be interpreted with caution.

As this was a biomarker discovery exercise and no proteins had q value <0.05 , findings need to be validated using ELISA. None the less, the changes in analytes and pathways linked to ECM organisation biologically fit with the clinical finding of increased AI with celecoxib use.

The changes in ECM related proteins were from serum samples and may not necessarily reflect what is happening at a local vascular level.

Transcriptomic analysis was performed on whole blood. Whilst this can give provide a detailed overview of overall physiological state, it may not represent direct changes to the vasculature and so should be interpreted accordingly.

5.6.2 Future Work

As this was a biomarker discovery exercise, significant proteins will need to be validated using single analyte platforms. The changes in AI and pathways relating to ECM organisation should be prioritised as an area for future work in this field. Whilst it is outside the scope of this thesis, as a next step, histological analysis of vascular tissue from both NSAID treated and COX-2 KO mice should be performed. This should be coupled with cell culture work to unpick the mechanism by which NSAIDs affect the ECM.

Finally, whilst it is striking that these changes are seen in young healthy volunteers after just 7 days of celecoxib use, it is not known if changes in these pathways translate to cardiovascular risk in NSAID users. In the following chapters I will address this by investigating biomarkers in NSAID users who go onto have cardiovascular events in a case-controlled study using the SCOT biobank.

Chapter 6: Clinical Drivers of Cardiovascular Events Amongst NSAID users

6.3 Rationale

I have already emphasised in previous chapters that there are no biomarkers used in clinical practice to predict non-steroidal anti-inflammatory drug (NSAID) induced cardiovascular events. As discussed in the introduction, there are two large scale randomised control trials, SCOT and PRECISION [31, 241], that demonstrated that the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib carries the same cardiovascular risk as non-selective COX Inhibitors (such as ibuprofen which is readily available over the counter in the UK) which aligns with meta-analysis of real world patient data[39]. Analysing samples from patients on NSAIDs who then go onto have cardiovascular events would provide novel insight into the mechanisms involved. To further explore this we obtained serum samples from the SCOT trial. As previously discussed, SCOT was a prospective, multi-centre, randomized, open-label trial comparing the gastrointestinal and cardiovascular safety of prescribed non-steroidal anti-inflammatory drug (NSAIDs with switching to celecoxib conducted in patients with low cardiovascular risk). Within the study there were 7,297 patients enrolled, of which, 249 patients had a cardiovascular event[31].

Based on outcome data, a nested case control study was designed. One of the major challenges was to determine how to match the events and controls appropriately.

Biomarkers are not only limited to molecules or genes but can also be physical characteristics. In fact, for cardiovascular disease in general, clinical characteristics such as age, blood pressure, BMI and gender are well recognised predictors of cardiovascular disease. These have been incorporated into cardiovascular risk prediction scores such as QRISK-3[242], which was developed to predict 10-year cardiovascular risk in the UK population and is used by healthcare professionals across the country to guide clinical care. I therefore began the search for biomarkers associated with cardiovascular events in SCOT by performing an exploratory analysis of the SCOT trial dataset to determine risk factors for cardiovascular events in this cohort. While a similar approach has now recently been taken by others using data from PRECISION[243], surprisingly, a comprehensive investigation of clinical drivers

of events amongst low cardiovascular-risk, NSAID users had not been previously explored.

6.3.1 Hypothesis

‘Clinical characteristics can be used to predict cardiovascular risk amongst NSAID users in the SCOT cohort.’

The aims of this chapter were to:

- Identify clinical drivers of cardiovascular events amongst NSAID users in the SCOT dataset.
- Inform decision making for the variable matching in the case control study of blood biomarkers.

6.4 Methods

6.4.1 Exploratory analysis using the SCOT dataset

6.4.1.1 Study Population

SCOT was a prospective, multi-centre, randomized, open-label clinical trial comparing the gastrointestinal and cardiovascular safety of a prescribed NSAID vs switching to prescribed celecoxib in patients with low cardiovascular risk with rheumatoid or osteoarthritis. A detailed protocol and primary results have been published[244] and are described in the **Chapter 1 Introduction**.

In brief, both men and women were eligible for inclusion if they were over 60 years of age, chronic NSAID users (≥ 90 days or at least 3 filled prescriptions in the last year) and were free from established cardiovascular disease. Exclusion criteria included ischaemic heart disease, myocardial infarction, angina or acute coronary syndrome, cerebrovascular disease including cerebrovascular accident or transient ischemic attack, peripheral vascular disease or moderate/severe heart failure, patients who had taken selective COX-2 inhibitors within 90 days of screening, life threatening co-

morbidity, clinically significant renal or hepatic disease, patients who were unlikely to comply with medications, allergy to NSAIDs, hypersensitivity to sulphonamide antibiotics, active peptic ulceration or gastrointestinal bleeding, patients scheduled to have arthritis surgery and those participating in another clinical trial.

The trial was conducted in the UK (England and Scotland), Netherlands and Denmark between January 2008 and March 2013 across 9 trial centres and 706 primary care practices. Eligible patients were identified in a primary care setting and were randomized to either switch to celecoxib or remain on their usual NSAID prescription. The medications were prescribed at approved doses and adjusted according to clinical indication.

6.4.1.2 Cohorts

I have used the intention-to-treat (ITT) cohort from the above population. Cases were identified as patients who had reached the primary endpoint which was a composite of hospitalisation for non-fatal myocardial infarction or biomarker positive acute coronary syndrome, non-fatal stroke or cardiovascular death. Controls were identified as patients who did not reach the primary endpoint.

In the primary publication, there were no statistically significant subgroup interactions of pre-defined covariates associated with cardiovascular disease between the celecoxib and the standard care group. This is in line with what we now know about the shared risk of cardiovascular side effects across all NSAID medications. In this analysis, I explored co-variables between patients who had a cardiovascular event and those that did not, to identify potential risk factors associated with cardiovascular events amongst chronic NSAID users. The methodology for this study was in accordance with the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) statement for developing and validating multivariable prediction models.[245]

6.4.1.3 Statistical analysis

I grouped patients according to the occurrence of the composite primary endpoint as previously described (cardiovascular event vs. no event). Analysis was performed

using IBM SPSS version 27. Least absolute shrinkage and selection operator (LASSO) method was performed by Dr Laura Portas using R version: 4.0.3; R package: 'glmnet'. The regression model was created by me using binary logistic regression (presented as odds ratios (ORs)) along with the 95% confidence intervals. Survival analysis was estimated with the Kaplan-Meier method using the log-rank test to compare differences between groups. A multivariable model was created using Cox regression analysis. A two-tailed significance level of $p < 0.05$ was deemed to be statistically significant.

6.4.1.4 Selection of Candidate Variables

As per the TRIPOD statement for developing multivariate prediction tools, the selection of variables is a key component and can ultimately influence outcomes. Ultimately there are several methods that can be used for candidate variable selection. I adopted a combination of clinical and statistical justification for variable selection.

6.4.1.5 Clinical Justification

Clinically plausible predictors of cardiovascular disease within the dataset included age, gender, ethnicity, smoking status (never smoker, current smoker, ex-smoker), chronic obstructive airway disease (COPD), rheumatoid arthritis, blood pressure, body mass index (BMI), waist hip ratio, diabetes, heart failure, total cholesterol, high density lipoprotein (HDL), triglycerides, renal disease, statins, aspirin, hypertension and uric acid. In the primary SCOT analysis[31], the type of NSAID was not a predictor of cardiovascular events which was confirmed in my own re-analysis so was not included as a predictor.

Following review of the literature including established cardiovascular risk scores in the general population[242], I examined causal relationships with the variables in the data using directed acyclic graphs (DAGs) **Figure 6.1**. DAGs is a method for visualising the relationships between multiple variables and can be used to identify potential biasing paths.

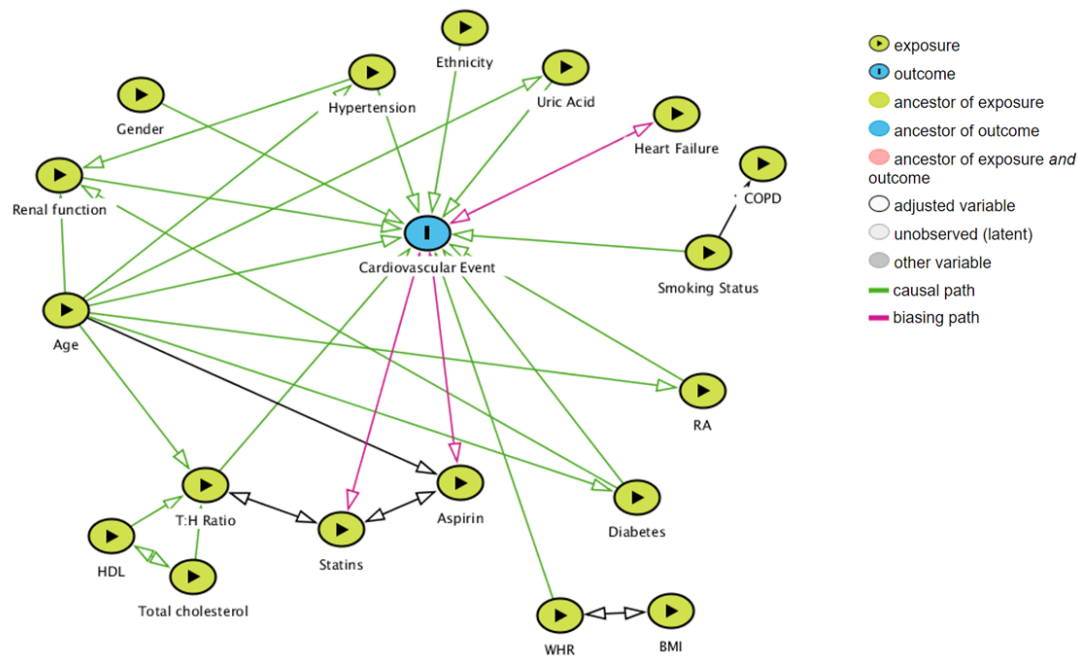


Figure 6.1 Directed Acyclic Graph assessing causal and biasing relationships between clinical predictors of cardiovascular events within the SCOT dataset. Abbreviations include: chronic obstructive pulmonary disease (COPD), rheumatoid arthritis (RA), high density lipoprotein (HDL), waist hip ratio (WHR) and body mass index (BMI).

Four variables were subsequently excluded:

- 1) COPD- smoking status was deemed to be a more reliable predictor as COPD is often underdiagnosed[246].
- 2) BMI- a waist hip ratio >1 has been reported to be a better predictor of cardiovascular risk[247, 248]
- 3) Total cholesterol, HDL, triglycerides- total cholesterol/HDL ratio >5 is a well-established predictor of cardiovascular risk and was deemed to be a better predictor than the individual lipid levels[249, 250].
- 4) Hypertension- data relating to the use of anti-hypertensives and blood pressure control was not available and therefore a decision to use the average of three blood pressure recordings at screening as a more reliable marker of uncontrolled hypertension was made. The median systolic blood pressure was 140mmHg in both groups. Within this dataset, Blood pressure was categorised into <130mmHg, 130-159mmHg and ≥160mmHg[251].

6.4.1.6 Statistical Justification

Predictors with less than 15 events per variable were not included in the model: ethnicity (n: 1 non-Caucasian), heart failure (n:1) and renal disease (n:10). As there is controversy in regard to the minimum number of events per variable in regression models[252], sensitivity analysis was performed with the inclusion of renal disease which did not affect the overall models and so was not included.

Next the candidate predictors of cardiovascular events for use in the logistic model were selected using the least absolute shrinkage and selection operator (LASSO) method[252]. LASSO simultaneously performs variable selection and regularisation utilising machine learning algorithms. The method works by shrinking the absolute size of the regression coefficients towards zero by placing a penalty to the sum of the estimated regression coefficients. Variables with regression coefficients that shrink to zero are excluded.

6.5 Results

6.5.1 Exploratory analysis using the SCOT dataset

We identified 7,297 NSAID users in the population dataset of which 249 patients (3.4%) reached the primary endpoint. The median ITT follow-up for the primary endpoint was 3 years (maximum 6.3 years, total 22,600 person years).

First, I compared baseline characteristics between the cardiovascular event and non-event groups **Figure 6.2** The groups were similar in terms of the type of NSAID following randomisation, country of residence, median age, and ethnicity (>99% Caucasian). There was a trend towards an increase in known cardiovascular risk factors in the cardiovascular event group including male gender, older age, rheumatoid arthritis, current smoker, diabetes, systolic blood pressure >160mmHg at screening, waist: hip ratio >1 and lipid profile.

Baseline Characteristic	n: 7048 No Event: (n or Median)	% or IQR	n: 249 Cardiovascular Events (n or Median)	% or IQR
Treatment Group (Celecoxib)	3522	49.97%	125	50.2%
Control (usual NSAID)	3526	50.03%	124	49.8%
Country				
Scotland	3817	54.2%	150	60.24%
Denmark	2135	30.3%	74	29.72%
England	1053	14.9%	25	10.04%
Netherlands	43	0.6%	0	0%
NSAID during Trial				
Ibuprofen	1114	15.80%	39	15.66%
Diclofenac	1359	19.30%	53	21.29%
Other NSAID	1053	14.90%	32	12.85%
Celecoxib	3522	50%	125	50.20%
Age (Years)	67	8.8	69.4	10.4
Age 60-70	4615	65.48%	130	52.20%
Age 70-80	2091	29.70%	86	34.50%
Age> 80	342	4.90%	33	13.25%
Male	2818	40%	141	56.63%
Non Caucasian	22	0.30%	1	0.40%
Current Smoker	1063	15.10%	67	26.91%
Never smoker	2929	41.60%	71	28.51%
Rheumatoid Arthritis	434	6.20%	20	8.03%
Statin therapy	1484	21.10%	38	15.26%
Aspirin therapy	810	11.50%	44	17.67%
Heart Failure	13	0.20%	1	0.40%

Baseline Characteristic	n: 7048 No Event: (n or Median)	% or IQR	n: 249 Cardiovascular Events (n or Median)	% or IQR
History of Diabetes	559	7.90%	27	10.84%
Hypertension	3136	44.50%	104	41.77%
Systolic BP *	139.5	22.5	140.5	24.5
Systolic BP < 130 mmHg *	1943	27.58%	59	23.69%
Systolic BP 130 – 160mmHg *	4178	59.31%	145	58.23%
Systolic BP> 160mmHg *	893	12.70%	45	17.67%
Diastolic BP *	77	14	75.5	15
Diastolic BP> 100mmHg *	163	2.30%	6	2.41%
Renal Disease	248	3.50%	10	4.02%
Asthma	699	9.90%	28	11.24%
COPD	296	4.20%	21	8.43%
BMI	28.7	6.8	28.5	6.7
Waist hip ratio	0.91	0.1	0.93	0.1
WHR >1	1012	14.40%	50	20.08%
Hypercholesterolaemia	2421	34.40%	58	23.29%
Total Cholesterol (mmol/l)**	5.23	1.48	5.31	1.59
Total cholesterol > 5.5**	2501	39.70%	97	42.92%
HDL cholesterol*	1.38	0.51	1.29	0.42
HDL<1	655	10.40%	33	14.60%
Triglycerides **	1.63	1.1	1.68	1.1
Tot Chol:HDL Ratio**	3.71	1.33	4	1.5
T:H Ratio >5 **	773	12.27%	49	21.68%
Uric Acid (mmol/l)***	319	110	327	125
Uric Acid<300umol/l***	2609	40.62%	82	35.96%
Uric Acid 300-400 umol/l***	2628	40.92%	92	40.35%
Uric Acid>400umol/l***	1186	18.46%	54	23.68%

*Figure 6.2 Baseline characteristics table comparing cases (cardiovascular event) and controls (no event). Abbreviations used: BP: blood pressure, COPD: chronic obstructive pulmonary disease, BMI: body mass index, WHR: waist hip ratio. HDL: high density lipoprotein, chol: cholesterol. *n: 7044 in non-event group and 249 in event group. **n:6300 in the non-event group and 226 in the event group. ***n: 6423 in non-event group and 228 in event group.*

Next, LASSO was used which did not identify any further variables for exclusion. The variables in the final model (11 variables) included age (60-70, 70-80, >80 years), gender, smoking status (never smoker, current smoker, ex-smoker), systolic blood pressure at screening (<130, 130-160, >160mmHg), rheumatoid arthritis, diabetes, waist: hip ratio>1, aspirin use, statin use, total cholesterol:HDL ratio > 5, serum uric acid (<300, 300-400, >400µmol/L). Each variable was adjusted for all other variables listed above.

There was at least one variable missing in 771 cases (10.6%). The total number of cases used in the final regression analysis was 6,300 Controls and 226 cases (3.6%).

6.5.1.1 Logistic Regression

A summary of the regression coefficients and odds ratios (OR) are presented in **Figure 6.3**. The factors significantly associated with a cardiovascular event were male gender, increasing age, current smoker, concomitant aspirin use and total cholesterol:HDL ratio ≥ 5 . The odds of a cardiovascular event were significantly lower in statin users compared to non-users (OR 0.68; 95% CI 0.46 to 0.98). There was a direction of effect towards an increased likelihood of cardiovascular events in patients with rheumatoid arthritis, diabetes and systolic blood pressure ≥ 160 mmHg although these did not reach statistical significance. Waist hip ratio and uric acid did not appear to be associated with a cardiovascular event in our cohort.

I also had access to time to event/last follow up and so I wanted to next look at the risk of a cardiovascular event using the aforementioned risk factors. Generating survival curves would also allow me to get a better visual understanding of the dataset.

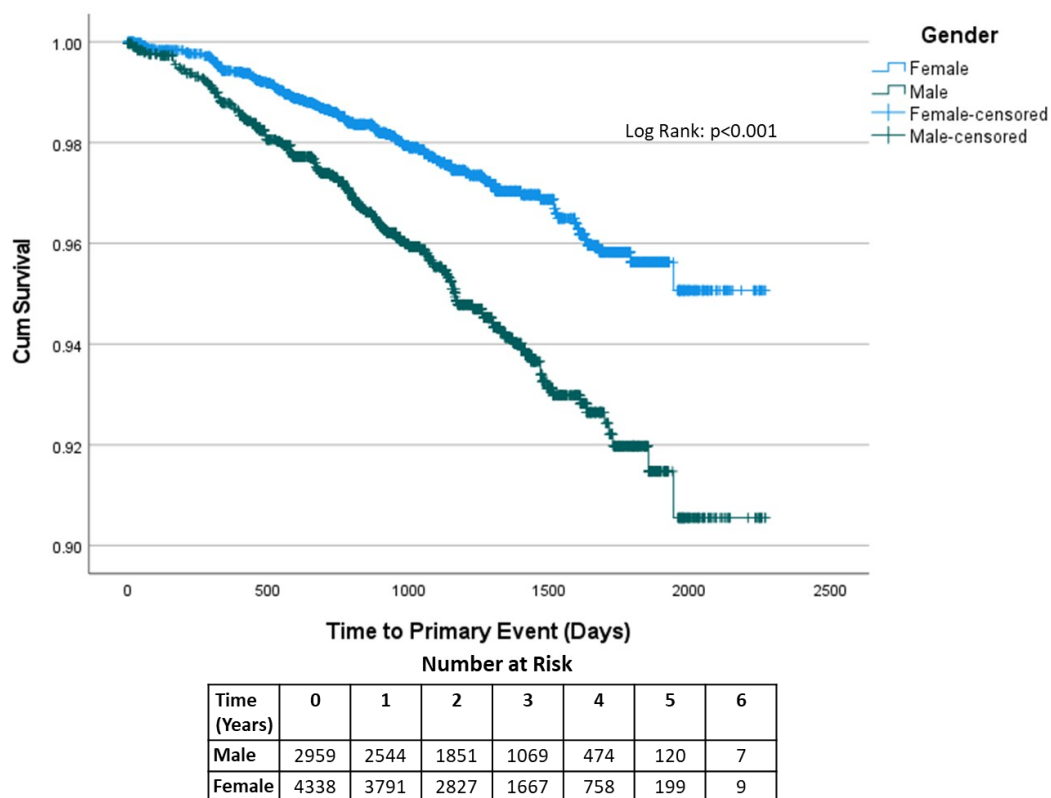
Variable	β Coefficient	S.E	OR	95% C.I. for OR		p value
				Lower	Upper	
Sex						
Female	Ref					
Male	0.57	0.156	1.77	1.303	2.404	0.0003
Smoking Status						
Non-Smoker	Ref					
Current Smoker	0.905	0.189	2.47	1.706	3.579	<0.0001
Former smoker	0.253	0.167	1.288	0.928	1.787	0.131
Systolic blood pressure at screening						
< 130mmHg	Ref					
130 – 159 mmHg	0.044	0.168	1.045	0.752	1.453	0.792
≥160mmHg	0.362	0.216	1.436	0.94	2.19	0.094
Arthritis						
Osteoarthritis	Ref					
Rheumatoid arthritis	0.478	0.252	1.613	0.985	2.642	0.058
Diabetes						
No	Ref					
Yes	0.438	0.228	1.55	0.99	2.425	0.055
Concomitant aspirin use						
No	Ref					
Yes	0.422	0.185	1.525	1.060	2.193	0.023
Total cholesterol : HDL ratio						
<5	Ref					
≥5	0.674	0.173	1.961	1.397	2.754	0.0001
Age						
60-69 years	Ref					
70-79 Years	0.499	0.152	1.647	1.223	2.219	0.001
≥80 years	1.401	0.22	4.059	2.637	6.248	<0.0001
Statin Use						
No	Ref					
Yes	-0.394	0.193	0.675	0.463	0.984	0.041
Uric acid						
Uric acid <300umol/l	Ref					
Uric acid 300 - 400umol/l	-0.085	0.162	0.919	0.669	1.262	0.601
Uric acid >400umol/l	0.078	0.192	1.082	0.742	1.576	0.683
Waist: Hip ratio						
<1	Ref					
≥1	0.051	0.183	1.053	0.736	1.506	0.779
Constant	-4.439	0.216	0.012			<0.0001

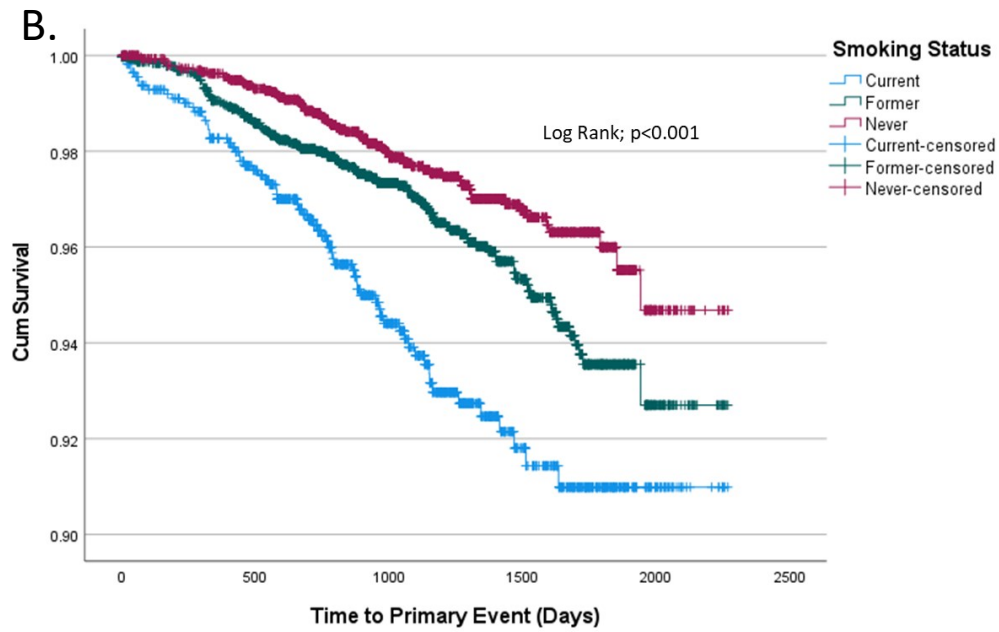
Figure 6.3 Factors associated with a cardiovascular event using binary logistic regression. The model includes all variables listed (Male gender, smoking status, systolic Blood pressure, arthritis, diabetes, waist: hip ratio, statin use, aspirin use, total cholesterol: HDL ratio, uric acid and age. Abbreviations: SE: standard error, OR: odds ratio, CI: confidence interval, Ref: reference. Each variable is adjusted for all other variables shown. The total number of cases used in the analysis was 6,300 Controls and 226 cases (3.6%).

6.5.1.2 Time to Event

First, I performed univariable analysis of risk factors using the log rank test. Kaplan Meier survival curves alongside a number at risk table is presented in **Figures 6.4a-j**.

A.

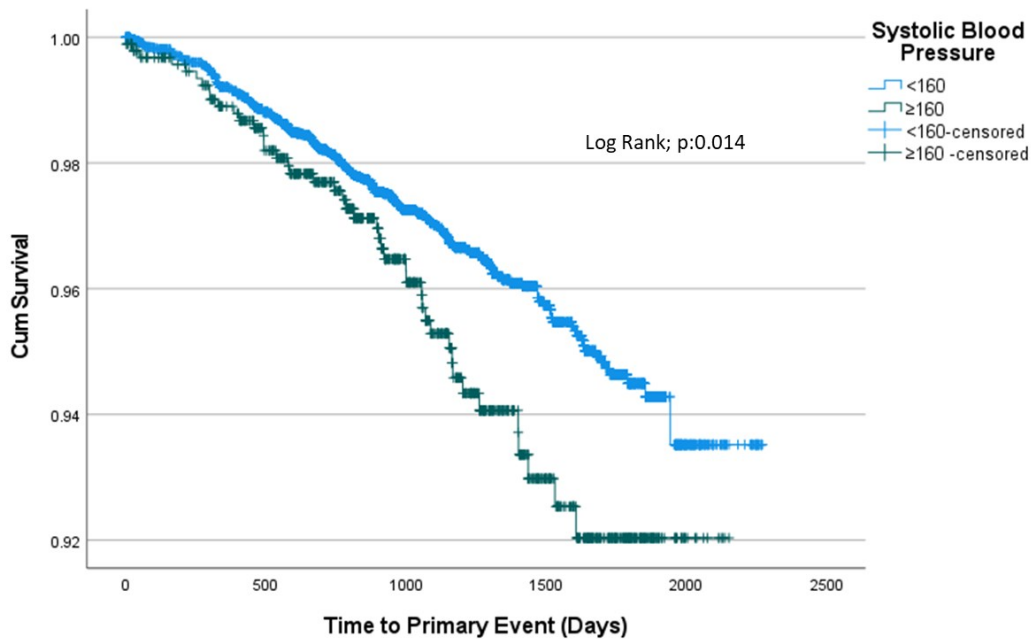




Number at Risk

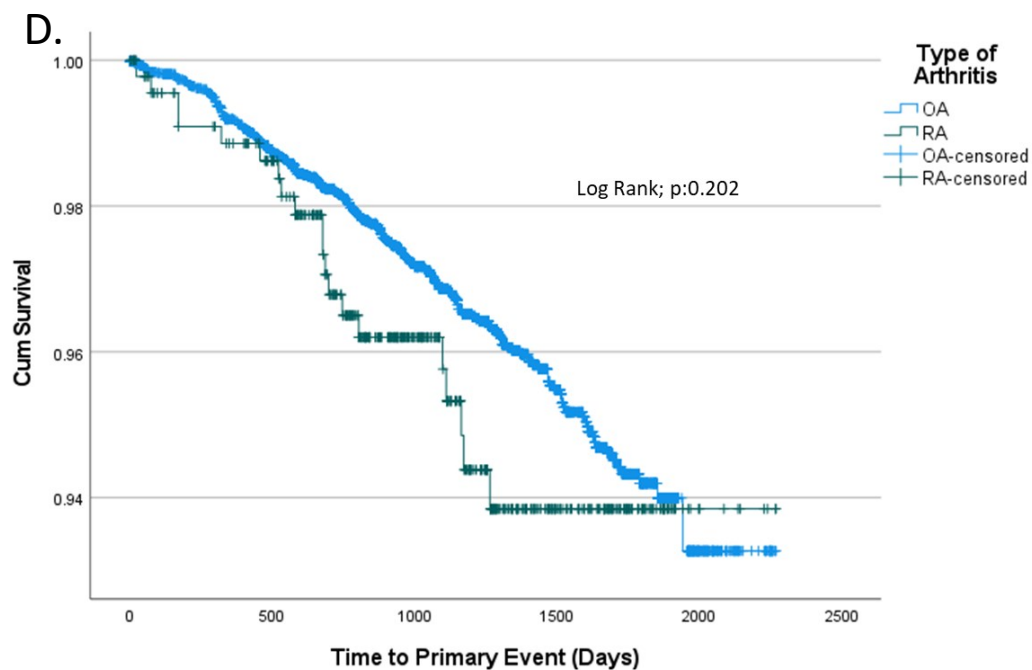
Time (Years)	0	1	2	3	4	5	6
Current	1130	962	712	412	186	53	4
Former	3167	2748	2033	1195	538	140	7
Never	3000	2624	1933	1129	508	126	5

C.



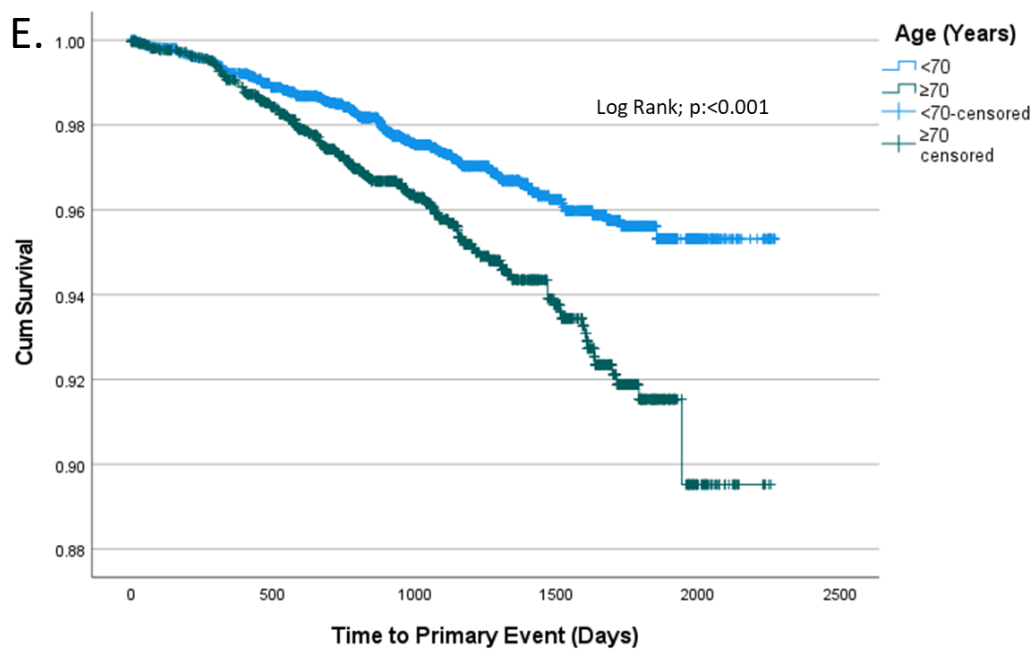
Number at Risk

Time (Years)	0	1	2	3	4	5	6
<160	6360	5541	4092	2388	1079	285	17
≥160	794	586	348	152	34		



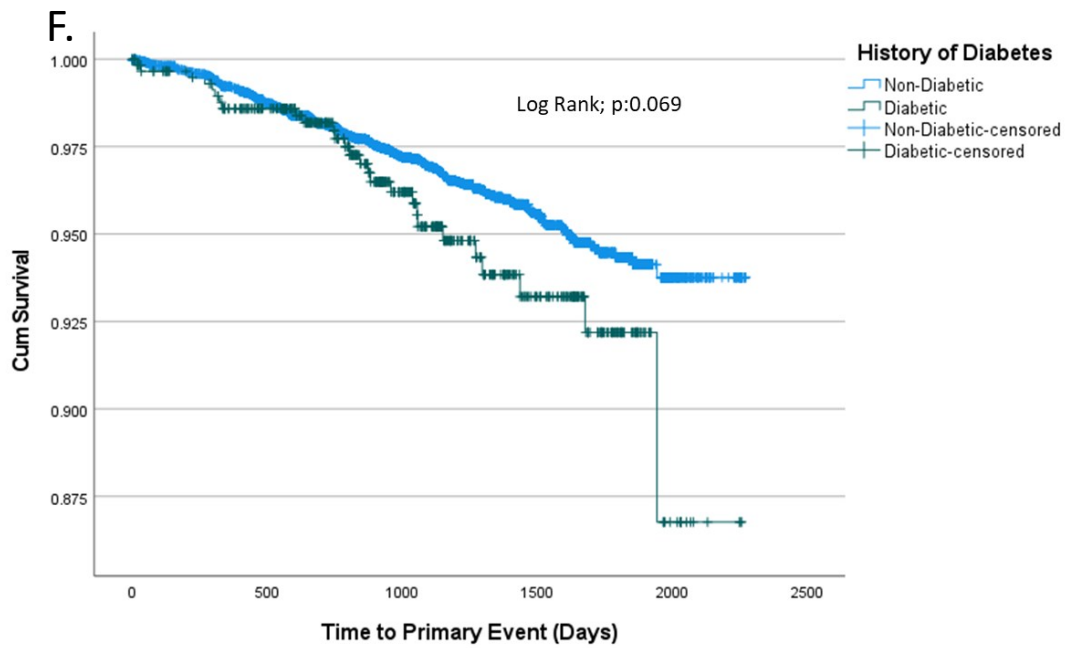
Number at Risk

Time (Years)	0	1	2	3	4	5	6
OA	6843	5948	4396	2564	1155	300	15
RA	454	387	282	172	77	19	2



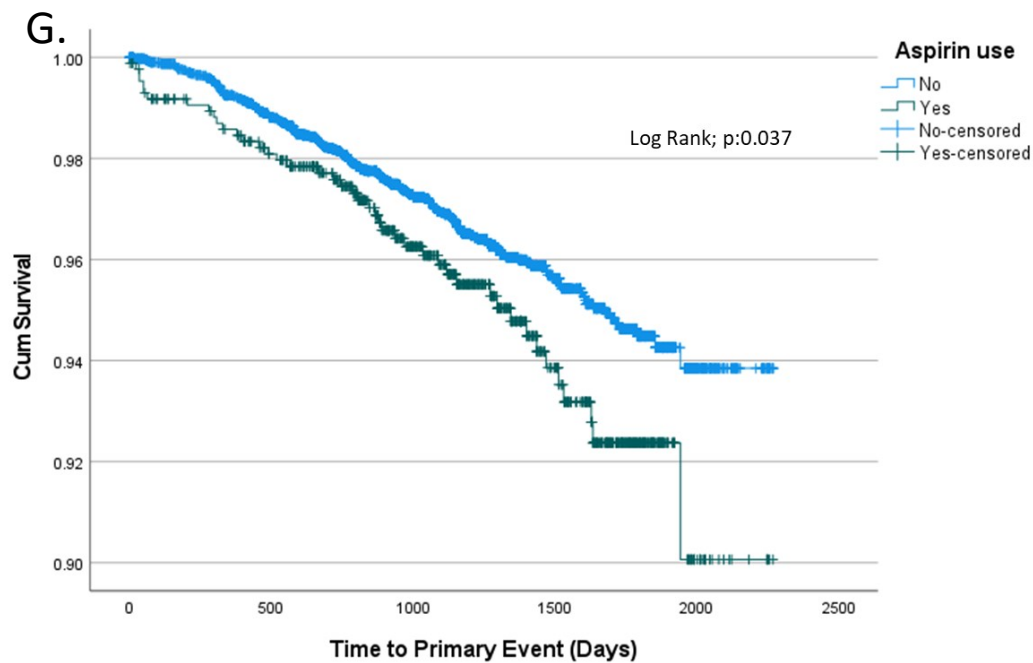
Number at Risk

Time (Years)	0	1	2	3	4	5	6
Age < 70	4751	4160	3080	1785	794	209	14
Age ≥ 70	2546	2174	1598	951	438	110	3



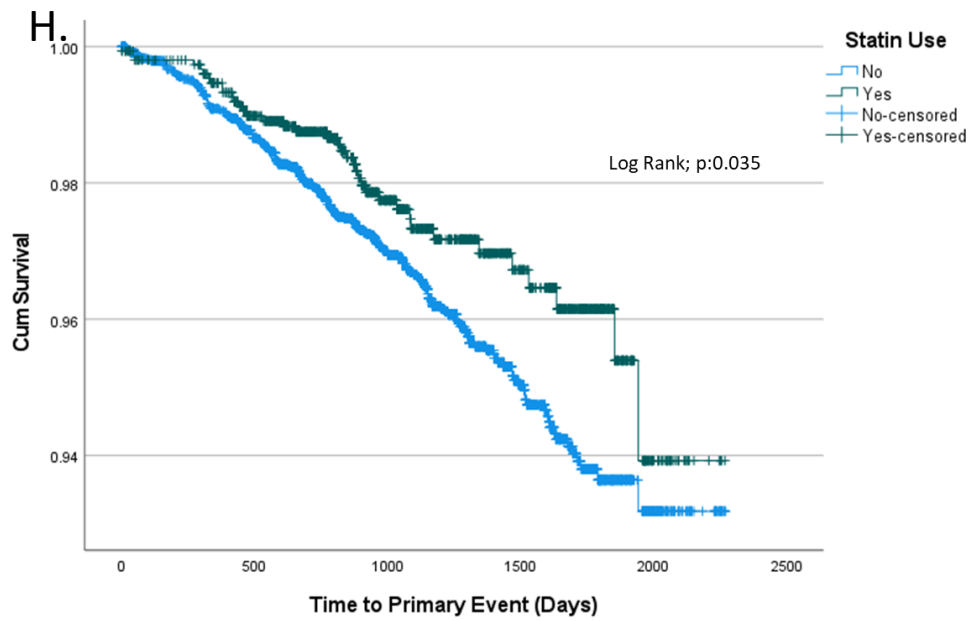
Number at Risk

Time (Years)	0	1	2	3	4	5	6
Diabetic	586	493	361	209	92	22	1
Non-diabetic	6711	5842	4318	2528	1139	297	15



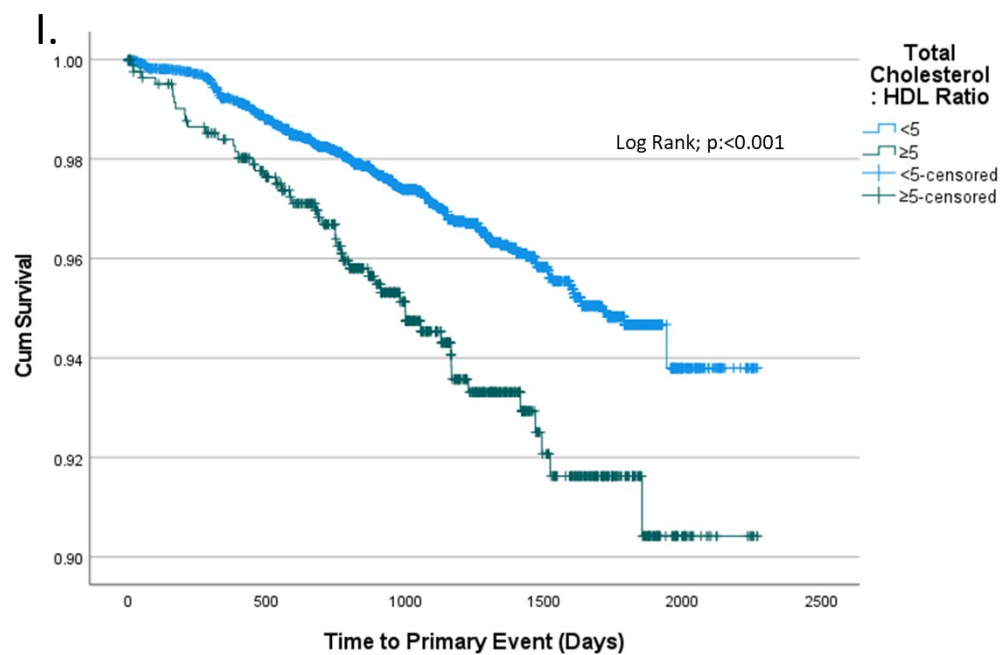
Number at Risk

Time (Years)	0	1	2	3	4	5	6
Aspirin	854	781	633	411	198	51	3
No Aspirin	6443	5553	4045	2326	1034	268	13



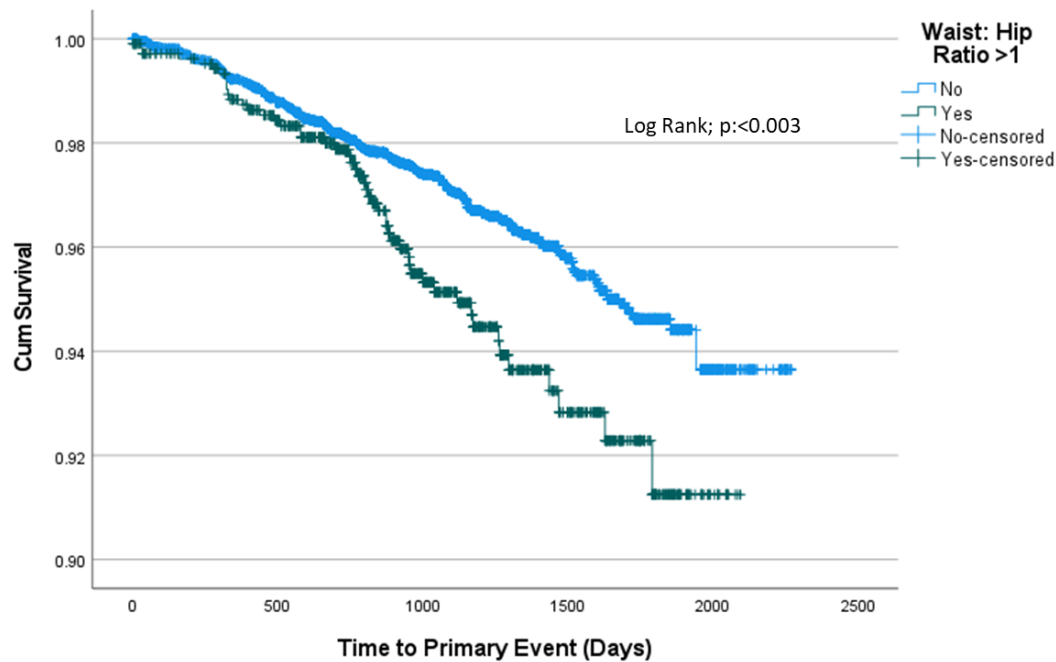
Number at Risk

Time (Years)	0	1	2	3	4	5	6
Statin	1522	1310	924	540	275	79	3
No Statin	5775	5025	3755	2197	956	240	14



Number at Risk

Time (Years)	0	1	2	3	4	5	6
TC:HDL <5	5704	4926	3630	2142	970	253	13
TC:HDL ≥5	822	736	559	328	155	48	4



Number at Risk

Time (Years)	0	1	2	3	4	5	6
WHR:<1	6325	5435	4033	2381	1083	286	17
WHR ≥1	1062	900	646	355	148	33	

Figure 6.4 Kaplan-Meier survival curves showing the impact of clinical variables on the occurrence of a cardiovascular event along with a number at risk table. Clinical variables assessed included (A) gender, (B) smoking status, (C) systolic blood pressure ≥ 160 mmhg, (D) rheumatoid arthritis, (E) age ≥ 70 years, (F) history of diabetes mellitus, (G) aspirin use, (H) statin use, (I) total cholesterol:HDL ratio ≥ 5 , (J) waist hip ratio ≥ 1 .

Next, I used Cox regression to determine the unadjusted risk of a cardiovascular event **Figure 6.5**. The suggested factors associated with an increased risk of a cardiovascular event included: male gender, smoking status, systolic blood pressure >160 mmhg, age >70 years, diabetes, aspirin use and a waist: hip ratio >1 . Statin use was associated with a reduced risk. I then entered these variables into a multivariable model using Cox regression whereby each variable was adjusted for all other variables. The risk factors that remained significant following adjustment included male gender (HR 1.78, 95%CI 1.8 to 1.3;), current smoking (HR 2.4, 95%CI 1.7 to 3.4), systolic blood pressure >160 mmhg (HR 1.46, 95%CI 1 to 2.1), age >70 years (HR 1.97, 95%CI 1.5 to 2.6), diabetes (HR 1.6, 95%CI 1 to 2.4;) and total cholesterol:HDL ratio >5 (HR 1.8, 95%CI 1.3 to 2.5). Interestingly Statin use remained protective with an

approximately 30% reduction in risk even after adjusting for cholesterol (HR 0.67, 95%CI 0.47 to 0.98). In the next section I will describe the matching strategy.

Factor	Univariable P-value	Hazard Ratio	95% CI upper	95% CI lower	Multi-variable P-value	Hazard Ratio	95% CI upper	95% CI lower
Male Gender**	<0.001*	1.98	1.5	2.5	<0.001**	1.78	1.33	2.37
Smoking Status (Ref: Never smoked)	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Former smoker	0.009*	1.49	1.11	2.01	0.136	1.28	0.926	1.76
Current Smoker**	<0.001*	2.6	1.86	3.62	<0.001**	2.38	1.66	3.41
Systolic BP >160mmhg **	0.015*	1.5	1.08	2.08	0.032**	1.46	1.03	2.05
Age>70 years**	<0.001*	1.75	1.36	2.24	<0.001**	1.97	1.51	2.57
Diabetes**	0.07	1.45	0.97	2.16	0.046**	1.55	1.01	2.4
Aspirin use*	0.037*	1.41	1.02	1.96	0.13	1.31	0.92	1.87
Statin use**	0.036*	0.69	0.49	0.98	0.04**	0.68	0.47	0.98
Total Cholesterol:HDL ratio>5**	<0.001*	1.83	1.34	2.52	<0.001**	1.81	1.31	2.51
Waist Hip Ratio >1	0.004*	1.58	1.16	2.16	0.46	1.14	0.81	1.6
Rheumatoid Arthritis (ref OA)	0.204	1.35	0.85	2.12	0.05	1.61	1	2.58

Figure 6.5 Factors associated with a cardiovascular event Cox regression. Each variable is adjusted for all other variables shown in the multivariable model. The total number of cases used in the analysis was 6,300 Controls and 226 cases (3.6%). **indicates statistical significance $p<0.05$. CI: confidence interval, Ref: reference.

6.5.2 Matching SCOT samples

Designing a case control study and matching events to controls was a crucial part of the serum biomarker studies described in the following chapters. I encountered numerous challenges along the way.

My first major challenge was to identify exactly how many samples I had available for analysis from the serum biobank. As the SCOT serum samples were taken over several years (2008-2013) from various centres internationally, the sample labelling and documentation was not optimal. With the aid of my colleague Dr Plinio Ferreira, we hand curated ~7000 samples to first identify how many event samples were present. For the purpose of the case control study, I decided to only use events from the on-treatment group. This would ensure that participants were on an NSAID at the

time of the event. I also excluded patients with rheumatoid arthritis. I had identified 118 samples from participants who had a cardiovascular event.

As discussed in the previous results section, I had identified potential drivers of cardiovascular risk in this cohort, but the next challenge was to determine how to best match the samples. On the surface, matching for all variables would seem like the most natural choice, however this can lead to selection bias. Over matching can also mean that potential biomarkers that are associated with clinical risk factors, e.g. blood pressure may be missed. The optimal strategy would be to match on a few variables and then perform post-hoc adjustment if necessary. I took the advice of experts in this field including my supervisors, the SCOT study team and Professor Jennifer Quint (Imperial College London) and decided to match on four variables:

1 & 2) Age $\pm$ 5 years and gender.

Age and gender are key risk factors for cardiovascular (CV) events and matching would ensure balanced groups.

3) Baseline NSAID

Samples taken from participants were at screening and therefore all participants were on a non-selective NSAID (ibuprofen, naproxen, diclofenac or other). Whilst there were no differences in CV events between type of NSAID, mechanistically there may be subtle differences that could introduce bias between the groups

4) Country

SCOT was an international multi-centre trial in 706 primary care practices across 9 trial centres in 4 Countries (England, Scotland, Denmark and Netherlands). There are multiple unknown confounders such as diet, lifestyle that may differ between regions. Furthermore, sample handling and processing may have also been different between regions. The most pragmatic approach was to match for the country.

6.5.2.1 Matching

The first step was to filter out control serum samples from my dataset that were not present in the biobank. Next, I used fuzzy matching which is an SPSS extension package to perform the matching. Variables that had to have an exact match were age, gender, country and baseline NSAID. For age I set a deviation of ± 5 years. The software first prioritises exact matches. When that is not possible it locates the closest remaining match from the pool (without replacement to ensure each participant can only act as one control). If there is more than one candidate match, the software will randomly identify one. I initially matched using 2:1, 3:1 and a 4:1 matching regime. After 3:1 matching there was loss of best match. It was pragmatic to use 2:1 matching with cost being one of the other limitations.

Once the matches had been identified, I wanted to compare the baseline characteristics between the cases and controls **Figure 6.6**.

Baseline Characteristic	n:236 No Event: (n or Mean)	% or standard deviation	n:118 Cardiovascular Events (n or mean)	% or standard deviation
Country				
Scotland	124	52.54%	62	52.5%
Denmark	84	35.59%	42	35.6%
England	28	11.86%	14	11.9%
Netherlands	0	0.00%	0	0.0%
Treatment Group (Celecoxib)	115	48.73%	53	44.9%
Control (Usual NSAID)	121	51.27%	65	55.1%
NSAID during Trial				
Celecoxib	115	48.73%	53	44.9%
Ibuprofen	47	19.92%	22	18.6%
Diclofenac	46	19.49%	29	24.6%
Other NSAID	28	11.86%	14	11.9%
Age (Years)	69.6	6.9	69.8	6.1
Age> 70	97	41.10%	51	43.2%
Age> 80	25	10.59%	13	11.0%
Male	142	60.17%	71	60.2%
Non Caucasian	0	0.00%	1	0.8%
Current Smoker	35	14.83%	31	26.3%
RA	0	0.00%	0	0.0%
Never smoker	98	41.53%	24	20.3%
Statins Therapy	56	23.73%	15	12.7%
Aspirin therapy	28	11.86%	22	18.6%
Heart Failure	0	0.00%	1	0.8%
History of Diabetes	18	7.63%	12	10.2%

Baseline Characteristic	n:236 No Event: (n or Mean)	% or Standard Deviation	n:118 Cardiovascular Events (n or Mean)	% or Standard deviation
Hypertension	101	42.80%	48	40.7%
Systolic BP	140	19.70	142.2	17.90
Systolic BP> 140 mmHg	117	49.58%	58	49.2%
Systolic BP> 150 mmHg	57	24.15%	39	33.1%
Systolic BP> 160 mmHg	33	13.98%	23	19.5%
Diastolic BP mmHg	77.9	11.90	76.9	10.70
DBP> 100 mmHg	6	2.54%	4	3.4%
Renal Disease	9	3.81%	5	4.2%
Asthma	26	11.02%	13	11.0%
COPD	10	4.24%	9	7.6%
BMI	29.3	5.40	29.6	5.5
BMI < 18.5	1	0.42%	1	0.8%
BMI>30	93	39.41%	49	41.5%
Waist hip ratio	0.93	8.00%	0.93	9.0%
WHR >1	42	17.80%	28	23.7%
Hypercholesterolaemia	80	33.90%	27	22.9%
Total Cholesterol (mmol/l)	5.1	1.16	5.4	1.1
TC> 6	43	18.22%	37	31.4%
HDL cholesterol	1.4	41.00%	1.37	40.0%
HDL<1	39	16.53%	19	16.1%
Triglycerides (mmol/l)	1.79	1.04	1.87	0.97
Total Chol:HDL Ratio	3.74	1.58	4.17	1.2
T:H Ratio >3.5	127	53.81%	81	68.6%
T:H Ratio >5	25	10.59%	28	23.7%
Uric Acid (mmol/l)	332	84.40	346	86.4

Figure 6.6 Baseline characteristics table comparing cases (cardiovascular event) and controls (no event). Abbreviations used: RA: rheumatoid arthritis, BP: blood pressure, COPD: chronic obstructive pulmonary disease, BMI: body mass index, WHR: waist hip ratio. HDL: high density lipoprotein, chol: cholesterol. T:H= total cholesterol:HDL ratio.

This shows that the matching had worked well, and that the distribution of co-variates was similar and therefore representative of the larger SCOT dataset **Figure 6.2**. Once I was happy with the matching, the 236 control samples were located from within the biobank. There were 8 control samples missing and so the total number of controls was 228.

6.5.2.2 Planning experiments and aliquoting

Due to the nature of the study, there was considerable heterogeneity in volumes between the samples, namely differences in the amount of volume available. The range of serum volumes within the biobank ranged from approximately 500µl for some to less than <250µl for others. I therefore had to prioritise experiments **Figure 6.7**. Due to limitations in sample volumes, for discovery, omic analysis (proteomics, amine mass spectrometry and multiplex arrays), which also carried significant costs I used 1:1 matching, with the intention that significant analytes could be validated using targeted platforms and 2:1 matching.

Priority	Assay	Format	Matching	Volume (uL)
1	Amine Array	Tube	1 to 1	30
2	ADMA-Arginine ELISA	Plate	2 to 1	40
3	Proteomics	Tube	1 to 1	100
4	Lipidomics	Tube	1 to 1	175
5	Multiplex Thrombosis + Kidney Arrays	Plate	1 to 1	20
6	IL-6 + MCP-1 ELISAs	Plate	2 to 1	120
7	ET-1 ELISA	Plate	1 to 1	65
8	Future Assays 1	Plate	2 to 1	65
9	Future Assays 2	Plate	2 to 1	65
10	Future Assays 3	Plate	2 to 1	65

Figure 6.7 Table of prioritisation of experiments with the volume of sample required. Abbreviations used: ADMA: Asymmetric dimethylarginine, IL-6: interleukin 6, MCP-1: Monocyte chemoattractant protein-1, ET-1: endothelin-1.

As I would be dealing with a large number of samples (354) it was important to take into consideration any potential of batch effect. To avoid this, I first randomly allocated event samples across 5 plates, each event had the 2 matched controls on the same plate so each plate could in theory be interpreted as a standalone experiment if necessary (Example plate layout: **Figure 6.8**). Each sample was defrosted slowly in the fridge and then aliquoted into a 2ml deep 96 well plate which was then centrifuged at 10,000g for 20 mins to remove any debris. Samples were then aliquoted into plates or tubes and re-frozen at -80°C. The final number of samples for each experiment is

presented in **Figure 6.9**. In the next chapter I will discuss the results of these experiments.

	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
	1	2	3	4	5	6	7	8	9	10	11	12
A	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
B	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
C	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
D	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
E	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
F	Event	Event	Event	Event	Control A	Control A	Control A	Control A	Control B	Control B	Control B	Control B
G	Event	Event	Event	Event	SC	SC	SC	SC	Blank	Blank	QC	QC
H	SC	SC	SC	SC	SC	SC	SC	SC	Blank	Blank	QC	QC

Figure 6.8 Example plate layout for case control study. Each plate is blocked with an event and the two matched controls (control A & control B). Abbreviations: QC: quality control, SC: standard curve.

Experiment (Total n:)	Sub Total		Plate 1		Plate 2		Plate 3		Plate 4		Plate 5	
	Events	Controls	Events	Controls	Events	Controls	Events	Controls	Events	Controls	Events	Controls
Amine Array (n:235)	118	117	24	24	24	23	24	24	24	24	22	22
ADMA-Arg ELISA (n:346)	118	228	24	45	24	43	24	48	24	48	22	44
Proteomics (n:235)	118	117	24	24	24	23	24	24	24	24	22	22
Lipidomics (n:234)	117	117	24	24	24	23	23	24	24	24	22	22
Multiplex (n:235)	118	117	24	24	24	23	24	24	24	24	22	22
IL-6 + MCP-1 ELISAs (n:344)	116	228	24	45	24	43	22	48	24	48	22	44
ET-1 ELISA (n:233)	116	117	24	24	24	23	22	24	24	24	22	22
Future Assays 1 (n:343)	115	228	23	45	24	43	22	48	24	48	22	44
Future Assays 2 (n:335)	109	226	21	44	24	42	21	48	22	48	21	44
Future Assays 3 (n:331)	106	225	21	44	24	42	20	47	22	48	19	44

Figure 6.9 Table of prioritisation of experiments with the volume and number of samples per experiment. Abbreviations used: ADMA: Asymmetric dimethylarginine, IL-6: interleukin 6, MCP-1: Monocyte chemoattractant protein-1, ET-1: endothelin-1.

6.6 Summary

The aim of this chapter was to inform decision making and design an effective matching strategy for the case control study. To my knowledge, this is the first exploratory analysis investigating clinical drivers of cardiovascular events amongst NSAID users with low cardiovascular risk. Whilst it is unsurprising that established predictors of cardiovascular events in all populations such as smoking, diabetes, rheumatoid arthritis, cholesterol, gender and age were also predictors amongst NSAID users, this analysis has identified several novel important findings including the potential cardioprotective role of statins in this population and the need to develop a personalised risk prediction tool.

6.6.1 Limitations

Data in the analysis was derived from a prospectively conducted randomised control trial with a low rate of missing values. Nonetheless these results have some important limitations that must be acknowledged. Although I have included relevant predictors of cardiovascular events within the dataset, there may be unknown drivers of cardiovascular events in this cohort that have not been captured. Within this dataset, the primary outcome is relatively unbalanced (<4% of cases) which can affect the quality of any predictions made. This is particularly true for the time to event analysis as the dataset has not fully matured (more than half of the cohort had not yet reached the primary endpoint) and therefore I was unable to ascertain median survival. Finally, the vast majority of the cohort (>99%) were Caucasian and so findings may not be generalisable. It is important to highlight that this work was exploratory, and any statistical relationship does not imply causation.

6.6.2 Future Work

Whilst results obtained from the SCOT dataset were useful for guiding the optimal matching strategy for the serum samples, as already discussed, there are several limitations namely a small sample size and event rate that would limit its clinical applicability. To overcome this I collaborated with Professor Jennifer Quint and her team to design and conduct a population-based cohort study; Statin use to mitigate the unwarranted cardiovascular side effects of non-steroidal anti-inflammatory drugs: A population-based case control study, SAMURAI. This will be performed using the Clinical Practice Research Datalink (CPRD). CPRD is a research service that encompasses primary healthcare data from 60 million UK patients including 16 million currently registered patients. Whilst the results will not be available for this thesis, the approved protocol is provided in **Appendix 4**. In brief the 3 main objectives are:

- 1) To identify whether NSAID use is an independent risk factor for cardiovascular disease we will use a matched case control study to identify predictors of cardiovascular disease (myocardial infarctions and strokes (excluding TIAs) using conditional logistic regression.

- 2) To identify what group of NSAID users are at the highest risk of a cardiovascular event we will identify risk factors for myocardial infarction or stroke in NSAID users using time to event data. We will use cox-regression. We hope this could allow clinicians to risk stratify patients who require longer-term NSAID use.
- 3) To assess if aspirin or statin use as a secondary preventative measure can offset the additional cardiovascular risk associated with NSAID use, again using Cox regression.

Chapter 7: Cardiovascular Biomarker Identification in the SCOT Trial Biobank (SCOT-1)

7.3 Rationale

As discussed in the previous chapter, an in-depth analysis of biomarkers from real world non-steroidal anti-inflammatory drug (NSAID) users who go onto have a cardiovascular event has not been undertaken and would provide novel insight into the mechanisms of NSAID cardiovascular events. In the previous chapter I discussed the decision-making process in carefully designing a nested case control study and in this chapter, I will present my findings of a multi-omics analysis of these serum samples.

7.3.1 Hypothesis

This chapter covers two primary hypotheses. The first is a direct testable hypothesis and the second is hypothesis generating.

Firstly: *‘Serum ADMA/arginine levels are different in NSAID users who go onto have a cardiovascular event compared to those what don’t’*

Secondly, *‘Using a multi-omic approach will identify serum biomarkers that predict cardiovascular events amongst NSAID users.’*

The aims of this chapter were to:

Assess if between NSAID users who go onto have a cardiovascular event, compared to those that do not, there is a difference in serum:

- a) Asymmetric dimethylarginine (ADMA), other methylarginines and amino acids, including arginine using mass spectrometry
- b) Proteins using mass spectrometry
- c) Renal markers measured using immunoassays
- d) Endothelial Inflammatory markers using immunoassays
- e) Thrombosis markers using immunoassays

7.4 Methods

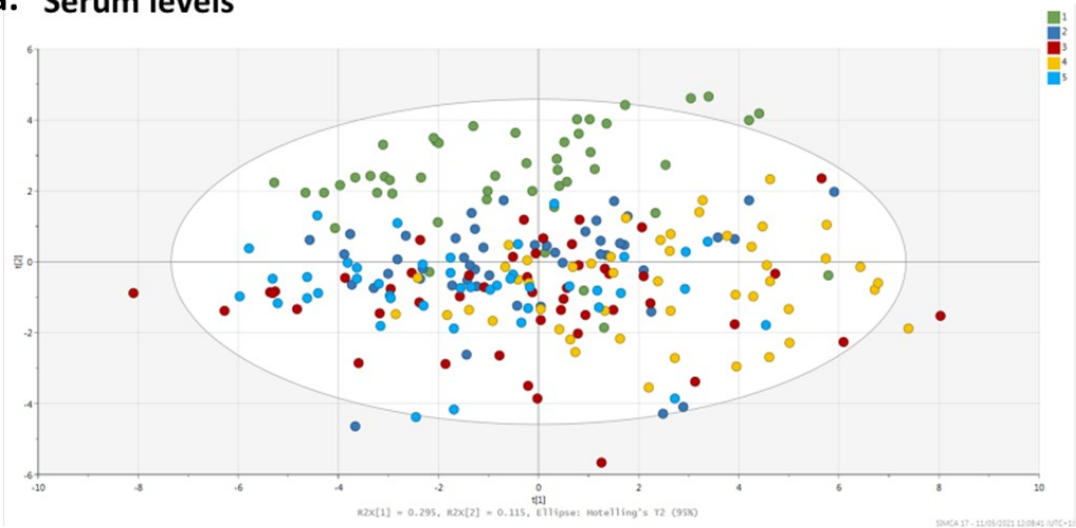
A detailed description of the Methods is provided in the methods section and in **Chapter 6**.

7.5 Results

7.5.1 Amines and methylarginines assessed using Mass spectrometry

Using UHPLC-MS/MS, in total 27 amines and 3 methylarginines were quantified in 235 samples using 1:1 matching (118 events and 117 controls). As discussed in the previous chapter these were batched across 5 plates. As discussed in **Chapter 3**, running a large number of plates introduces the potential for a batch effect. The principal component analysis (PCA) in **Figure 7.1a** shows distinct clustering of samples based on the plate. To correct for this, as in **Chapter 3**, samples were transformed into Z-score for each plate and then the results combined. After converting to Z-score, the samples were no longer clustering according to plate **Figure 7.1b**.

a. Serum levels



b. Z-score

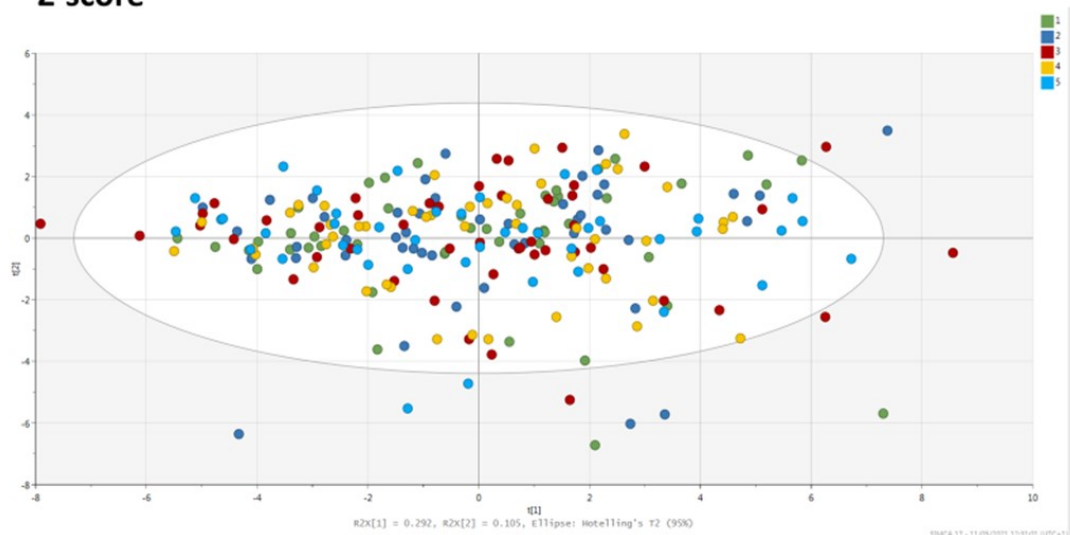


Figure 7.1 Principal Component Analysis Plot (PCA). There is clear batch effect demonstrated by the separation of samples according to plate 1a. Following transformation using Z-score, samples no longer cluster according to plate 1b.

Next the samples in the PCA were labelled according to event/control which showed that there was no real separation between the groups **Figure 7.2**. This was also evident from the loadings plot shown in **Figure 7.3**. The 6 analytes involved in nitric oxide (NO) synthesis (ADMA, symmetric dimethyl arginine (SDMA), NG-Methyl-L-arginine (LNMA), arginine, ornithine and citrulline) grouped together as one would expect in normal physiological conditions.

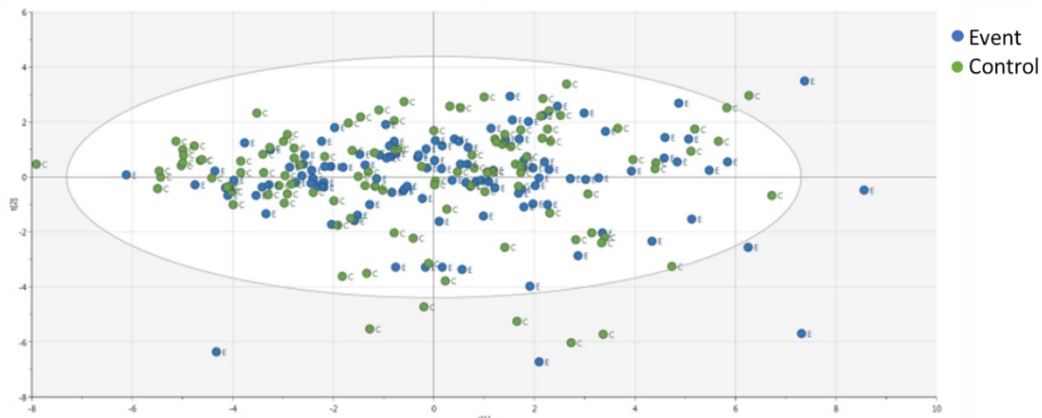


Figure 7.2 Principal Component Analysis Plot (PCA). Samples were labelled according to group. There is no separation between controls and events.

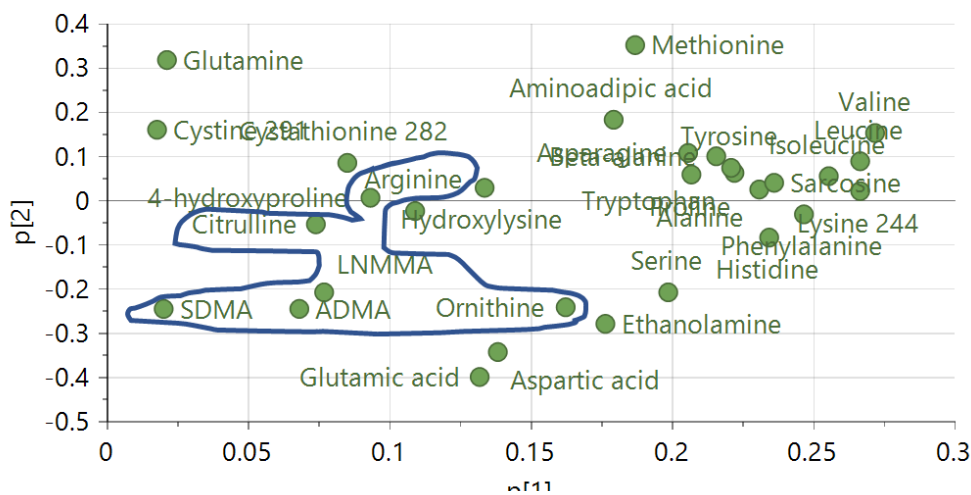


Figure 7.3 Loadings Plot. There are no standout amines that influence the principal components. The Nitric oxide amines and methylarginines cluster together as indicated by the blue line.

Next, I wanted to run a supervised analysis. My primary focus was on ADMA alongside the other 5 NO amines **Figure 7.4**. There was no difference in ADMA, arginine, Arginine/ADMA ratio, SDMA, LNMMA, ornithine or global arginine availability ratio (GABR) (arginine/(ornithine+citrulline)) levels between the groups. Citrulline was significantly higher in the event group although the effect size was small to medium (Cohens D: 0.347). Primary concentration data for these amines and ratios are shown in supplementary **Appendix 6**.

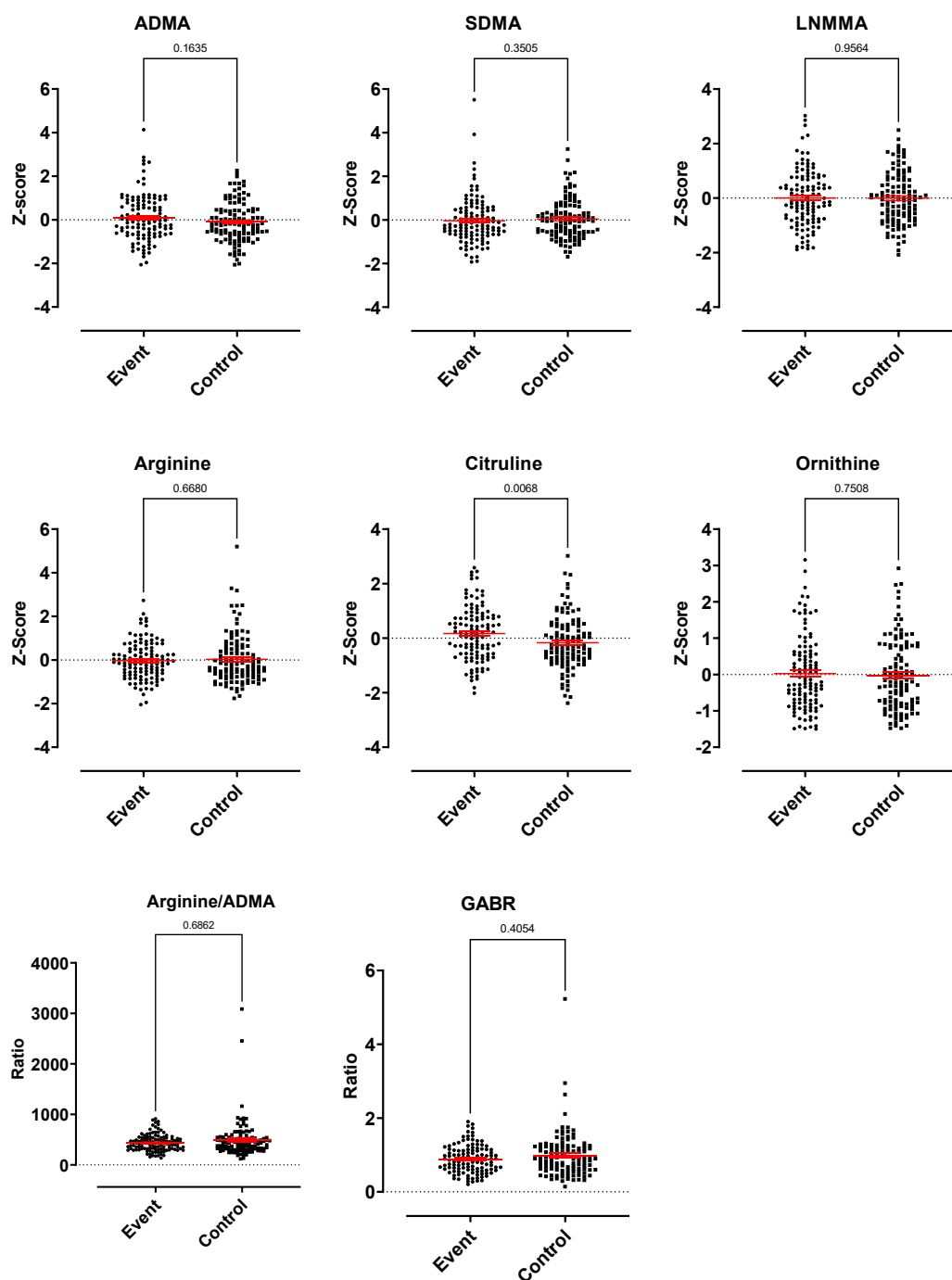


Figure 7.4 Scatter Plot of Nitric Oxide Amines. There was higher citrulline in the event group. There was no significant difference in any of the other analytes. Red bars represent mean $\pm$ Standard error.

I then looked at the differences in the remaining amines. A summary of the p-values and effect sizes is provided in **Figure 7.5**. The mean concentrations; for the individual analytes is provided in **Appendix 6**. Alanine, cystine, histidine, phenylalanine and beta-alanine were significantly higher in the event group with a small to medium effect

size. Given the relatively large sample size and small to medium effect size, these differences could be due to a type 1 error.

Amine	P Value	Effect Size
LNMMA	0.956	0.001
ADMA	0.163	0.203
SDMA	0.35	-0.076
Arginine	0.667	-0.063
Glutamic acid	0.079	0.134
Glutamine	0.76	-0.005
Citrulline	0.007	0.347
Ornithine	0.75	0.050
4-hydroxyproline	0.875	0.079
Alanine	0.007**	0.353
Aspartic acid	0.065	0.214
Asparagine	0.112	0.137
Cystine	0.01	0.360
Ethanolamine	0.063	0.291
Histidine	0.035	0.266
Isoleucine	0.623	0.045
Leucine	0.855	0.024
Lysine	0.607	-0.040
Methionine	0.437	0.084
Phenylalanine	0.024**	0.296
Proline	0.088	0.223
Serine	0.169**	0.180
Tryptophan	0.931**	0.011
Tyrosine	0.3	0.151
Valine	0.776	0.057
Cystathionine	0.717	0.034
Hydroxylysine	0.189	0.097
Aminoadipic acid	0.75	-0.030
Sarcosine	0.004**	0.379
Beta-alanine	0.012	0.308
Arginine/ADMA	0.15	-0.189
Global Arginine Bioavailability	0.6862	-0.194

*Figure 7.5 Table Summarising the difference in amines between the event and control group. Cells highlighted in green indicate a p-value of <0.05. ** Indicates samples that were normally distributed and were analysed using a T-Test. Remaining comparisons made using Mann Whitney U test.*

Next, I wanted to look at the interaction between amines within the dataset. There was a strong correlation between ADMA and SDMA, alanine and beta-alanine and leucine and isoleucine **Figure 7.6**. These are correlations that we would biologically expect to

see as both ADMA and SDMA share some common pathways of synthesis and excretion while alanine is the substrate for beta-alanine and leucine and isoleucine are both formed from pyruvate.

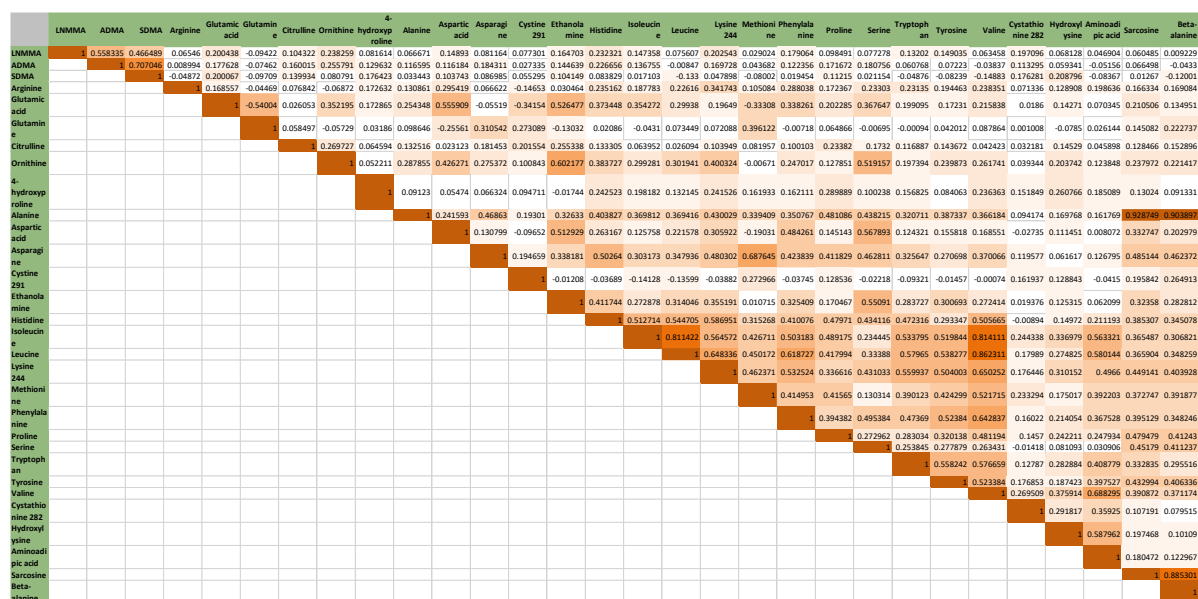


Figure 7.6 Correlation Matrix of Amines. Correlations were assessed using Pearson's correlation efficient. Score of -1 indicates strong negative correlation, 0 indicates no correlation and 1 indicates a strong positive correlation.

As amines interact with each other in discrete and more complex cycles, I wanted to see if a combination of amines could better identify those at a higher cardiovascular risk. To do this I performed regression analysis on the data set. In **Chapter 6**, we used Least Absolute Shrinkage and Selection Operator (LASSO) for candidate variable selection. As some amines are highly correlated (multi co-linearity) in this dataset, LASSO will tend to select a single correlated variable whilst shrinking the others to zero and so is not the best method to use. Instead with the help of Dr Portas (data scientist, Imperial College) we used ElasticNet regression. ElasticNet, like LASSO and ridge regression looks at the sum of the squared residuals but uses a combination of the LASSO and ridge regression penalty. This makes it better at handling datasets with multi co-linearity compared to LASSO or RIDGE alone.

ElasticNet identified 7 candidate variables for selection: citrulline, alanine, cystine, ethanolamine, histidine, phenylalanine and sarcosine. This analysis identified similar candidates to the significant amines identified in the supervised analysis in **Figure 7.5**. I then performed binary logistic regression using these candidate variables. Cystine

remained the only variable that was associated with an increased odds of having a cardiovascular event. There was a trend towards increased odds of a cardiovascular event in those with higher levels of citrulline although this was not statistically significant **Figure 7.7**. Overall the model was a very poor fit as indicated by the model summary This suggests that overall, amines, including methylarginines are poor indicators of cardiovascular events in this cohort.

	Beta	Standard error	Wald	df	Sig.	Odds Ratio (OR)	95% CI for OR	
							Lower	Upper
Citrulline	.261	.151	2.974	1	.085	1.298	.965	1.745
Alanine	.047	.384	.015	1	.902	1.048	.494	2.223
Cystine	.317	.153	4.282	1	.039	1.373	1.017	1.854
Ethanolamine	.095	.163	.337	1	.561	1.099	.798	1.514
Histidine	.156	.171	.833	1	.361	1.169	.836	1.635
Phenylalanine	.162	.163	.985	1	.321	1.175	.854	1.617
Sarcosine	.145	.386	.140	1	.708	1.156	.542	2.463
Constant	.057	.138	.172	1	.679	1.059		

Model Summary			
Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	297.571 ^a	.088	.117

a. Estimation terminated at iteration number 3 because parameter estimates changed by less than .001.

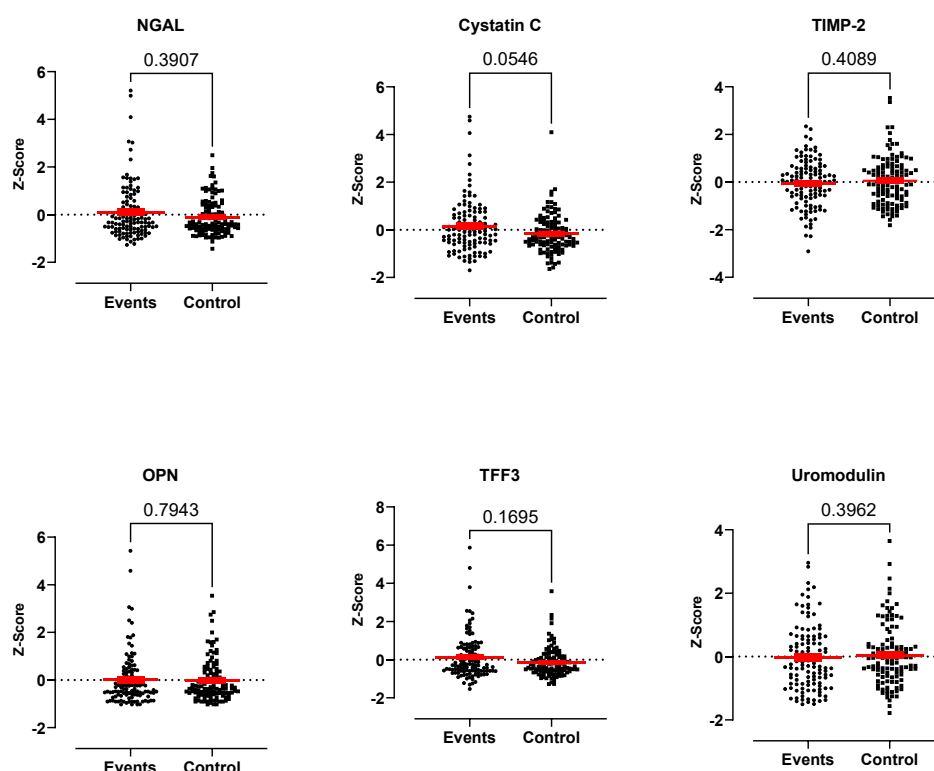
Figure 7.7 Amines associated with an increased odds of a cardiovascular event using binary logistic regression. Cystine was the only variable that was associated with an increased odds of having a cardiovascular event. The model summary indicates a poor overall fit.

As discussed in **Chapter 3** the kidney plays a key role in cardiovascular homeostasis and there is evidence to suggest that COX-2 in the kidney is cardioprotective. Furthermore, cardiovascular events are also often linked to increases thrombotic tone. We therefore measured a panel of renal and thrombosis markers using the LegendPlex assay (See **Methods** for full details)

7.5.2 Renal and Thrombosis Markers Measured Using Multiplex.

7.5.2.1 Renal Markers

Six renal markers were quantified (NGAL, cystatin C, TIMP-2, OPN, TFF3, Uromodulin) using 1:1 matching (118 events and 117 controls). Following quality control, 230 samples (117 events and 113 controls) were included in the final analysis (**See Methods**). Samples were batched across 3 plates and a standard curve was generated for each plate (**See Methods**). As previously discussed, data was transformed into Z-score with non-normalised concentration data shown in **Appendix 6**. There was a strong trend ($p=0.0546$) towards a modest increase in cystatin C in the event group, in line with renal function playing a role in cardiovascular risk in NSAID users as well as in the wider population. None of the other markers were significantly different between the groups. **Figure 7.8**.



Analyte	Effect Size
NGAL	0.219
Cystatin C	0.307
TIMP-2	-0.109
OPN	0.033
TFF3	0.283
Uromodulin	-0.091

Figure 7.8 Scatter plot showing differences in renal markers between event and control group. There is a trend towards increased cystatin C in the Events group. No difference in other markers. Mann Whitney U test was used for all comparisons except TIMP-2 which was analysed using a t-test.

7.5.2.2 Thrombosis Markers

Seven thrombosis markers were quantified (P-selectin, D-Dimer, PSGL-1, tPA, sCD40L, PAI-1 & Factor IX) USING 1:1 matching (118 events and 117 controls). Samples were batched across 3 plates and a standard curve was generated for each plate (**See methods**). Data was transformed into Z-Score **Figure 7.9** with non-normalised concentration data shown in **Appendix 6**. There was significantly higher P-selectin levels in the event group although the effect size was small. P-selectin is important marker of endothelial dysfunction and platelet activation. This observation is in line with the idea that peripheral blood P-selectin levels are increased in patients at risk of cardiovascular events and provides novel insights showing that this is the case in NSAID-user populations[253].

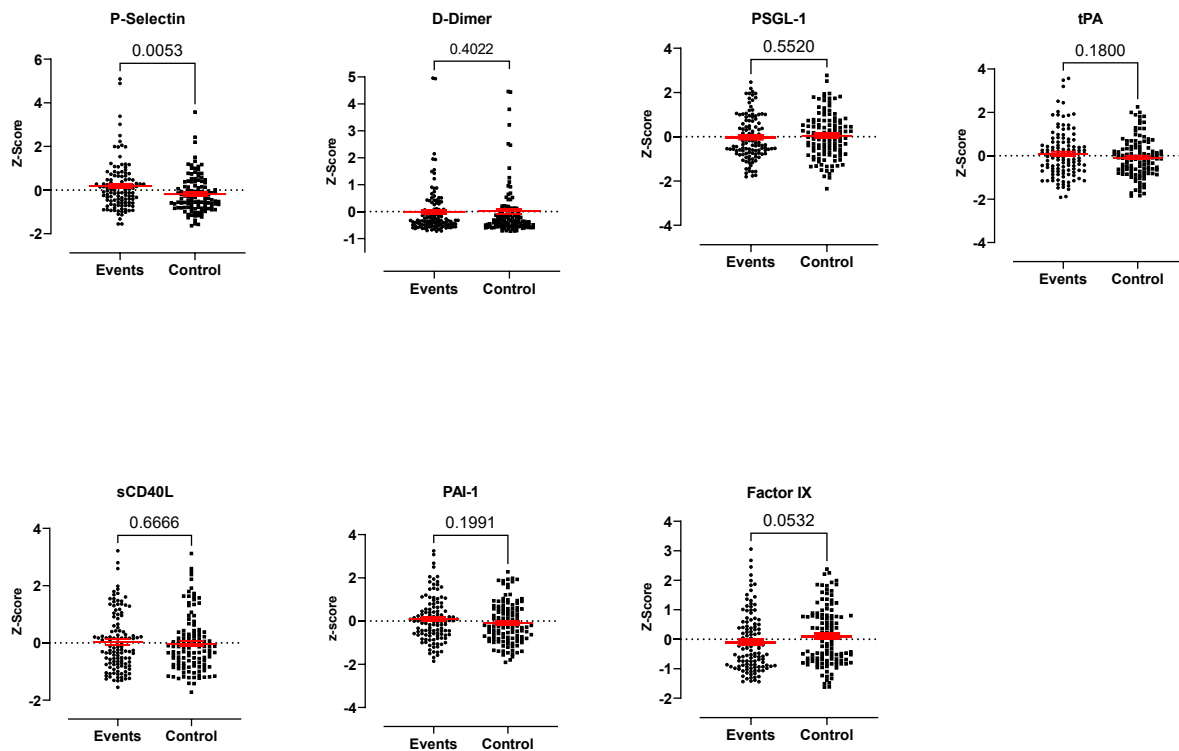


Figure 7.9 Scatter plot showing differences in thrombosis markers between event and control group. There is significantly lower P-selectin in the Events group and a trend towards lower Factor IX in the Events group. No difference in other markers. Mann Whitney U test was used for all comparisons except PSGL-1, t-PA and PAI-1 which was analysed using a t-test.

Analyte	Effect Size
P-selectin	0.371
D-Dimer	-0.021
PSGL-1	-0.078
tPA	0.175
sCD40L	0.067
PAI-1	0.168
Factor IX	-0.206

There was a strong trend ($p=0.0532$) towards lower levels of Factor IX in the event group although this did not reach statistical significance. There was no difference in any of the other markers **Figure 7.9**. Whilst these findings are novel, interesting and relevant to the scientific community, it should be noted that due to the small effect size they could be due to a type 1 error.

7.5.3 Analytes measured using ELISA

We have previously used a specific ADMA/L-arginine ELISA to confirm results obtained using MS. In this study therefore, SCOT samples were separately assayed for ADMA and L-arginine using ELISA. In addition, key vascular mediators associated with cardiovascular disease, specifically interleukin-6 (IL-6), Monocyte chemoattractant protein-1 (MCP-1) and endothelin-1 (ET-1) were measured using single analyte ELISAs. Details on the methods and standard curves are provided in the **Chapter 2 Methods**.

7.5.3.1 ADMA and Arginine

As was seen using MS, there was no difference in ADMA, arginine or ADMA/Arginine ratio measured using ELISA between the groups. **Figure 7.10**.

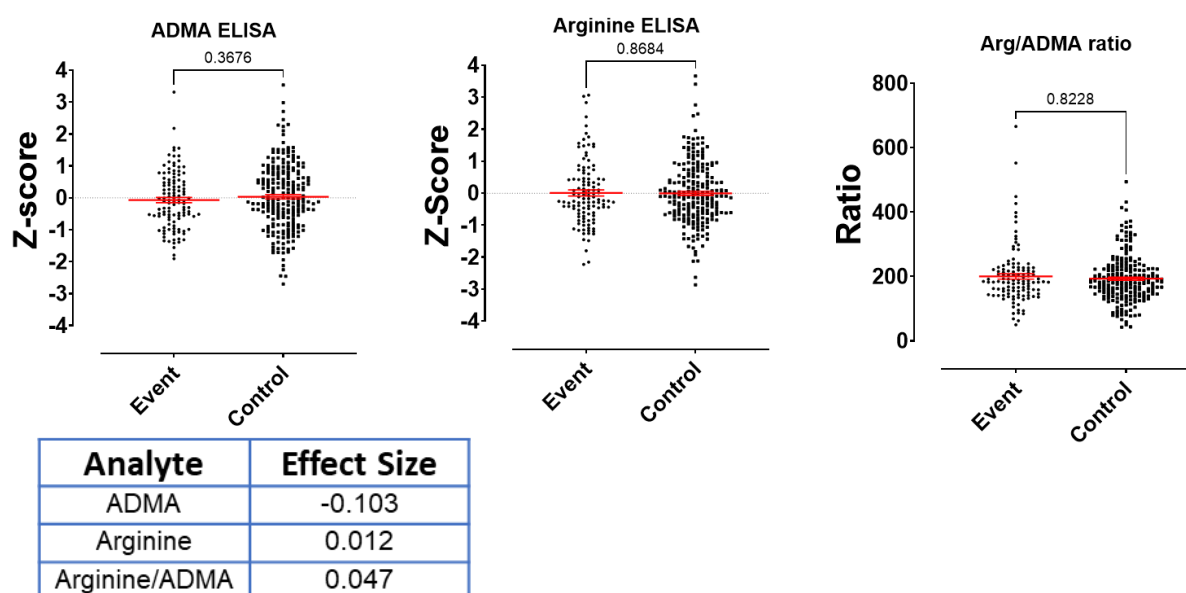


Figure 7.10 Differences in ADMA and Arginine between groups assessed using ELISA. No differences in ADMA, arginine or Arginine/ADMA ratio between the groups. ADMA was analysed using a T Test and the remaining analytes were assessed using Mann Whitney U test. ADMA n: 117 events, 228 controls. Arginine: 117 events 219 controls, Arg/ADMA: 116 events, 217 controls.

7.5.3.2 ET-1

ET-1 is a potent endogenous vasoconstrictor that is produced by endothelial cells and is implicated in the pathogenesis of endothelial dysfunction. It acts as a natural counterpart to the vasodilator NO and so could mechanistically be implicated in NSAID induced cardiovascular events. There was no difference in ET-1 levels between the groups. **Figure 7.11a.**

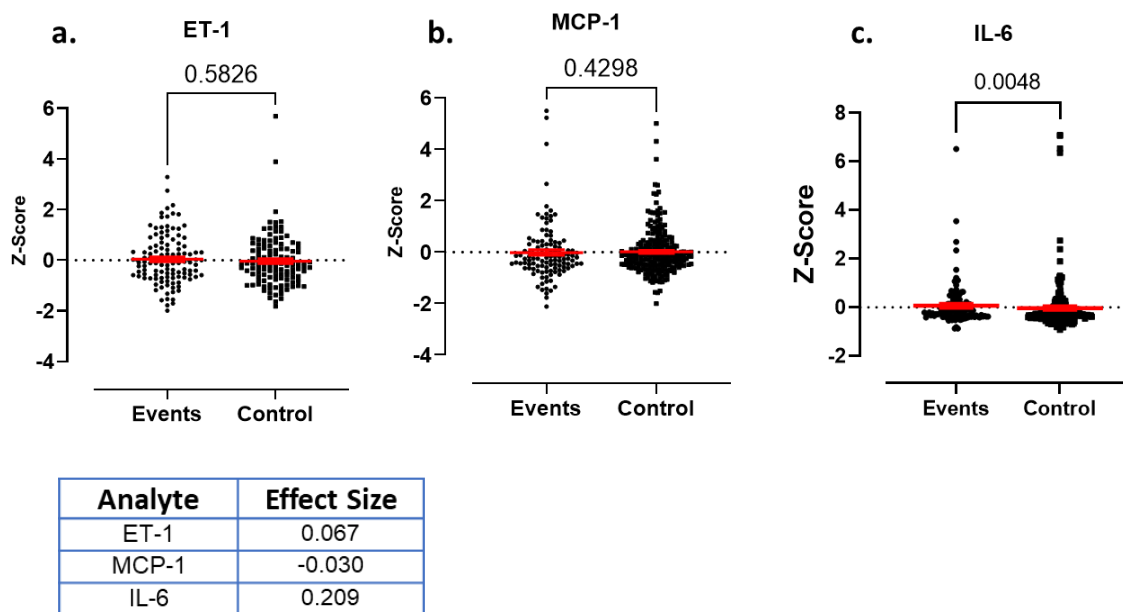


Figure 7.11 11 Differences in (a) ET-1, (b) IL-6 and (c) MCP-1 between groups assessed using ELISA. Significant increase in IL-6 in the event group. No differences in MCP-1, or ET-1. ET-1 was analysed using a T-test and the remainder using Mann Whitney U test. IL-6 and MCP-1 n: 116 events, 227 controls. ET-1 n: 116 events and 117 controls.

7.5.3.3 IL-6 and MCP-1

IL-6 and MCP-1 are key cytokines associated with inflammation and atherosclerosis. Furthermore, whilst NSAIDs are anti-inflammatory, their use can prolong the resolution of inflammation and increase release of some cytokines in vitro vascular cell models[254-256]. In total 116 events and 227 controls were analysed across 5 plates. There was no difference in MCP-1 between the groups **Figure 7.11b**. However, there were significantly higher IL-6 levels in the event group **Figure 7.11c**. It should be noted that one sample in the control group fell outside the top standard on the IL-6 ELISA (>500pg/ml) and was a clear outlier based on a Grubbs test. Moreover, this value is not biologically plausible for someone enrolling in this type of study and would only be

seen in someone with extreme sepsis in intensive care who would be unlikely to survive the hospital admission[257]. The outlier was therefore removed from the analysis.

Whilst increased IL-6 has been associated with an increased risk of cardiovascular disease in epidemiological studies [258], this finding needs to be interpreted cautiously due to the following reasons:

A) The lowest standard in the ELISA kit is 7.8pg/ml and as we expected values to fall on the lower end of the standard, two further serial dilutions were made to extrapolate the curve. (See **Methods**). The median IL-6 was around 7pg/ml in both groups and therefore below the lowest recommended kit standard. Whilst I had extrapolated the curve, the linearity of the standard at these lower concentrations was not good and so should be interpreted cautiously (See **Methods**).

B) The effect size is small and could be due to a type 1 error

Nevertheless, this could mechanistically be an interesting finding and potentially important and are in line with the idea that, through the inhibition of anti-inflammatory functions of COX-2, longer term NSAID use could create a pro-inflammatory state that could predispose individuals to cardiovascular events. To test this we measured a panel of Inflammatory cytokines in a mouse model from samples previously generated by the group. Wild type mice were given a Proprotein convertase subtilisin/kexin type 9 (PCSK9) recombinant adeno-associated viral vector and fed a high fat diet creating an atherosclerotic model without germline genetic engineering. They were given either parecoxib or control for 17 weeks. In essence this extreme model would mimic longer term NSAID use in the presence of cardiovascular insult (atherosclerosis). A panel of 12 cytokines was measured using MultiPlex from plasma samples taken at 17 weeks. There were 15 mice in each group.

There was a significantly higher level of IL-17A and a trend towards higher IL-27 and interferon gamma in the parecoxib group **Figure 7.12** all of which have been implicated in the pathogenesis of atherosclerosis and cardiovascular disease [236, 259, 260]. Whilst these results may support the hypothesis of a pro-inflammatory state with longer term NSAID use, this is an extreme animal model and so subtle differences in cytokine profile following NSAID use may not be detectable using this model.

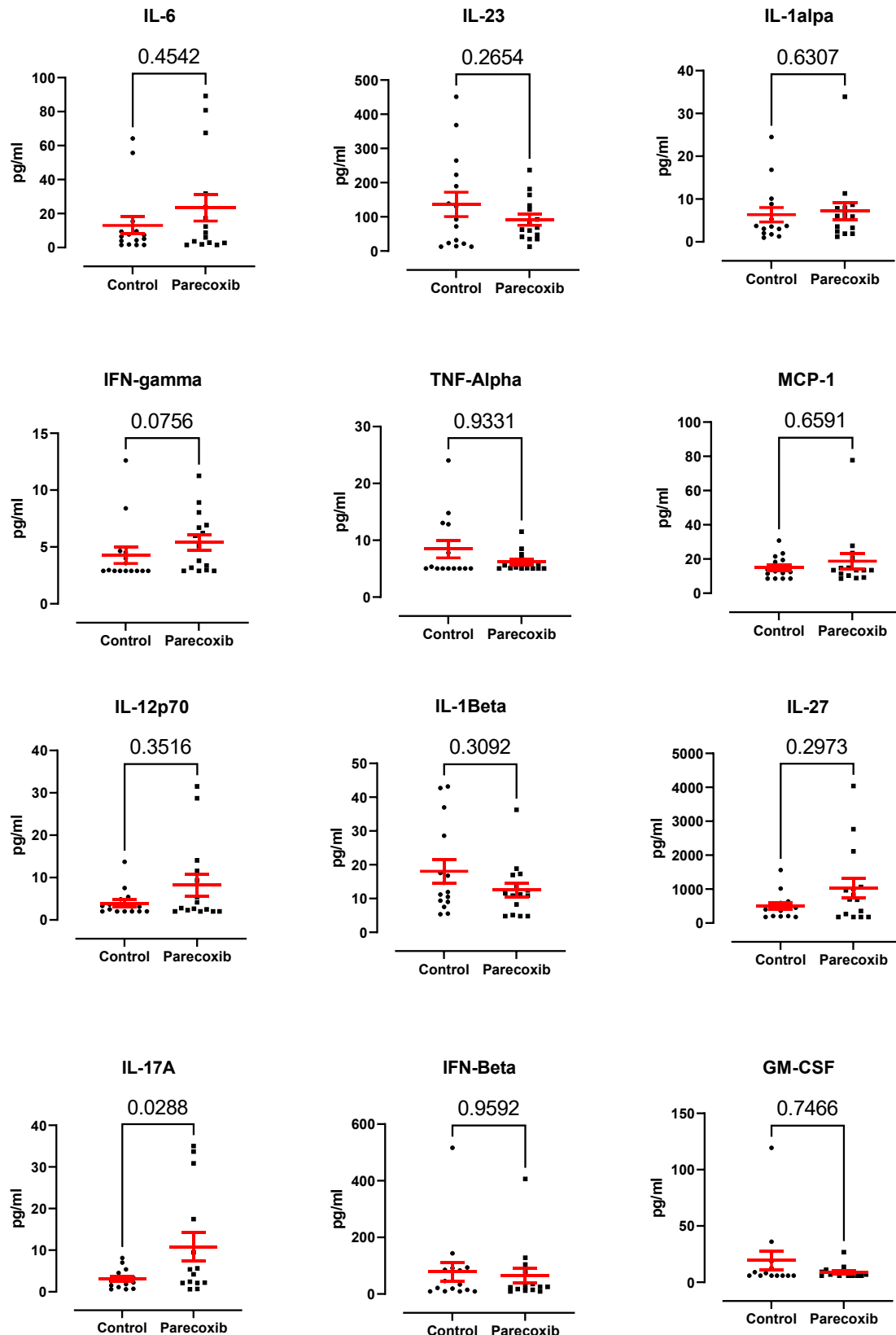


Figure 7.12 . Scatterplot of differences in cytokine levels between parecoxib treated and control mice in an atherosclerotic model. There was significantly higher IL-17A and a trend towards higher IL-27 and interferon gamma in the parecoxib treated group. IL7-A & IL-23 were assessed using a T-Test. All other analytes were assessed using a Mann Whitney U test.

7.5.4 Proteomics using MS SWATH

Proteomics was performed and analysed by the Manchester Biomarker Discovery centre. Details of Methodology are provided in **Chapter 2**.

In total 235 samples (117 controls and 118 events) were submitted for analysis. Five samples (2 controls and 3 events) were excluded due to haemolysis and a further 2 samples (1 from each group) failed quality checks after repeat measurement. A total of 228 samples (114 in each group) were included in the analysis.

In total 416 proteins were quantified in at least 30% of the samples and were included in the PCA which showed no separation between the event and control group **Figure 7.13**. There were 28 proteins that were differentially expressed between the event and control group although no proteins remained significant after false discovery correction ($Q < 0.05$) **Figure 7.14**.

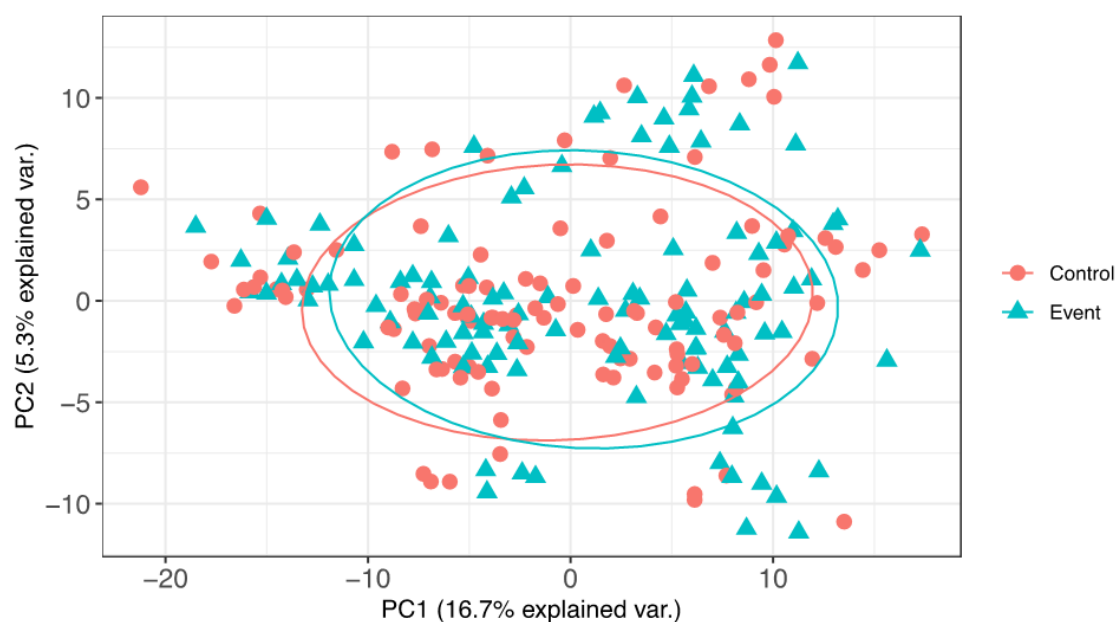


Figure 7.13 Principal Component Analysis plot. Samples were labelled according to group. There is no separation between events and controls.

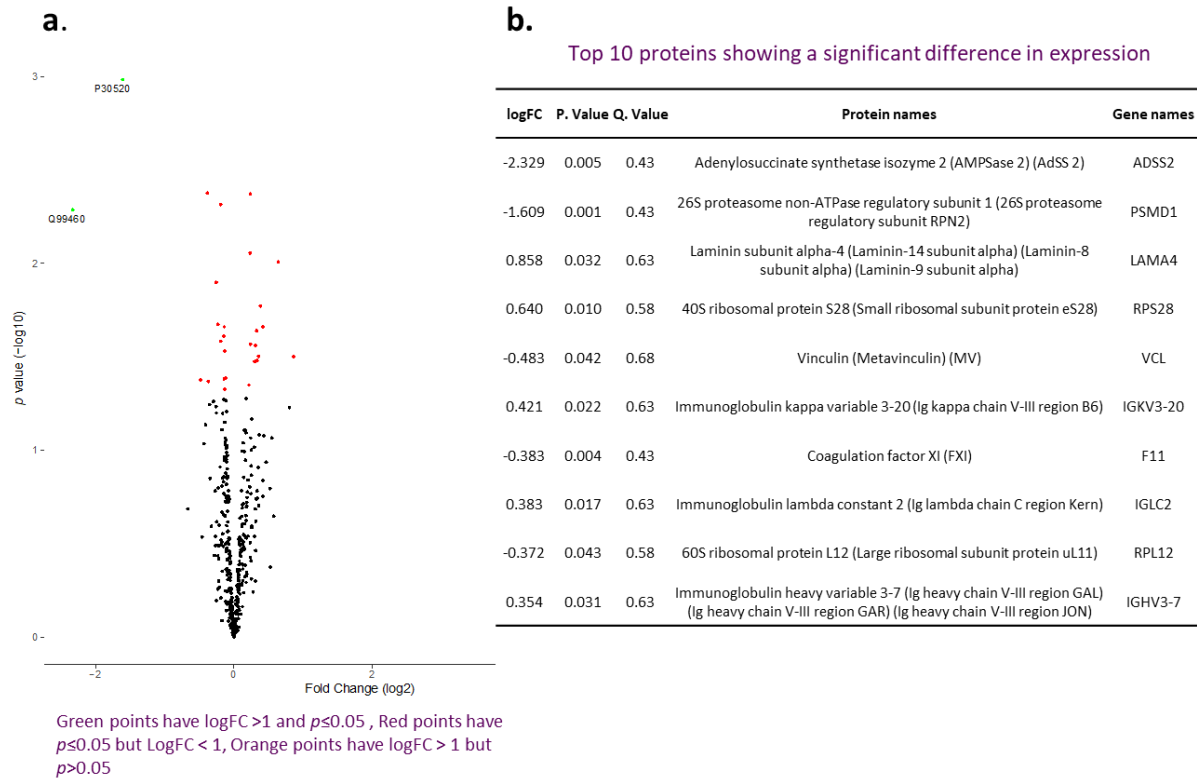


Figure 7.14 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups. In total, 28 proteins were significantly different between the groups.

On further inspection, there was evidence of a sizeable batch effect as samples were clustering according to the instrument the samples were run on; each mass spectrometry machine was given a name as depicted in **Figure 7.15**.

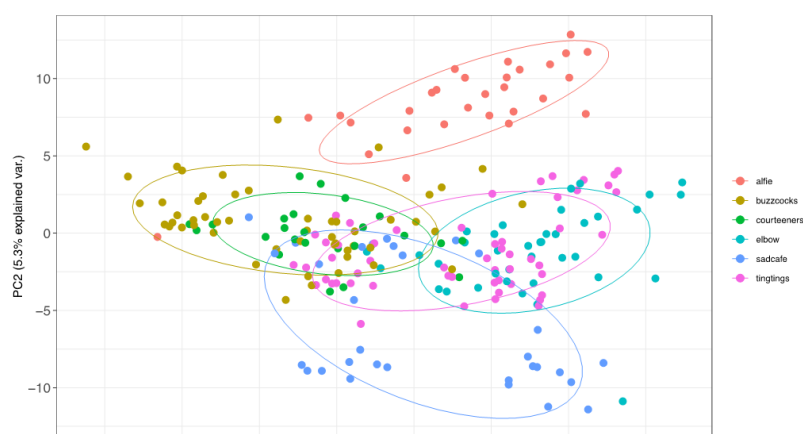


Figure 7.15 Principal Component Analysis plot. Samples were labelled according to the mass spec instrument they were ran on. There is a batch effect as evidenced by separation on the PCA.

Data was reanalysed sequentially removing results from the key outlier device (Alfie) of the six MS machines used for the analysis **Figures 7.16ab**.

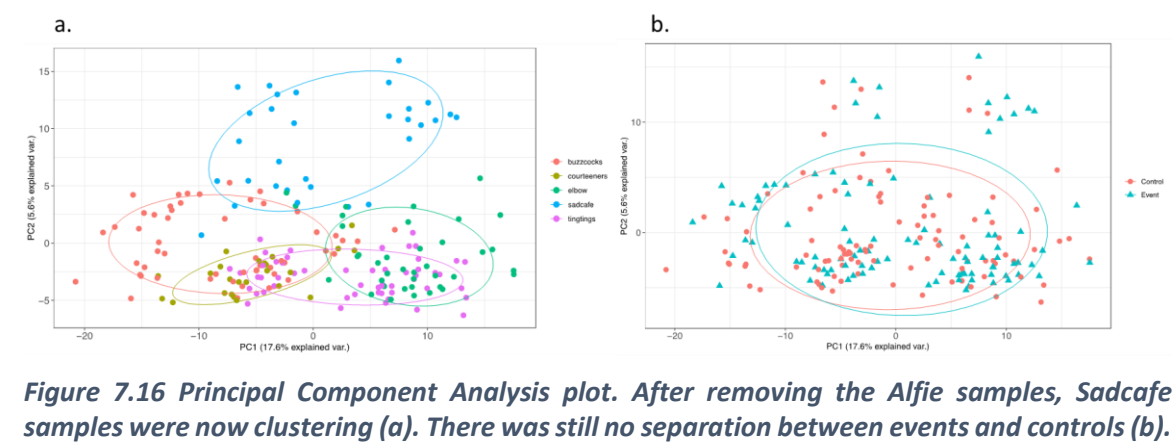


Figure 7.16 Principal Component Analysis plot. After removing the Alfie samples, Sadcave samples were now clustering (a). There was still no separation between events and controls (b).

After this step, while there was still no separation on PCA between events and controls (Figure 7.16b) 26 proteins were differentially expressed between the control and event group but again no proteins remained significant after false discovery correction (Figure 7.17).

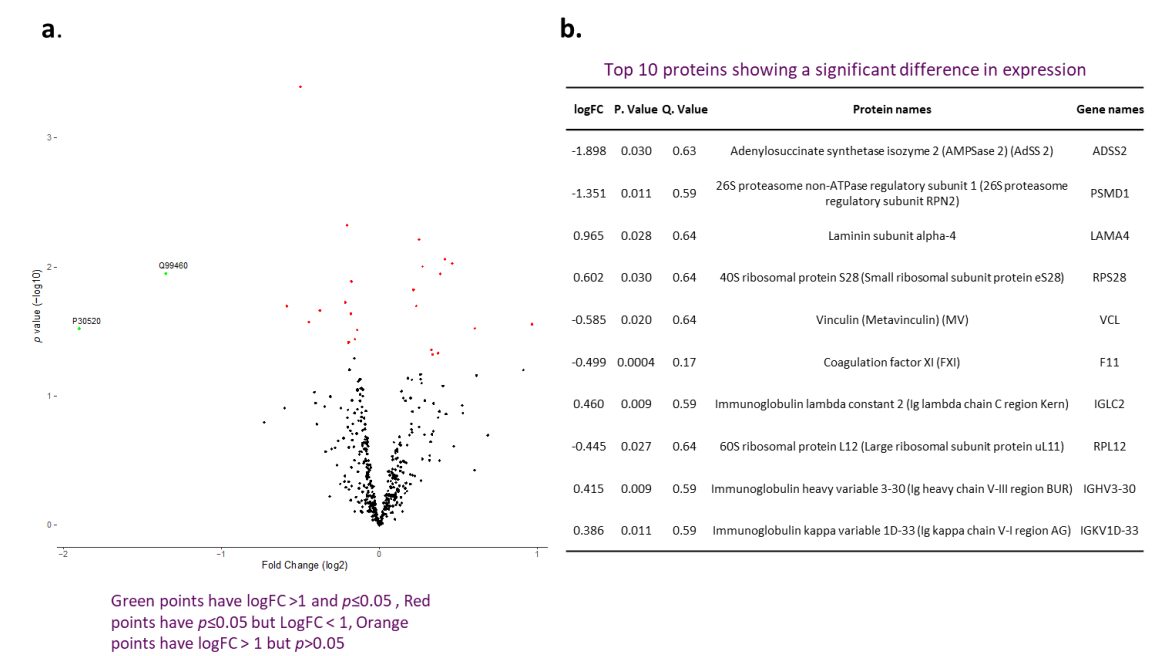


Figure 7.17 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after removing the Alfie instrument. In total, 26 proteins were significantly different between the groups.

Next data was removed from the second key outlier (Sadcafe) of the remaining five MS machines used for the analysis. This step did not improve the instrument clustering on PCA **Figure 7.18a** and again there was no real separation between events and controls **Figure 7.18b** although there were 20 proteins that were differentially expressed between the control and event group but again no proteins remained significant after false discovery correction **Figure 7.19**.

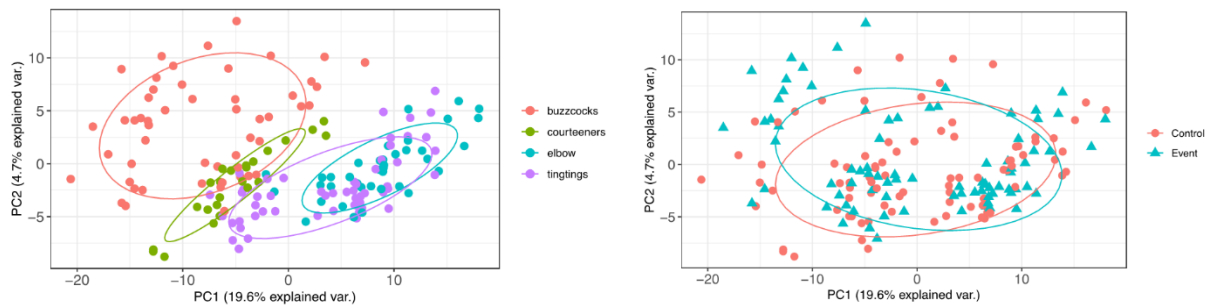


Figure 7.18 Principal Component Analysis plot after removing the Alfie and SadCafe samples. There was still clustering according to instrument(a). There was still no separation between events and controls (b).

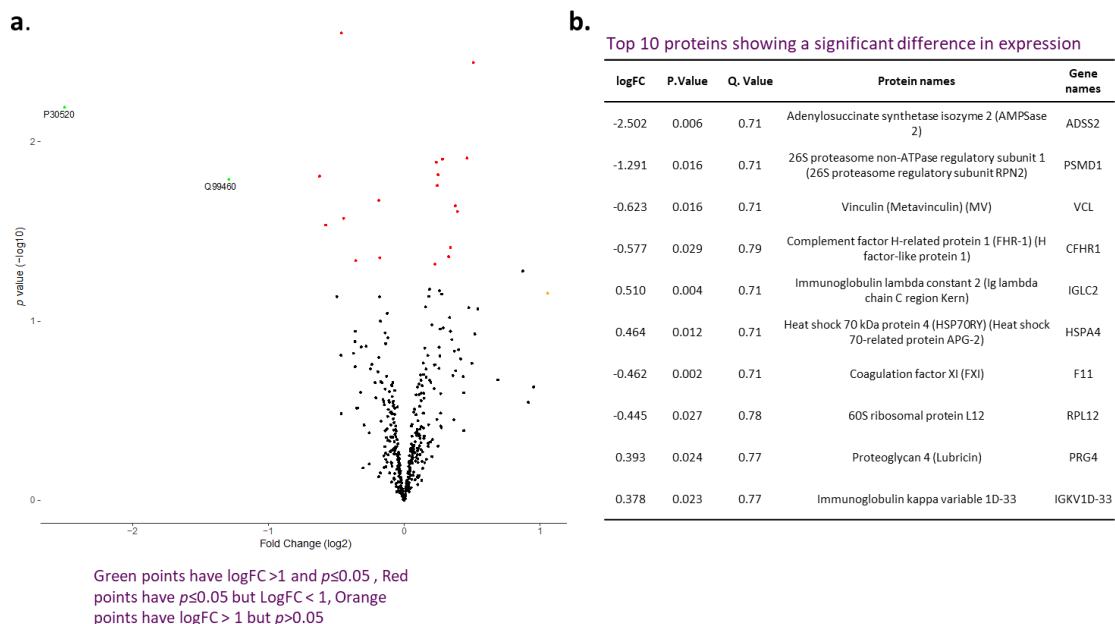


Figure 7.19 Figure 7.19. Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after removing the Alfie and Sadcafe instruments. In total, 20 proteins were significantly different between the groups.

Finally an attempt to correct for batch effects the full data set was reanalysed at the transition-level quantification in R using proBatch with quantile normalisation and per-feature median centering. Following correction, samples on PCA still appeared to cluster according to the instrument **Figure 7.20a** and again while there was no real separation on PCA between events and controls **Figure 7.20b** 26 proteins were differentially expressed between the control and event group. No proteins remained significant after false discovery correction **Figure 7.21**.

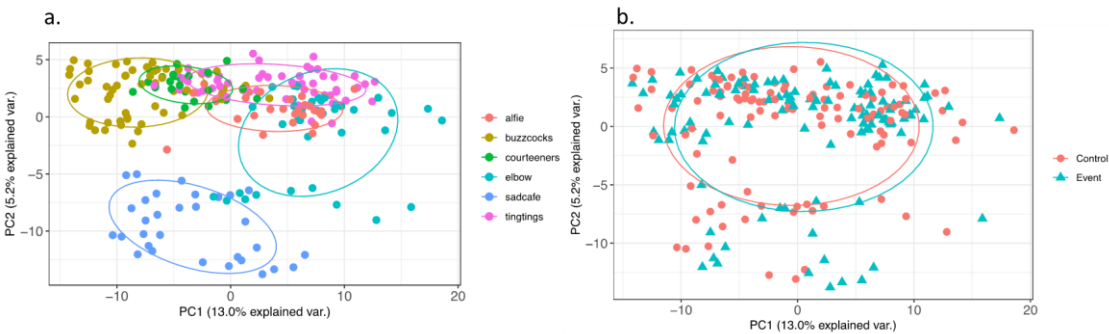


Figure 7.20 Principal Component Analysis plot after correcting for batch effect. The Alfie samples were more consistent with other instruments. The SadCafe samples were still separate (a). There was still no separation between events and controls (b).

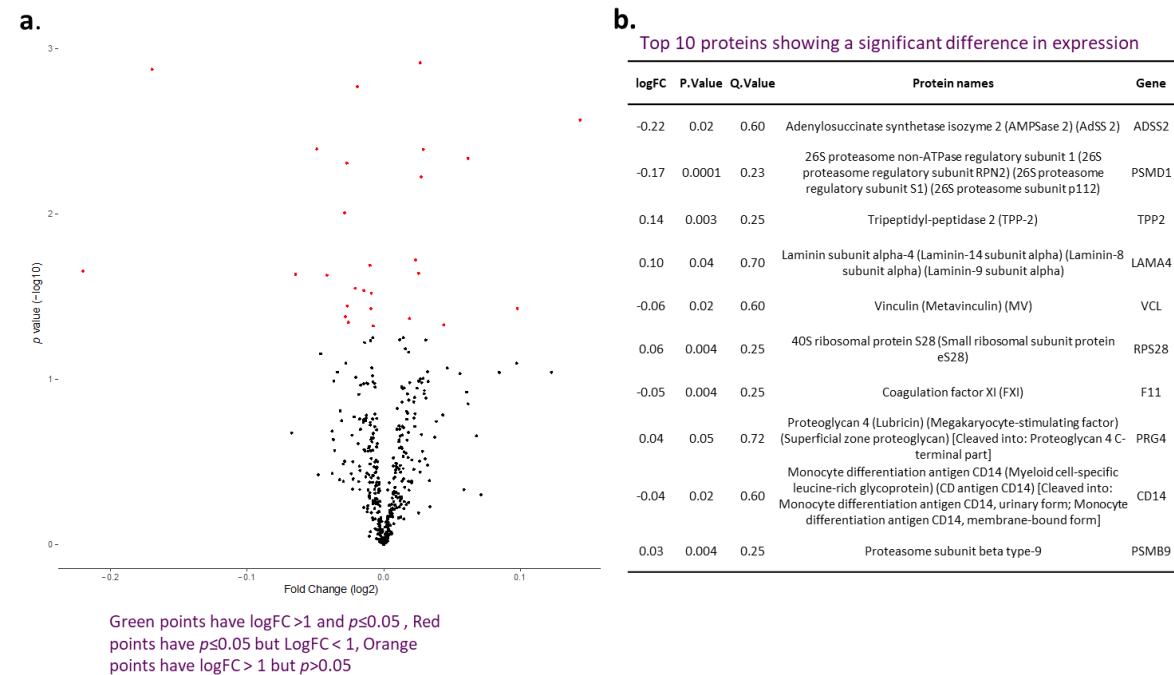


Figure 7.21 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after correcting for batch effect. In total, 26 proteins were significantly different between the groups.

As the SadCafe instrument was still separate on PCA **Figure 7.20a**, a final sensitivity analysis was performed with the removal of the SadCafe samples which slightly

improved the separation according to instrument **Figure 7.22a**. However, samples still did not separate

according to group **Figure 7.22b** but there were 21 proteins that were differentially expressed between the control and event group but again no proteins remained significant after false discovery correction **Figure 7.23**.

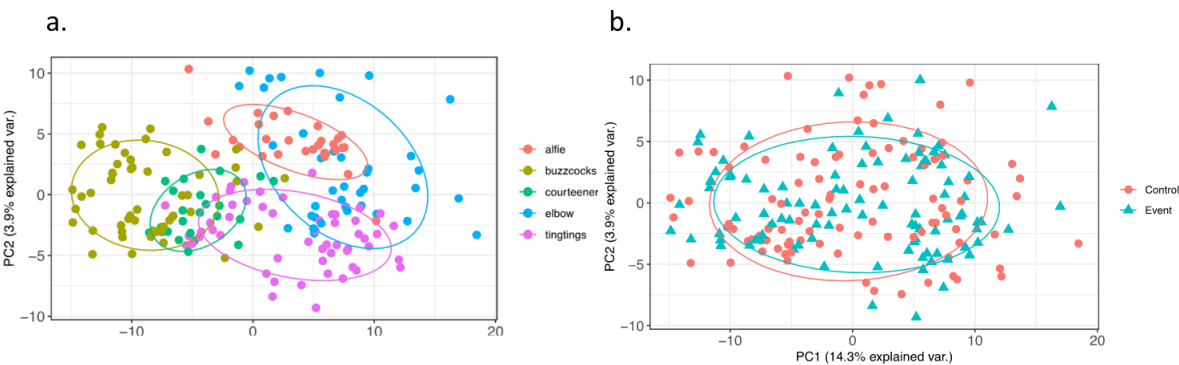


Figure 7.22 Principal Component Analysis plot after correcting for batch effect and removing the SadCafe instrument. There is still clustering according to instrument (a). There was still no separation between events and controls (b).

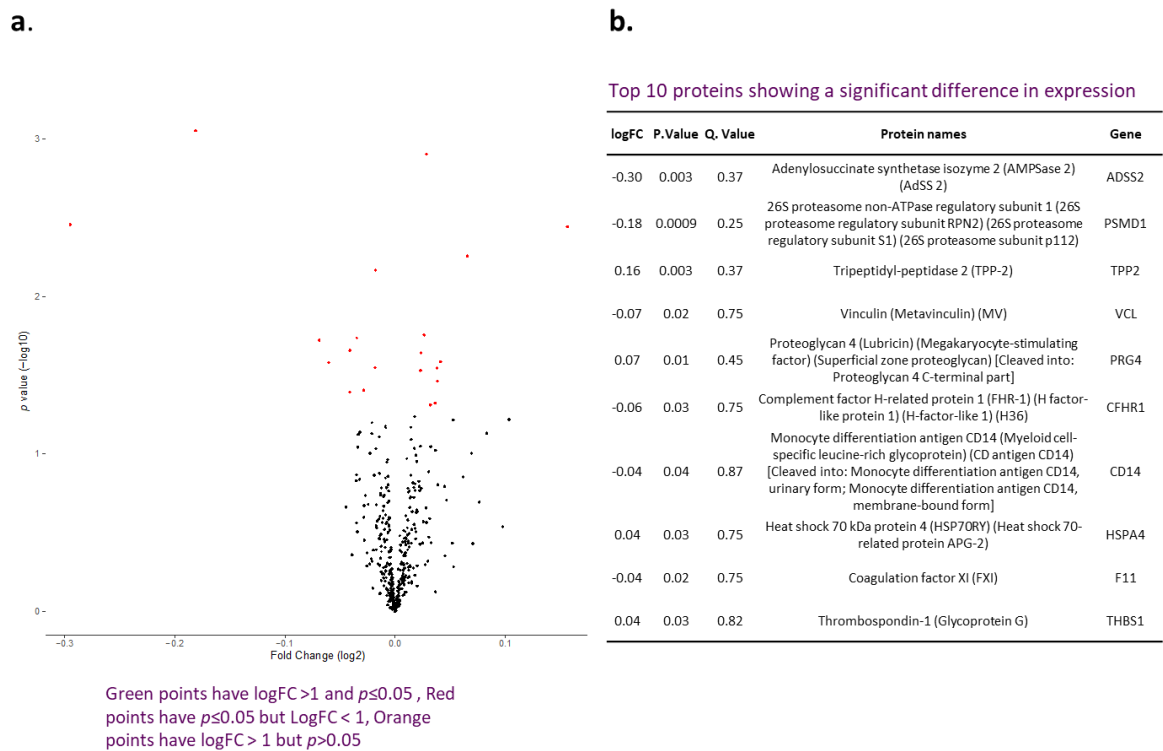


Figure 7.23 Volcano plot (a) and table (b) showing the top proteins that are significantly different between groups after correcting for batch effect & removing SadCafe instrument. In total, 21 proteins were significantly different between the groups.

It was evident that using this method, less than 5% of total proteins quantified were different between the event and control groups. There were 7 proteins that were consistently significantly different ($p < 0.05$) regardless of analytical approach:

Protein Names	Genes
Adenylosuccinate synthetase isozyme 2 (AMPSase 2)	ADSS2 ADSS
Acylamino-acid-releasing enzyme (AARE)	APEH D3F15S2 D3S48E DNF15S2
Coagulation factor XI (FXI)	F11
Glutathione peroxidase 3 (GPx-3) (GSHPx-3)	GPX3 GPXP
Prohibitin	PHB PHB1
Vinculin (Metavinculin) (MV)	VCL
Exportin-1 (Exp1) (Chromosome region maintenance 1 protein homolog)	XPO1 CRM1

7.5.5 Subgroup Analysis of Early Events

In our main analysis of all samples, we found modest changes in a restricted number of biomarkers in events compared to control. However, the SCOT study design involved blood samples taken **only** at screening with a median follow up of 3 years with a maximum follow up of 6.3 years. We therefore hypothesised that biochemical differences between the groups would be more likely seen closer to the event as opposed to 3-5 years later. We therefore decided to perform an exploratory subgroup analysis with a focus on the early events.

In the first Instance data was split into 2 subgroups, (i) those that had an event within 6 months vs all non-events and (ii) those that had an event within 1 year vs all non-events. For the purpose of this, anyone that did not have an event within the specified time point was considered a control even if they went onto have an event at a later point. This minimises selection bias.

First, potential differences in NO amines between the groups were considered. Interestingly at 6 months, there was a trend towards an increase in ADMA in the event group ($p = 0.079$). There were no other amines or methylarginines that were different between the groups **Figure 7.24**.

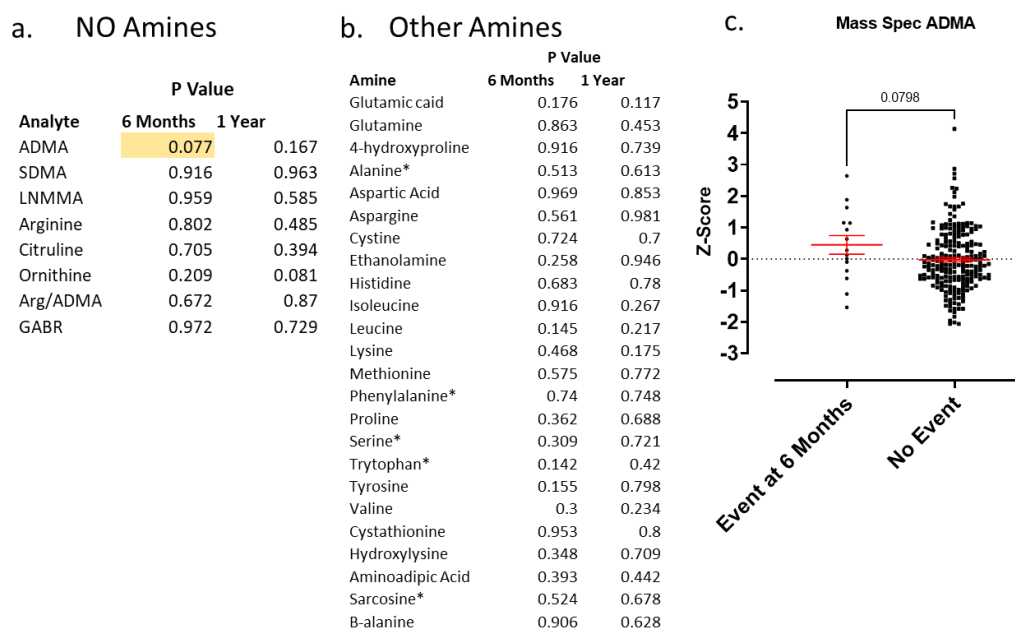


Figure 7.24 Subgroup analysis assessing differences in amines in earlier events vs controls. There was a trend towards an increase in ADMA in the 6-month group (a) and no difference in any other amines (b). (c) is a scatterplot showing trend towards an increase in ADMA in the early event group. *Indicates samples analysed using a T-test, all other samples analysed using Mann Whitney U test.

To further explore this we next looked at the non-cumulative events across time **Figure 7.25**. The ADMA levels appeared to be higher closer to the event and appeared to reduce over time.

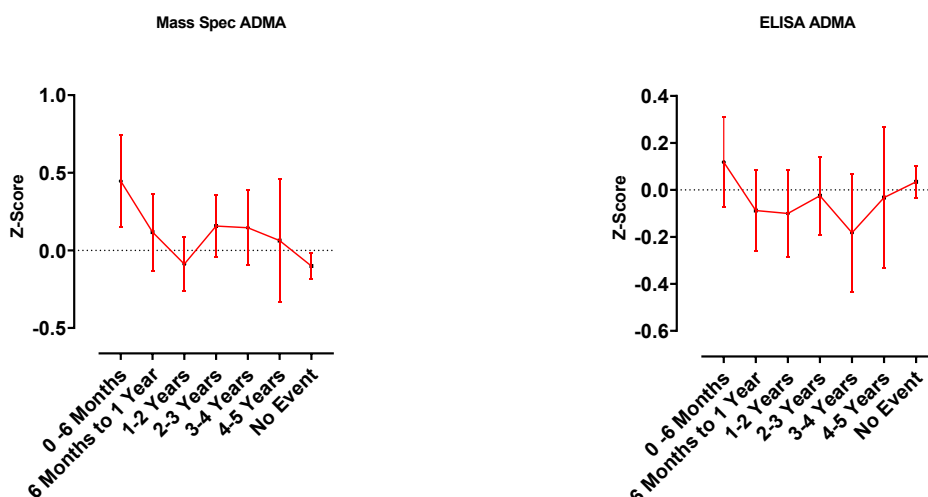


Figure 7.25 Graph showing ADMA levels as non-cumulative events over time using Mass spectrometry (a) and ELISA (b). ADMA levels appear to be higher closer to the time of the event and reduce over time.

Next, the other analytes at these earlier time points were reassessed to look for any trends in the data (**Figure 7.26a**). Interestingly, there was higher cystatin C and lower uromodulin in those that had an event at 6 months (**Figures 7.26bc**) indicative of impaired renal function in the events group.

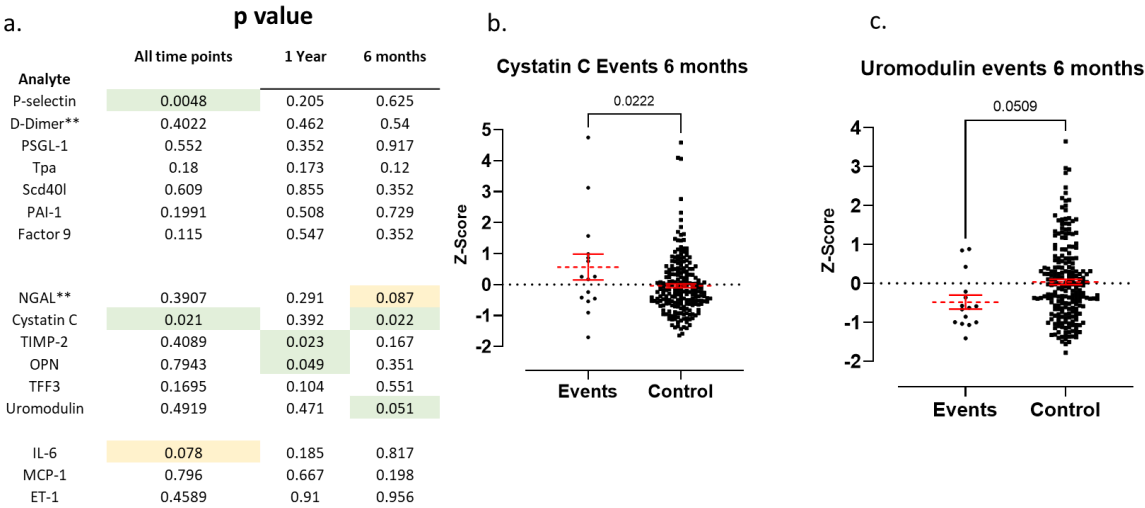


Figure 7.26 Subgroup analysis assessing differences in analytes in earlier events vs controls. There were differences in renal markers in the 6 month and 1 year event groups (a). Scatterplots show an increase in Cystatin C (b.) and a reduction in Uromodulin (c). **indicates samples analysed using a T-test, all other samples analysed using Mann Whitney U test.

A similar subgroup analysis of the proteomic data without correction for batch effect or removal of any machines revealed some interesting leads. PCA showed some clustering of those who had an event within 6 months (**Figure 7.27**). There were 88 proteins that were differentially expressed between the groups. There were 7 proteins that remained significant after FDR correction ($Q < 0.05$). Cystatin C was the top protein that was increased in the event group (**Figure 7.28**) substantiating the results obtained with Biologend MultiPlex shown **Figure 7.26**.

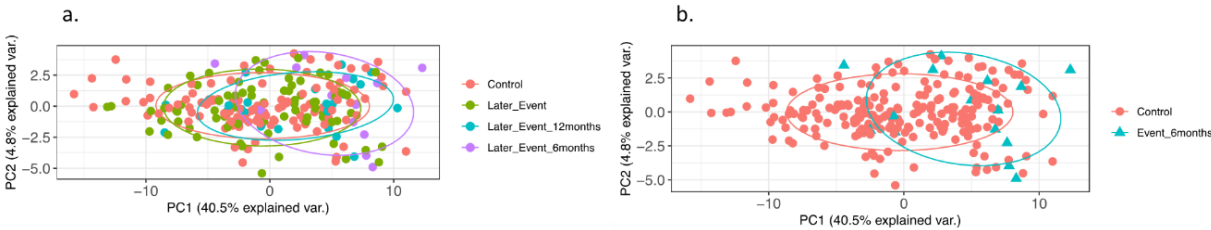


Figure 7.27 Principal Component Analysis plot. There appears to be some separation in the samples with earlier events (a). The separation was more evident in the 6-month group (b).

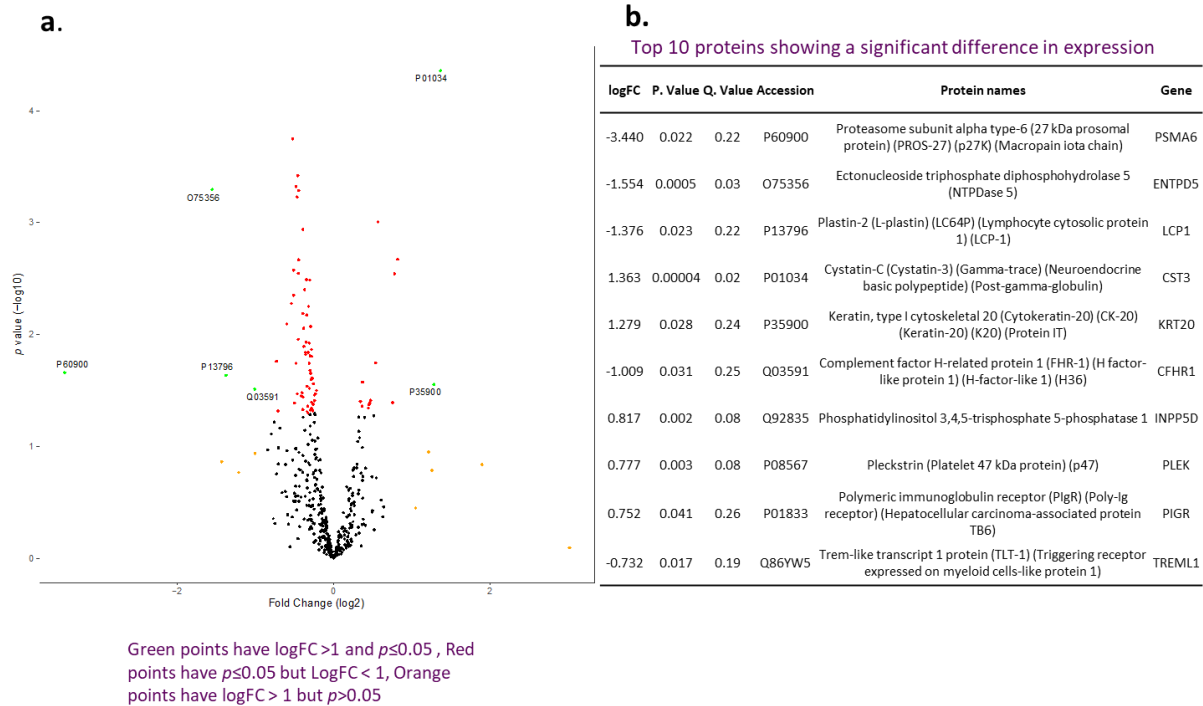


Figure 7.28 Volcano plot (a) and table (b) showing the top proteins that are significantly different between events at 6 months vs controls. In total, 78 proteins were significantly different between the groups.

Whilst these changes seen in the earlier time points are promising, the approach is not a formal subgroup analysis and so results were interpreted with extreme caution. However, based on the trends seen in analytes measured in samples from those having events within 6 months/ 1year, In the next chapter I will discuss how I validate these leads.

7.6 Summary

The aims of this chapter were to see if serum ADMA and other “multi-omic” biomarkers were different in NSAID users who go onto have a cardiovascular event compared to controls. Whilst there was no real difference in ADMA, there was significantly higher citrulline, other amines (alanine, cystine, ethanolamine, histidine, phenylalanine, sarcosine), P-selectin, IL-6 and cystatin C in the event group. What was particularly interesting was the differences in renal biomarkers in those that had an event closer to the time of blood sampling measured by both proteomics and MultiPlex. Whilst we

must be extremely cautious of how we interpret this (**See limitations**), the biology makes sense. In **Chapter 3** I discussed how COX-2 in the kidney maybe cardioprotective and showed a correlation between COX-2, renal function and methylarginines. If the findings in this subgroup analysis are due a real effect, this further supports the notion that COX-2 in the kidney is cardioprotective and highlights the idea that renal function is an important mechanistic driver in patients having a cardiovascular event while taking NSAIDs and, in line, that renal biomarkers could potentially be used to predict cardiovascular events in NSAID users. I also found an increase in IL-6 in the event group and in a follow up study using an extreme animal model of atherosclerosis, mice treated with parecoxib for 17 weeks had higher IL-17A, IL-27 and interferon gamma compared to controls which supports a hypothesis of a pro-inflammatory state with chronic NSAID use.

One of the most important points from this chapter is the importance of study design. My initial aim was to consider cardiovascular events occurring at any time within the study, post baseline sample collection. Patients were followed for a maximum of 6.3 years and a median of 3 years. The post hoc analysis, considering events that occurred early in time (following baseline sample collection), suggested that, for this group, individuals' markers may be present at highest confidence within the first 12 months of sampling. This makes the most biological sense. However, as there is so much uncertainty in interpreting this subgroup effect, principally around adequate matching of events to controls, a further second nested case-controlled study was required (see below).

In hindsight, focusing on individuals who had an event closer to the time the sample was taken makes the most biological sense. As there is so much uncertainty in interpreting this subgroup effect, further investigation is required.

7.6.1 Limitations

Whilst this is the first time a multi-omics approach has been used in real world NSAID users who go onto have cardiovascular events, there are several important limitations that need to be taken into consideration.

Whilst every effort had been taken to minimise confounding, this is still a case control study from a cohort of NSAID users enrolled in a randomised controlled trial and so there maybe unknown confounders that have not been taken into account.

As this was an international multi-centre trial there is considerable variation in how the serum samples were collected and stored. Some of these samples were also taken over 10 years ago and whilst we attempted to control for some of the heterogeneity by matching to the country the sample was taken in, the quality of our results could still be affected.

Due to the relatively large sample size we had a clear batch effect in the amine mass spectrometry which required correction with transformation of data in Z-scores. Whilst this is a widely acceptable method, caution needs to be undertaken and any statistical relationship does not imply causality.

We also experienced a batch effect in the proteomics data which was still apparent even after using multiple methods for correction. Whilst there were no standout proteomic hits in the overall cohort, if there were any hits, these could potentially have been missed due to the batch effect.

The biggest limitation is in interpreting the findings of altered renal biomarkers (ADMA, uromodulin and cystatin C) in the subgroup analysis. Whilst these biologically make sense, these should be interpreted with caution until they can be validated.

7.6.2 Future Work

In the next chapter I have addressed the areas of uncertainty in the subgroup analysis with a follow-on validation study.

Chapter 8: Biomarkers of Early Events in the SCOT Cohort (SCOT-2)

8.1 Rationale

In the previous chapter, where samples from patients having cardiovascular events at any time during follow up period of the SCOT trial, I found relatively few analytes differed in the events group compared to controls. However, as mentioned previously, serum samples used for my analysis were collected at baseline, where all patients were taking a non-steroidal anti-inflammatory drug (NSAID), but not yet allocated to their study group. In the previous chapter I reasoned that the likelihood of finding a biomarker or mechanistic clue in samples from the SCOT Trial would be increased in those who had a cardiovascular event closer to the time of serum collection. In the previous chapter I tested this idea by conducting a post-hoc sub-group analysis of samples from those patients that had cardiovascular events within 6 or 12 months after providing their baseline serum sample. This followed from the hints in the subgroup analysis performed in **Chapter 7** that some biomarkers and in particular renal markers may be different at the earlier time points. However, as this analysis in **Chapter 7** was a post-hoc, subgroup analysis, we cannot be confident of the results. For that reason, in this chapter I designed an entirely new study which focused on identifying biomarkers in serum samples taken from patients having events at early time points. Patient samples from those having events within 12 months were carefully matched to relevant controls and all measurements performed de novo using a randomised, blocked plate layout design.

8.1.1 Hypothesis

This chapter tests two primary hypotheses. The first is a direct testable hypothesis and the second is hypothesis generating.

Firstly: 'Serum ADMA levels are different in NSAID users who go on to have a cardiovascular event within 1 year of serum collection, compared to those that don't'

Secondly: 'Using a targeted proteomic approach will identify serum biomarkers in NSAID users who have cardiovascular events within 1 year of serum collection'

The aims of this chapter are to:

Assess if NSAID users who go onto have a cardiovascular event within 1 year of sample collection have differences in serum levels of:

- f) Asymmetric dimethylarginine (ADMA), symmetric dimethyl arginine (SDMA), NG-Methyl-L-arginine (LNMMA and/or other amines measured using mass spectrometry
- g) Proteins using the O link proximity extension assay multiplex platform

In addition to the 1-year group, a subgroup analysis of results from samples of patients having events within 6 months of serum collection as also performed.

8.2 Methods

A detailed description of the Methods is provided in the methods section and in **Chapter 6**.

8.2.1 Designing the new study (SCOT-2)

The first challenge was to determine the optimal study design. In an ideal world one would perform a cohort study using the whole SCOT cohort. As discussed in **chapter 6**, this was not feasible as there are over 7000 samples. Furthermore, based on the data from **chapter 7**, I performed a power calculation using the ADMA data. In order to achieve a hazard ratio of 1.2, with an event rate of 4% within the SCOT cohort at an alpha of 0.05, we would require a sample size of 11,300 to reach 80% power.

The most pragmatic way forward was to design a new case control study with a primary research question to determine if a biomarker(s) of events exists/could be detected in those who had events in the first 12 months after sample collection. In the previous chapter we only used the on-treatment cohort but considering that in SCOT-2 only early events were being studied and knowing that at the time of screening (when the sample was taken), all participants are on an NSAID it was considered that using an intention to treat cohort would be a valid approach with the added advantage of increasing the sample size. First, 50 participants were identified who had an event within 1 year of which, 17 had an event within 6 months. The next step was to find matched controls. As discussed extensively In **Chapter 6**, matching was based on

age $\pm$ 5 years, gender, baseline NSAID and Country. As the focus was on participants who had an event within 1 year, it was important that the pool of patients for sampling had a follow up of at least 1 year to minimise the risk of selection bias. Furthermore, participants who went on to have an event after 1 year were still included in the pool of controls to minimise the risk of selection bias. As per **Chapter 6**, I used the fuzzy matching extension in SPSS to perform 2:1 (controls: events) matching. Once the matches had been identified, I compared the baseline characteristics between the groups to ensure the groups were representative of the overall SCOT cohort before proceeding **Figure 8.1**. As the matching was sufficient, next the samples were identified from the freezer. There was 1 event sample and 1 control sample missing from the biobank so in total there are 49 events and 97 matched controls.

Baseline Characteristic	n:97 No Event; (n or Median)		n:49 Cardiovascular Events (n or Median)	
	% or IQR		% or IQR	
Treatment Group	52	53.61%	30	61.22%
Control	45	46.39%	19	38.78%
NSAID during Trial				
Ibuprofen	23	23.71%	8	16.33%
Diclofenac	8	8.25%	8	16.33%
Other NSAID	14	14.43%	3	6.12%
Celecoxib	52	53.61%	30	61.22%
Age (Years)	67.02	8.51	67.54	10.96
Age > 70 years	36	37.11%	19	38.78%
Age > 80 years	4	4.12%	4	8.16%
Male	57	58.76%	29	59.18%
Non Caucasian	0	0%	0	0%
Current smoker	16	16.49%	17	34.69%
Never smoked	42	43.30%	7	14.29%
Previous smoker	39	40.21%	25	51.02%
Statin therapy	15	15.46%	7	14.29%
Aspirin therapy	16	16.49%	11	22.45%
Heart Failure	0	0%	1	2%
Hypertension	46	47.42%	16	32.65%
Systolic BP	143	24.25	136.5	24.25
Systolic BP > 140	52	53.61%	20	40.82%
Systolic BP > 150	32	32.99%	14	28.57%
Systolic BP > 160	20	20.62%	8	16.33%
Diastolic BP	80	15.25	75.5	14.5
Diastolic BP > 100	1	1.03%	1	2.04%

Baseline Characteristic	n:97 No Event; (n or Median) % or IQR		n:49 Cardiovascular Events (n or Median) % or IQR	
Hypercholesterolaemia	35	36.08%	6	12.24%
Renal Disease	5	5.15%	3	6.12%
Asthma	22	22.68%	5	10.20%
COPD	7	7.22%	2	4.08%
BMI	29.05	6.28	27.34	5.36
BMI < 18.5	0	0.00%	0	0.00%
BMI>30	41	42.27%	14	28.57%
Waist hip ratio	0.94	0.1	0.92	0.13
Waist hip ratio >1	20	20.62%	11	22.45%
Total Cholesterol (mmol/l)**	5.34	1.77	5.43	1.47
Total Cholesterol >6**	26	27.66%	16	32.65%
HDL cholesterol**	1.33	0.47	1.35	0.52
HDL cholesterol<1**	13	27.66%	4	8.16%
Triglycerides (mmol/l)**	1.64	1.07	1.68	1.11
Total Chol:HDL ratio**	3.99	1.3	3.89	1.71
T:H Ratio >5**	16	27.66%	12	24.49%
Uric Acid	329	125	331	139

Figure 8.1 . Baseline characteristics table comparing cases (cardiovascular event) and controls (no event). The treatment group refers to patients randomised to celecoxib and control group is the usual NSAID. Abbreviations used: BP: blood pressure, COPD: chronic obstructive pulmonary disease, BMI: body mass index, WHR: waist hip ratio. HDL: high density lipoprotein, chol: cholesterol. (** indicates n:94 controls, 49 events)

8.2.2 Planning experiments and aliquoting

As discussed in **Chapter 6**, these samples are a precious resource, and we had a limited serum volume remaining given we had already used considerable sample volume to perform the assays within our first analysis (SCOT-1) in **Chapter 7**. Furthermore, the samples used in **Chapter 7** had already been freeze-thawed at least once and so this had to be taken into consideration. We had previously planned to perform a lipidomic analysis as part of in the primary SCOT-1 analysis but due to issues with contracts, specifically our collaborators in the US were not able to commit to the stipulations within our sample sharing agreement with the SCOT study team, this was not possible. We therefore had a 175µl aliquot of control and event samples that had only been freeze-thawed once. These aliquots were therefore used to perform the remaining analysis. As we were now using the intention to treat group and rematching these to controls, there were several new “untouched” samples relating to

events and appropriate controls in the freezer. To minimise any bias due to differences in freeze thawing, any unthawed samples were subject to freeze thaw cycle to ensure parity between all samples tested.

In total 147 samples were retrieved from the 7000, organised and aliquoted. To accommodate the samples in each assay, samples were split across two 96 well layouts. To account for potential batch effects, events were evenly spread across the two plates alongside the matched controls. Within this plan, samples were randomised on each plate and aliquoted for the future assays **Figure 8.2**.

	1	2	3	4	5	6	7	8	9	10	11	12
A	control	control	Event	control	control	control	Event	control	control	control		
B	Event	control	control	control	Event	control	control	control	Event	Event		
C	control	control	control	control	control	control	Event	Event	Event	control		
D	Event	control	control	control	control	Event	control	Event	control			
E	Event	control	Event	control	Event	control	control	control	control			
F	control	control	Event	Event	control	control	Event	Event	control			
G	control	Event	control	control	Event	control	control	control	Event			
H	control	Event	control	control	control	Event	control	control	Event			

Priority	Assay	Vol for Assay	Vol for Aliquot
1	O-link	1ul	40
2	Amine Array	20ul + additional for extra runs	50
3	Future Validation	unknown	80

Figure 8.2 Example randomised plate layout and aliquots for experiments. Events and controls were randomised on a 96 well plate. Experiments were prioritised according to the volume (vol) of sample available (μ L).

Statistical analysis

Regression analysis and receiver operator characteristics (ROC) analysis was performed using IBM SPSS. Details of general statistical methods can be found in **Chapter 3 Methods**.

8.3 Results

8.3.1 Amines and methylarginines assessed using Mass spectrometry.

8.3.1.1 Events within 1 year

Using UHPLC-MS/MS, in total 33 amines and 3 methylarginines were quantified in 146 samples (49 events and 97 controls). First an unsupervised approach was used to interrogate the results. Using PCA-X, there was no distinct separation between the controls and events at 1 year **Figure 8.3**.

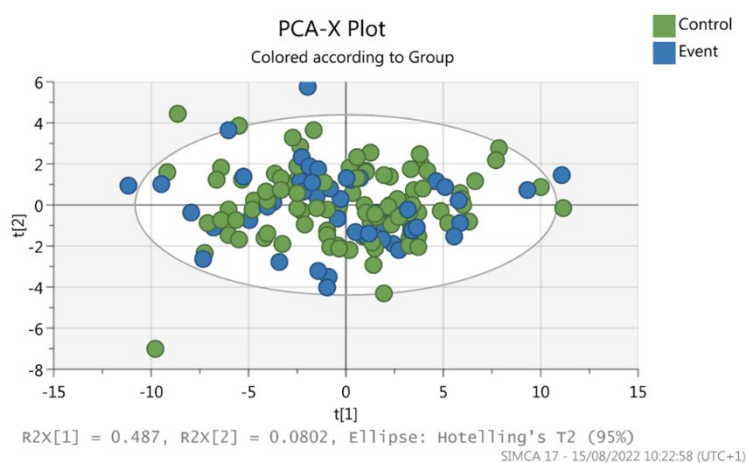


Figure 8.3 Principal Component Analysis Plot (PCA). Samples were labelled according to group (event and control). There was no separation between the groups.

Next, we ran a supervised analysis. The first focus was again the 6 nitric oxide (NO) amines and associated ratios **Figure 8.4**. There was significantly higher ADMA in the 1-year event samples and lower Arg/ADMA ratio, compared to matched controls. There was no difference in any of the other analytes tested. The mean concentrations for all analytes are provided in **Appendix 7**.

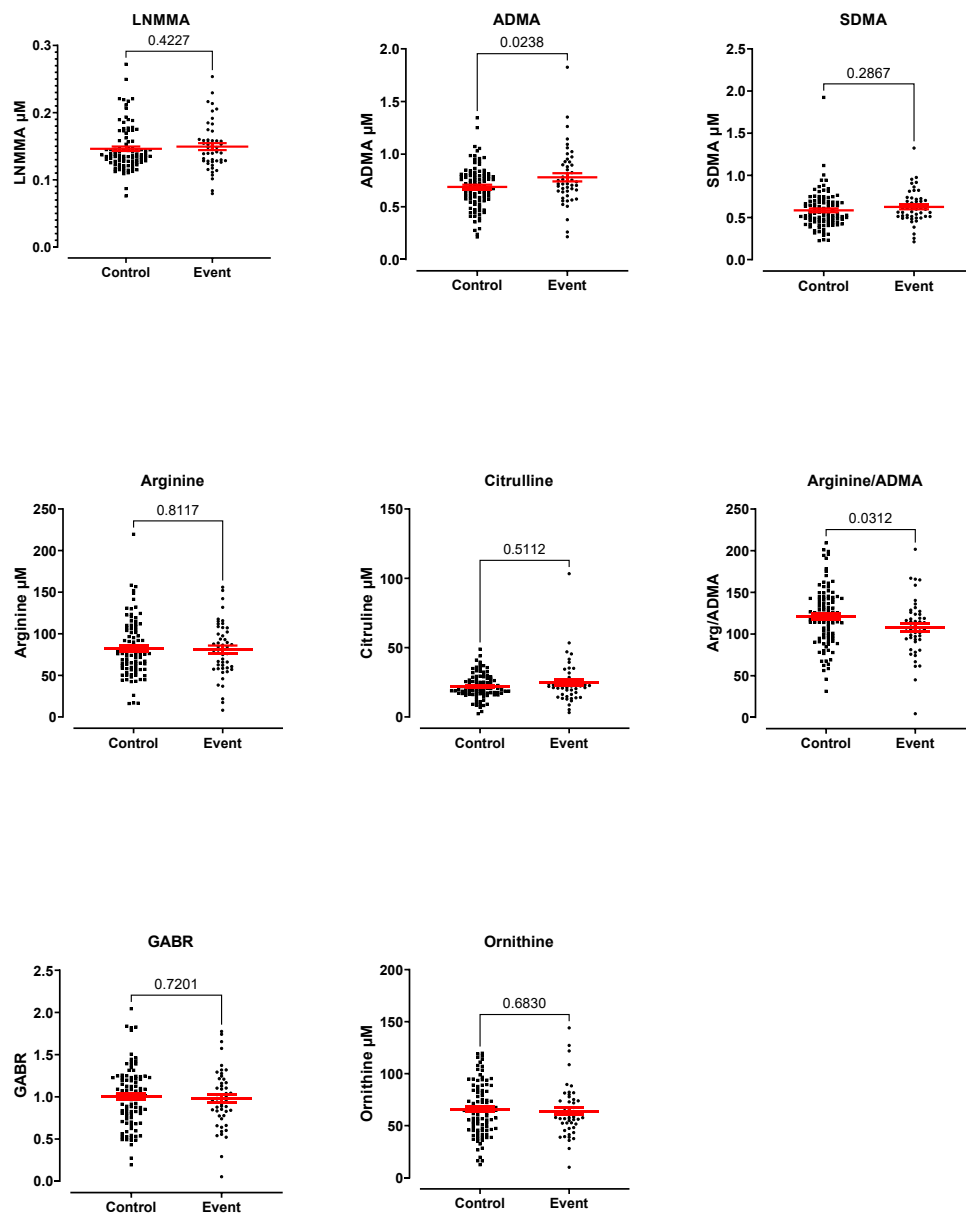


Figure 8.4 Scatter Plot of Nitric Oxide Amines. There was higher ADMA and lower Arginine/ADMA ratio in the event group. There was no significant difference in any of the other analytes. All samples were analysed using an unpaired t-test except LNMA, arginine and citrulline which were analysed using Mann Whitney U test.

It should be noted that the Kolmogorov-Smirnov test for ADMA and SDMA did not reach significance ($p > 0.05$) but was close (0.04 ADMA and 0.02 SDMA). I therefore examined the QQ plots **Figure 8.5** which suggested the data was normally distributed and so t-tests were used for the comparisons.

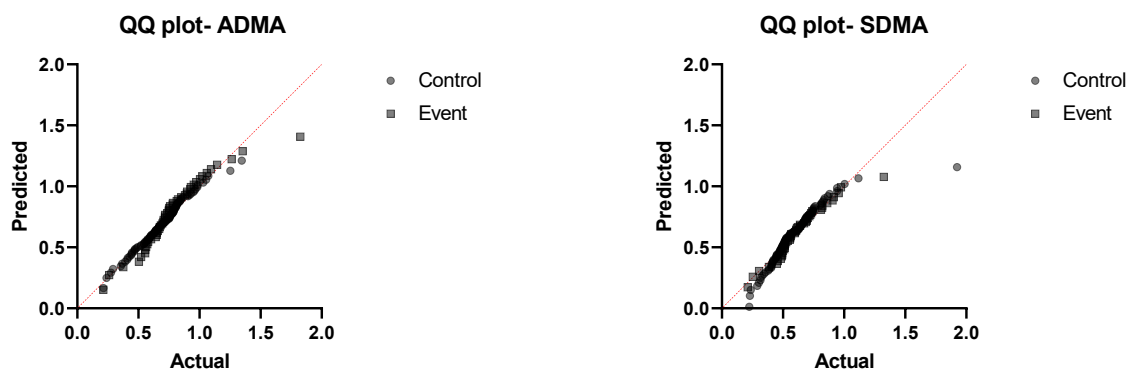


Figure 8.5 QQ plots for ADMA and SDMA. The QQ plots suggests that the data is normally distributed for ADMA and SDMA

8.3.1.2 Subgroup Analysis of Events within 6 months

Next, we looked at the 6-month subgroup. There was no separation in events at 6 months with matched controls (n:17 events vs 34 controls) on PCA **Figure 8.6**. Using a supervised approach we found a trend for increased ADMA ($p=0.169$), SDMA ($p=0.36$) but not LNMMA. However, as was seen with samples from the 12-month group, there was significantly lower Arg/ADMA ratio in event group **Figure 8.7**. There was no difference in any of the anther analytes. A table of mean concentrations for all amine analytes in provided in **Appendix 8**.

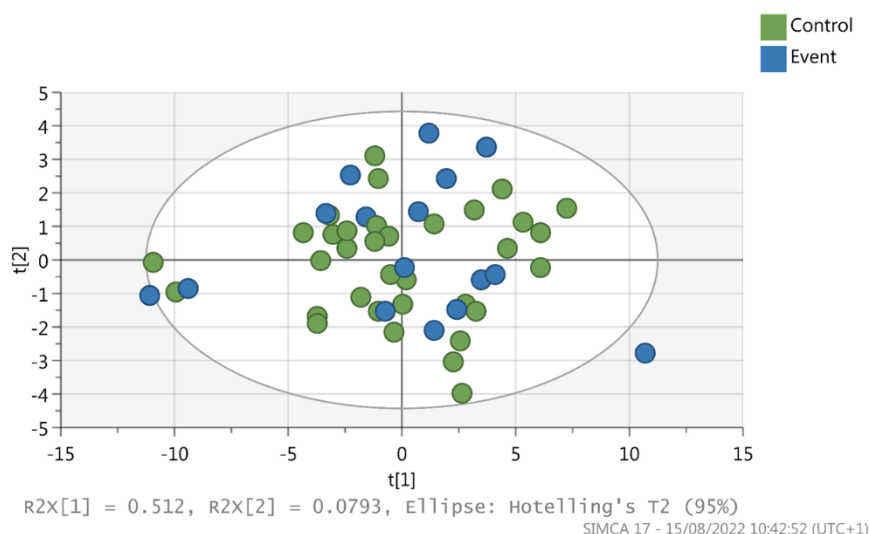


Figure 8.6 Principal Component Analysis Plot (PCA). Samples were labelled according to group (event within 6 months and control). There was no separation between the

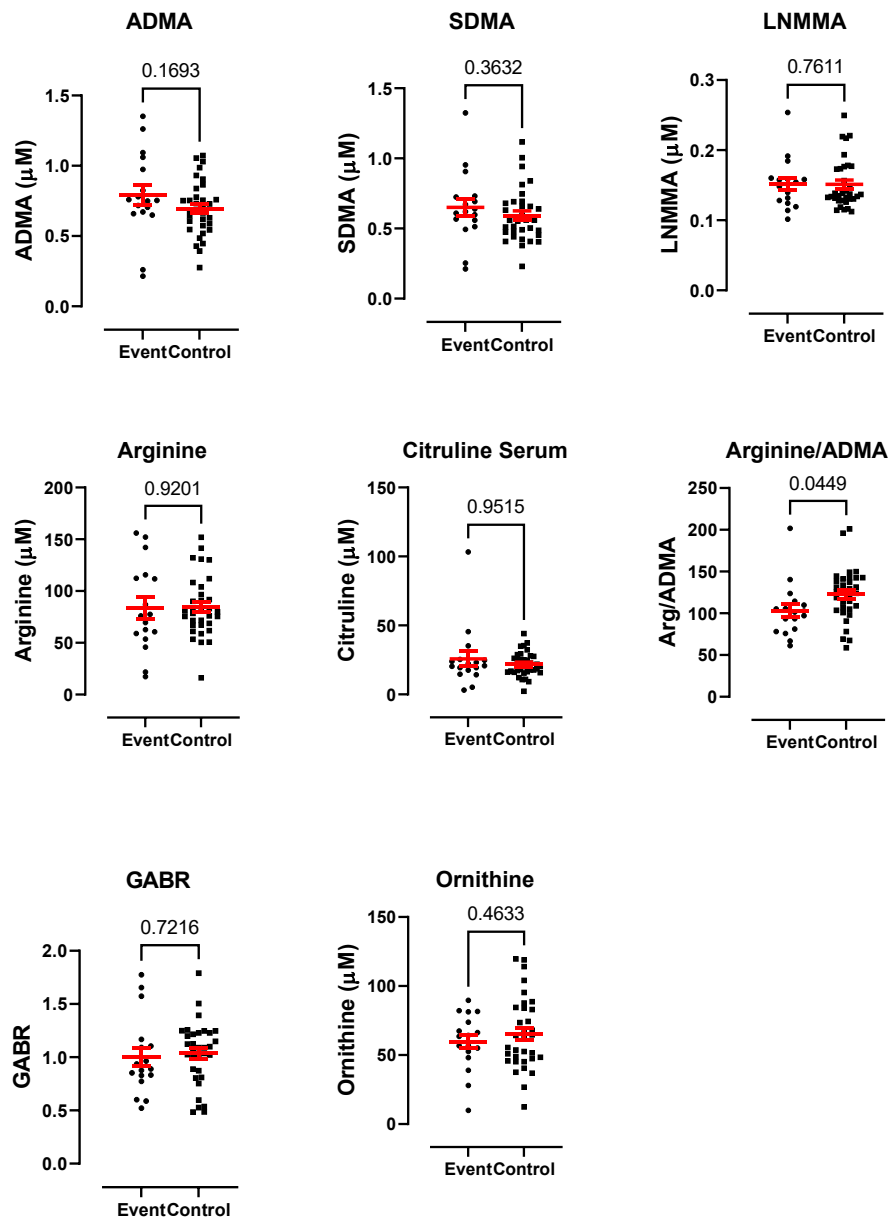


Figure 8.7 Scatter Plot of Nitric Oxide Amines. There was higher ADMA and lower Arginine/ADMA ratio in the event group. There was no significant difference in any of the other analytes. Comparisons were made using t-test for all analytes except LNMA and citrulline which were analysed using Mann Whitney U test.

8.3.2 Proteomics measured using O link

Given the issues with batch effect, quantification and coverage of proteins using SWATH in the previous chapter, a decision was made to use a more targeted approach which could also allow for low/undetectable levels of analyte to be confirmed and included within the analysis. A full description of the methodology and principles behind Olink are provided in the methods section (**Chapter 2**). Samples were analysed by the Olink laboratory in Sweden who were blinded to the groups. The raw data generated was analysed by me in house. In total, 369 proteins were quantified. The full list is presented in **Appendix 2**.

8.3.2.1 Events within 1 year

In the first instance, PCA-X was used as an unsupervised approach which showed no separation between the controls and events at 1 year **Figure 8.8**.

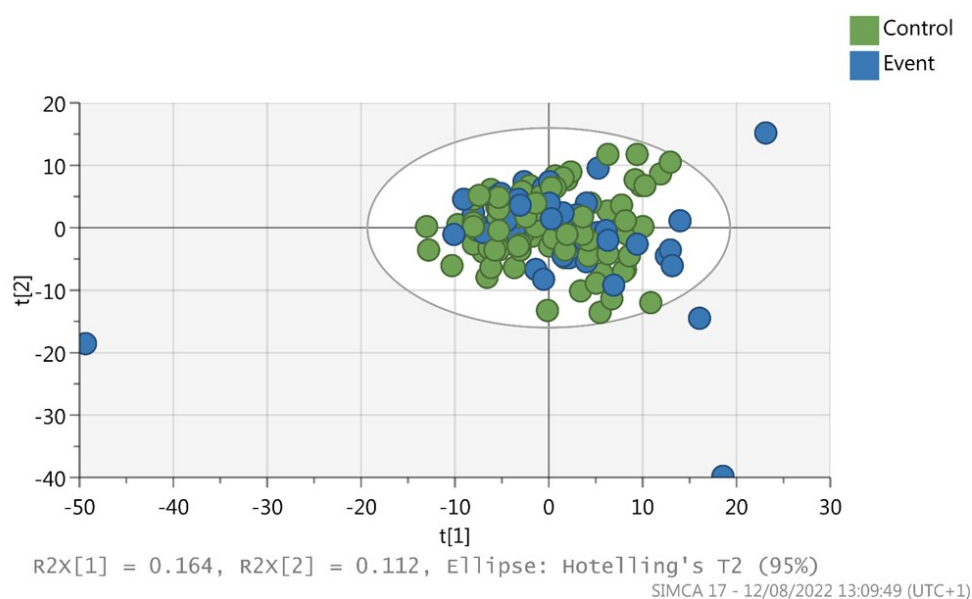


Figure 8.8 Principal Component Analysis Plot (PCA). Samples were labelled according to group (event and control). There was no separation between the groups.

Next, a supervised analysis was performed by screening individual analytes using a T-test. In total there were 34 proteins that were significantly different ($p < 0.05$) including 4 downregulated and 30 upregulated proteins which are highlighted on the volcano plot in (**Figure 8.9a**). Although q-values (using the Benjamini, Krieger and Yekutieli)

were >0.05 in all cases except for GDF15 ($q < 0.01$), (**Figure 8.9b**). GDF15, also known as NSAID activated gene, is clearly a fundamental observation in this study since it was found to be increased significantly as defined both by p and q-values and it is activated by NSAIDs. As such, GDF15, will be discussed in detail later in the chapter.

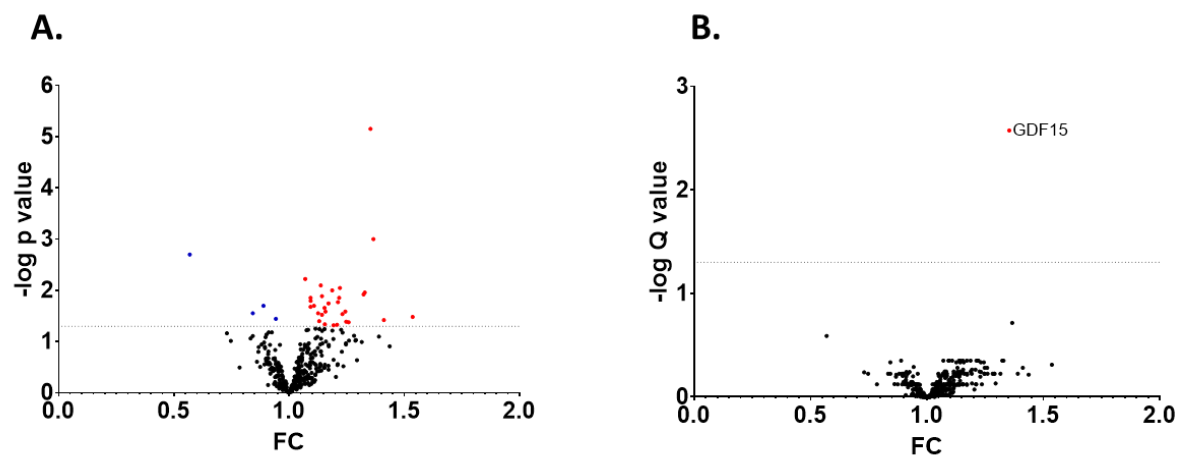


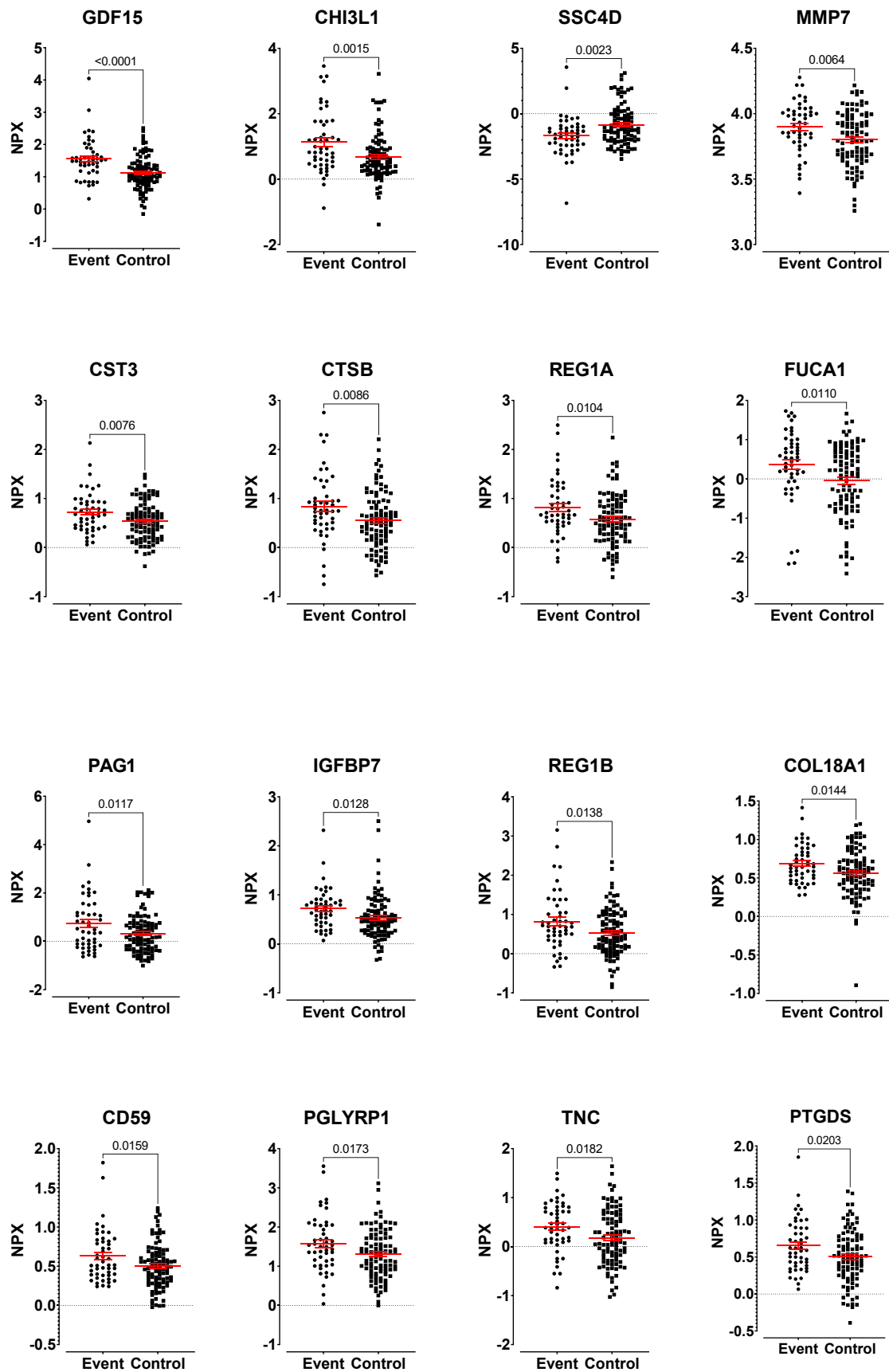
Figure 8.9 Volcano plots showing proteins that are significantly different between event and non-event groups before and after false discovery rate (FDR) correction. (A) At a p-value cut off of <0.05 (dotted line), 34 proteins were significantly different including 4 down (blue) and 30 up regulated (red). After applying FDR at a Q value cut off <0.05 (dotted line), only GDF15 remained significant.

A table of the significant proteins with fold change is provided in **Figure 8.10** and is sorted by Q-Value.

Protein	P Value	Q Value	Fold Change
GDF15	0.000	0.000	1.35350
CHI3L1	0.001	0.193	1.36624
SSC4D	0.002	0.258	0.56937
MMP7	0.006	0.447	1.07036
CST3	0.008	0.447	1.13787
CTSB	0.009	0.447	1.22083
REG1A	0.010	0.447	1.18711
FUCA1	0.011	0.447	1.32884
PAG1	0.012	0.447	1.32341
IGFBP7	0.013	0.447	1.14275
REG1B	0.014	0.447	1.21741
COL18A1	0.014	0.447	1.09369
CD59	0.016	0.447	1.09393
PGLYRP1	0.017	0.447	1.21233
TNC	0.018	0.447	1.17144
PTGDS	0.020	0.447	1.10834
COL1A1	0.020	0.447	0.88876
ENTPD6	0.021	0.447	1.09342
CD14	0.022	0.447	1.15395
TFF3	0.026	0.464	1.15804
MSMB	0.026	0.464	1.24434
ANGPTL3	0.028	0.464	1.12608
KITLG	0.028	0.464	0.84260
TGFBR3	0.029	0.464	1.23127
CCL15	0.030	0.464	1.14282
NTproBNP	0.033	0.490	1.53643
EGFR	0.036	0.515	0.94267
IGFBP1	0.038	0.524	1.41158
IL2RA	0.040	0.524	1.13109
REG3A	0.041	0.524	1.24785
PRTN3	0.042	0.524	1.25868
LBP	0.046	0.546	1.15520
CSTB	0.047	0.546	1.20844
ACOX1	0.048	0.546	1.19313

Figure 8.10 Table showing proteins that are different between 12-month events and matched controls with fold change. Data is sorted by Q value. GDF15 is the only analyte that remained significant after false discovery rate correction ($Q < 0.05$).

Individual Scatterplots for the top 16 upregulated and 4 downregulated proteins is presented in **Figure 8.11**.



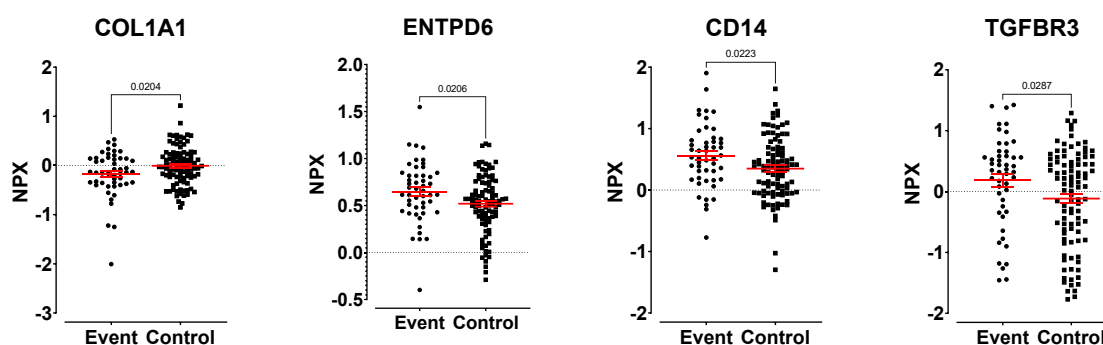


Figure 8.11 Scatter plot of top 20 significantly changed proteins between events at 12 months and matched controls. The Figures represent NPX scores with mean $\pm$ -SEM (red error bars).

Next pathway analysis was performed using Reactome which is a free web-based tool which supports pathway analysis of proteins using over-representation. Whilst false discovery rate (FDR) correction is useful in identifying biomarkers, when performing discovery experiments, it can sometimes be too strict and could lead to us missing some important pathways, therefore a decision to use p-values as a reference for ‘significant proteins’ for pathway analysis was made. The 34 significant proteins were entered into the tool. The top 10 pathways are presented in **Figure 8.12**.

Pathway name	Entities				Reactions	
	found	ratio	P-Value	Entities FDR	Found	Reactions ratio
Neutrophil degranulation	11/480	0.0317	9.91E-08	7.53E-05	8/10	7.18E-04
Assembly of collagen fibrils and other multimeric structures	5/67	0.004425	3.28E-06	8.30E-04	16/26	0.001866207
Collagen degradation	5/69	0.004557	3.28E-06	8.30E-04	13/34	0.002440425
Transfer of LPS from LBP carrier to CD14	2/3	1.98E-04	1.39E-04	0.026483	2/2	1.44E-04
Post-translational protein phosphorylation	4/109	0.007199	2.68E-04	0.039583	1/1	7.18E-05
Collagen formation	5/104	0.006868	3.14E-04	0.039583	38/77	0.005526845
Degradation of the extracellular matrix	5/148	0.009774	4.76E-04	0.051433	40/105	0.007536606
Activation of Matrix Metalloproteinases	3/35	0.002311	0.001245	0.110022	13/27	0.001937984
Antimicrobial peptides	4/123	0.008123	0.00131	0.110022	8/58	0.004163078
PI3K events in ERBB2 signalling	2/22	0.001453	0.00182	0.138342	5/7	5.02E-04

Figure 8.12 Top 10 pathways identified in 1 year event group using Over-representation analysis in REACTOME. Broad characterisation identifies two key pathway themes: 1) inflammation and immunity, 2) Extra-cellular matrix organisation.

Next, the proteins were characterised into broad groups:

- i) Pathways relating to inflammation and the immune system (*neutrophil degranulation, transfer of LPS from LBP carrier to CD14, antimicrobial peptides*)
- ii) Pathways relating to Extra-cellular Matrix organisation (ECM) (assembly of collagen fibrils and other multimeric structures, collagen degradation, collagen formation, degradation of ECM, activation of metalloproteinases)
- iii) Other Pathways (post-translational protein phosphorylation, PI3K events in ERBB2 signalling)
- iv) Proteins not found in Reactome.

A protein summary of each of these groups is provided in **Figure 8.13** in the form of a heatmap.

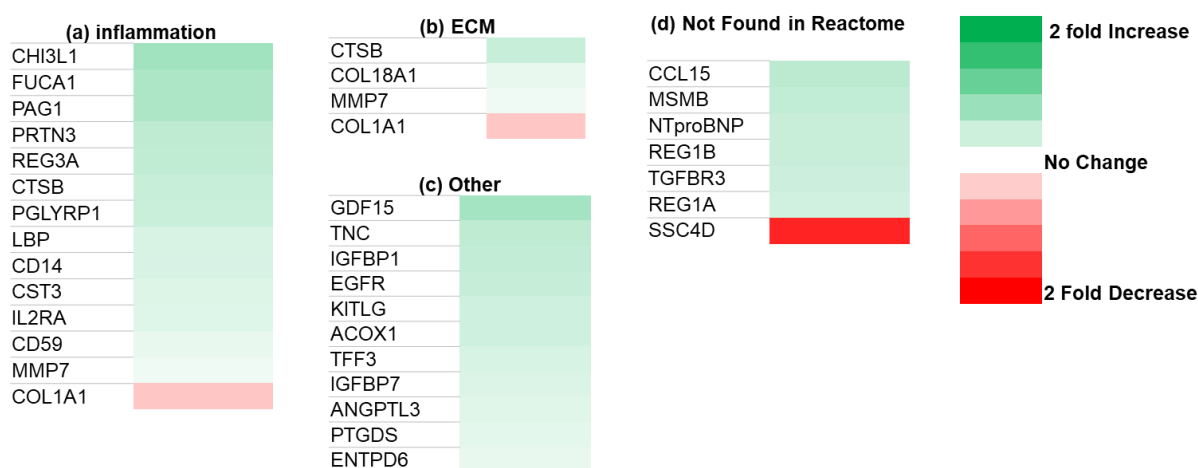


Figure 8.13 Heatmaps of top 36 proteins in events at 1 year grouped according to pathways highlighted in REACTOME. Dark green indicates a 2-fold increase and dark red indicates a 2-fold decrease.

It is important to note that some of the proteins in the 'other' or 'not found group' may be involved in ECM and inflammatory pathways when looking in the literature but for the sake of simplicity, they were grouped based on where they feature in the Reactome pathway database.

Pathways relating to inflammation and the immune system

In total, 14 proteins were identified in the inflammation and the immune system pathway **Figure 8.13a**. The protein with the highest increased fold change in this group was chitinase-3 like-protein1 (CHI3L1) and with the largest decreased fold change was Collagen type 1 alpha 1 chain (COL1A1). Interestingly, cystatin C (CS3) was among the markers that was increased in this event group **Figure 8.11**. This is in line with findings from the exploratory analysis in **Chapter 7** where cystatin C was found to be increased in the larger cohort included within SCOT-1. This finding together with increased ADMA and/or arginine ADMA ratio, shown earlier in this chapter within SCOT-2, further supports the notion of renal COX-2 as an important site for cardio protection.

Pathways relating to Extracellular Matrix Organisation

In total 4 proteins were identified in the ECM Organisation pathway **Figure 8.13b**. The protein with the highest fold increase was cathepsin B (CTSB) and with the greatest decreased fold change was collagen type1 alpha1 chain (COL1A1) which was also highlighted in the inflammation and immunity pathways. Interestingly, similar pathways related to ECM organisation were also highlighted as the top hit in the ASCENT analysis **Chapter 5** and therefore warrant further investigation.

Other Pathways

There were 11 proteins classified into 'other pathway's according to Reactome **Figure 8.13c**. One of the limitations of pathway analysis is that whilst it can be useful for identifying proteins within well-established pathways, it can be more limited when trying to assess interactions with lesser-known pathways or pathways that do not feature within Reactome at that time. For example, when searching through GeneCards.org, tenascin C (TNC) and epidermal growth factor receptor (EGFR) are both linked to ECM organisation.

GDF15 (which is our primary biomarker candidate) is part of the transforming growth factor beta (TGF-beta) superfamily of proteins and could play a role in ECM organisation and collagen turnover[261]. Of course depending on the number of degrees of separation, almost all proteins will eventually be associated with each other, but these findings should not be ignored

Not Found in Reactome

There were 7 proteins that were not identified within the Reactome database **Figure 8.13d**. Of these, one protein in particular, stood out: N-Terminal pro-brain natriuretic peptide (NT-NT-proBNP). This is cardiovascular biomarker that is extensively used in clinical practice to aid in the diagnosis of or rule out heart failure [262]. Whilst there is now some emerging data to suggest it can predict cardiovascular death in the general population independent of cardiac remodelling [263], it is best known as a marker of ventricular remodelling and stretch secondary to increased pre-load. This could therefore be relevant as both a biomarker and provides some mechanistic insight into the patho-physiology of participants who go onto have an event.

8.3.2.2 Similarities between ASCENT and SCOT

A key original aim of my thesis was to use data from SCOT and ASCENT to cross-inform results from each study and progress the ambition of better understanding the mechanisms involved in NSAID associated cardiovascular risk. To achieve this, I went on to identify any significant overlapping proteins and methylarginines between SCOT and ASCENT which are provided in the Venn diagram in **Figure 8.14**. I noticed that collagen type 6 alpha 3 chain (COL6A3) was altered in both the ASCENT study ($p < 0.05$) and in the SCOT-2 analysis ($p = 0.056$). Given that COL1A1 was the only protein that was highlighted in both groups, COL6A3 should be considered a potentially important mechanistic clue and should be looked at in future validation or mechanistic studies.

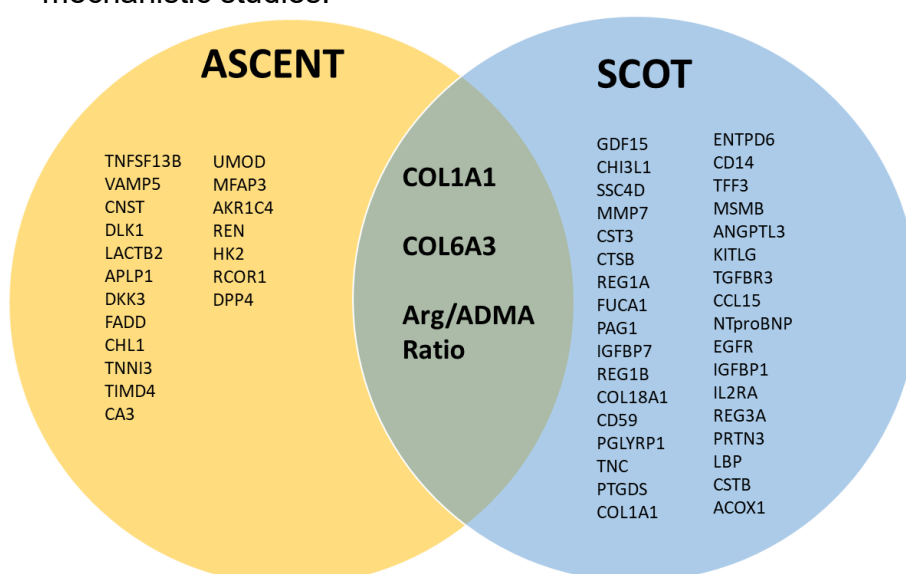


Figure 8.14 Venn diagram showing overlap in significant proteins and methylarginines between ASCENT and SCOT-2 study. COL1A1, COL6A3, ADMA and Arginine/ADMA were significantly different in both SCOT-2 and ASCENT.

Obviously both SCOT-2 and ASCENT are two distinctive populations (young healthy volunteers, with and without short term NSAID use vs older patients with osteoarthritis who are all on long term NSAIDs and so any direct comparisons of individual proteins are difficult to make. What was interesting is that pathways relating to ECM organisation were highlighted in both studies. Given the biological plausibility and the established role of ECM in cardiovascular disease, ECM should be prioritised as an area for future work in this field.

8.3.2.3 Subgroup Analysis of Early Events within 6 months.

Next, I wanted to look at the 6-month subgroup. As this is a subgroup analysis that has a smaller sample size than the 1-year events, it is important not to over interpret these findings in Isolation, but results should be interpreted with the 1-year data in mind.

First, PCA was used as an unsupervised approach which showed no separation between the controls and events at 6 months **Figure 8.15**.

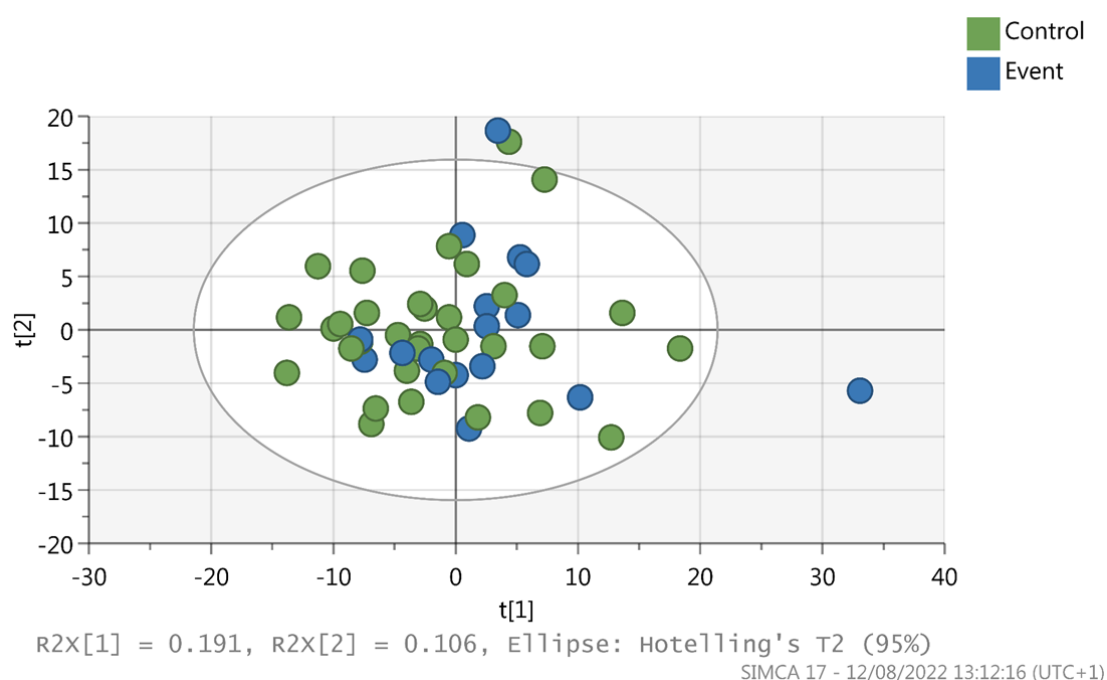


Figure 8.15 Principal Component Analysis Plot (PCA). Samples were labelled according to group (event at 6 months and matched controls). There was no separation between the groups.

Next, a supervised analysis was performed by screening individual analytes using a T-test. In total there were 28 proteins that were significantly different ($p < 0.05$) including 2 downregulated and 26 upregulated proteins which are highlighted on the volcano plot (**Figure 8.16a**). Although q-values (using the Benjamini, Krieger and Yekutieli) were > 0.05 in all cases (**Figure 8.16b**). A table of the top 20 proteins is provided in **Figure 8.17** and is sorted by Q-Value.

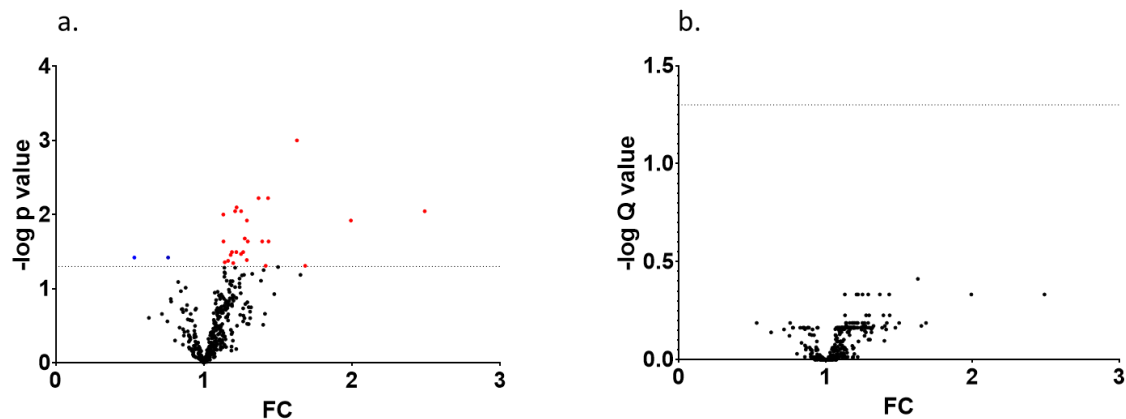


Figure 8.16 Volcano plots showing proteins that are significantly different between 6-month event and non-event groups before and after false discovery rate (FDR) correction. (A) At a p -value cut off of < 0.05 (dotted line), 28 proteins were significantly different including 2 down (blue) and 25 up regulated (red). After applying FDR at a Q value cut off of < 0.05 (dotted line), no analytes remained significant.

Protein	P Value	Q Value	Fold Change
GDF15	0.001	0.387	1.62826
FCGR2A	0.006	0.465	1.43315
CTSB	0.006	0.465	1.36938
ENTPD6	0.008	0.465	1.22124
COL18A1	0.009	0.465	1.21058
LILRB2	0.009	0.465	1.25136
NTproBNP	0.009	0.465	2.49096
MMP7	0.010	0.465	1.13105
CNPY2	0.012	0.465	1.29041
TSLP	0.012	0.465	1.99262
TNC	0.021	0.594	1.27629
ENTPD5	0.023	0.594	1.13259
CHI3L1	0.023	0.594	1.43629
NPDC1	0.023	0.594	1.29630
DPP7	0.023	0.594	1.39355
TFF3	0.032	0.650	1.21938
NECTIN2	0.032	0.650	1.18935
ADA2	0.032	0.650	1.26546
ANGPTL3	0.034	0.650	1.25105
IGSF8	0.035	0.650	1.18120

Figure 8.17 Table showing proteins that are different between 6-month events and matched controls with fold change. Data is sorted by Q value. No proteins remained significant after false discovery correction ($Q < 0.05$).

Next pathway analysis was again performed using Reactome. The 28 significant proteins were entered. The top pathway hits again related to inflammation and ECM **Figure 8.18**.

As done previously, the proteins were characterised into broad groups according to how they were classified in Reactome and a heatmap of proteins within each group are presented in **Figure 8.19**: Interestingly, NTproBNP was almost 2.5-fold higher in the 6-month event group compared to controls.

Pathway name	Entities				Reactions	
	Found	Ratio	P-Value	Entities FDR	Found	Reactions ratio
Collagen degradation	4/69	0.004557	1.73E-05	0.001937	9/34	0.00244
Assembly of collagen fibrils and other multimeric structures	4/67	0.004425	1.73E-05	0.001937	1/26	0.001866
Neutrophil degranulation	6/480	0.0317	3.03E-04	0.022746	6/10	7.18E-04
Activation of Matrix Metalloproteinases	3/35	0.002311	4.06E-04	0.022755	13/27	0.001938
Collagen formation	4/104	0.006868	6.94E-04	0.031249	20/77	0.005527
Degradation of the extracellular matrix	4/148	0.009774	9.75E-04	0.036078	36/105	0.007537
Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	6/136	0.020869	0.001623	0.051928	5/44	0.003158
Phosphate bond hydrolysis by NTPDase proteins	2/34	0.002245	0.002007	0.054513	2/12	8.61E-04
Extracellular matrix organization	7/326	0.021662	0.002181	0.054513	66/319	0.022897
NR1H2 & NR1H3 regulate gene expression linked to lipogenesis	2/17	0.001123	0.012864	0.275969	2/8	5.74E-04

Figure 8.18 Top 10 pathways identified in 6-month event group using Over-representation analysis in REACTOME. Broad characterisation again identifies two key pathway themes: 1) Extracellular matrix organisation, 2) inflammation and immunity.

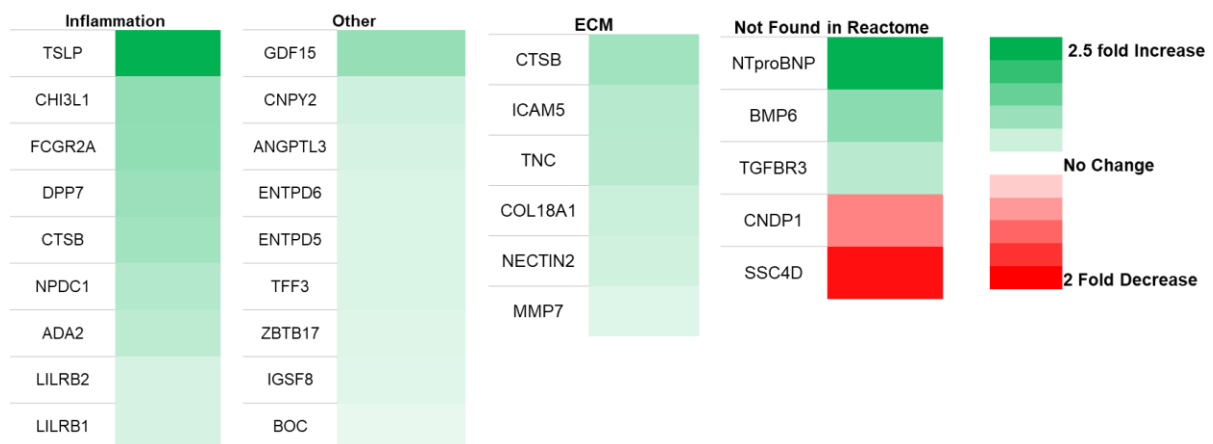


Figure 8.19 Heatmaps of top 28 proteins in events at 6 Months year grouped according to pathways highlighted in REACTOME. Dark green indicates a 2-fold increase and dark red indicates a 2-fold decrease.

Next, I wanted to identify common proteins between the 1 year and 6-month subgroup analysis. There were two reasons for this:

- 1) As previously discussed, biologically it would make sense for some biomarkers to be time dependant and be higher closer to an event.
- 2) Whilst it has several limitations, this subgroup analysis could also be used as a validation cohort (for the 12-month data set), providing confidence in the data seen within analysis of either group (i.e. 6 or 12 month).

A Venn diagram in **Figure 8.20** identified 10 common proteins. Going forward I believe these should be prioritised and taken into consideration in future validation or mechanistic studies.

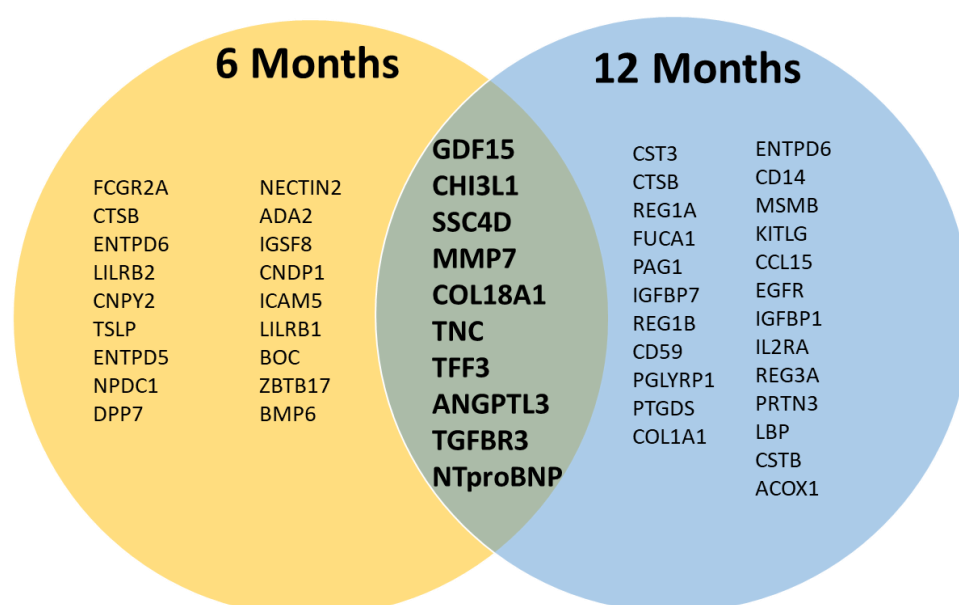


Figure 8.20 Venn diagram showing overlap in significant proteins at 6 months and 1 year. Ten proteins were significantly different in both the 1 year and 6-month events versus matched controls.

8.3.3 Biomarkers of Cardiovascular Events

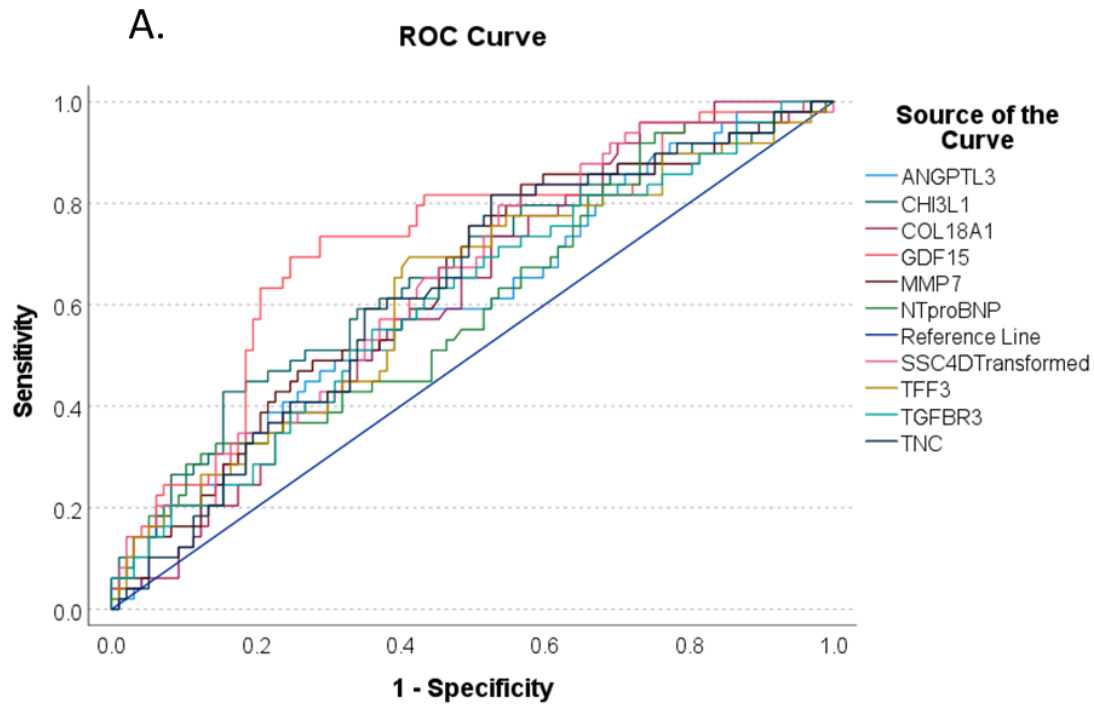
Whilst the proteins that were significantly different between the groups provides some mechanistic insight into NSAID cardiovascular events, they may not necessarily be suitable as biomarkers.

In order to explore potential biomarkers, as an exploratory exercise, I performed receiver operating characteristic (ROC) analysis on the 10 common proteins identified

above within the 12-month event group. ROC analysis is routinely used in clinical practice to determine how accurately a diagnostic test or, in this case, a circulating biomarker, can discriminate between two groups (cardiovascular event and no event). To do this, a subsequent ROC curve of sensitivity vs 1-specificity is plotted and an area under the curve (AUC) is generated which gives an indication of how well the biomarker can discriminate between two groups. An AUC of 0.5 indicates there is 50% chance of the marker predicting the right group (same as chance) and would indicate poor discrimination. Therefore to determine if an AUC is meaningful, it is compared to an AUC of 0.5. An AUC of 1 would indicate a perfect biomarker delivering a prediction rate of 100% [264].

A ROC curve along with a table sorted by area under the curve is provided in **Figure 8.21**. As SSC4D was reduced in the event group, (higher levels would be expected in those that do not have an event), the data was transformed by multiplying the NPX scores for SSC4D by -1 to display all the variables on a single graph. The raw NPX scores were used in all other analysis.

GDF15 was the best performing biomarker overall with an AUC of 0.715 (95% CI 0.63 TO 0.81). This means that within this dataset, GDF15 levels can be used to discriminate between events at 1 year and non-events, 71.5 % of the time. ROC curves are also useful in determining a cut off value depending on the required sensitivity and specificity of a biomarker. For example, the point that offers the best balance between sensitivity and specificity (the closest point to the top left corner of the Y axis) in **Figure 8.21a** equates to a cut off NPX score of 1.3 which as a biomarker would provide a sensitivity and specificity of 71%. If we wanted to improve sensitivity, a cut off NPX score of 1.12 would provide a biomarker with 80% sensitivity but at a cost of 57% specificity. Of course these are just exploratory analysis and larger studies would need to be performed to determine what a clinically important cut off should be. As a comparison, NTproBNP, which is a clinically used biomarker for heart failure was extremely poor at discriminating between groups with an AUC of 0.596.



B.

				Asymptotic 95% Confidence Interval	
Variable	Area	Std. Error	Asymptotic Significance	Lower Bound	Upper Bound
GDF15	0.715	0.046	0.000	0.625	0.805
CHI3L1	0.658	0.049	0.001	0.563	0.753
SSC4D (Transformed)	0.648	0.047	0.002	0.556	0.740
MMP7	0.638	0.048	0.004	0.544	0.732
TNC	0.629	0.048	0.007	0.535	0.722
TFF3	0.620	0.049	0.016	0.523	0.716
COL18A1	0.615	0.047	0.014	0.524	0.707
TGFR3	0.607	0.049	0.030	0.510	0.703
ANGPTL3	0.605	0.049	0.033	0.509	0.702
NTproBNP	0.596	0.050	0.053	0.499	0.693

Figure 8.21 Receiver Operating Characteristic (ROC) Curves (A.) and table of Area Under Curve (AUC) analysis (B.) for 10 proteins used to predict cardiovascular events within 1 year. GDF15 has the highest AUC and could be a candidate biomarker.

Next, I wanted to see if a combination of proteins would be able to better discriminate groups. To do this I performed binary logistic regression. To determine candidate variables, I used the forward stepwise conditional method for candidate selection. This works by starting with a model with no variables (constant only) and adds variables in

a stepwise fashion. For the purpose of this exploratory exercise, a condition for entry was a $p < 0.05$ and removal from the model was $p > 0.1$.

This identified 2 variables for inclusion in the model, GDF15 and SSC4D **Figure 8.22**. The group probabilities were generated by the model and defined as the combined GDF15 and SSC4D score. This was compared to GDF15 in isolation **Figure 8.23**. The addition of SSC4D improved the AUC to from 0.715 to 0.75.

Variable	β Coefficient	Standard error	Odds Ratio (OR)	95% Confidence Interval for OR		p value
				Lower	Upper	
GDF15	1.507	0.39	4.514	2.103	9.69	<0.001
SSC4D	-0.419	0.143	0.657	0.497	0.870	0.003
Constant	-3.203	0.612	0.041			<0.001

Figure 8.22 Proteins associated with a cardiovascular event within 1 year using binary logistic regression. The probabilities generated by the model were used as a combined GDF15 + SSC4D score.

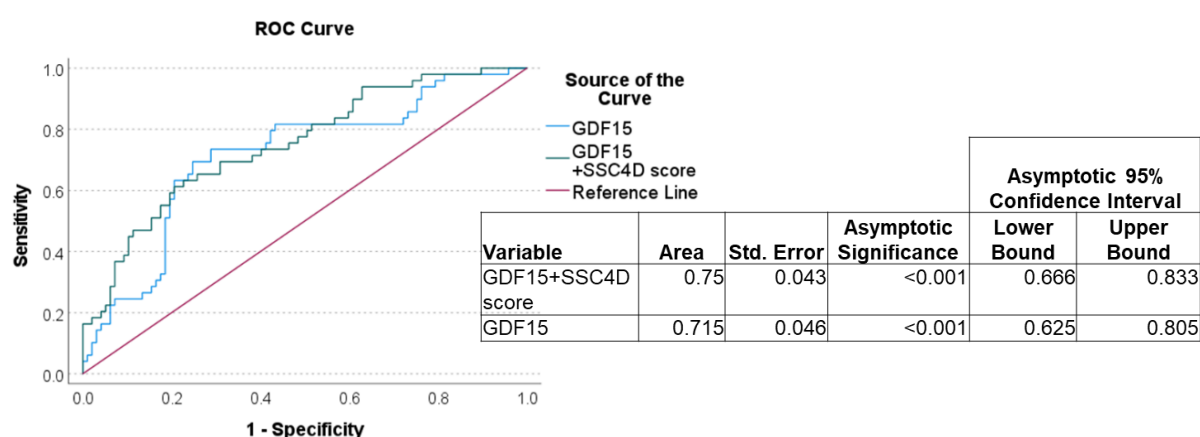


Figure 8.23 Receiver Operating Characteristic (ROC) Curves and table of Area Under Curve (AUC) analysis for GDF15 +SSC4D score to predict events within 1 year. The combined score offered better discrimination than GDF15 alone.

Next, I wanted to see if GDF15 or the GDF15+SSC4D score was useful as a biomarker in the 6-month subgroup **Figure 8.24**. GDF15 alone had an AUC of 0.768 and the addition of SSC4D increased the AUC to 0.804 which would suggest a very good discriminator in this cohort. However, due to the small sample size (n:17 events and n:34 controls), there is of course a very real possibility of overfitting a model and whilst these findings are exploratory, they suggest that GDF15 either in isolation or in

combination with other proteins has the potential to be used as a biomarker to predict cardiovascular events in NSAID users.

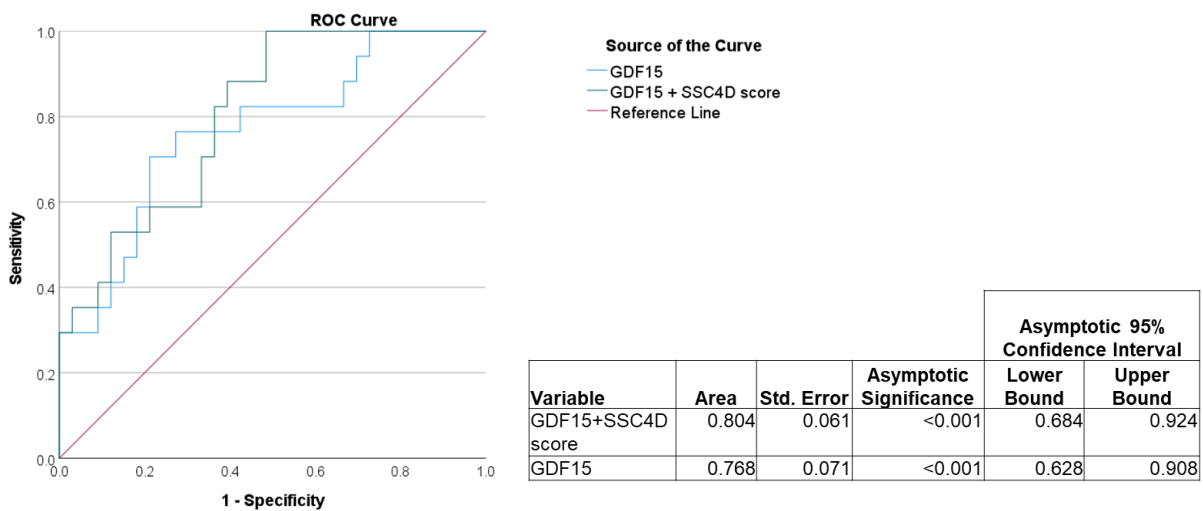


Figure 8.24 Receiver Operating Characteristic (ROC) Curves and table of Area Under Curve (AUC) analysis for GDF15 + SSC4D score to predict cardiovascular events within 6 months. Both GDF15 in isolation and in combination with SSC4D as a score demonstrate potential as a biomarker of early events.

8.3.3.1 How do these candidate biomarkers compare to clinical variables in dataset

Next, I wanted to see how these biomarkers compared to conventional clinical risk factors within the dataset. In **Chapter 6**, Using LASSO regression we identified several clinical co-variates that were associated with a cardiovascular event in the overall SCOT cohort (~7000 patients) **Figure 8.25**. The probabilities generated by this model were used as a NSAID risk score with ROC curve presented in **Figure 8.26**.

Variable	β Coefficient	S.E	OR	95% C.I. for OR		p value
				Lower	Upper	
Sex						
Female	Ref					
Male	0.57	0.156	1.77	1.303	2.404	0.0003
Smoking Status						
Non-Smoker	Ref					
Current Smoker	0.905	0.189	2.47	1.706	3.579	<0.0001
Former smoker	0.253	0.167	1.288	0.928	1.787	0.131
Systolic blood pressure at screening						
< 130mmHg	Ref					
130 – 159 mmHg	0.044	0.168	1.045	0.752	1.453	0.792
≥160mmHg	0.362	0.216	1.436	0.94	2.19	0.094
Arthritis						
Osteoarthritis	Ref					
Rheumatoid arthritis	0.478	0.252	1.613	0.985	2.642	0.058
Diabetes						
No	Ref					
Yes	0.438	0.228	1.55	0.99	2.425	0.055
Concomitant aspirin use						
No	Ref					
Yes	0.422	0.185	1.525	1.060	2.193	0.023
Total cholesterol : HDL ratio						
<5	Ref					
≥5	0.674	0.173	1.961	1.397	2.754	0.0001
Age						
60-69 years	Ref					
70-79 Years	0.499	0.152	1.647	1.223	2.219	0.001
≥80 years	1.401	0.22	4.059	2.637	6.248	<0.0001
Statin Use						
No	Ref					
Yes	-0.394	0.193	0.675	0.463	0.984	0.041
Uric acid						
Uric acid <300umol/l	Ref					
Uric acid 300 - 400umol/l	-0.085	0.162	0.919	0.669	1.262	0.601
Uric acid >400umol/l	0.078	0.192	1.082	0.742	1.576	0.683
Waist: Hip ratio						
<1	Ref					
≥1	0.051	0.183	1.053	0.736	1.506	0.779
Constant	-4.439	0.216	0.012			<0.0001

Figure 8.25 Factors associated with a cardiovascular event using binary logistic regression. The model includes all variables listed (Male gender, smoking status, systolic Blood pressure, arthritis, diabetes, waist: hip ratio, statin use, aspirin use, total cholesterol: HDL ratio, uric acid, and age. Abbreviations: SE: standard error, OR: odds ratio, CI: confidence interval, Ref: reference. Each variable is adjusted for all other variables shown. The total number of cases used in the analysis was 6,300 Controls and 226 cases (3.6%).

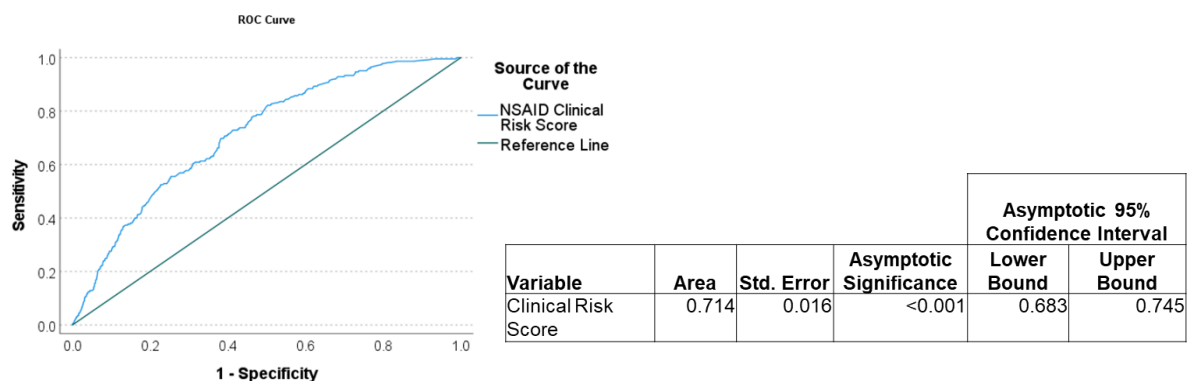


Figure 8.26 Receiver Operating Characteristic (ROC) Curve and table of Area Under Curve (AUC) analysis for clinical risk score to predict cardiovascular events in NSAID users. Clinical risk score of 11 variables was comparable to GDF15 in terms of discrimination.

Using a combination of 11 clinical variables provided an AUC of 0.714 which is comparable to GDF15 alone (AUC 0.715) in the 1-year group. As a comparison of clinical variables within the 1-year subgroup, I used total cholesterol: HDL cholesterol ratio (THRatio) as an example as this is a well-established risk factor for cardiovascular disease. The ROC curve in **Figure 8.27** shows that in isolation, THRatio is a poor discriminator (AUC .512) compared to GDF15.

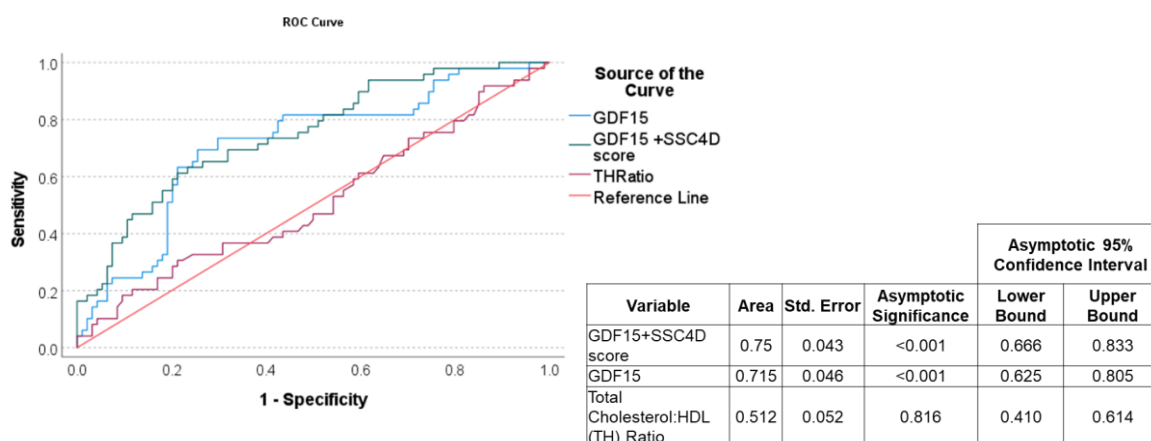


Figure 8.27 Receiver Operating Characteristic (ROC) Curve and table of Area Under Curve (AUC) analysis for clinical risk score to predict cardiovascular events in NSAID users. Total cholesterol:HDL ratio (THRatio) is poor at discriminating between events and non-events at 1 year.

8.4 Summary

The aims of this chapter were to assess serum levels of ADMA and other multi-omic analytes in osteoarthritis patients within the SCOT trial who had an ‘early’ cardiovascular event (within 1 year) and compare them with levels in serum from matched controls. This study was inspired by data found in chapter 7 (SCOT-1) but an entirely separate analysis (SCOT-2) where ‘fresh’ samples were, matched and re-measured before data analysis was performed. We found a significant increase in the cardiovascular/renal biomarker ADMA and a reduction in Arginine/ADMA ratio in the event group as well as higher levels of the renal biomarker cystatin C in the event group. This mechanistically ties in with the hypothesis that renal COX-2 is cardioprotective and suggests that renal dysfunction maybe a key driver of cardiovascular events amongst NSAID users. Future studies should assess if changes in renal function using serial measurements in NSAID users could be used to guide clinicians. From the proteomic study we identified GDF-15 as the key analyte changed (increased) in those with early events taking NSAIDs. This is a remarkable discovery, not least because GDF-15 is both a cardiovascular risk factor and is derived from a gene that is specifically induced by NSAIDs[265]. This observation, provides another novel mechanism of NSAID cardiovascular events and should be a priority for future work

In this chapter we also found several serum protein markers especially relating to ECM organisation pathways that were significantly different between the groups. Similar pathways were also seen in ASCENT **Chapter 5**. These similarities in pathways coupled with the fact that celecoxib increased AI in healthy volunteers’ points towards a key role of the ECM in NSAID cardiovascular events which is a novel finding that warrants further investigation.

8.4.1 Limitations

Whilst there are novel, and potentially important findings described in this chapter, there are several limitations that need to be taken into account.

Whilst every effort had been taken to minimise confounding, this is still a case control study from a cohort of NSAID users enrolled in a randomised controlled trial and so there maybe unknown confounders that have not been considered.

All the patients in SCOT at screening were already on an NSAID and although we know 'chronic NSAID user' was an inclusion criterion for the study, we do not have data defining exactly how long they were on the NSAID for.

Our Proteomic analysis using Olink was semi targeted towards 369 known cardiometabolic proteins. This could potentially bias any downstream pathway analysis and so should be interpreted with caution.

As this is a case control study from a larger cohort, there is a risk of overfitting when performing any regression or ROC analysis and whilst the finding that GDF15 has the potential to be a novel biomarker that could have clinical relevance is promising, these findings will need to be externally validated in a separate larger cohort.

8.4.2 Future Work

The role of inhibited renal COX-2 in cardiovascular events associated with NSAIDs has been highlighted in both animal models and in real world NSAID users who go onto have a cardiovascular event. Furthermore, we have now found a mechanistic role of the ECM and have identified GDF15 and (possibly) other proteins as potential biomarkers. In an ideal world a new prospective cohort study in which new NSAID users are enrolled and have serial serum samples taken over a defined follow up period (e.g. blood sample every 6 months for 5-10 years) would help to both validate findings and provide novel insight into how changes in renal function and biomarkers over time effect cardiovascular risk. However, this would both be costly and unpragmatic. Internal validation using the whole SCOT cohort is again not an option due to the limited sample volume and so a natural next step would be to approach the PRECISION trial biobank. As discussed in the introduction, the population for the PRECISION trial is inherently different from SCOT in that they had patients with higher cardiovascular risk. This could work in our favour as this study had more overall events which would provide a more enriched cohort however, the results may not be generalisable to patients with low to moderate risk.

Finally cell culture and animal work should be used to unpick relevance of GDF15 and the ECM in NSAID cardiovascular risk.

Chapter 9: Discussion

9.1 Discussion

The overarching aim of this thesis was to use an ‘omics’ approach to identify biomarkers that predict cardiovascular risk in individuals taking non-steroidal anti-inflammatory drugs (NSAIDs) and understand mechanistic pathways through which cyclooxygenase-2 (COX-2) protects the cardiovascular system. A summary of my key findings is provided in the **General Conclusions (9.3)**.

What is remarkable is that NSAIDs like ibuprofen were first introduced in the 1950’s but that it wasn’t until the early 2000’s that the cardiovascular risks came to light. Considering that billions of doses of NSAIDs are taken each year with an estimated 30 million people taking NSAIDs each day[39], even in the pre-2000 era, it is remarkable that an increased cardiovascular risk went unrecognised. In the Coxib and traditional NSAID trialists’ collaboration meta-analysis [39], the risk of a cardiovascular event was similar if not higher than the risk of major upper GI complications across NSAIDs (except Naproxen). Pre the 2000s, the main focus of NSAID side effects had been around reducing GI side effects and the advent of proton pump inhibitors has in some way helped to reduce these risks [266]. However, post-2000s, the main focus of concerns has been on cardiovascular risks associated with NSAID usage. Despite this, beyond a likely role of prostacyclin, there has been no real progress in our understanding of the mechanisms that cause cardiovascular side effects associated with NSAIDs usage. This lack of understanding is the limiting factor in the development of new drugs in this class. One must not forget that NSAIDs are extremely effective analgesic, anti-inflammatory, anti-pyretic and even chemo preventative in some cases so furthering our understanding of how COX-2 protects the cardiovascular system could have wide reaching implications for millions of people world-wide. Given the scale of the problem, it is surprising that little progress has been made over the last 20 years and since starting my thesis, while over 100 publications (2019-2022) return on a PubMed search using the terms ‘COX-2’ and ‘Cardiovascular Risk’, no studies have aimed to systematically address novel mechanisms using unbiased approaches.

In my thesis I set out to use ‘Omics’ approaches in healthy volunteers and in real world NSAID users who go onto have a cardiovascular event but before getting into these subtle models, I set out to explore changes in extreme human and animal models.

9.1.1 COX-2 Methylarginine Axis and Renal Function in Extreme Models

In **Chapter 3**, global loss of prostacyclin synthase (PGIS) in mice or COX-2 in mice and humans significantly increased plasma levels of the methylarginine ADMA and these changes in methylarginines correlated with the renal dysfunction marker cystatin C and urea.

These findings are in line with previously published work demonstrating that PGIS [15] or COX-2 [178, 184] deletion in mice is associated with renal dysmorphia and dysfunction.

This further adds to the growing body of evidence that COX-2 derived prostacyclin is a critical regulator of renal function increased levels of methylarginines and in particular ADMA are linked to renal homeostasis. These findings support the notion that the kidneys could play a key role in NSAID cardiovascular side effects. Previous work from the group identified ADMA as a novel explanation for NSAID cardiovascular side effects and so in the following chapters I sought to explore the role of ADMA amongst other multi-omic markers in COX-2 inhibition in more subtle models.

9.1.2 Effects of Celecoxib on Endothelial Function in Healthy Volunteers

In chapter 4 I set out to assess the effects of COX-2 inhibition on endothelial function and augmentation index (AI), blood pressure and cardiovascular & renal function in healthy volunteers in a randomised controlled trial. As previously described in **Chapter 1**, the effects of NSAIDs on endothelial function appear to be inconsistent with studies reporting an increase, decrease or no difference. This in part could be due to the heterogeneity of the studies in terms of interventions (drug, dose and duration), population and outcome assessment (e.g. ultrasound flow mediated dilatation which is operator dependant). The EndoPAT device which is operator independent, and FDA approved (for the hyperaemic readout) had not been used in this setting before. Furthermore, there was an unmet need to fully understand the effect of blocking COX-2 on endothelial function in the context of a complete assessment of multi-omic readouts. In our study, I found that after 7 days of treatment with 200mg BD celecoxib, there was no difference in reactive hyperaemia index compared to placebo. We did however find that celecoxib increased AI assessed by pulse wave analysis (PWA).

This was surprising as increased AI is a surrogate for vascular stiffness which has been reported to be an independent risk factor for cardiovascular risk. For example, in a study of 4,561 participants from the Copenhagen City Heart Study, increased AI was associated with an increase in cardiovascular risk score [267]. Whilst AI measured by EndoPAT is not FDA approved for predicting cardiovascular risk, there have been at least 10 publications that have associated EndoPAT AI in cardiovascular disease [268-276].

To date there have been no randomised controlled trials that have assessed the effects of NSAIDs on AI. There are however two observational studies that have hinted at associations between NSAID use and vascular stiffness.

In a cross sectional study of 60 users of non-selective NSAIDs and 25 non users with rheumatoid arthritis, chronic diclofenac use and the level of aldosterone glucuronidation inhibition was associated with higher AI [158]. In a study of 122 Ehlers-Danlos syndrome patients, univariate regression suggested that NSAID use maybe associated with increased AI (Coefficient 7 (95% CI -0.07 to 14.19; p:0.052)). Of course there are several limitations in interpreting these small observational subgroup analysis but none the less their findings fit with my results.

Given that there is limited literature in this area, our finding of celecoxib increasing AI in healthy volunteers in a blinded, placebo-controlled trial provides a novel mechanism of NSAID cardiovascular events and should be validated and explored as an area of priority in this field.

9.1.3 Effects of Celecoxib on Cardiovascular Biomarkers in Healthy Volunteers

As previously described, there was an unmet need to fully understand the effect of blocking COX-2 on endothelial function in the context of a complete assessment of multi-omic readouts to determine associations with unappreciated signalling pathways involved in vascular protection of COX-2. In **Chapter 5** I addressed this knowledge gap using a multi-omics approach as part of the ASCENT study. I found significantly reduced arginine/ADMA ratio in the celecoxib group compared to placebo. This fits with the findings in extreme human and animal models in **Chapter 3** that described

the role of COX-2 methylarginine axis and renal function in NSAID cardiovascular events. Furthermore, as well as being an independent risk factor for cardiovascular risk and all-cause mortality, increased ADMA has also been shown to increase arterial stiffness [277, 278] which mechanistically fits with our findings.

It is well established that NSAIDs can cause renal dysfunction but within our study of healthy volunteers, there was no difference in the renal function markers creatinine, urea, GFR or cystatin C which is probably attributable to our population of young healthy participants. There was however a reduction in uromodulin and renin which suggests there could be subclinical effects occurring in the kidneys. What is also interesting is that SDMA which is a far more sensitive renal biomarker than ADMA was not changed between the groups. This could suggest that the decrease in arginine:ADMA ratio in the celecoxib group was driven by changes in metabolic factors rather than a reduction in renal filtration. Further studies focusing on the effects of blocking/knocking out COX-2 on tissue specific levels of methylarginines could help to uncover the mechanism behind reduced arginine:ADMA ratio. These findings also fit with previous work from my group, showing that ADMA, but not SDMA, was increased in healthy male volunteers taking celecoxib. There were, however, some differences. In the earlier study, which was not placebo controlled, creatinine and ADMA were increased, while in my study, which was a follow-on including a placebo arm, arginine:ADMA ratio was decreased (without significant changes in renal function markers) but ADMA *per say* was not affected.

From our proteomic and mass spectrometry amine analysis, I identified differences in analytes relating to extracellular matrix (ECM) organisation and in particular increase in serum collagen markers (COL1A1, COL6A3 and 4-hydroxyproline) and a reduction in MFAP3 which is involved in elastin formation in healthy volunteers taking celecoxib. Mechanistically, this again fits with the finding of increased AI with celecoxib use and mechanistically provides a novel role of ECM in NSAID cardiovascular events. However, it is important to remember that as this was a biomarker discovery exercise where no proteins had $Q\text{-value} < 0.05$, these markers will need to be validated using specific analyte platforms, in future studies.

9.1.4 Clinical Predictors of Cardiovascular Events Amongst NSAID Users.

Whilst it is well established that NSAIDs increase cardiovascular risk generally, the actual degree of risk for an individual patient calculated along with other risk factors, remains largely unknown. Recently in 2019, the PRCISION trialists attempted to develop an NSAID major toxicity risk score [279]. However, this was in a high cardiovascular risk group, and they used a composite of both cardiovascular and renal events and so results may not be generalisable. It is surprising, therefore, that mine was the first attempt to identify clinical drivers of cardiovascular events in a lower risk cohort given the widespread use of NSAIDs and potential scale of the problem. In **Chapter 6** using LASSO regression we identified 11 clinical variables as part of a risk prediction model and in **Chapter 8** showed that this model has an AUC of 0.714 which suggests that there is the potential for the clinical use of risk prediction tools in this population.

9.1.4.1 Aspirin use Amongst NSAID users, Friend or Foe?

Whilst the benefits of aspirin in secondary prevention of cardiovascular events is well established, the FDA have stated there is a lack of evidence to support its use as a primary preventative agent in the general population where the risks of bleeding may outweigh any potential benefits[280].

As described in **Chapter 6**, concomitant aspirin use was associated with an increased likelihood of cardiovascular events compared to non-users (OR 1.5; 95% CI 1.1 to 2.2). Whilst the adjusted risk of a cardiovascular event was non-significant (HR 1.3. 95%CI 0.9 to 1.9) the direction of effect was consistent. Although this might be considered an “unusual” finding given the cardioprotective effect of aspirin, I believe the most likely explanation for this is protopathic bias. Given the inclusion criteria for enrolment into the SCOT trial was “low cardiovascular risk”, patients initiated on aspirin may inherently have a higher cardiovascular risk although the exact reason for aspirin use was unknown to me.

To my knowledge, there are no randomised trials investigating the role of aspirin as a primary preventative agent in chronic NSAID users however, a secondary analysis of the PRECISION study [281] found that aspirin exposure amongst rheumatoid arthritis patients with chronic NSAID use demonstrated no significant increase in NSAID gastric or renal toxicity (HR 1.08, 95% CI 0.69 to 1.69) or the risk of major adverse cardiac events (HR 1.23, 95% CI: 0.72 to 2.10). Furthermore a systematic review by Alqahtani et al in 2018 identified 20 molecular interaction studies and 12 clinical observational studies and concluded that aspirin appears to maintain its cardioprotective effectiveness in the presence of naproxen, ibuprofen, meloxicam and rofecoxib with conflicting data on in vitro/ in vivo/ ex vivo platelet aggregation studies[282]. Given the low quality of the available evidence (no randomised trials) there remains a level of uncertainty surrounding aspirin use with NSAIDs and cardiovascular events. This will be addressed as part of the future SAMURAI study.

9.1.4.2 Statin Use

Interestingly in my analysis in **Chapter 6**, the odds of a cardiovascular event were significantly lower in statin users compared to non-users (OR 0.68; 95% CI 0.46 to 0.98). This finding was also seen in Cox regression, even after adjustment for cardiovascular risk factors and cholesterol (HR 0.7, 95%CI 0.47 to 0.98, p:0.04).

There are currently no studies that have investigated the potential cardioprotective benefits of statins amongst chronic NSAID users in a clinical trial. Trial of Atorvastatin for the Primary Prevention of Cardiovascular Events in Patients with Rheumatoid Arthritis (TRACE RA)[283] was a randomised, double-blind placebo-controlled study. It found a reduction in cardiovascular events with statin use (HR 0.66, 95% CI 0.39 to 1.11) although this was not statistically significant as the study was underpowered primarily due to a low event rate which led to premature termination of the study and did not explore the cardiovascular risk amongst NSAID users.

Statins are cholesterol lowering drugs which work by competitively inhibiting HMG-CoA reductase. However, the protective effect of statins on cardiovascular events in NSAID users was not simply explained by adjusting for cholesterol:HDL ratios. This suggests one or more of the pleiotropic effects of statins in cardiovascular protection which

include protection of the endothelium[284] and a direct inhibition of platelet activation[285], may function to directly mitigate the increased risk of cardiovascular events associated with NSAID usage. Furthermore, there are now a number of studies including a meta-analysis of randomised trials suggesting statins have a beneficial effect on vascular stiffness [286].

Thus our finding in my thesis that the cardio-protective effects of statins are not mitigated by NSAIDs suggests that their use should be explored as a potential primary preventative therapy in patients requiring long term NSAID therapy, irrespective of cholesterol levels. Whilst a blanket policy of statin prescription amongst NSAID users is probably not the solution, the development of a personalised risk prediction tool may allow for a more targeted approach which could be cost effective and have an increased risk/benefit ratio for this group of patients.

9.1.4.3 Blood Pressure

The effect of NSAIDs on blood pressure elevation is well established, particularly in those with compromised renal function and is due to a combination of mechanisms relating to prostaglandin inhibition, vasoconstriction and salt and water retention[287]. Numerous studies have demonstrated that NSAID use can increase blood pressure in both normotensive and hypertensive patients and varies according to the type and dose of NSAID [51, 288-291]. In arthritis patients with a high cardiovascular risk, the PRECISION ABPM study[292] demonstrated that allocation to the non-selective COX-2 inhibitor ibuprofen was associated with a significant increase in systolic blood pressure and a higher incidence of new onset hypertension when compared to the selective COX-2 inhibitor celecoxib.

The UK NICE guidelines recommend that etoricoxib and high-dose ibuprofen should be avoided in patients with a blood pressure persistently above 140/90 [293]. Furthermore, the European hypertension guidelines highlight NSAIDs as a cause of secondary hypertension which can also antagonise the blood pressure lowering effects of antihypertensive medications leading to a loss of blood pressure control [294].

In **Chapter 6** I found there was an increased risk of cardiovascular events in patients who had a systolic BP of ≥ 160 mmHg (HR 1.46 95%CI 1 to 2.1). If blood pressure is indeed a key driver of cardiovascular events amongst NSAID users, these patients may require stricter blood pressure control, lower threshold for initiation of anti-hypertensives, referral to a blood pressure specialist or discontinuation of the NSAID.

9.1.4.4 Risk Stratification

Clearly there are significant knowledge gaps and areas of uncertainty in this field that warrant further investigation. The development of cardiovascular risk prediction tools in this population could potentially address some of these uncertainties which could:

- 1) allow clinicians and patients to make a more informed choice when commencing NSAIDs
- 2) facilitate the re-introduction of celecoxib as a chemo preventative agent
- 3) allow for the targeted use of primary preventative drugs like statins
- 4) identify clinical drivers of cardiovascular events which may provide mechanistic insight into how COX-2 protects the cardiovascular system.

Future work in the SAMURAI study will help to try answer some of these uncertainties.

9.1.5 Biomarkers of NSAID Cardiovascular Events

In **chapter 7**, I sought to identify biomarkers that predict cardiovascular events in NSAID users. In the SCOT-1 analysis (where all events were included regardless of time to event from sample collection), there were no standout markers when comparing all events vs controls but there appeared to be differences in renal markers closer to the time of the event. This prompted the validation study in **Chapter 8** (SCOT-2) which focused on biomarkers in participants who had an “early” event (within 1 year).

Interestingly I found an increase in ADMA and reduction in arginine/ADMA ratio as well as higher cystatin C in the event group. This again ties in mechanistically with the findings in the extreme models in **Chapters 3**. One could argue, for example that ADMA is a well-known marker for cardiovascular events and so may be non-specific. Our findings in **Chapter 5** that arginine/ADMA is reduced after just 7 days of celecoxib in healthy volunteers adds further weight to the hypothesis that ADMA

could be a key mechanistic driving force behind NSAID cardiovascular side effects. The finding of higher cystatin C suggests that renal dysfunction could also be a driver of cardiovascular events. Future studies should assess if changes in renal function using serial measurements in NSAID users could be used to guide clinicians

Using proteomics, I also found that GDF-15 was significantly increased in those with early events even after FDR correction. Furthermore, GDF-15 was shown to be a potential candidate biomarker to predict events at 1 year (AUC 0.715) or 6 months (AUC 0.77). What was truly remarkable is that GDF-15 is also known as non-steroidal anti-inflammatory drug activated gene-1 (NAG-1). In 2001, Baek and colleagues found that NSAIDs stimulated expression of a TGF- β superfamily protein in colorectal cells that they called NAG-1[265]. NAG-1 has a broad range of roles in both inflammation and malignancy. Numerous studies have shown the NAG-1/GDF-15 are drivers of apoptosis and cell growth arrest and have hypothesised this as a mechanism for the chemo preventative effects of NSAIDs [265]. Under physiological conditions in humans, GDF-15 is barely expressed in tissues (except the placenta), however, under pathological cardiovascular conditions, GDF-15 is produced by numerous cell types but with differing roles [265]. In acute MI models in mice, GDF-15 has anti-inflammatory effects [295] whereas in models of atherosclerosis, GDF-15 appears to have pro-inflammatory effects [296, 297].

It has now become apparent that GDF-15 is also expressed in response to oxidative stress, hypoxia and inflammation and cardiovascular disease has been suggested to be a major driver of increased GDF-15[298]. Furthermore, GDF-15 has been investigated as a cardiovascular biomarker in over 20 clinical studies [298]. Given that GDF-15 is a biomarker of cardiovascular disease and is also induced by NSAIDs, this should be a priority area for future work.

9.2 Priority Areas for Future Research

The three key areas for future studies are

- I. Validating GDF-15 as a biomarker and investigating its mechanistic role in NSAID cardiovascular events
- II. Exploring the effects of NSAIDs on the ECM and vascular stiffness
- III. Developing a clinical risk stratification tool for NSAID users (SAMURAI)

Due to time restraints, a limitation of my thesis was the validation of the biomarker GDF-15. In order to translate this into clinical care, it now needs to be externally validated in a larger cohort such a PRECISION biobank[243]. The finding of increased vascular stiffness with NSAID use was unexpected and as AI was not our primary outcome, this now needs to be validated in a follow-on study using additional measures of vascular stiffness such as magnetic resonance imaging and echocardiography. Finally the development of a risk prediction tool could help clinicians to better risk stratify NSAID users, the future SAMURAI study previously described will address this.

9.3 General Conclusions

Despite their widespread use, we do not fully understand how NSAIDs increase cardiovascular risk and in this thesis, I set out to use a fresh multi-omics approach to try and answer this age-old question. As part of a clinical trial (ASCENT), NSAID use was associated with a significant increase in AI (vascular stiffness) in healthy volunteers and this was linked to changes in serum protein markers relating to ECM as well a reduction in arginine/ADMA ratio. Furthermore, similar changes were also seen in serum samples from real world NSAID users who go onto have a cardiovascular event within 1 year (SCOT-2).

In extreme animal and human models of COX-2 deletion, changes in plasma methylarginines were associated with changes in renal biomarkers. Renal dysfunction was also seen in NSAID users who go onto have cardiovascular events (SCOT-2) supporting the notion that COX-2 in the kidneys is a site for cardio protection.

Finally GDF-15 was identified as a candidate biomarker to predict NSAID cardiovascular events. What makes this a remarkable discovery is that GDF-15 is induced by NSAIDs providing a novel mechanism for NSAID cardiovascular events.

Overall, using a multi-omics approach has provided novel mechanistic insight into how COX-2 protects the cardiovascular system and has highlighted several key areas for future research. Whilst there are several limitations as this was a discovery exercise, validating and translating these findings into clinical care could help to prevent unwarranted cardiovascular events in millions of people worldwide.

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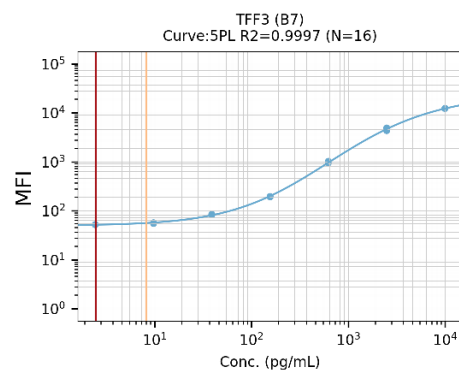
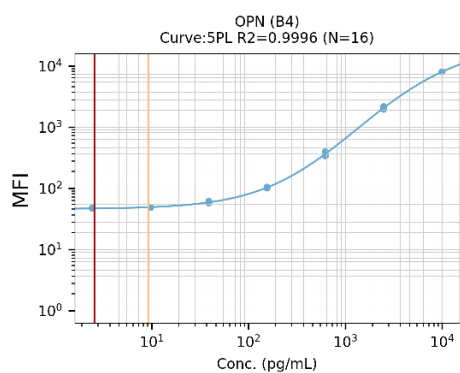
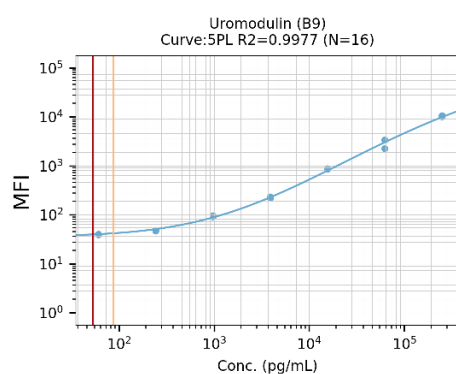
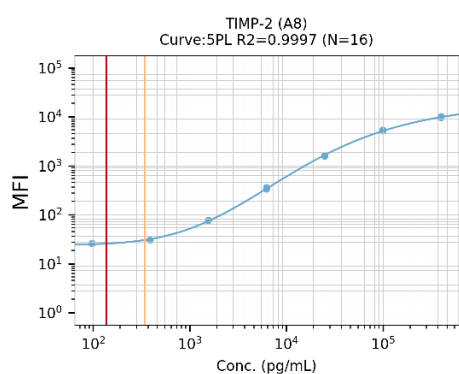
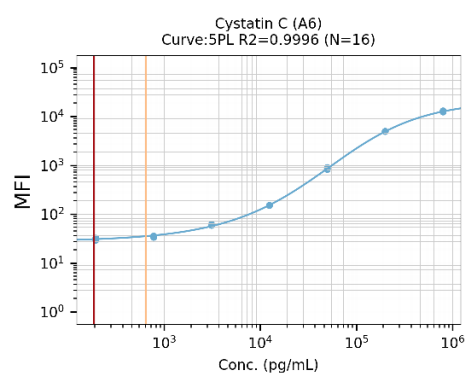
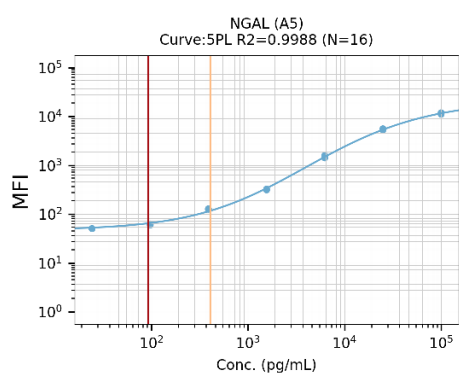
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Appendix 1- Example Standard Curves

Multiplex Assay

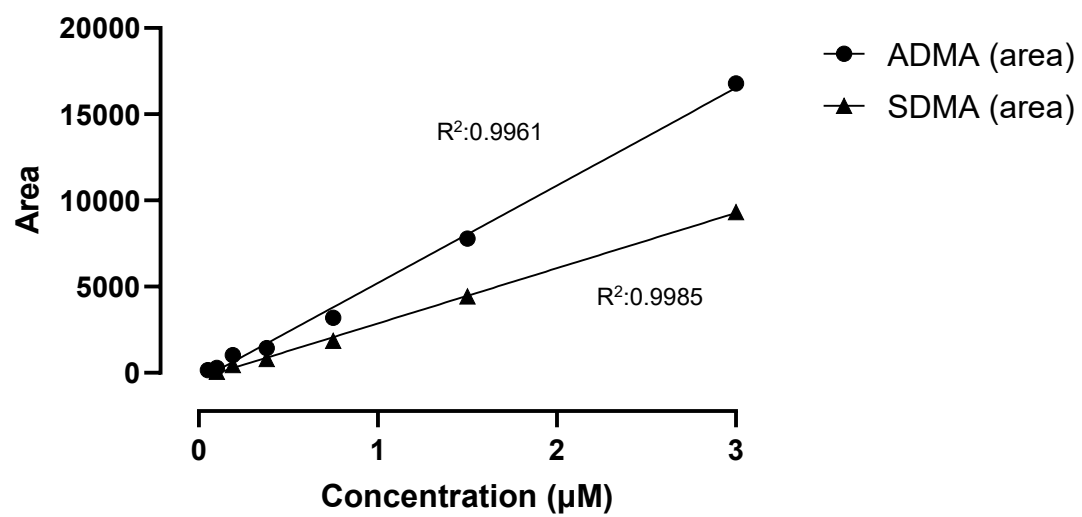


Appendix 2- List of Proteins Measured using Olink

Protein	Uniprot ID	Protein	Uniprot ID	Protein	Uniprot ID	Protein	Uniprot ID	Protein	Uniprot ID
NPPB	P16860	COMT	P21964	AK1	P00568	IL19	Q9UHD0	THOP1	P52888
TNNI3	P19429	MMP7	P09237	ANXA4	P09525	ACAN	P16112	GLRX	P35754
HNRNPK	P61978	PON2	Q15165	ACOX1	Q15067	PLXNB3	Q9ULL4	KITLG	P21583
CEBPB	P17676	ADAMTS16	Q8TE57	NTproBNP	NTproBNP	TYRO3	Q06418	PILRB	Q9UKJ0
VIM	P08670	GP2	P55259	CLC	Q05315	EIF4EBP1	Q13541	CLUL1	Q15846
CHEK2	O96017	WASF1	Q92558	CNST	Q6PJW8	PDCD6	O75340	AHCY	P23526
CRHR1	P34998	MPHOSPH8	Q99549	LEPR	P48357	APLP1	P51693	FABP2	P12104
TSLP	Q969D9	DNAJB8	Q8NHS0	TSHB	P01222	CLEC5A	Q9NY25	REN	P00797
MFAP3	P55082	SLITRK6	Q9H5Y7	S100A11	P31949	GSTA1	P08263	GDF2	Q9UK05
TCL1B	O95988	GPR37	O15354	VSIR	Q9H7M9	IGSF8	Q969P0	RNASE3	P12724
DUOX2	Q9NRD8	GZMH	P20718	PCDH17	O14917	MEP1B	Q16820	SOST	Q9BQB4
CTF1	Q16619	GYS1	P13807	PDGFRA	P16234	SDC1	P18827	NPDC1	Q9NQX5
AKR1C4	P17516	THPO	P40225	CDH17	Q12864	CDH2	P19022	CD69	Q07108
HEBP1	Q9NRV9	CLEC1A	Q8NC01	SNX9	Q9Y5X1	MCFD2	Q8NI22	CXCL5	P42830
SERPINB5	P36952	ENTPD6	O75354	NTRK2	Q16620	FCRL1	Q96LA6	COL4A1	P02462
HK2	P52789	IL6	P05231	ACY1	Q03154	GPNMB	Q14956	LILRA5	A6NI73
GRK5	P34947	CEACAM8	P31997	DOK2	O60496	QDPR	P09417	MARCO	Q9UEW3
VSTM2L	Q96N03	S100P	P25815	AGXT	P21549	GHRL	Q9UBU3	ANGPTL1	O95841
TIA1	P31483	ENTPD5	O75356	STK4	Q13043	PPP1R2	P41236	PRSS27	Q9BQR3
CNPY2	Q9Y2B0	ACE2	Q9BYF1	TGM2	P21980	CD2AP	Q9Y5K6	SORT1	Q99523
CRX	O43186	DCN	P07585	DPP7	Q9UHL4	NADK	O95544	SFTPD	P35247
VAMP5	O95183	GLO1	Q04760	PLIN3	O60664	CBLIF	P27352	DDC	P20711
SUSD1	Q6UWL2	PAG1	Q9NWO8	PLPBP	O94903	TINAGL1	Q9GZM7	SDC4	P31431
RCOR1	Q9UKL0	ADAM15	Q13444	ICAM5	Q9UMF0	CANT1	Q8WVQ1	HMOX1	P09601
TNF	P01375	USP8	P40818	PRKAR1A	P10644	SSC4D	Q8WTU2	SNAP23	O00161
CA5A	P35218	BMP6	P22004	IL6ST	P40189	OLR1	P78380	SERPINA1	Q8IW75
FABP6	P51161	ITGB1BP2	Q9UKP3	DCTPP1	Q9H773	DIABLO	Q9NR28	GH1	P01241
STK11	Q15831	CTSH	P09668	PM20D1	Q6GTS8	LRP11	Q86VZ4	SPON2	Q9BUD6
TSPAN1	O60635	BAG6	P46379	LACTB2	Q53H82	SEMA3F	Q13275	NECTIN2	Q92692
ACTA2	P62736	MSTN	O14793	FADD	Q13158	LEP	P41159	CHRD2	Q6WN34
MTPN	P58546	BOC	Q9BWV1	CGREF1	Q99674	LPL	P06858	ROR1	Q01973
EDIL3	O43854	ADH4	P08319	CXCL8	P10145	ADGRG2	Q8IZP9	CA13	Q8N1Q1
ZBTB17	Q13105	CLTA	P09496	LGALS1	P09382	SIGLEC7	Q9Y286	CCDC80	Q76M96
PTN	P21246	EPHX2	P34913	IGFBPL1	Q8WX77	NPTXR	O95502	FBP1	P09467
CEP43	O95684	TP53INP1	Q96A56	MNDA	P41218	GRAP2	O75791	CTSL	P07711
IRAG2	Q12912	CCL27	Q9Y4X3	CDH6	P55285	CDHR5	Q9HBB8	NRCAM	Q92823
APOM	O95445	CD14	P08571	FCN2	Q15485	SOD1	P00441	NRP1	O14786
TIMD4	Q96H15	SELP	P16109	HSPG2	P98160	FCGR3B	O75015	FCGR2A	P12318
ITGB1	P05556	CD46	P15529	PCOLCE	Q15113	TFPI	P10646	LCN2	P80188
CD55	P08174	GP1BA	P07359	IL6R	P08887	AOC3	Q16853	CR2	P20023
ICAM1	P05362	CCL5	P13501	IGFBP6	P24592	EFEMP1	Q12805	REG1A	P05451

Protein	Uniprot ID	Protein	Uniprot ID	Protein	Uniprot ID	Protein	Uniprot ID
HSPB1	P04792	REG1B	P48304	ST6GAL1	P15907	PGLYRP1	O75594
THBD	P07204	IL2RA	P01589	TIE1	P35590	CD59	P13987
KYAT1	Q16773	PDGFRB	P09619	SIRPA	P78324	AMY2B	P19961
FAM3C	Q92520	MET	P08581	CASP3	P42574	TCN2	P20062
TYMP	P19971	ESAM	Q96AP7	PTPRS	Q13332	SERPINE1	P05121
PCSK9	Q8NBP7	CA3	P07451	CNTN1	Q12860	MCAM	P43121
IGFBP7	Q16270	TNFSF13B	Q9Y275	BPIFB1	Q8TDL5	DEFA1_DE	P59665
UMOD	P07911	ALCAM	Q13740	AZU1	P20160	VASN	Q6EMK4
PLTP	P55058	PLA2G2A	P14555	PTPRF	P10586	ART3	Q13508
MFAP5	Q13361	MSMB	P08118	NOTCH1	P46531	CNDP1	Q96KN2
LDLR	P01130	CTSB	P07858	CHIT1	Q13231	IL18BP	O95998
CA4	P22748	ICAM3	P32942	PDGFA	P04085	F9	P00740
EPHB4	P54760	DPT	Q07507	ACP5	P13686	ANPEP	P15144
CDH5	P33151	CRTAC1	Q9NQ79	ITGB2	P05107	SSC5D	A1L4H1
CES1	P23141	CPA1	P15085	FAS	P25445	ITIH3	Q06033
FABP4	P15090	SERPINA1	Q86U17	PLXNB2	O15031	CDH1	P12830
IGFBP1	P08833	CXCL16	Q9H2A7	GAS6	Q14393	NCAM1	P13591
PLAT	P00750	SEMA7A	O75326	EGFR	P00533	LBP	P18428
ICAM2	P13598	LGALS3	P17931	QPCT	Q16769	OSMR	Q99650
ADAMTS1	Q76LX8	MEGF9	Q9H1U4	RNASET2	O00584	HYAL1	Q12794
IL1RL1	Q01638	CORO1A	P31146	SPP1	P10451	CTSD	P07339
GDF15	Q99988	ENG	P17813	LILRB1	Q8NHL6	TNC	P24821
PLA2G1B	P04054	ENPP2	Q13822	LILRB5	O75023	CD163	Q86VB7
NOTCH3	Q9UM47	CPB1	P15086	IGFBP2	P18065	SPARCL1	Q14515
LTBP2	Q14767	SELE	P16581	PI3	P19957	NID1	P14543
TGFBR3	Q03167	PAM	P19021	RETN	Q9HD89	AXL	P30530
VWF	P04275	COL6A3	P12111	CCL15	Q16663	PRSS2	P07478
PEAR1	Q5VY43	CST6	Q15828	PRTN3	P24158	CTSZ	Q9UBR2
REG3A	Q06141	CSTB	P04080	FAP	Q12884	CHL1	O00533
XG	P55808	DLK1	P80370	PPIB	P23284	C1QTNF1	Q9BXJ1
ADGRE5	P48960	GUSB	P08236	COL18A1	P39060	THBS4	P35443
FUCA1	P04066	SCARF1	Q14162	AMY2A	P04746	TFRC	P02786
CD209	Q9NNX6	CELA3A	P09093	CCL16	O15467	KIT	P10721
MB	P02144	CCN3	P48745	COL1A1	P02452	TFF3	Q07654
DKK3	Q9UBP4	HYOU1	Q9Y4L1	BLMH	Q13867	F7	P08709
TNFRSF10	O14798	LILRB2	Q8N423	PRCP	P42785	RARRES2	Q99969
ADA2	Q9NZK5	IGFBP3	P17936	PROC	P04070	DPP4	P27487
CCL18	P55774	CHI3L1	P36222	ANG	P03950	ANGPTL3	Q9Y5C1
VCAM1	P19320	CST3	P01034	FETUB	Q9UGM5	PTGDS	P41222
CD93	Q9NPY3	CCL14	Q16627	COMP	P49747	CA1	P00915
GGH	Q92820	TGFBI	Q15582	TIMP1	P01033	C2	P06681

Appendix 3- Example Standard Curves for ADMA and SDMA Measured using UHPLC/MS/MS



Appendix 4- SAMURAI Study Protocol

Statin use to mitigate the unwarranted cardiovascular side effects of non-steroidal anti-inflammatory drugs: A population-based case control study.

SAMURAI

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Information on Data

Primary care data- CPRD Aurum

Patient level data- HES Admitted Patient Care, ONS Death Registration Data

Area Level data- Patient Level of Index of Multiple Deprivation

Lay Summary

Non-steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen are some of the most commonly taken drugs around with around 20% of people in the UK taking them at any one time. NSAIDs are good at treating inflammation and pain and can prevent some cancers but they increase a person's chance of having a heart attack or a stroke by as much as 30%. It is surprising that given the scale of use, we currently do not know:

- 1) If NSAID use is an independent risk factor of cardiovascular disease.
- 2) Enough about what groups of patients have the highest risk.
- 3) If there are any treatments to reduce the risk.

We want to therefore conduct a large population based study to try and address these questions in patients in routine clinical care. We hope that this study will give researchers and

clinicians around the world a better understanding of what groups of patients are at the highest risk and highlight potential treatment options to reduce this risk.

Technical summary

Non-steroidal anti-inflammatory drugs (NSAIDs) which include ibuprofen, naproxen and celecoxib, are amongst the most commonly used medications world-wide to treat fever, pain and inflammation. With the exception of aspirin, all NSAIDs carry a significant risk of heart attacks and strokes which has led to increased anxiety amongst physicians and patients and the withdrawal of celecoxib as a preventive treatment for colon cancer.

Despite the widespread use of NSAIDs, we currently do not know:

- 1) If NSAID use is an independent risk factor of cardiovascular disease.
- 2) Enough about what groups of patients have the highest risk.
- 3) If there are any treatments to reduce the risk.

Our objective is to identify risk factors that might predict cardiovascular events in patients who take NSAIDs. We want to assess whether cardio-protective drugs including statins or aspirin may be protective against additional cardiovascular disease associated with NSAID use. Our study will be split into three stages:

Stage 1: To identify whether NSAID use is an independent risk factor for cardiovascular disease we will use a matched case control study to identify predictors of cardiovascular disease (myocardial infarctions and strokes (excluding TIAs) using conditional logistic regression.

Stage 2: To identify what group of NSAID users are at the highest risk of a cardiovascular event we will identify risk factors for myocardial infarction or stroke in NSAID users using time to event data. We will use cox-regression. We hope this could allow clinicians to risk stratify patients who require longer-term NSAID use.

Stage 3: We will assess if aspirin or statin use as a secondary preventative measure can offset the additional cardiovascular risk associated with NSAID use, again using Cox regression.

Outcomes to be measured

Myocardial infarction which will include STEMI, NSTEMI and Acute coronary syndromes as a composite outcome and if numbers allow individually.

Stroke which will include ischaemic and haemorrhagic but not TIA given the inaccuracy of recording of TIA events. Codes will be used according to the stroke validation study undertaken (Morgan et al Br J GP 2020).

We will identify these outcomes in HES and ONS so death by these causes will be included. If numbers allow, we will look at mortality separately as an outcome.

Objectives, specific aims and rationale

Our study has three objectives.

Our primary objective is to assess if NSAID use is an independent risk factor for cardiovascular disease

Our secondary objective is to identify clinical risk factors that may predict the risk of having a cardiovascular event in patients taking NSAIDs.

Our tertiary objective is to determine if aspirin or statin use can offset any additional cardiovascular risk seen with NSAID use.

Given the scale of NSAID use and the uncertainties relating to the increased cardiovascular risk, we hope this work will allow clinicians to risk stratify NSAID users who maybe at the highest risk of cardiovascular disease and explore any potential benefits of aspirin and or statins as a secondary preventative measure in this cohort.

Study background

The scale of use of NSAIDs (estimated to be regularly taken by 20-30% of the population) and the degree of associated risk (increased by 30%) suggests that these drugs are a significant risk factor for heart attacks and strokes in their own right, potentially accounting for up to 50,000 of the 458,000 heart attacks and strokes in the UK each year.(1-4) Moreover, we now know that this risk emerges with just 1-2 weeks of use.(5) It was initially thought that cardiovascular side effects were limited to drugs that selectively target COX-2 such as rofecoxib and celecoxib. However, as a result of subsequent epidemiology analyses (6,7) and,

most recently, the publication of two large clinical cardiovascular outcome studies, SCOT(8) and PRECISION(9), it is clear that traditional NSAIDs, including ibuprofen and naproxen, carry at least as great a cardiovascular risk as COX-2 selective drugs. This is particularly concerning since ibuprofen is still available over the counter.

Concern over adverse cardiovascular events caused by NSAIDs has led to cautious prescribing of COX-2 selective NSAIDs. However, the relative contribution of NSAIDs as a class of drugs, the specific drug used and duration of use as independent risk factors for cardiovascular events is not known. Thus, despite more than 15 years of research in the area we still do not fully understand:

- 1) If NSAID use is an independent risk factor of cardiovascular disease.
- 2) Enough about what groups of patients have the highest risk.
- 3) If there any treatments to reduce the risk.

Study type

Hypothesis testing

Study design

Case control study for objective 1 and a population-based cohort study for objectives 2 and 3

Feasibility counts

Our preliminary analyses revealed approximately 244,280 heart failure (HF) patients with at least one NSAID prescription in the CPRD-Aurum between 1/1/2008 and 31/12/2019. We expect that approximately around 60% would be eligible for HES linkage. Since the prevalence of HF in the UK is very similar to that of MI and stroke, we consider these figures as a reliable measure of the expected sample size.

Therefore, the sample size will be sufficient to undertake a meaningful analysis as proposed within this protocol.

Sample size considerations

To assess the feasibility of undertaking this study, we have obtained an approximate count of N=119 as the recommended sample size for the NSAIDs non-users group, taking into account the following parameters:

Relative precision: 50%

Confidence interval: 95%

Expected prevalence of the outcome in the controls group: 15%

Expected odds ratio: 1.2

Case-controls ratio: 1

Recommended sample size for the controls group: 119

We have also calculated alternative scenarios by changing all the above parameters (i.e. relative precision from 10% to 90%; confidence level from 90% to 99%; expected prevalence of the outcome in controls from 5% to 25%; expected odds ratio from 1.0 to 2.5; cases-controls ratio from 0.5 to 2.5) obtaining a minimum recommended sample size of 11 (with a relative precision of 90%) and a maximum recommended sample size of 5,114 (with a relative precision of 10%).

Based on our preliminary estimates, we conclude that, even in the worst-case scenario, we will have a sufficient sample size to undertake a meaningful analysis as proposed within this protocol.

Planned use of linked data and/or withheld concepts

HES APC - to identify MI and stroke

ONS - to identify MI and stroke

IMD - to adjust for SES in the model

The outputs generated from this study will have a significant impact on a large number of patients in England. It is estimated that NSAIDs are used by approximately 30% of the population and can increase the risk of a cardiovascular event by up to 30%. Given that they are readily available over the counter they could potentially account for tens of thousands of heart attacks and strokes each year in the UK alone. NSAIDs are extremely effective anti-inflammatory drugs are vital for patients with chronic pain and inflammatory disease.

This study will Investigate if NSAID use should be considered an independent risk factor for cardiovascular disease and what groups of patients are at the most risk. This could allow healthcare professionals to risk stratify patients prior to commencing longer term NSAID therapy which could reduce the number of NSAID related cardiovascular events.

This study will also investigate whether concomitant treatment with statins +/- aspirin can mitigate the additional risk and may allow patients who rely on NSAIDs for chronic pain to continue using them whilst minimising risk.

The results from this study could give us insight into some of the key mechanistic drivers of cardiovascular events in this cohort. Follow on mechanistic studies could allow for new drug discovery.

Finally, although this study will primarily be applicable to the UK population, the findings could be applicable to patients worldwide.

Definition of the study population

The minimum eligibility criteria for inclusion is as follows:

- Acceptable patient status in CPRD
- Registration with GP for minimum of 1 year before study start.
- Male or female gender
- Aged 18 years or over at study entry
- At least 1 day of “useable” follow up during the study period (nominally 1 January 2008 – 31 December 2019).

Study 1

Our cases will be individuals who have a medcode for stroke or MI in their primary care record or a stroke or MI ICD-10 code in their hospitalization record (either as the primary reason for hospitalisation or as a comorbidity) and/or a stroke/MI ICD-10 code in their mortality record in the last year of study (2018-2019).

For the cohort studies, people will be followed from 1 January 2008, date of current registration, date of their 18th birthday, or practice up to standard date, whichever is later. Follow up will end at death, transfer out date, last collection date from the practice, or 31 December 2019, whichever is earliest.

Study 2

Our study population will include individuals who have a newly commenced NSAID

prescription (Appendix A). Cohort entry will be defined as the date of the first NSAID prescription. Anyone with evidence of prior NSAID prescription at any point will be excluded.

Study 3

Our NSAID users will be the same population as Study 2. Our Non-NSAID users will be drawn from subjects who have never had an NSAID prescription over the same time period.

Selection of comparison group(s) or controls

Study 1

We will use concurrent sampling to identify controls; i.e. find controls who haven't had a stroke or MI at the point the case has theirs. This would mean controls can go on to become cases themselves later on, but this would mimic what would happen if we did the study prospectively. Controls will be matched 1control:1case by GP practice, age (ideally +/- 1 year) and sex. We will not match cases and controls on other factors but will adjust for important covariates in the analysis.

Control Group:

Inclusion criteria

- Adult patients (>18 years age)

Exclusion criteria

- History of cardiovascular disease defined as a fatal or non-fatal myocardial infarction or stroke (TIAs excluded)

Study 2

Does not have a control group

Study 3

We will compare

- a) Cohort of NSAID users with no statin or NSAID use with

- Cohort of NSAID users + Aspirin and statin use
- Cohort of NSAID users + Aspirin
- Cohort of NSAID users + statin use

b) Cohort of Non NSAID users with no aspirin or statin use with

- Cohort of Non NSAID users + Aspirin and statin use
- Cohort of Non NSAID users + Aspirin use
- Cohort of Non NSAID users + statin use

Exposures, outcomes and covariates

Study 1

Cases will be defined as people who have had a MI or stroke in primary care, HES or ONS. We will exclude TIAs as previous validation work has shown that TIA is inaccurately measured (Morgan et al Br J GP 2020).

NSAID use will be defined as at least one prescription of NSAIDs (including Ibuprofen, diclofenac, naproxen, celecoxib) during the study period.

Study 2

Cohort entry will be defined as the date of the first NSAID prescription (Ibuprofen, diclofenac, naproxen, celecoxib) during the study period. We will use an as-treated exposure definition in which subjects will be considered continually exposed to NSAIDs if the duration of prescriptions overlap. We will use a grace period of 2 months in between prescriptions. If NSAID use is terminated (defined as absence of prescription after 2-month grace period), 6 months from the date of end of the last prescription will be used as the censor date for time to event analysis.

Study 3

We will use the same exposure criteria as study 2 for NSAID use. A similar as-treated exposure definition will be used for aspirin and statin use.

Outcomes

MI, Stroke

1) Cardiovascular events defined as a fatal or non-fatal myocardial infarction , fatal or non-fatal stroke . We will not include Transient ischaemic attacks in our definition of a cardiovascular event for reasons stated above.

List of pre-defined Co-variates

Age (Continuous)

Gender (dichotomous)

Ethnicity (White or not stated, Indian, Pakistani, Bangladeshi, other Asian, black Caribbean, black African, Chinese, other ethnic group)

SES

Smoking status (Non, current, ex-smoker)

Smoking Pack Years (Continuous)

Diabetes status (type 1 or type 2)

HBA1C (Continuous)

Angina or heart attack in a first degree relative (dichotomous)

Chronic kidney disease (stage 3,4 or 5) (dichotomous)

Atrial fibrillation(dichotomous)

On blood pressure treatment (dichotomous)

Migraines (dichotomous)

Rheumatoid arthritis (dichotomous)

Systematic lupus erythematosus (SLE) (dichotomous)

Severe mental illness (schizophrenia, bipolar disorder, moderate/severe depression) (dichotomous)

On atypical antipsychotic medication (dichotomous)

Regular steroid use (dichotomous)

Diagnosis or treatment for erectile dysfunction (dichotomous)

Cholesterol/ HDL ratio (Scale)

Systolic Blood pressure (continuous)

Standard deviation of at least two most recent systolic BP readings

BMI (continuous)

Type of NSAID (celecoxib, ibuprofen, diclofenac, naproxen)

Chronic pain (dichotomous)

For continuous variables e.g. HBA1C we will attempt to categorise the data and combine with diabetes to assess the effects of glycaemic control on outcomes. We will also do this with other risk factors if appropriate. E.g. Assessing BP measurements.

Data/statistical analysis

For the case-control study

We will generate simple descriptive statistics for all risk factors, and depending on their frequency and distribution, decide whether any should be re-classified before further analysis. Some categories may be aggregated if numbers are small.

For the cases as a whole, associations with potential risk factors will then be examined by conditional logistic regression for matched sets and summarised by odds ratios with 95% confidence intervals. We will first analyse each risk factor independently, and then include those with potentially important associations in a single, multivariate regression model. Calculation of confidence intervals will take into account the possibility that a minority of individuals contribute to more than one matched set (Cousineau et al).

If appropriate, we will calculate the number needed to harm. We will perform subgroup analysis to look at the effects of different types of NSAID used (ibuprofen, diclofenac, naproxen, celecoxib), dosage and duration of use if numbers allow.

For the cohort study

In the cohort study, follow-up of cases will start from the date of first NSAID prescription and will continue until the earliest of death, transfer out, or the end of the follow-up period. We will generate simple statistics for outcome variables and depending on their frequency in the study sample as a whole, decide which should be included in further analysis.

All data will be analysed using STATA 16.

Continuous data will be presented as median +- IQR, dichotomous data will be expressed at n (%) and the standardised mean differences (SMD) between the groups. We will not present p

values when presenting baseline characteristics between groups.

We will present data as OR (95% CI). If appropriate, we will calculate the number needed to harm. We will perform subgroup analysis to look at the effects of different types of NSAID used (ibuprofen, diclofenac, naproxen, celecoxib), duration of use.

To identify what group of NSAID users are at the highest risk of a cardiovascular event (as defined above) we will identify risk factors for myocardial infarction or stroke in NSAID users (as defined above) using time to event data.

Cohort entry will be defined as the date of the first NSAID prescription (Ibuprofen, diclofenac, naproxen, celecoxib) during the study period. We will use an as-treated exposure definition in which subjects will be considered continually exposed to NSAIDs if the duration of prescriptions overlap. We will use a grace period of 2 months in between prescriptions. If NSAID use is terminated (defined as absence of prescription after 2-month grace period), 6 months from the date of end of the last prescription will be used as the censor date for time to event analysis.

Time to event analysis will be estimated using the Kaplan Meier method, with log-rank tests to compare the differences between the groups. A cardiovascular event will be defined as the first (fatal or non-fatal) myocardial infarction or stroke. Patients with no events will be censored at the last known time to be alive (if the death was not due to a cardiovascular death) the end of the study period or 6 months after termination of NSAID prescription. A multivariable model will be created using COX regression analysis. Adjusted models will be built iteratively, by adding potential confounders one-at-a-time, retaining the strongest confounder and repeating the process until no covariate changed the effect estimate by 10% or more or the model was saturated (number of outcomes/number of predictors <10). By building the model iteratively, it will be possible to identify multicollinearity that causes instability in model covariates. Where there is doubt, multicollinearity will be assessed using variance inflation factor (multicollinearity if >10). Depending on the variable and degree of multicollinearity, the variable may be dropped from the model.” We will test the Cox proportional hazard assumptions using Schoenfeld residual analysis. We will assess both the model in totality and the individual components of the model.

Study 3

Finally, we will assess if aspirin or statin use as a secondary preventative measure can offset the additional cardiovascular risk associated with NSAID use. To do this we will use the same populations outlined earlier. Time to event data will be obtained from CPRD Aurum, HES and ONS data and be defined as a fatal or non-fatal myocardial infarction (codes), fatal or non-fatal stroke (codes). We will then use univariate analysis using log rank tests to establish if there is any reduction in cardiovascular risk with aspirin and or statin use in new NSAID users.

We will compare any effects in NSAID users to a similar cohort of non-NSAID users. We will compare hazard ratios in both groups and compare number needed to treat.

Time to event analysis will be estimated using the Kaplan Meier method, with log-rank tests to compare the differences between the groups. A cardiovascular event will be defined as the first (fatal or non-fatal) myocardial infarction or stroke. Patients with no events will be censored at the last known time to be alive (if the death was not due to a cardiovascular death) the end of the study period or 6 months after termination of NSAID prescription. A multivariable model will be created using COX regression analysis. Adjusted models will be built iteratively, by adding potential confounders one-at-a-time, retaining the strongest confounder and repeating the process until no covariate changed the effect estimate by 10% or more or the model was saturated (number of outcomes/number of predictors <10). By building the model iteratively, it will be possible to identify multicollinearity that causes instability in model covariates. Where there is doubt, multicollinearity will be assessed using variance inflation factor (multicollinearity if >10). Depending on the variable and degree of multicollinearity, the variable may be dropped from the model. We will test the Cox proportional hazard assumptions using Schoenfeld residual analysis. We will assess both the model in totality and the individual components of the model.

Plan for addressing confounding See list of covariates to be considered in the models. As discussed in the statistical analysis section, we will build models which take into account confounding by adding variables to the outcome model one-at-a-time, each time retaining the strongest confounder until the model is saturated or no covariate changes the effect estimates by $\geq 10\%$.

Plans for addressing missing data

We are not expecting significant amounts of missing data apart from the covariates of BMI, smoking status, height, blood pressure variables and ethnicity. If data is missing at random complete case analysis could be performed or multiple imputation. If the data is not missing at random, complete case analysis will be used.

We will check for missing and implausible data. From previous experience, we anticipate smoking, height, BMI, blood pressure variables, ethnicity and smoking status will have missing data, and that within CPRD, the incompleteness is unlikely to be random. We expect to make a complete case analysis, but are mindful that we may need unknown categories for some variables, multiple imputation of missing values, or exclusion of subjects with missing data from some analyses. The potential for bias because of missing data will be discussed when results are reported.

Patient or user group involvement

Patients have not directly been involved in this study thus far. As part of a larger piece of work, the findings from this study will be shared with relevant patient groups. When disseminating the results, we will work with patients to produce a lay summary.

Plans for disseminating and communicating study results

The study findings will be submitted for publication in peer-reviewed scientific journals, and will be presented at appropriate conferences and other meetings; the latter will include scientific meetings externally, for example American Heart Association and internally within Imperial College London.

Conflict of interest statement The investigators have no conflicts of interest to declare.

Limitations of the study design, data sources, and analytic methods

Observational studies allow understanding of disease progression and associations using real world data sources, in this case electronic health data. However, strengths and limitations are associated with this study design.

Firstly, it is important to note that although CPRD is broadly representative of the UK population in terms of age, sex, and socio-economic status, it may not be representative of all GPs across the UK. Furthermore, our patient group is limited to England only as linked data is only available for these patients. Selection of patients into the study will be based on prospectively recorded diagnoses of CVD or NSAID prescription and may not be representative of individuals across the whole UK.

In addition, it is important to note that studies using CPRD cannot prove causality, and merely find results that are consistent with casual hypotheses backed up by trials. Even when accounting for many confounders, there can always be residual confounding.

The major limitation of this study is that ibuprofen is readily available over the counter and this study cannot account for un-prescribed use in the control groups

References

Morgan et al 2020- A Morgan, SJ Sinnott, L Smeeth, C Minassian, J Quint. Concordance in the recording of stroke across UK primary and secondary care datasets: a population-based cohort study. BJGP Open in Press.

Appendix 5- Raw Concentrations for Analytes Measured in Chapter 3

COX-2 knockout (KO) and wild type (WT) mice

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Arginine ELLISA	WT	9	91.61	38.23	12.74
	KO	9	61.64	41.99	14.00
ADMA ELISA	WT	9	0.88	0.28	0.09
	KO	9	0.60	0.30	0.10
Cystatin C	WT	8	372.35	101.42	35.86
	KO	9	258.15	117.84	39.28
Urea	WT	9	57.92	19.73	6.58
	KO	9	16.22	3.08	1.03
ADMA	WT	9	0.83	0.46	0.15
	KO	9	0.59	0.38	0.13
Alanine	WT	9	252.82	127.30	42.43
	KO	9	254.16	136.08	45.36
Arginine	WT	9	74.72	30.11	10.04
	KO	9	43.93	26.74	8.91
Aspartic acid	WT	9	8.50	3.50	1.17
	KO	9	9.56	5.13	1.71
Asparagine	WT	9	49.03	35.84	11.95
	KO	9	42.17	19.00	6.33
Ethanolamine	WT	9	16.83	6.06	2.02
	KO	9	14.98	3.42	1.14
Glutamic acid	WT	9	49.44	23.38	7.79
	KO	9	48.62	13.19	4.40
Glutamine	WT	9	657.73	342.77	114.26
	KO	9	655.77	238.59	79.53
Glycine	WT	9	217.14	51.48	17.16
	KO	9	197.31	37.97	12.66
Histidine	WT	9	59.70	17.89	5.96
	KO	9	56.91	11.12	3.71
Isoleucine	WT	9	75.95	24.03	8.01
	KO	9	66.81	17.86	5.95
Leucine	WT	9	104.63	34.65	11.55
	KO	9	92.71	27.63	9.21
Lysine	WT	9	221.78	98.06	32.69
	KO	9	174.57	48.82	16.27
Methionine	WT	9	55.77	19.22	6.41
	KO	9	48.07	7.53	2.51
Phenylalanine	WT	9	77.34	25.73	8.58
	KO	9	68.56	15.89	5.30
Serine	WT	9	111.91	41.04	13.68
	KO	9	96.84	20.53	6.84
Threonine	WT	9	136.68	42.17	14.06
	KO	9	122.79	23.32	7.77
Tryptophan	WT	9	77.62	21.96	7.32
	KO	9	61.55	12.21	4.07
Tyrosine	WT	9	100.15	53.79	17.93
	KO	9	91.93	18.38	6.13
Valine	WT	9	129.16	43.93	14.64
	KO	9	116.82	31.51	10.50
Citrulline	WT	9	59.12	23.21	7.74
	KO	9	38.28	6.87	2.29
Alpha-amino-n-butyric acid	WT	9	4.15	1.53	0.51
	KO	9	4.45	0.89	0.30
Sarcosine	WT	9	2.55	0.74	0.25
	KO	9	1.87	0.58	0.19
Taurine	WT	9	490.25	188.60	62.87
	KO	9	372.88	123.32	41.11

PGIS knockout (KO) and wild type (WT) mice

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Cystatin C	KO	8	870.14	297.05	105.02
	KO	7	391.53	80.43	30.40
UREA	KO	8	69.38	26.25	9.28
	KO	7	16.51	3.74	1.41
ADMA	KO	8	0.73	0.21	0.08
	KO	7	0.50	0.10	0.04
Alanine	KO	8	759.81	249.56	88.23
	KO	7	591.82	143.28	54.15
Arginine	KO	8	136.73	54.16	19.15
	KO	7	76.85	21.46	8.11
Aspartic acid	KO	8	10.40	4.18	1.48
	KO	7	7.03	2.25	0.85
Asparagine	KO	8	62.22	20.49	7.25
	KO	7	47.23	12.55	4.74
Ethanolamine	KO	8	18.49	6.34	2.24
	KO	7	11.82	3.32	1.25
Glutamic acid	KO	8	60.18	31.68	11.20
	KO	7	32.10	13.82	5.22
Glutamine	KO	8	715.06	253.85	89.75
	KO	7	532.27	95.02	35.92
Glycine	KO	8	593.08	184.97	65.40
	KO	7	325.28	73.75	27.87
Histidine	KO	8	115.54	32.35	11.44
	KO	7	67.99	14.33	5.42
Isoleucine	KO	8	78.55	25.34	8.96
	KO	7	57.11	9.96	3.76
Leucine	KO	8	77.98	26.53	9.38
	KO	7	57.50	9.06	3.42
Lysine	KO	8	174.56	55.44	19.60
	KO	7	144.26	24.23	9.16
Methionine	KO	8	79.93	23.62	8.35
	KO	7	49.76	11.79	4.46
Phenylalanine	KO	8	81.14	25.19	8.91
	KO	7	48.71	7.56	2.86
Serine	KO	8	193.07	60.76	21.48
	KO	7	146.62	37.46	14.16
Threonine	KO	8	226.98	81.84	28.94
	KO	7	149.55	27.30	10.32
Tryptophan	KO	8	117.61	34.84	12.32
	KO	7	80.36	14.57	5.51
Tyrosine	KO	8	86.92	23.43	8.28
	KO	7	68.87	9.72	3.68
Valine	KO	8	125.60	41.74	14.76
	KO	7	91.18	15.14	5.72
Citrulline	KO	8	189.65	67.70	23.94
	KO	7	63.59	22.47	8.49
Alpha-amino-n-butyric acid	KO	8	6.00	1.88	0.66
	KO	7	3.44	0.94	0.35
Sarcosine	KO	8	2.59	1.01	0.36
	KO	7	1.27	0.36	0.13
Taurine	KO	8	872.81	401.49	141.95
	KO	7	478.85	178.77	67.57

PLA₂ Deficient Patient before and after renal transplant

Analyte	Control				Pre Transplant				Post Transplant			
	N	Mean	Std. Deviation	Std. Error of Mean	N	Mean	Std. Deviation	Std. Error of Mean	N	Mean	Std. Deviation	Std. Error of Mean
Cystatin C	9	303.2756	50.83735	16.94578	8	3446.0044	864.20790	305.54363	5	474.2190	60.46669	27.04153
Urea	9	10.8812	3.77094	1.25698	8	55.8016	14.47984	5.11940	5	18.1503	1.53038	0.68441
ADMA	9	0.6396	0.22677	0.07559	8	0.9323	0.32589	0.11522	5	0.5334	0.15246	0.06818
Alanine	9	336.6058	107.95058	35.98353	8	455.6040	150.29086	53.13584	5	431.6644	88.44235	39.55262
Arginine	9	58.2562	25.74314	8.58105	8	49.2080	15.06917	5.32776	5	48.0176	9.67335	4.32605
Aspartic acid	9	4.4782	1.01379	0.33793	8	5.3565	0.96837	0.34237	5	4.7740	1.13510	0.50763
Asparagine	9	83.9807	19.17480	6.39160	8	91.8790	30.92989	10.93537	5	69.2180	4.46095	1.99500
Ethanolamine	9	9.3531	1.46434	0.48811	8	6.7023	1.66191	0.58757	5	5.9516	0.25799	0.11538
Glutamic acid	9	52.2733	19.53613	6.51204	8	59.2215	13.07953	4.62431	5	71.7492	15.53674	6.94824
Glutamine	9	882.1082	151.42066	50.47355	8	1015.7425	170.94993	60.43993	5	864.3312	121.69910	54.42549
Glycine	9	211.5411	73.80839	24.60280	8	438.7778	68.31650	24.15353	5	274.1804	38.99055	17.43710
Histidine	9	74.3920	13.53101	4.51034	8	76.5190	25.64916	9.06835	5	59.6868	4.00240	1.78993
Isoleucine	9	63.7338	18.18931	6.06310	8	61.2675	7.79924	2.75745	5	54.5268	3.23120	1.44504
Leucine	9	97.1180	14.56207	4.85402	8	62.0658	17.92692	6.33812	5	78.0192	4.92525	2.20264
Lysine	9	149.4402	31.19766	10.39922	8	146.3255	39.86904	14.09583	5	147.3992	6.96670	3.11560
Methionine	9	21.8013	6.09638	2.03213	8	17.3985	3.72618	1.31741	5	17.9208	2.34159	1.04719
Phenylalanine	9	56.8527	9.95717	3.31906	8	52.7615	14.51530	5.13193	5	43.0336	4.84347	2.16607
Serine	9	110.1053	22.24216	7.41405	8	120.0860	28.95837	10.23833	5	101.6648	12.12343	5.42176
Threonine	9	105.5322	29.85742	9.95247	8	69.6568	25.11857	8.88076	5	99.6744	6.64399	2.97128
Tryptophan	9	55.0911	14.69225	4.89742	8	14.4065	1.33908	0.47344	5	35.7108	5.93122	2.65252
Tyrosine	9	63.9567	11.54571	3.84857	8	37.8308	12.44140	4.39870	5	49.1772	4.58096	2.04867
Valine	9	156.5591	18.76039	6.25346	8	108.6668	21.42048	7.57328	5	118.2776	11.98329	5.35909
Citrulline	9	25.8409	10.27501	3.42500	8	110.6848	16.85303	5.95845	5	45.2492	3.72003	1.66365
Alpha-amino-n-butyric acid	9	17.6007	2.58821	0.86274	8	17.1505	3.03215	1.07203	5	19.0516	4.02956	1.80208
Sarcosine	9	1.5147	0.56227	0.18742	8	2.0040	0.29813	0.10541	5	1.6016	0.21812	0.09754
Taurine	9	68.7947	14.31075	4.77025	8	84.3363	43.05710	15.22298	5	50.8852	9.79860	4.38207

Appendix 6- Raw Concentrations for Analytes measured in Chapter 7

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Arginine	Event	117	122.52	52.37	4.84
	Control	219	116.72	38.93	2.63
ADMA	Event	117	0.63	0.13	0.01
	Control	228	0.65	0.18	0.01
IL-6 Cleaned	Event	107	8.31	5.10	0.49
	Control	219	7.17	4.63	0.31
MCP-1	Event	116	133.70	154.28	14.32
	Control	228	120.49	55.37	3.67
NGAL	Event	117	604733.45	486102.93	44940.23
	Control	113	512745.65	306459.01	28829.24
Cystatin C	Event	117	637462.12	267658.50	24745.04
	Control	113	569949.90	150245.60	14133.92
TIMP-2	Event	117	58948.81	12656.06	1170.05
	Control	113	59849.54	12367.86	1163.47
OPN	Event	103	1222.27	1448.60	142.74
	Control	101	1111.23	1029.07	102.40
TFF3	Event	117	5370.42	3500.55	323.63
	Control	113	4754.58	1790.91	168.47
Uromodulin	Event	117	74902.15	35682.29	3298.83
	Control	113	79009.48	34350.73	3231.44
P-selectin	Event	118	12351.08	7448.38	685.68
	Control	117	10103.37	5347.39	494.37
D-Dimer	Event	118	101444.86	110980.58	10216.59
	Control	117	106236.54	149391.57	13811.26
PSGL-1	Event	118	17641.06	3640.85	335.17
	Control	117	17989.74	3753.40	347.00
Tpa	Event	118	24292.36	10641.79	979.66
	Control	117	22547.93	8551.94	790.63
sCD40L	Event	118	19456.54	13154.17	1210.94
	Control	117	18305.73	12116.06	1120.13
PAI-1	Event	118	447148.90	129815.41	11950.48
	Control	117	425331.99	116265.67	10748.77
Factor IX	Event	118	1075482.00	569140.08	52393.59
	Control	117	1199079.10	579962.68	53617.57
LNMNA	Event	118	0.07	0.02	0.00
	Control	117	0.07	0.02	0.00
ADMA	Event	118	0.13	0.04	0.00
	Control	117	0.12	0.04	0.00
SDMA	Event	118	0.26	0.09	0.01
	Control	117	0.27	0.09	0.01
Arginine	Event	118	53.59	19.59	1.80
	Control	117	55.43	25.93	2.40
Glutamic acid	Event	118	78.62	41.25	3.80
	Control	117	73.13	39.74	3.67
Glutamine	Event	118	213.81	74.04	6.82
	Control	117	214.73	62.20	5.75
Citrulline	Event	118	21.67	12.81	1.18
	Control	117	19.67	12.93	1.20
Ornithine	Event	118	43.07	13.84	1.27
	Control	117	41.99	12.97	1.20
4-hydroxyproline	Event	118	5.10	2.39	0.22
	Control	117	4.91	2.03	0.19

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Alanine	Event	118	187.75	36.82	3.39
	Control	117	176.14	35.00	3.24
Aspartic acid	Event	118	18.53	5.38	0.50
	Control	117	17.35	5.43	0.50
Asparagine	Event	118	19.07	3.60	0.33
	Control	117	18.49	4.57	0.42
Cystine	Event	118	10.02	5.98	0.55
	Control	117	7.98	5.02	0.46
Ethanolamine	Event	118	5.02	1.53	0.14
	Control	117	4.63	1.33	0.12
Histidine	Event	118	34.69	5.82	0.54
	Control	117	33.24	6.02	0.56
Isoleucine	Event	118	37.00	22.65	2.09
	Control	117	36.79	21.39	1.98
Leucine	Event	118	64.08	15.77	1.45
	Control	117	63.77	15.13	1.40
Lysine	Event	118	75.74	15.52	1.43
	Control	117	76.40	15.46	1.43
Methionine	Event	118	9.18	2.93	0.27
	Control	117	8.98	3.52	0.33
Phenylalanine	Event	118	45.66	8.85	0.81
	Control	117	43.19	10.27	0.95
Proline	Event	118	101.23	32.28	2.97
	Control	117	93.46	25.98	2.40
Serine	Event	118	63.03	13.10	1.21
	Control	117	60.93	14.05	1.30
Tryptophan	Event	118	28.76	7.63	0.70
	Control	117	28.74	7.67	0.71
Tyrosine	Event	118	38.46	10.99	1.01
	Control	117	37.05	11.54	1.07
Valine	Event	118	101.80	20.63	1.90
	Control	117	100.78	20.78	1.92
Cystathionine	Event	118	0.30	0.23	0.02
	Control	117	0.29	0.21	0.02
Hydroxylysine	Event	118	1.39	0.53	0.05
	Control	117	1.35	0.54	0.05
Aminoadipic acid	Event	118	0.65	0.27	0.02
	Control	117	0.66	0.28	0.03
Sarcosine	Event	118	217.23	55.22	5.08
	Control	117	200.49	52.52	4.86
Beta-alanine	Event	118	220.65	53.71	4.94
	Control	117	206.88	52.54	4.86
Arg/ADMA Ratio	Event	118	439.00	162.67	14.97
	Control	117	491.15	356.09	32.92
GABR	Event	118	0.89	0.38	0.03
	Control	117	0.99	0.61	0.06

Appendix 7- Raw Concentrations for Analytes Measured in Chapter 8 (1-year events)

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
4-hydroxyproline	Event	49	7.14	3.09	0.44
	Control	97	7.53	3.60	0.37
alanine	Event	49	324.02	108.19	15.46
	Control	97	313.18	111.77	11.35
Arginine	Event	49	81.21	32.40	4.63
	Control	97	82.61	33.80	3.43
Aspartic acid	Event	49	30.71	16.87	2.41
	Control	97	27.28	10.71	1.09
Asparagine	Event	49	29.27	9.57	1.37
	Control	97	27.43	9.44	0.96
Cystine	Event	49	12.68	8.99	1.28
	Control	97	12.20	11.22	1.14
Ethanolamine	Event	49	7.79	3.00	0.43
	Control	97	7.25	2.51	0.25
Glutamic acid	Event	49	109.64	54.29	7.76
	Control	97	99.44	40.77	4.14
Glutamine	Event	49	229.99	67.57	9.65
	Control	97	220.33	71.98	7.31
Glycine	Event	49	127.83	37.58	5.37
	Control	97	123.54	26.55	2.70
Histidine	Event	49	55.88	22.02	3.15
	Control	97	53.80	18.33	1.86
Isoleucine	Event	49	37.92	14.94	2.13
	Control	97	39.50	16.49	1.67
Leucine	Event	49	47.49	13.23	1.89
	Control	97	48.44	12.39	1.26
Lysine 244	Event	49	84.27	22.29	3.18
	Control	97	86.40	23.36	2.37
Methionine	Event	49	14.80	5.95	0.85
	Control	97	14.44	6.62	0.67
Phenylalanine	Event	49	49.67	13.60	1.94
	Control	97	49.25	12.70	1.29
Proline	Event	49	160.62	63.35	9.05
	Control	97	154.40	65.56	6.66
Serine	Event	49	94.15	33.82	4.83
	Control	97	93.19	25.75	2.61
Threonine	Event	49	58.20	17.29	2.47
	Control	97	56.96	14.25	1.45
Tryptophan	Event	49	44.63	18.24	2.61
	Control	97	47.61	16.01	1.63

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Tyrosine	Event	49	50.78	17.08	2.44
	Control	97	50.85	15.45	1.57
Valine	Event	49	62.62	14.74	2.11
	Control	97	62.89	13.93	1.41
Beta-amino-iso-butyric acid	Event	49	4.97	4.54	0.65
	Control	97	4.88	4.58	0.47
Citrulline	Event	49	24.73	15.35	2.19
	Control	97	22.09	8.69	0.88
Cystathione	Event	49	0.36	0.21	0.03
	Control	97	0.40	0.31	0.03
3-methylhistidine	Event	49	2.66	3.82	0.55
	Control	97	2.92	6.03	0.61
1-methylhistidine	Event	49	2.27	1.05	0.15
	Control	97	2.25	1.27	0.13
Ornithine	Event	49	64.07	24.69	3.53
	Control	97	65.88	25.41	2.58
Aminoadipic acid	Event	49	1.08	0.60	0.09
	Control	97	1.15	0.59	0.06
Alpha-amino-n-butyric acid	Event	49	9.69	4.35	0.62
	Control	97	10.49	4.03	0.41
Taurine	Event	49	67.37	36.45	5.21
	Control	97	67.66	34.75	3.53
beta-alanine	Event	49	122.65	29.61	4.23
	Control	97	120.96	26.43	2.68
LNMA	Event	49	0.15	0.03	0.00
	Control	97	0.15	0.03	0.00
ADMA	Event	49	0.78	0.27	0.04
	Control	97	0.69	0.20	0.02
SDMA	Event	49	0.63	0.20	0.03
	Control	97	0.58	0.22	0.02
L-homoarginine	Event	49	1.90	1.03	0.15
	Control	97	1.77	1.00	0.10
Arg/ADMA	Event	49	107.64	33.18	4.74
	Control	97	121.05	36.12	3.67
GABR	Event	49	0.98	0.35	0.05
	Control	97	1.00	0.36	0.04

Appendix 8- Raw Concentrations for Analytes Measured in Chapter 8 (6- month subgroup)

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
4-hydroxyproline	Event	17	7.24	4.35	1.06
	Control	33	7.20	3.53	0.61
alanine	Event	17	331.58	116.42	28.23
	Control	33	322.98	99.36	17.30
Arginine	Event	17	83.60	42.14	10.22
	Control	33	84.63	29.27	5.10
Aspartic acid	Event	17	30.65	21.92	5.32
	Control	33	27.73	11.30	1.97
Asparagine	Event	17	30.53	12.16	2.95
	Control	33	28.00	9.53	1.66
Cystine	Event	17	12.18	8.07	1.96
	Control	33	11.68	11.60	2.02
Ethanolamine	Event	17	7.52	2.41	0.58
	Control	33	7.22	2.34	0.41
Glutamic acid	Event	17	107.68	47.94	11.63
	Control	33	103.45	39.84	6.94
Glutamine	Event	17	225.04	69.79	16.93
	Control	33	215.72	60.45	10.52
Glycine	Event	17	127.21	46.97	11.39
	Control	33	117.75	25.00	4.35
Histidine	Event	17	57.41	29.39	7.13
	Control	33	54.93	18.89	3.29
Isoleucine	Event	17	38.37	14.46	3.51
	Control	33	41.17	15.10	2.63
Leucine	Event	17	47.39	13.26	3.22
	Control	33	48.65	9.53	1.66
Lysine	Event	17	81.58	26.13	6.34
	Control	33	85.79	19.03	3.31
Methionine	Event	17	15.68	6.55	1.59
	Control	33	14.46	6.24	1.09
Phenylalanine	Event	17	48.94	13.65	3.31
	Control	33	49.69	9.90	1.72
Proline	Event	17	169.95	75.74	18.37
	Control	33	169.97	77.92	13.56
Serine	Event	17	92.77	45.19	10.96
	Control	33	92.04	25.65	4.46
Threonine	Event	17	56.25	20.73	5.03
	Control	33	56.61	12.75	2.22

Analyte	Group	N	Mean	Std. Deviation	Std. Error Mean
Tryptophan	Event	17	43.63	14.90	3.61
	Control	33	50.13	13.52	2.35
Tyrosine	Event	17	47.11	15.96	3.87
	Control	33	52.15	15.22	2.65
Valine	Event	17	63.64	15.62	3.79
	Control	33	63.05	10.62	1.85
Beta-amino-iso-butyric acid	Event	17	5.79	5.91	1.43
	Control	33	5.26	5.26	0.92
Citrulline	Event	17	25.80	22.22	5.39
	Control	33	21.84	8.99	1.57
Cystathione	Event	17	0.38	0.24	0.06
	Control	33	0.35	0.19	0.03
3-methylhistidine	Event	17	2.19	2.84	0.69
	Control	33	2.48	2.50	0.43
1-methylhistidine	Event	17	2.41	1.42	0.34
	Control	33	2.17	1.11	0.19
Ornithine	Event	17	59.78	20.64	5.01
	Control	33	65.21	26.34	4.59
Aminoadipic acid	Event	17	1.02	0.51	0.12
	Control	33	1.13	0.50	0.09
Alpha-amino-n-butyric acid	Event	17	9.66	4.17	1.01
	Control	33	10.46	3.41	0.59
Taurine	Event	17	61.31	32.06	7.77
	Control	33	69.18	34.08	5.93
beta-alanine	Event	17	119.70	31.80	7.71
	Control	33	120.94	21.59	3.76
LNMMA	Event	17	0.15	0.04	0.01
	Control	33	0.15	0.04	0.01
ADMA	Event	17	0.79	0.30	0.07
	Control	33	0.70	0.19	0.03
SDMA	Event	17	0.65	0.25	0.06
	Control	33	0.59	0.19	0.03
L-homoarginine	Event	17	1.77	0.81	0.20
	Control	33	1.93	1.00	0.17
Arg/ADMA	Event	17	103.06	32.47	7.88
	Control	33	122.50	31.20	5.43
GABR	Event	17	1.00	0.36	0.09
	Control	33	1.04	0.30	0.05