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UROCORTIN 2:
AN INTERESTING ROLE IN HUMAN
ABDOMINAL AORTIC ANEURYSM

Thesis submitted by

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MSc. (Distinction) Nottingham

in June 2013

for the degree of Doctor of Philosophy

in the School of Medicine and Dentistry

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FINANCIAL SUPPORT

James Cook University Postgraduate Research Scholarship

School of Medicine and Dentistry Top-up Scholarship

CONTENTS

CONTENTS.....	i
DECLARATION	v
STATEMENT OF ACCESS TO THESIS	vi
ACKNOWLEDGEMENTS	vii
COMMUNICATIONS, AWARDS AND GRANTS.....	ix
STATEMENT ON THE CONTRIBUTION OF OTHERS	xiv
LIST OF ABBREVIATIONS	xvii
ABSTRACT.....	xxvi

CHAPTER 1..... 1

INTRODUCTION AND LITERATURE REVIEW 1

1.1 General Introduction..... 2

1.2 Literature Review..... 6

1.2.4.1 Chronic Inflammation and the immune response in AAA 12

1.2.4.2 Proteolytic degradation of the aortic wall..... 13

1.2.4.3 Genetic predisposition to AAA and stresses within the aortic wall..... 16

1.2.7.1 Chemically induced AAA mouse models..... 19

1.2.7.2 Genetically modified AAA mouse models..... 19

1.2.7.3 Combined gene knockout and chemically induced AAA..... 20

1.2.10.1 Synthesis and localisation of UCNs 28

1.2.10.2 Urocortin receptors 29

1.2.10.3 Urocortin signalling..... 30

1.2.10.4 Urocortins: Protective or destructive force in the cardiovascular system?..... 32

1.2.10.5 Studies in animal models 33

1.2.10.6 *In vitro* investigations of the UCNs..... 35

1.2.10.7 Circulating UCNs concentration in CVD 37

1.2.10.8 The effect of UCNs administration in human subjects..... 39

CHAPTER 2..... 45

MATERIALS AND METHODS 45

2.1 Materials..... 46

2.1.1 Mice..... 46

2.1.2 Western blot and immunoprecipitation 46

2.1.3 Real time PCR..... 46

2.1.4 Histology and immunohistochemistry..... 47

2.1.5 ELISA..... 47

2.1.6 Tissue culture 48

2.1.7 Proliferation/viability, apoptosis and cell cycle assays	48
2.2 Mouse Studies - The role of urocortin 3 (UCN3) in a mouse model of AAA.....	48
2.3 Human studies.....	49
2.3.1 Study Design.....	49
2.3.2 Patients.....	50
2.3.3 Plasma samples	51
2.3.4 Aortic and atheroma samples.....	51
2.3.5 Gene expression studies.....	53
2.3.6 Histology.....	55
2.3.7 Immunohistochemistry	56
2.3.8 Assessment of UCN2 by ELISA.....	57
2.4 In vitro investigations	58
2.4.1 Explant culture	58
2.4.2 Cell culture.....	58
2.4.3 Determination of human aortic smooth muscle cell counts.....	59
2.4.4 Akt signalling assessment.....	60
2.4.5 Examination of cytokine secretion.....	61
2.4.6 Determination of HASMC proliferation.....	63
2.4.7 Apoptosis investigations.....	63
2.4.8 Cell cycle investigations using flow cytometry	65
2.5 Statistical analysis	66
CHAPTER 3.....	68
THE ASSOCIATION OF UROCORTIN 3 (UCN3) WITH ABDOMINAL AORTIC ANEURYSM	68
3.1 Introduction.....	69
3.2 Materials and methods.....	72
3.2.1 Effects of angiotensin II (AII) infusion on ApoE ^{-/-} mouse aortic diameter and blood pressure.....	72
3.2.1.1 Mice.....	72
3.2.1.2 Angiotensin II treatment to induce AAA.....	72
3.2.1.3 Blood pressure and heart rate determination.....	73
3.2.1.4 Ultrasound and plasma assessment of aneurysm progression	73
3.2.1.5 Aorta morphometry	74
3.2.2 Comparison of urocortin 3 (UCN3) levels in aneurysmal versus non-aneurysmal mice.....	76
3.2.2.1 Extraction of aortic proteins.....	76
3.2.2.2 Mouse UCN3 ELISA.....	77
3.2.3 Expression of the UCN3 receptor CRFR2 expression in aortas from aneurysmal and non-aneurysmal mice	78
3.2.3.1 Extraction of aortic proteins.....	78
3.2.3.2 Immunoprecipitation	78

3.2.3.3 Western blotting	79
3.2.4 Statistics.....	80
3.3 Results.....	82
3.3.1 Blood pressure is increased in ApoE ^{-/-} mice following AII infusion	82
3.3.2 Aneurysm progression in angiotensin II (AII) infused ApoE ^{-/-} mice.....	84
3.3.3 Aortic diameter morphometry in the aneurysmal and non-aneurysmal mice.....	87
3.3.4 UCN3 protein is not upregulated in mice with AAA.....	90
3.3.5 Expression of CRFR2 in mouse aorta	92
3.4 Discussion	97
 CHAPTER 4.....	 101
 UROCORTIN 2 AND THE PRESENCE OF HUMAN ABDOMINAL AORTIC ANEURYSM	 101
4.1 Introduction.....	102
4.2 Materials and methods.....	105
4.2.1 Study design and patients	105
4.2.2 Aortic and atheroma samples:.....	106
4.2.3 Expression of the UCNs and CRFR1/ 2 genes in human AAA biopsies	106
4.2.4 Immunohistochemical staining for UCN2, lymphocytes and neutrophils	106
4.2.5 UCN2 secretion from AAA biopsies.....	107
4.2.6 Circulating concentration of UCN2 in AAA	110
4.2.7 Determination of plasma UCN2 by ELISA.....	110
4.2.7 Statistics.....	111
4.3 Results.....	112
4.3.1 Primer design and real time PCR optimisation.....	112
4.3.2 UCN2 and its receptor, CRFR2 expression are upregulated in human AAA.....	115
4.3.3 Assessment of UCN2 protein expression in AAA biopsies	117
4.3.4 UCN2 is secreted by AAA explant biopsies.....	120
4.3.5 Circulating UCN2 is significantly elevated in AAA and correlates with aortic diameter.....	123
4.4 Discussion	127
 CHAPTER 5.....	 132
 UROCORTIN 2 (UCN2) FUNCTIONAL EFFECT ON SMOOTH MUSCLE CELLS IN VITRO IN HUMAN ABDOMINAL AORTIC ANEURYSM	 132

5.1 Introduction.....	133
5.2 Materials and methods.....	136
5.2.1 Determination of UCN2 secretion from AAA thrombus explant cultures and concentration used in the <i>in vitro</i> investigations	136
5.2.2 Cell culture.....	136
5.2.3 Determination of the efficacy of the AlamarBlue® cell proliferation and viability assay.....	137
5.2.4 Determination of the optimal concentration range of UCN2 and Ast-2B for <i>in vitro</i> studies.....	137
5.2.5 HASMC proliferation assay.....	138
5.2.6 Akt signaling in HASMC	139
5.2.7 Cytokine assays.....	140
5.2.8 Apoptosis investigations.....	140
5.2.9 Cell cycle progression analysis	141
5.2.10 Statistics	142
5.3 Results.....	143
5.3.1 Efficacy of the AlamarBlue® cell proliferation and viability assay	143
5.3.2 Optimal concentration range of UCN2 and Ast-2B for <i>in vitro</i> studies.....	144
5.3.3 UCN2 inhibited Akt phosphorylation in HASMC.....	145
5.3.4 Effect of UCN2 on HASMC cytokine secretion.....	148
5.3.5 UCN2 inhibited HASMC proliferation	150
5.3.6 AAA thrombus conditioned media inhibits HASMC proliferation	152
5.3.8 UCN2 had no significant effect on HASMC apoptosis.....	155
5.3.10 UCN2 induces a G1 arrest in HASMC.....	157
5.4 Discussion	161
CHAPTER 6.....	169
GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	169
6.1 General Discussion	170
6.2 Limitations and future directions	176
6.3 Summary	178
APPENDIX 1- ETHICS APPROVAL.....	181
APPENDIX 2- STANDARD CURVES	189
APPENDIX 3- LIST OF SUPPLIERS	193
APPENDIX 4- REAGENTS AND SOLUTIONS.....	196
REFERENCES	200

DECLARATION

I, the undersigned declare that this research investigation is carried out on my own and it has not been previously submitted anywhere for another degree or diploma at any university or institution of tertiary education in or out of Australia. Information derived from the published or unpublished works of others has been acknowledged in the text and a list of references is given.

Theophilus I Emeto

June 2013

STATEMENT OF ACCESS TO THESIS

I, the undersigned, author of this thesis, understand that James Cook University will make this work available for use within the university library and, by microfilm or other photographic means, allow access to users in other approved libraries. All users consulting this thesis will have to sign the following statement.

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Theophilus I Emeto

June 2013

ACKNOWLEDGEMENTS

First, all glory to God for mercies and grace and for teaching me to appreciate the important things in life. Most PhD candidature involves a convoluted dance balancing between rewards and pitfalls, whilst hoping that one has more gains than frustration. Unlike most PhD candidature, mine came with extra obstacles which I call ‘stepping stones’ towards greatness. The last two years of my candidature was undertaken whilst being the sole carer for my darling wife who had been diagnosed with Philadelphia positive B-cell acute lymphoblastic leukaemia just before our first wedding anniversary. She went on to have an allogeneic stem cell transplant from a generous and kind hearted German girl. Emma is more than one year cancer free today due to this kind girl, the amazing doctors and to the glory of God. I could not have completed this PhD without the amazing support, love and prayers from everyone I know.

My sincere thanks to my supervisors: Prof. Jonathan Golledge, Drs Catherine Rush, Lynn Woodward and Joseph Moxon for their constant encouragement, guidance and the confidence they have in me to complete this work. Thanks to the School of Medicine and Dentistry, James Cook University, for providing a friendly, stimulating and enjoyable environment that allowed me undertake a worthwhile research project within the doctoral program and for financial and administrative support. I also thank all the members of the Vascular Biology Unit both past and present for help and support within the confines of my PhD candidature and beyond. I can’t fail to express my profound gratitude to all the friends who made Townsville home.

I wish to express my heart-felt gratitude to my parents, brothers, and sisters for their blind faith in me, and their loving support all through my candidature. To my big brother, Dede- you are amazing, Emma and I will always be grateful for all you have done. Finally, to my gorgeous wife Emma (Obiiyke), thank you for your patience and never ending love and support through it all. You are God's unique gift specially crafted for me, my miracle. I thank God for blessing me with you and for his amazing grace upon us. I love you.

COMMUNICATIONS, AWARDS AND GRANTS

1. CONFERENCES AND PRESENTATIONS

Moxon JV, Liu D, Crossman D, Moran CS, **Emeto TI**, Krishna SM, Yonglitthipagon P, Mulvenna J and Golledge J. *Profiling protein expression in the mouse aorta using 8-plex iTRAQ proteomics*. North Queensland Festival of Life Sciences, so you think you can do research? (NQFLS); Townsville, Australia; October 2012 (Poster presentation)

Emeto TI, Moxon JV, Biro E, Clancy P, Moran C, Rush C, Woodward L, and Golledge J. *Urocortin 2 Inhibits Human Aortic Smooth Muscle Cell Proliferation Via Corticotrophin Releasing Hormone Receptor-2 In Abdominal Aortic Aneurysm*. 26th Annual Vascular Research Initiative Conference (VRIC); Chicago, Illinois, USA; 17th, April 2012. (Oral presentation)

Emeto TI, Moxon JV, Biro E, Clancy P, Rush C, Woodward L and Golledge J. *TP2- a putative biomarker for abdominal aortic aneurysm*. North Queensland Festival of Life Sciences, so you think you can do research? (NQFLS); Townsville, Australia; September 2011(Oral and poster presentation)

Moxon JV, Padula MP, Clancy P, **Emeto TI**, Herbert BR, Norman PE, Golledge J. *Improving the depth of proteomic analysis for biomarker screening studies of complex clinical tissues*. The North Queensland Medical Research Symposium; Townsville, Australia; October 2011 (Poster presentation)

Emeto T.I, Moxon J.V, Clancy C, Biroš E, Rush C, and Golledge J. *Ø\$#- a novel biomarker for abdominal aortic aneurysm*. Celebrating research at James Cook University; Townsville, Australia; August 2011 (Oral presentation)

Emeto T.I, Moxon J.V, Rush C, Woodward L, and Golledge J. *Aneurysm resistance in the angiotensin II (AII) infused Apo E^{-/-} mouse model*. Townsville Festival of Life Sciences, so you think you can do research? Townsville, Australia; November 2010 (Oral and poster presentation)

Emeto T.I, Moxon J.V, Rush C, Woodward L, and Golledge J. *Abdominal aortic aneurysm (AAA) detection: the way forward*. Celebrating 40 years of research, James Cook University; Townsville, Australia; August 2010 (Oral presentation)

Emeto T.I, Moxon J.V, Rush C, Woodward L, and Golledge J. *Diagnostics and biomarkers of abdominal aortic aneurysm presence and outcomes*. Townsville Festival of Life Sciences, so you think you can do research? Townsville, Australia; November 2009 (Oral and poster presentation)

2. PAPERS ARISING DIRECTLY FROM THIS THESIS

Emeto TI, Moxon JV, Biroş E, Rush C, Clancy P, Woodward L, Moran CS, Jose RJ, Nguyen T, Walker PJ, Golledge J. Urocortin 2 is associated with abdominal aortic aneurysm and mediates anti-proliferative effects on vascular smooth muscle cells via corticotrophin releasing factor receptor 2. *Clin Sci* 2013 (under review)

Emeto TI, Moxon JV, Rush C, Woodward L, Golledge J. Relevance of urocortins to cardiovascular disease. *J Mol Cell Cardiol* 2011. **51**(3): p299-307

3. PAPERS ARISING FROM OTHER PROJECTS CONTRIBUTED TO DURING THIS THESIS

Krishna SM, Seto SW, Clancy P, Jose RJ, Wang Y, **Emeto TI**, Moxon JV, Norman P, Golledge J. Deficiency of thrombospondin-1 accelerates angiotensin II induced aortic dilatation and atheroma progression in the mouse model of abdominal aortic aneurysm. *J Clin Invest.* 2013 (In preparation)

Moxon JV, **Emeto TI**, Golledge J. Further evidence to support a role for urocortin 2 in heart failure-Editorial comment. *Anadolu Kardiyol Derg.* 2012 **12**(2): p121-122

Moxon JV, Padula M, Clancy P, **Emeto TI**, Herbert B, Norman PE, Golledge J. Proteomic analysis of intra-arterial thrombus secretions reveals a negative association of clusterin and thrombospondin-1 with abdominal aortic aneurysm. *Atherosclerosis* 2011. **219**(2):p432-439

Moxon JV, Parr A, **Emeto TI**, Walker P, Norman PE, Golledge J. Diagnosis and Monitoring of Abdominal Aortic Aneurysm: Current Status and Future Prospects. *Current problems in cardiology* 2010. **35**(10): p512-548.

4. AWARDS

- James Cook University Doctoral Completion Award 2013
- James Cook University Postgraduate Research Scholarship (JCUPRS)
- James Cook University School of Medicine Top Up Scholarship
- James Cook University Tuition fee waiver scholarship
- North Queensland Festival of Life Sciences (NQFLS) 2011- Best Poster Prize (HDR)
Medical and Allied Medical Sciences
- James Cook University Townsville Festival of Life Sciences (TFLS) 2010- Best
Poster Prize Medical and Allied Medical Sciences

5. GRANTS

- James Cook University Faculty (FAIG) Grant 2011 \$8000.00 '*Association of cerebral cytokines with abdominal aortic aneurysm*' Golledge J; **Emeto TI**
- Travel and workshop grant up to \$1400 to attend the 2011 UniQuest research commercialisation workshop at the Gold Coast, Australia
- James Cook University Graduate Research Scheme (GRS) Grant 2010 \$3,000.00 '*Diagnostics and biomarkers of abdominal aortic aneurysm presence and progression*' **Emeto TI**
- James Cook University Faculty (FAIG) Grant 2010 \$8000.00 '*Biomarkers of abdominal aortic aneurysm*' Moxon JV; Anwar Am; Golledge J; **Emeto TI**
- James Cook University Research and Infrastructure Building Grant (RIBG) 2010. '*Isoelectric focussing*'. \$12,500. Moxon JV, Chora S, **Emeto TI** and J Golledge
- James Cook University Research Infrastructure Block Grant (RIBG) 2009 \$ 12,800.00 '*Laboratory equipments-proteomics analysis*' Moxon JV; Golledge J; Rush C; **Emeto TI**

STATEMENT ON THE CONTRIBUTION OF OTHERS

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Financial support		Golledge J, Prof, JCU
Data collection	Research assistance	Moxon JV, Dr, JCU

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Data collection	Research assistance-IHC	Nguyen T, Mr, JCU

Chapter 5:

Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-contributors
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Data collection	Research assistance	Moran CS, Dr, JCU

Chapter 6:

Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-contributors
Intellectual Support	Editorial assistance	Golledge J, Prof, JCU Moxon JV, Dr, JCU Rush CM, Dr, JCU Woodward L, Dr, JCU

LIST OF ABBREVIATIONS

µg/kg: Microgram/ kilogram

µl: Microlitre

AAA: Abdominal aortic aneurysm

ACE: Angiotensin converting enzyme

AI: Angiotensin I

AII: Angiotensin II

Akt: Serine threonine specific protein kinase (also known as protein kinase B)

ALP: Alkaline phosphatase

AMI: Acute myocardial infarction

ANP: Atria natriuretic peptide

AOD: Atherosclerotic occlusive diseases

ApoE^{-/-}: Apolipoprotein E deficient

Ast-2B: Astressin2-B

ATP: Adenosine triphosphate

BAD: Bcl-2-associated death promoter

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

Ca²⁺: Calcium ion

CaCl₂: Calcium chloride

cAMP: Cyclic adenosine monophosphate

cDNA: Complementary deoxyribonucleic acid

CD40L: Cluster of differentiation 40 ligand

CHD: Coronary heart disease

CI: Confidence interval

cm: Centimetre

COPD: Chronic Obstructive Pulmonary Disease

Cp: *Chlamydia pneumoniae*

CRF: Corticotrophin-releasing factor

CRFR: Corticotrophin-releasing factor receptor

CRFR2^{-/-}: Corticotrophin-releasing factor receptor 2 deficient

CRP: C-reactive protein

CT: Connective tissue

CTA: Computed topographic angiography

CT-1: Cardiotrophin-1

CVD: Cardiovascular diseases

DAB: 3, 3'-Diaminobenzidine

DEVD: Asp-Glu-Val-Asp

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dL: Decilitre

DPBS: Dulbecco's Phosphate Buffered Saline

ECM: Extracellular matrix

EDTA: Ethylene diamine tetra-acetic acid

ELISA: Enzyme linked immunosorbent assay

EP: Serum elastin peptide

ERK1/2: Extracellular signal Regulated Kinase 1/2

EtOH: Ethanol

EVAR: Endovascular aneurysm repair

FasL: Fas ligand

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPCR: G-protein coupled receptors

h: Hour

H₂O₂: Hydrogen peroxide

HASMC: Human aortic smooth muscle cells

HCL: Hydrochloric acid

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HF: Heart failure

HLA: Human leukocyte antigen

HR: Heart rate

hsp90: Heat shock protein 90

HUVEC: Human umbilical cord vascular endothelial cell

I-CAM-1: Intercellular adhesion molecule-1

IFN γ : Interferon gamma

IgG: Immunoglobulin G

IHD: Ischaemic heart disease

IL- 6: Interleukin 6

IL-1 α : Interleukin 1- alpha

IL-1 β : Interleukin 1- beta

IL-8: Interleukin 8

IL-17: Interleukin 17

IL-23: Interleukin 23

IL-32: Interleukin 32

ILT: Intraluminal thrombus

IP: Immunoprecipitation

iPLA2: Calcium insensitive phospholipase A₂

IRA: Infrarenal aorta

IQR: Inter-quartile range

i.v: Intravenous

K_{ATP}: Sensitive potassium channel

LDL^{-/-}: Low density lipoprotein receptor deficient

LDLR: Low-density lipoprotein receptor

Lox: Lysyl oxidase

LPC: Lysophosphatidylcholine

LVEF: Left ventricular ejection fraction

M: Molar

MAPK: Mitogen-activated protein kinase

MBP: Mean blood pressure

mg: Milligrams

MGP: Matrix gamma-carboxyglutamic acid protein

MIF: Macrophage migration inhibitory factor

min: Minutes

mL: Millilitre

mM- Millimolar

mm³: Cubic millimetre

nmol/L: Nanomole/ litre

MMP: Matrix metalloproteinase

MNC: Mononuclear cells

mPTP: Mitochondrial permeability transition pore

n: Number

ng/g/24h: Nanogram/gram/ 24 hours

ng/mL: Nanogram/milliliter

NGAL: Neutrophil gelatinase associated lipocalin

NK: Natural killer

nm: Nanometre

nM: Nanomolar

NP-40: Tergitol®- type NP-40/ Nonyl phenoxyethoxyethanol

NYHA: New York Heart Association

°C: Degrees centigrade

OPG: Osteoprotegerin

OPN: Osteopontin

PAD: Peripheral artery disease

PAI: Plasminogen activator inhibitor

PAP: P-plasmin antiplasmin complex

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

PE: Phycoerythrin

pg/ml: picogram/millilitre

PI: Propidium iodide

PIIINP: Pro-collagen type II N-terminal peptide

PI3K: Phosphoinositide-3-kinase

PKA: Protein kinase A

PKC ϵ : Protein kinase C epsilon

pM: Picomolar

PPi: Pyrophosphate

PVDF: Polyvinylidene difluoride

qPCR: Quantitative real-time polymerase chain reaction

QTL: Quantitative trait loci

RBC: Red blood cells

RDU: Relative density unit

RLU: Relative luminescence unit

RNA: Ribonucleic acid

RNASE: Ribonuclease

ROC: Receiver operator characteristic

ROS: Reactive oxygen species

RT-PCR: Reverse transcriptase polymerase chain reaction

s.c: Subcutaneous

SBP: Systolic blood pressure

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOD: Superoxide dismutase

SRA: Suprarenal aorta

SVS: Society for Vascular Surgery

TAO: Thromboangitis obliterans

TBST: Tris-buffered Saline Tween-20; 0.05% Tween-20

Th1: Helper T-cell type 1

Th2: Helper T-cell type 2

TIMP: Tissue inhibitor of matrix metalloproteinase

TNF α : Tumor necrosis factor- alpha

tPA: Tissue plasminogen activator

UCN: Urocortin

uPA: Urokinase-type plasminogen activator

V-CAM-1: Vascular cell adhesion molecule-1

VEC: Vascular endothelial cell

VEGF: Vascular endothelial growth factor

VSMC: Vascular smooth muscle cells

v/v: Volume/ volume

w/v: Weight/ volume

wk: Week

Zn²⁺: Zinc ion

ZrO₂: Zirconium oxide

ABSTRACT

Acquired cardiovascular diseases such as coronary heart disease, peripheral artery disease, abdominal aortic aneurysm (AAA) and related vascular problems contribute to more than one-third of worldwide morbidity and mortality. In many instances, particularly in the under developed world, cardiovascular diseases are diagnosed at a late stage limiting the scope for improving outcomes. AAA accounts for thousands of deaths in the western world including Australia annually. It is estimated to be the tenth leading cause of death in those aged over 60 years. Hundreds of millions of dollars are spent annually to treat and manage AAA in Australia. Current methods of diagnosing and managing AAA are expensive and not very efficient, necessitating research into identifying blood borne markers involved in AAA pathology that could offer a cheap diagnostic and/or prognostic alternative, or serve as a therapeutic target for disease management.

The urocortins (UCNs) are a group of recently defined protein members of the corticotrophin-releasing factor family. Previous pre-clinical work and human association studies suggest that UCNs have potential to exert either beneficial or detrimental effects on the heart and major blood vessels. Current evidence favours beneficial effects of UCNs within the cardiovascular system, for example, these proteins have been shown to inhibit production of reactive oxygen species and apoptosis, implying a potential to antagonise the progression of cardiovascular disease.

Loss of medial smooth muscle cells is believed to be a critical feature of AAA. The UCNs have been previously reported to regulate the proliferation and survival of various cell types.

Urocortin 2 (UCN2) particularly was reported to inhibit the proliferation and survival of Lewis lung carcinoma cells. The objective of the work discussed in this thesis was to elucidate the expression and functional role of UCNs in human AAA development and progression both *in vivo* and *in vitro*.

To this end, urocortin 3 (UCN3) was demonstrated to play no role in aneurysm development in a mouse model of AAA. More importantly, UCN2 and its receptor, corticotrophin releasing factor receptor 2 (CRFR2), were shown to be significantly over expressed in biopsies taken from the main dilated region of human AAAs compared to non-aneurysmal aortic tissues. Plasma concentrations of UCN2 were demonstrated to be significantly higher in patients with AAA compared to patients with non-aneurysmal peripheral artery disease even after adjusting for confounding cardiovascular risk factors. *In vitro*, UCN2 was shown to promote AAA pathogenesis by stimulating an aneurysmal phenotype on human aortic smooth muscles cells (HASMC); an effect that was significantly abrogated by Astressin-2B the selective CRFR2 antagonist. The work described in this thesis demonstrated for the first time an association between UCN2 and AAA in man. This effect may be restricted to UCN2 alone, but it is unlikely given that other analogues of the peptide-urocortin1 (UCN1) and UCN3 have been found in the disease environment. It is proposed that UCN2 may serve as a possible therapeutic target in AAA, although further work is required to validate these findings.

LIST OF FIGURES

Figure 1. 1 A cross section through the aortic wall showing the different layers	7
Figure 1. 2 Course of the aorta and its major branches.....	8
Figure 1. 3 A cross section through the aneurysmal aorta showing infiltration of inflammatory cells and VSMC degradation.....	11
Figure 1. 4 AAA formation in ApoE ^{-/-} mice infused with AII	22
Figure 1. 5 Regions of amino acid similarity between the urocortin related peptides.....	26
Figure 1. 6 Proposed signalling mechanism of urocortins as relevant to cardiovascular disease	32
Figure 2. 1 Schematic diagram showing the study design applied in the human studies for the association of UCN2 with human AAA.....	49
Figure 2. 2 Diagram of the aorta showing an abdominal aortic aneurysm and regions from which aortic biopsies were collected	53
Figure 3. 1 Diagram of the mouse aorta showing the different regions used in morphometric analysis	75
Figure 3. 2 Blood pressure and heart rate measured at days 0, 14 and 27 in ApoE ^{-/-} mice that received AII or vehicle.....	83
Figure 3. 3 Ultrasound comparison of aneurysm growth in mice that received AII or vehicle.	86
Figure 3. 4 Study design showing the experimental groups and dissected aortas from mice that received AII with and without aneurysm, and mice that received vehicle at necropsy	87

Figure 3. 5 Kaplan Meier plot showing overall survival curves according to study treatment.	88
Figure 3. 6 Comparison of maximum aortic diameter measured from 4 regions of aortas (arch, thoracic, suprarenal and infrarenal) at necropsy in mice that received AII or vehicle	89
Figure 3. 7 Comparison of aortic and plasma UCN3 protein concentrations in mice that received AII with and without AAA.....	92
Figure 3. 8 Representative images of Western blot comparison of CRFR2 protein expression within the SRA and IRA of ApoE ^{-/-} mice that received AII	93
Figure 3. 9 CRFR2 protein distribution within the SRA and IRA of ApoE ^{-/-} mice that received AII with AAA.....	94
Figure 3. 10 CRFR2 protein expression within the SRA of mice that received AII with and without AAA	95
Figure 3. 11 CRFR2 protein expression within the aortas (SRA + IRA) of ApoE ^{-/-} mice that received AII with and without AAA.....	96
Figure 4. 1 Melt curves showing primer optimisation for the UCNs and their receptors on a two-step qPCR thermal cycling program	113
Figure 4. 2 Melt curves showing primer optimisation for the UCN2 and CRFR2 on a three- step qPCR thermal cycling program	114
Figure 4. 3 Representative fluorescence versus cycle number showing amplified peaks for UCN2 in AAA neck and AAA body on a three-step qPCR thermal cycling program..	116
Figure 4. 4 Examples of histological findings in human AAAs.....	118
Figure 4. 5 Examples of UCN2 expression in human AAA	119

Figure 4. 6 T lymphocyte and neutrophil populations in inflammatory infiltrates of human AAA body biopsies.....	120
Figure 4. 7 Comparison of UCN2 secretion from aortic aneurysm and femoral atheroma explants over 24 hours	122
Figure 4. 8 Correlation between plasma UCN2 concentration and aortic diameter ROC curve for UCN2 in predicting AAA presence.....	125
Figure 5. 1 Validation of the AlamarBlue® assay <i>in vitro</i>	143
Figure 5. 2 Determination of UCN2 dose used in the <i>in vitro</i> studies.....	144
Figure 5. 3 Determination of Astressin 2B (Ast-2B) dose used in the <i>in vitro</i> studies	145
Figure 5. 4 Effect of UCN2 on healthy HASMC Akt phosphorylation <i>in vitro</i>	147
Figure 5. 5 Effect of UCN2 on HASMC cytokine secretion <i>in vitro</i>	149
Figure 5. 6 Effect of UCN2 on HASMC proliferation <i>in vitro</i>	150
Figure 5. 7 Effect of CRFR2 blockade on UCN2 induced inhibition of HASMC proliferation <i>in vitro</i>	152
Figure 5. 8 Effect of AAA thrombus conditioned media on HASMC proliferation <i>in vitro</i>	153
Figure 5. 9 Effect of CRFR2 blockade on AAA thrombus conditioned media induced inhibition of HASMC proliferation <i>in vitro</i>	154
Figure 5. 10 Effect of UCN2 on HASMC apoptosis <i>in vitro</i>	156
Figure 5. 11 Cell cycle distribution analysis of HASMC exposed to UCN2 by flow cytometry	160
Figure 5. 12 Putative mechanisms by which UCN2 promotes an aneurysm phenotype	167

LIST OF TABLES

Table 1. 1 Expression profiles of matrix metalloproteinases in abdominal aortic aneurysm	15
Table 1. 2 Risk factors associated with AAA.....	18
Table 1. 3 Examples of mouse models of abdominal aortic aneurysm.....	21
Table 1. 4 Examples of biomarkers associated with abdominal aortic aneurysm	25
Table 1. 5 Summary of the possible stages of AAA & atherosclerosis progression with relevance to urocortins	28
Table 1. 6 Expression of the UCNs in the heart and vasculature	29
Table 1. 7 Animal studies assessing the role of urocortins in cardiovascular diseases	35
Table 1. 8 <i>In vitro</i> studies assessing the role of urocortins in cardiovascular diseases	37
Table 1. 9 Studies assessing the association of UCNs with cardiovascular diseases in patients	39
Table 1. 10 Studies assessing the effect of administering urocortins in subjects with or without cardiovascular diseases.....	41
Table 1. 11 Potential cardiovascular protective and cardiovascular destructive effects of urocortins.....	44
Table 2. 1 Patients recruitment criteria applied for the human studies.....	50
Table 4. 1 Risk factors of the patients providing AAA biopsies used to assess expression of UCNs and their receptors in qPCR and immunohistochemistry	107
Table 4. 2 Risk factors of the patients providing aortic and femoral atheroma biopsies used to assess expression of UCN2 in explant cultures	109
Table 4. 3 Details of the thermal cycling steps used in the gene expression studies.....	112

Table 4. 4 Relative expressions of the urocortins and their receptors in six matched AAA body and neck control biopsies.....	117
Table 4. 5 Comparison of subjects with and without AAA used for plasma UCN2 measurement.....	124
Table 4. 6 Independent association of plasma UCN2 with AAA in 134 patients	126

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Acquired cardiovascular diseases (CVD) including coronary heart disease (CHD), peripheral artery disease (PAD), abdominal aortic aneurysm (AAA) and other vascular problems in which atherosclerosis is implicated contribute to more than one-third of worldwide morbidity and mortality.¹⁻⁵ For example, the Heart Disease and Stroke Statistics update, reported that CVD was responsible for 33.6% of overall deaths in 2007 in the USA, 10.4% higher than cancers.⁶ The incidence of CVD is rising in women,⁷ possibly associated with the prevalence of smoking in this gender.^{7,8,9} Roughly 80% of total CVD deaths are estimated to occur in under developed countries where the incidence is increasing rapidly.¹⁰ In contrast, the age-adjusted CVD mortality in developed countries has been halved between 1960 and 2010.⁴ Amongst CVD, abdominal aortic aneurysm (AAA), a local dilation in the infrarenal aortic wall,^{11,12} often occurring in a milieu of inflammation, atherosclerosis and thrombosis,¹³⁻¹⁵ accounts for ~ 1000 deaths in Australia, 8000 in the UK and 15000 in the USA annually.^{8,16-20} Death from AAA is often associated with an increased risk of developing other major cardiovascular events. For instance, the UK small aneurysm trial (UKSAT) reported that only 16% of deaths in patients with 4 - 5.5cm AAAs was related to AAA repair or rupture while ~50% were due to other cardiovascular causes (mainly myocardial infarction and stroke).²¹ AAA is estimated to be the tenth leading cause of death in men in industrialised nations and affects ~ 8% of men over 65 years.^{14,18,22}

Despite these alarming figures, the only treatment option currently available for AAA is surgical repair.²³ The absence of non-invasive pharmacological interventions to slow aneurysm progression, especially at the early stages of development, stems from an inability to identify the biochemical and physiological mechanisms underpinning AAA pathogenesis

despite extensive pre-clinical and clinical research. Ideally, effective drug therapies or health behaviour changes based on the biology of AAAs should be implemented at an early stage to impede disease progression. The in-hospital cost for surgical management of AAA per individual per annum is estimated to be ~ \$ 25,000 in Australia, \$16,000 in Canada and \$23,000 in the USA (US dollars).^{20,24} Thus, with increasing prevalence of AAA with the worldwide ageing population, global disease management is estimated to cost billions of dollars each year.^{14,18,22}

The characteristic features of AAA include the loss of vascular smooth muscle cells (VSMC) following the infiltration of inflammatory cells that are major sources of proteolytic enzymes responsible for degradation of aortic wall and associated thrombus formation.²⁵⁻²⁷ These immune cells are thought to be implicated in the pathogenesis of AAA through their ability to produce apoptosis-inducing factors and extracellular matrix (ECM) degrading proteolytic enzymes such as matrix metalloproteinase-2 (MMP-2) and MMP-9.^{28,29} In this way, the aortic wall is weakened and less likely to withstand arterial blood pressure which promotes vessel wall expansion.

Urocortin 2 (UCN2) is a member of the corticotrophin-releasing factor (CRF) family of peptides,^{30,31,32} and is constitutively expressed by endothelial cells, smooth muscle cells, fibroblasts and myocytes.^{33,34} UCN2 has been demonstrated to be upregulated under inflammatory conditions and associated with cardiovascular diseases.^{35,36} *In vitro* and animal studies demonstrate that UCN2 inhibits cell proliferation, increases proinflammatory cytokine secretion, and encourages apoptosis.³⁷⁻³⁹ In contrast, reports of anti-oxidant and anti-inflammatory effects of the urocortins (UCNs) suggest that UCN2 may play a more

protective role in modulating cell physiology.^{33,40} These observations suggest that UCN2 may be relevant to processes involved in AAA pathogenesis.

The focus of this study is to elucidate the role of the UCNs in AAA pathogenesis. Hypotheses proposed were that:

1. UCN3 protein is decreased within the aorta of apolipoprotein E deficient (ApoE^{-/-}) mice that develop AAA;
2. UCN2 expression is upregulated in human AAA;
3. UCN2 promotes an aneurysm phenotype by modulating the aortic wall milieu interior.

Specifically, the aims of this study were to:

1. Investigate the association of UCN3 with AAA in ApoE^{-/-} mice;
2. Define the relationship between UCN2 and human AAA presence;
3. Elucidate the possible mechanism by which UCN2 may promote an aneurysm phenotype;
4. Determine the effect of blocking UCN2 receptor on AAA development.

The predominant method of detecting and monitoring AAA currently is through imaging, which is limited in its ability to identify “at risk” AAA that may expand to a size that may rupture or require intervention.¹⁹ To prevent rupture significantly associated with mortality, when the aneurysm is $\geq 5\text{cm}$,²³ available treatment options are restricted to surgery which is associated with significant perioperative risks.^{41,42} Consequently, these significant shortfalls

in current AAA management procedures necessitate the need to develop better diagnostic, prognostic and therapeutic tools for the disease. Ideally, the investigative process will be based on understanding disease pathology rather than on morphological parameters such as aortic diameter alone so that non-surgical interventions aimed at impeding AAA progression can be developed. In order to accomplish this however, current research in this area is aimed at understanding AAA pathology enough so that non-surgical therapeutic targets can be identified. The basis of the research presented herein is to elucidate the role of the UCNs, specifically UCN2, in AAA pathogenesis and determine its therapeutic potential as a pharmacological target for AAA.

1.2 LITERATURE REVIEW

1.2.1 The aorta in health

The aorta is the largest blood vessel in the body and transports oxygenated blood from the left ventricle of the heart to the rest of the systemic circulation. A normal aortic wall consists of three layers: the innermost *tunica intima*, a thick central *tunica media* and the outer *tunica adventitia* (Figure 1.1). The *tunica intima* has vascular endothelial cells, the endothelium resting on a basal lamina and a thin layer of connective tissue (CT) which helps maintain vascular tone and metabolic homeostasis.⁴³ The *tunica media* consists of elastin fibers which are organised into numerous concentrically layered lamellae, which are in turn separated by numerous concentric layers of VSMC.⁴⁴ The elastic and cohesive attributes of the *tunica media* are invaluable in maintaining haemodynamic equilibrium for normal blood flow and pressure. The *tunica adventitia* comprises some collagen and elastin fibers, stroma with a network of nerves and blood vessels which supplies the aorta.⁴⁵ These layers are interlaced in an abundant network of extracellular matrix (ECM), which together with the collagen and elastin fibers, is responsible for the resistance of the aorta to blood flow and pressure.^{46,47} Collagen fibres provide tensile strength to the vessel wall, whilst elastin fibres provide the consistent distensible attribute key in maintaining the pulsating nature of blood flow.⁴⁸ Other cell types found in the adventitia include fibroblasts, dendritic cells, endothelial cells, progenitor cells, macrophages and pericytes.⁴⁹

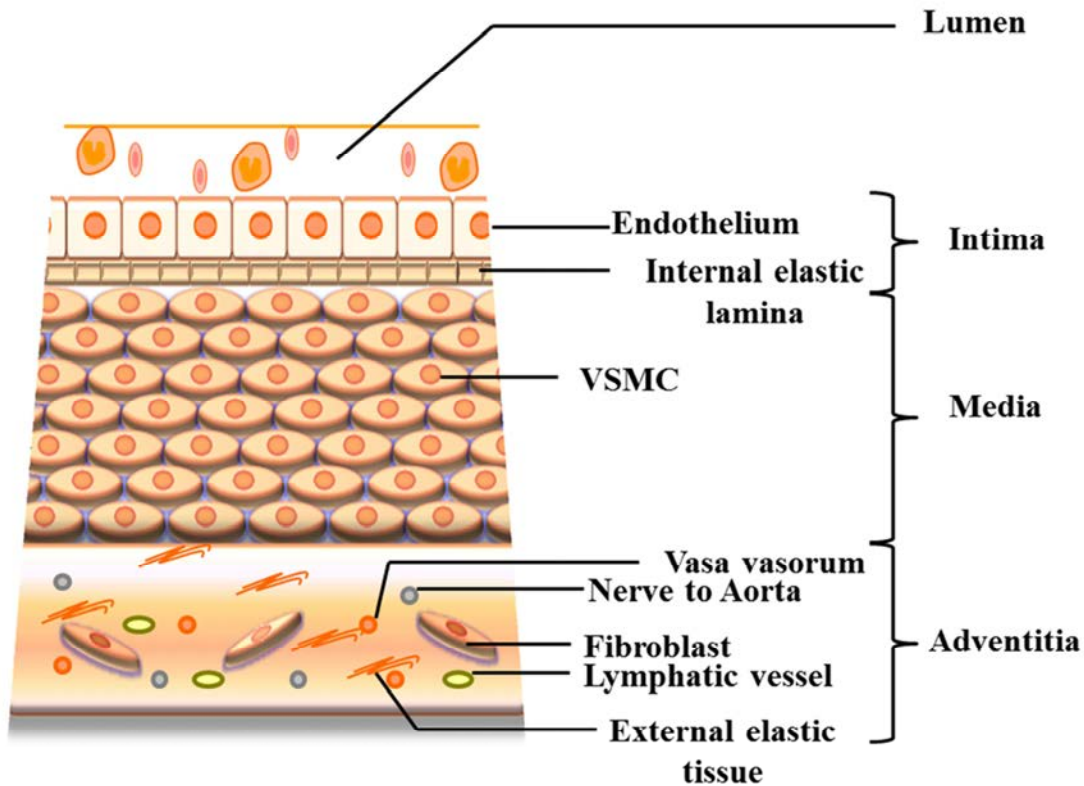


Figure 1. 1 A cross section through the aortic wall showing the different layers

1.2.2 *Abdominal aortic aneurysm*

The Society of Vascular Surgery (SVS) defines an ‘*aneurysm as- a permanent localized dilation of an artery with at least a 50% increase in diameter compared with the expected normal diameter of the artery, or diameter of the segment proximal to the dilation.*’⁵⁰

Aneurysms commonly affect major blood vessels of the body including the aorta, femoral artery, popliteal artery, iliac artery and basilar artery.⁵¹ The definition of AAA varies, but a generally accepted description is a permanent maximal infrarenal aortic diameter of $\geq 30\text{mm}$.⁵²⁻⁵⁶ Other designations include an infrarenal to suprarenal diameter ratio of 1.2 to 1.5,^{11,52} and a 1.5 fold increase in the expected infrarenal aortic diameter adjusted for age and sex.⁵⁰ AAA is due to degeneration and weakening of the aortic wall and the resultant progressive dilation of the blood vessel (Figure 1.2). It is often fatal and the main cause of

death is associated opportunistic cardiovascular events like myocardial infarction and stroke; or massive hemorrhage following rupture of the dilated aorta if left without surgical intervention.⁵⁷ There is a $\geq 70\%$ mortality rate when an AAA rupture occurs before surgery.^{16,18,58}

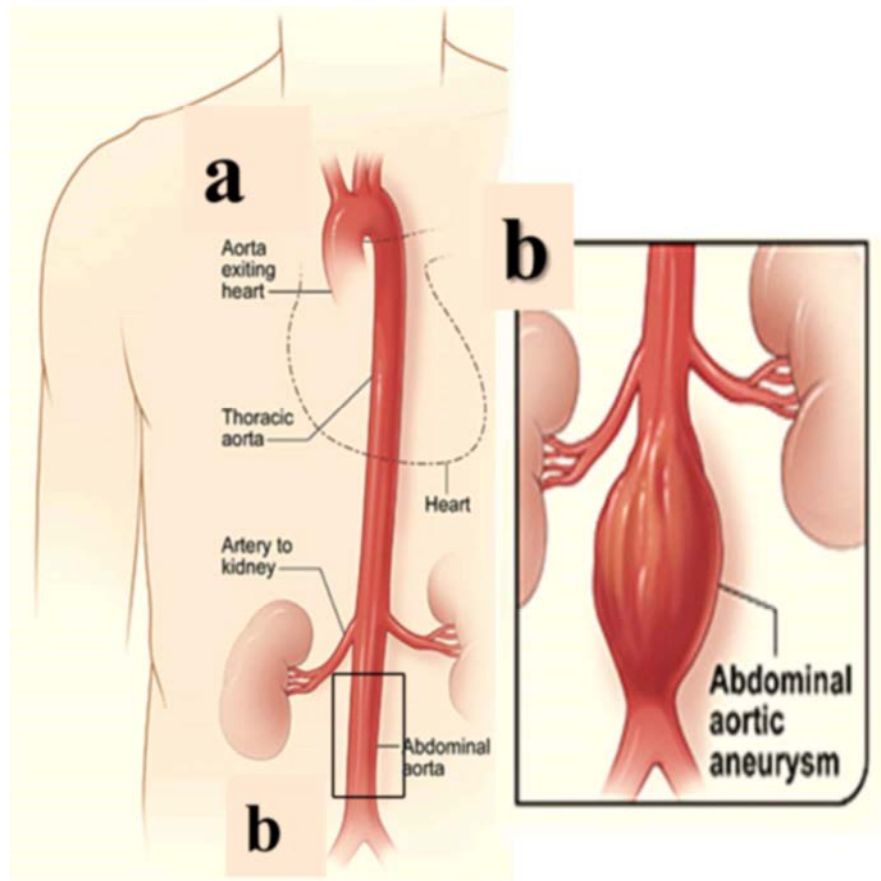


Figure 1. 2 Course of the aorta and its major branches

(a). Diagram showing the position of the aorta in relation to major organs in the body. Insert (b) expanded to show an infrarenal abdominal aneurysm.

Modified from http://www.nhlbi.nih.gov/health/dci/Diseases/arm/arm_types.html

1.2.3 AAA aetiology

Aortic aneurysms have been linked to a range of pathologies including connective tissue disorders (Ehlers-Danlos Syndrome type IV, Marfan Syndrome),^{59,60} infection by pathogens (*Brucella melitensis*, *Mycobacterium tuberculosis*, *Salmonella choleraesuis*, *Chlamydia pneumoniae*),⁶¹ inflammatory diseases (Behçet disease, Takayasu disease, Procollagen mutation type III),⁶¹⁻⁶⁴ and trauma.⁶⁵ Aortic aneurysms have been associated with atherosclerotic damage, so some researchers have suggested that atherosclerosis may be a possible cause of AAA.⁶⁶ More recent data however indicates that AAA is a result of other pathogenic mechanisms, which differ from those implicated in atherosclerotic occlusive diseases (AOD),^{46,67,68} for example Xu *et al.* suggested that arterial plaque deposition associated with localised dilation, thinning and loss of medial elastic lamellae predispose to aneurysm formation, whereas plaque deposits without thinning and loss of media elastic lamellae and without artery wall dilation predisposes to AOD.⁶⁸ Furthermore, findings suggest that protease inhibitors play a significant role in the divergent initial plaque developmental pathway towards AAA or AOD.¹⁸ For instance, matrix metalloproteinase 3 (MMP-3) and tissue inhibitor of metalloproteinase 3 (TIMP-3) are over expressed in AAA relative to AOD,⁶⁹ whereas TIMP-2 and plasminogen activator inhibitor-1 (PAI-1) are under expressed.⁷⁰ Therefore, the exact aetiology of AAA remains elusive.

1.2.4 Pathogenesis and presentation

So far, the exact mechanisms underpinning AAA formation remain unclear; nonetheless, it is accepted that aortic aneurysms appear to result from interactions between environmental risk factors and genetic predisposition which exacerbate the normal ageing processes.⁷¹ Macroscopically, the aneurysmal aorta is dilated at the infrarenal region often

permanently.^{54,56,72-74} Aortic dilation is usually progressive, and is frequently associated with the formation of a laminated, non-occlusive, intraluminal thrombus (ILT).⁷⁵⁻⁷⁷ The arterial wall may be partly or wholly covered by the thrombus which varies in size and location between patients,⁷⁸ but is continually remodelled and remains in permanent contact with circulating blood.^{79,80} Regions of the aorta in contact with the thrombus are associated with localised hypoxia which has been suggested to exacerbate the physiological stresses within the arterial wall.⁸¹ Akin to other cardiovascular diseases, calcification is common in AAA tissues, though the degree of calcification varies between patients, which may complicate surgical intervention.⁸²⁻⁸⁴ Evidence supports the presence of ILT and vascular calcification in AAA formation,^{85,86} progression,^{55,87-89} and rupture.⁹⁰⁻⁹² Most AAA with a diameter ≥ 5 cm have an increased ILT compared to AAA with diameters < 5 cm,^{89,93} and are at risk of rupture.^{90,91} Neutrophil gelatinase associated lipocalin (NGAL) found in all layers of ILT,⁹³ is reported to prevent MMP-9 inactivation,⁹⁴ and the NGAL-MMP-9 complex may contribute to the enhanced proteolytic degradation of the aortic ECM by preserving the MMP-9 enzymatic activity.⁹³ The ILT encourages migration of inflammatory cells such as neutrophils,⁹³ macrophages and T-lymphocytes,⁹² which promotes VSMC apoptosis and thinning of the aortic wall that leads to rupture.⁹² A recent report indicated that patients with $\geq 50\%$ calcification in the AAA wall had less aneurysm expansion rates when compared to patients with $< 50\%$ calcification.⁵⁵ These results indicate that calcification may be protective against aneurysm rupture, since the size of aneurysm is an indication of rupture.¹⁶⁻¹⁸ In contrast however, earlier publications have failed to show any association between calcification and AAA expansion.^{88,89} Thus, it is hypothesised that the weakening of the aorta as a result of structural degeneration allows vessel dilation,^{95,96} and eventual rupture if left untreated.^{16-18,92}

Histological examination demonstrates that the pathological processes in AAA involve all layers of the aortic wall including the aortic media, in contrast to those observed for AOD.^{80,97} Characteristically, AAA biopsies reveal degradation and fragmentation of the media ECM, elastin and collagenous fibres,^{98,99} significant reductions in VSMC density,^{100,101} and invasion of the *tunica media* and *tunica adventitia* by infiltrating macrophages and mononuclear lymphocytes which are hallmarks of the disease (Figure 1.3).¹⁰²⁻¹⁰⁵

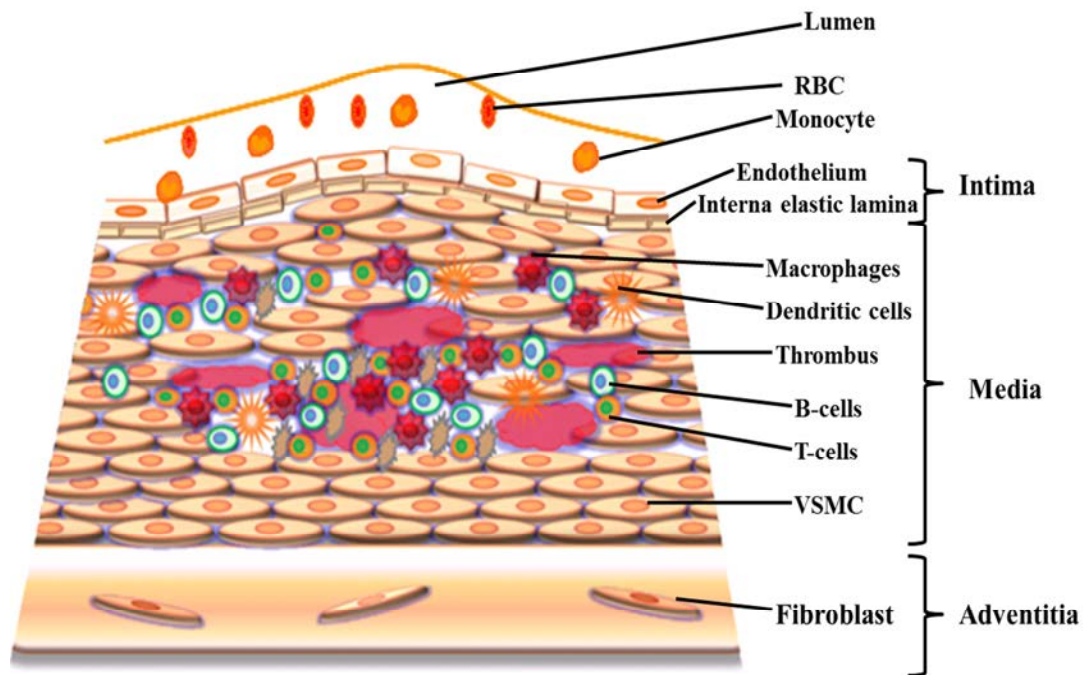


Figure 1. 3 A cross section through the aneurysmal aorta showing infiltration of inflammatory cells and VSMC degradation

Abbreviations used in figure not explained in text: red blood cell (RBC)

A series of putative mechanisms to explain the pathogenesis of AAA have been proposed.¹⁰⁶

These mechanisms fall into three broad groups:¹⁰⁶

I) Chronic inflammation and the immune response in AAA;

II) Proteolytic degradation of the aortic wall;

III) Genetic predisposition to disease and stresses within the aortic wall.

1.2.4.1 Chronic inflammation and the immune response in AAA

Inflammation is a common feature of most acquired CVD including AAA, and is thought to be vital in their development and subsequent complications. Secretion of proinflammatory cytokines by foam cells in early atherosclerosis encourages aortic infiltration by inflammatory cells leading to subsequent degeneration of the ECM.^{107,108} Similarly, inflammation is implicated in atherosclerotic plaque rupture underlying acute myocardial infarction and stroke.^{27,109-114} A defining feature of AAA is inflammation and an extensive infiltration of lymphocytes and macrophages in the aneurysmal tissue.^{102,103,115} The hypothesis behind aneurysm formation is that these cells release a cascade of cytokines including monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) that activate proteases and thereby degrade the vessel wall. Substantial evidence from chronic conditions such as atherosclerosis and AAA documents transmural infiltration of a range of inflammatory cells, including macrophages, mast cells, lymphocytes and neutrophils.²⁵⁻²⁷ Chronic inflammation is associated with high concentrations of proteases, cytokines and chemokines that have been linked to medial collagen and elastic fibre degradation, and aortic cell apoptosis.^{76,116,117} Also, mast cells are reported to release TNF- α and IL-6 that encourages the expression of vascular adhesion molecules implicated in inflammatory heart disease.^{118,119} It is important to note that although the major pathophysiological alterations that lead to AAA and atherosclerosis for example have been well characterised (Table 1.5), the initial trigger for the inflammatory response is still under debate. Studies also established that the medial VSMC trigger apoptotic depletion of the cell population capable of maintaining connective tissue repair,¹²⁰ which leads to infiltration of macrophages and T-cells into the aneurysmal wall and the consequent release

of cytotoxic mediators such as perforin, tumour necrosis factor-alpha (TNF- α), Fas/FasL which can eliminate VSMC, destroy ECM and cause aneurysm formation.^{120,121}

Experimental evidence reveals that elastin and collagen degradation products in the aortic wall promote the recruitment of inflammatory cells.^{103,122,123} These cells are a source of proinflammatory cytokines, chemokines, prostaglandin derivatives; cysteine and serine elastases which mediate aortic wall degradation and VSMC apoptosis.^{124,125} Infiltration of proinflammatory cells and the purification of IgG from AAA tissue reactive to ECM proteins supports the concept that AAA development is an autoimmune response.¹²⁶ Both innate (natural killer/NK cells) and adaptive (cytotoxic lymphocytes) immune effectors are elevated in the circulation of patients with AAA where they exhibit VSMC apoptosis.^{127,128} Helper T-cell type-1 (Th1) and type-2 (Th2) cytokines were confirmed in both human AAA and animal models.¹²⁹ Inflammatory molecules: IL-6, interferon gamma (IFN- γ), TNF- α , IL-8 and MCP-1 were also reported to be higher in AAA tissue and are responsible for ECM remodeling which is a characteristic feature of AAA.^{103,130,131} Interestingly, infections such as that from *Chlamydia pneumoniae* have been suggested as the underpinning trigger for aneurysm formation.¹³² This claim was supported by investigations that demonstrated that more than half the patients with AAA had an elevated serum IgG titre against *C. pneumoniae* compared to those without aneurysm.¹³³

1.2.4.2 Proteolytic degradation of the aortic wall

Proteolytic degradation of the elastin and collagenous components of the arterial media in the pathogenesis of AAA has been well documented.⁹⁶ Animal based investigations,¹³⁴ as well as

in vitro studies on human aortic tissue,^{69,135} suggest that proteases including MMPs, cathepsins, and neutrophil elastase secreted by inflammatory cells and VSMC are implicated in the destruction of the aortic media and subsequent AAA formation.^{69,135-137} MMPs are Zn²⁺ and Ca²⁺ dependent enzymes,^{136,138,139} and are secreted in the inactive zymogen form.¹³⁵ They are then activated by mast cells and plasmin generated from plasminogen by the action of plasminogen activating factors such as urokinase-type plasminogen activator (uPA) and tissue plasminogen activator (tPA). Physiologically, MMP activity is strictly regulated by inhibitors such as α 2-macroglobulin, α 1-antitrypsin and TIMP, which help maintain the connective tissue balance. However, aberrant protease expression can result in unbalanced MMP activity, and lead to pathological destruction of the aortic media.^{135,140,141} There is an extensive body of work to suggest that this is a contributing factor in AAA pathogenesis (Table 1.1). MMP-9 (gelatinase B), and MMP-12 (macrophage elastase) are elevated in animal models of AAA as well as plasma and sera from patients with AAA.^{141,142} MMP-3 (stromelysin-1), MMP-7 (matrilysin), are also increased in aneurysmal tissue.⁶⁹ Over expression of the collagenases MMP-1 (interstitial collagenase) and MMP-13 (collagenase 3) results in interstitial collagen degradation in AAA. Additionally, high levels of MMP-2 (gelatinase A), are found in small AAAs, which suggests a role for MMP-2 in early aneurysm formation.¹⁰⁶ Some investigators have reported that blocking the active form of MMP-2 and MMP-9 with doxycycline, a tetracycline derivative, in both *in vitro* and *in vivo* studies suppresses aneurysm expansion rate.^{135,143} In contrast, another study demonstrated that while proMMP-9 plasma levels decrease in patients with small AAA with doxycycline treatment, there was no decrease in AAA development.¹⁴⁴ More recent studies however report that doxycycline treatment decreases aortic wall cytotoxic T-cell and neutrophil content,^{145,146} supporting a beneficial role of this drug in AAA. The variations in results suggest that there

is a need for an appropriate, sensitive and specific marker for AAA whose effect can be reproducible across borders.

Table 1. 1 Expression profiles of matrix metalloproteinases in abdominal aortic aneurysm

Protease	Technique Used	Producing Cells	Expression in AAA
MMP-1 ¹⁴⁷	ELISA ¹⁴⁷	VSMC, endothelial Cells	Increased
MMP-2 ^{106,134,135,143}	rtPCR, ^{135,143} Southern Blot ¹⁴³	VSMC, fibroblasts and mononuclear phagocytes	Increased
MMP-3 ^{69,143,148}	rtPCR ⁶⁹	Macrophages	Increased
MMP-7 ^{69,106,143,148}	rtPCR, ^{69,143} Southern Blot ¹⁴³	Macrophages	Increased
MMP-8 ¹⁴⁹⁻¹⁵¹	ELISA ¹⁴⁹⁻¹⁵¹	VSMC, endothelial Cells, and mononuclear phagocytes	Increased *
MMP-9 ^{106,134,135,141-144,147,148}	ELISA, ^{141,142,144,147} PCR, ¹⁴² rtPCR ¹⁴³ , Southern Blot ¹⁴³	VSMC, mononuclear phagocytes	Increased
MMP-10 ⁶⁹	rtPCR ⁶⁹	Mononuclear cells, endothelial cells, and fibroblasts	Not expressed *
MMP-11 ⁶⁹	rtPCR ⁶⁹	Fibroblasts, B-cells, astrocytes	Increased
MMP-12 ^{106,141,142}	ELISA ¹⁴¹ , PCR ¹⁴²	Macrophages, stromal cells	Increased
MMP-13 ^{152,153}	Southern Blot, ¹⁵² rtPCR ^{152,153}	VSMC, fibroblasts, and endothelial cells	Increased *
MMP-14 ¹⁴⁸	Unpublished. ¹⁴⁸	VSMC, macrophages, Langerhans cells	Increased
MMP-15 ⁶⁹	rtPCR ⁶⁹	T-cells, VSMC, and endothelial cells	Not expressed *
MMP-16 ⁶⁹	rtPCR ⁶⁹	VSMC, and macrophages	Not expressed *
MMP-17 ⁶⁹	rtPCR ⁶⁹	Mononuclear cells, endothelial cells, and macrophages	Increased

* Shown not to be expressed by Carrell *et al.*, 2002 on their comparison of 14 MMPs and MMP inhibitors expression patterns between 8 AOD and 8 AAA patient tissue samples.⁶⁹ Abbreviations and alternate names given in text: MMP-3 (Stromelysin-1), MMP-8 (Neutrophil Collagenase), MMP-10 (Stromelysin-2), MMP-11 (Stromelysin-3), MMP-14 (Membrane type-1 metalloproteinase), MMP-15 (Membrane type-2 metalloproteinase), MMP-16 (Membrane type-3 metalloproteinase), MMP-17 (Membrane type-4 metalloproteinase), rtPCR (reverse transcriptase polymerase chain reaction)

1.2.4.3 Genetic predisposition to AAA and stresses within the aortic wall

AAA has a significant familial component and ~ 20 % of patients have relatives who develop the disease.¹⁵⁴ Although AAA is heterogeneous, quantitative trait loci (QTL) have been identified on chromosome 19q13^{155,156} and 4q31.¹⁵⁵ Current polymorphisms associated with AAA match known pathogenesis of the disease demonstrated by inflammation (HLA-DQA1),¹⁵⁷ MMP-9,¹⁵⁸ PAI-1,¹⁵⁹ and TIMP-1 genes.¹⁶⁰ The prevalence for aneurysms to form in the infrarenal aorta suggests potential differences in the physiology, anatomy and biomechanical stress along the aorta. For example, evidence suggests that the distal infrarenal aorta has lower levels of elastin compared to the proximal suprarenal, thoracic and arch of the aorta.¹⁶¹ The decreased elastin mass in the infrarenal aorta may potentially result in a weaker aortic wall and an increased tendency to develop aneurysms at this site.^{28,120}

1.2.5 AAA prevalence

Incidence of AAA has significantly increased over the years; however more recent data suggest that the incidence of clinically relevant AAA may be on the decline due to a 5-10 year increase in the age at which AAA presents clinically.^{18,162} Screening studies for AAA show predominance in males with a male to female ratio of 5:1.^{18,163,164} However, recent data indicate a surge in the number of females diagnosed and dying from the disease.^{9,18,165,166} In addition, with an increasing ageing global population, AAA has been predicted to become an increasingly important socio-economic disease as its incidence rises in parallel with increased life expectancy.^{18,167}

1.2.6 AAA risk factors

Epidemiological screening studies provide the best insight into the risk factors for AAA development (Table 1.2).¹⁴ Male gender and age are established risk factors while peripheral vascular disease; diabetes mellitus and female gender seem to be protective.^{8,53,168-170} Smoking is strongly correlated to AAA development,^{22,167,171} and active smokers are more susceptible to developing AAA than non-smokers or those who have previously smoked.^{167,172} Prevalence of AAA in lifelong smokers is also ≥ 4 times that in non-smokers.²² The recent rise in women diagnosed with and dying of AAA may be due to the increasing number of female smokers,^{8,9} some yet to be elucidated hormonal factor, or the tendency of women to live longer than men. Aneurysms have been shown to progress faster and are more liable to rupture in smokers compared to non-smokers probably because smoking increases the degradation of aortic connective tissue.⁵³ Also linked to AAA predisposition is ethnicity, and the disease is more common in white northern Europeans than in their Asian or African counterparts.^{11,163,173} A positive family history is indicative of AAA risk, and occurs in ~ 20% of cases in patients with first-degree relatives with the disease.^{11,61,173} Other risk factors for AAA include hypertension, hyperlipidaemia, and chronic obstructive pulmonary disease.^{53,174,175}

Table 1. 2 Risk factors associated with AAA

Predisposing Factors	Protective Factors
Smoking ^{11,22,53,167,171}	Diabetes mellitus ^{11,169-171}
Male gender ^{8,11,22,53,169,170}	Africans and Asians ^{11,14,173}
≥ 60 years of age ^{8,11,22,53,169,174}	Female gender ^{ψ11,22,53,169}
Northern european/caucasian ^{11,14,173}	≤ 50 years of age ¹⁷⁴
Family history ^{11,168,169,173}	
Hypertension ^{11,22}	
Hyperlipidaemia ^{φ11,174}	
COPD ^{*;φ11}	

* Chronic Obstructive Pulmonary Disease

φ Not conclusive

ψ Women tend to have poor prognosis^{9,18,165,166}

1.2.7 Experimental models of AAA

There is limited information on the pathogenesis of AAA because it is ethically and practically inappropriate to obtain aneurysmal tissue for analysis in the developmental stages of the disease from human subjects. Researchers are thus restricted to the analysis of pathology of tissues harvested during AAA repair to understand the disease process. Experimental models of AAA (Table 1.3) resemble the essential physiological and biochemical characteristics of the progression of the human disease,¹³⁴ thus enabling a better understanding of AAA formation, development and progression. Mice are the experimental animal of choice in AAA research because they are easy to handle, reproduce quickly, are low-priced, readily available, small, and have ~ 99% genetic similarity to humans.¹⁷⁶ Some of the AAA models demonstrate several aspects of the disease including atherosclerosis, medial degeneration, inflammation, thrombus formation, and rupture.¹³⁴ Current methods of inducing aneurysm in mice are broadly classified into three approaches:^{134,177,178}

1. Chemically induced AAA mouse models;
2. Genetically modified AAA mouse models;
3. Combined gene knockout and chemically induced AAA.

1.2.7.1 Chemically induced AAA mouse models

Brief intraluminal infusion of porcine pancreatic elastase into the infrarenal aorta in rats was the first chemically-induced AAA model developed.¹⁷⁹ This elastase model was later modified for mice,^{180,181} and leads to delayed dilation of the aorta present with medial degeneration and inflammation.^{134,179,181,182} Another model present with medial degeneration and inflammation involves the application of calcium chloride (CaCl₂) on the periaortic wall for ~ 10 minutes.¹⁸³ Most mice treated with CaCl₂ develop AAA within 2 - 4 weeks.^{134,183}

1.2.7.2 Genetically modified AAA mouse models

Defective cross-linking of collagen and elastin due to a decrease in lysyl oxidase (Lox) following aberrant copper absorption as a result of mutations on the X-chromosome is the classic scenario in 'Blotchy' mice.^{184,185} These mice develop aneurysms with medial degeneration and a gender bias towards males,^{186,187} but the aneurysm is not restricted to the aorta in the abdominal region thereby limiting the usefulness of this model.^{134,185,187} Genetically engineered defects in MMP-3¹⁸⁸ and TIMP-1,^{189,190} are associated with aortic aneurysm development with medial degeneration. The lack of specificity to the abdominal part of the aorta in aneurysm development of the MMP-3 and TIMP-1 models has constrained their exploitation in AAA research. The apolipoprotein E deficient (ApoE^{-/-}),¹⁹¹ and low density lipoprotein receptor knock-out (LDLR^{-/-}) mouse models,¹⁹² which were originally developed for atherosclerosis investigations,¹⁹³ are also linked to a suprarenal

aorta restricted AAA development.¹³⁴ These mice are hyperlipidaemic and present with medial degeneration (LDLR^{-/-}), or medial degeneration and atherosclerosis (ApoE^{-/-}).¹³⁴

1.2.7.3 Combined gene knockout and chemically induced AAA

Subcutaneous infusion of angiotensin II (AII) has been demonstrated to induce aortic aneurysm formation in mice,¹⁹⁴ and appears to aggravate a disposition for small aneurysm development in the hyperlipidaemic ApoE^{-/-} or LDLR^{-/-} mouse strains.¹⁹⁵ Subcutaneous infusion of AII at a dose of ~ 1.0 ug/kg/min via an implanted osmotic pump into ApoE^{-/-} mice over 28 days leads to AAA formation in the suprarenal aorta (Figure 1.4).^{134,196,197} AAA formation in this mouse model is similar to the human disease in terms of site specificity, medial degeneration, atherosclerosis, inflammation and thrombus formation,¹³⁴ and gender preference for males.¹⁹⁸ Increased expression of MMP-9, MMP-12, chymase, and ACE have also been reported in late-stage aneurysms following AII infusion in hyperlipidaemic mice.¹⁹⁹ Saraff *et al.* reported that aortic dissection is a common cause of rupture of large aneurysms within the first week following AII infusion in these models.²⁰⁰ However, evidence implies that AII infusion may exacerbate the formation of traditional cardiovascular risk factors such as atherosclerosis, suggesting that it is difficult to study AAA as a separate disease in this model.^{201,202} Nevertheless, AII infusion of hyperlipidaemic mice is widely employed for studying AAA, and has generated relevant therapeutic and mechanistic data globally,^{199,202-205} and within the Vascular Biology Unit of the James Cook University.^{206,207}

Table 1. 3 Examples of mouse models of abdominal aortic aneurysm

Mouse model	Comments	Pathology
Genetically modified		
Lox ^{-/-}	Gender bias, rupture, foetal death	Medial degeneration
MMP-3 or TIMP-1 defects	Not site selective	Medial degeneration
LDL receptor ^{-/-}	AAA with high fat diet only	Medial degeneration
ApoE ^{-/-}	Gender bias	Medial degeneration, atherosclerosis
Chemically-induced		
Topical elastase	Difficult technique, not site selective	Medial degeneration, inflammation
Topical calcium chloride	Difficult technique, not site selective	Medial degeneration, inflammation
s.c AII	~40% suprarenal aneurysm	Inflammation
Combined method		
LDL receptor ^{-/-} + AII infusion		Medial degeneration, inflammation, thrombus
ApoE ^{-/-} + AII infusion	~80% suprarenal aneurysm, site selectivity, gender bias	Medial degeneration, atherosclerosis, inflammation, thrombus, rupture

* Apolipoprotein E deficient (ApoE^{-/-}), Low density lipoprotein (LDL), Lysyl oxidase deficient (Lox^{-/-}), Matrix metalloproteinase-2 (MMP-2), Subcutaneous (s.c), and Angiotensin II (AII)

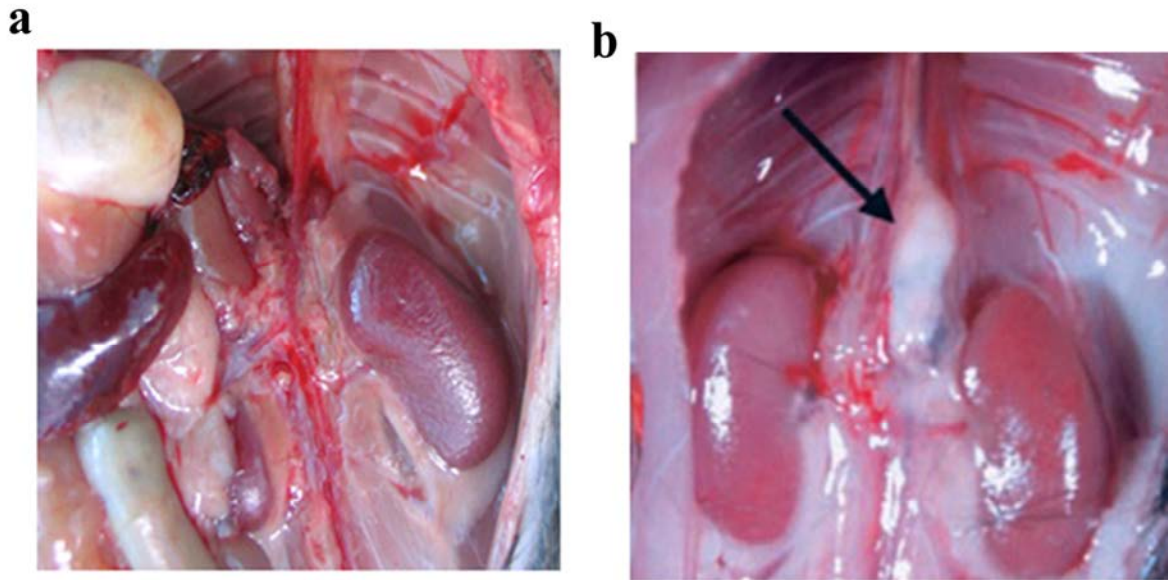


Figure 1. 4 AAA formation in ApoE^{-/-} mice infused with AII

Control mice infused with saline (a), Arrow indicates SRA restricted AAA in AII infused mice (b). Modified from Daugherty *et al.*, 2004.

1.2.8 Clinical management of AAA

A detailed practice guideline for the clinical management of AAA has been provided by the SVS.⁵ A generalised summary of the AAA management protocol is presented here. Current AAA diagnosis is determined by imaging.^{11,208} Initial aortic diameter has been shown to predict subsequent increase in AAA diameter and risk of rupture,^{53,54,56} but there are variations in the rates of expansion among patients.⁵³ Usually AAA is clinically managed by regular ultrasound monitoring and surgery based on the expansion ratio of the normal to aneurysmal aortic diameter, history, operative morbidity rate, and when the aneurysm is ≥ 5 cm.⁴² Currently, there are two surgical means of repairing AAA: open surgical repair and endovascular aneurysm repair (EVAR) which involves the insertion of a stent graft to bridge

the healthy parts of the aorta.^{14,209} Around 5000 AAA repairs are carried out in Australia annually at a cost of ~ \$100 million and in other western countries such as the UK and USA.²⁰ Open surgical repair has been the traditional means of managing AAA while EVAR is relatively new and rapidly becoming the preferred method of AAA repair. Both treatment options are limited by a significant peri-operative morbidity and mortality rate. There is however, a lower incidence of myocardial ischemia using EVAR in contrast to open surgical repair.²¹⁰ Although long term follow up of patients post EVAR indicate that ~ 20% require re-intervention within 5 years, and a proportion of them suffer late terminal complications such as rupture.²¹¹⁻²¹³

Ultrasound screening programs have been introduced in some countries and have been shown to be effective in reducing mortality due to aortic rupture; initial appraisal indicates that screening programs detection of AAA incidence is lower than expected.^{214,215} In addition following diagnosis, prognostic determinants for AAA are poorly defined.⁵³ Furthermore, overall evidence suggest that imaging-based screening trials have not been very successful in reducing AAA-related mortality,²¹⁶⁻²¹⁸ and randomised clinical trials suggest that elective open surgical repairs of small AAAs ($\leq 5\text{cm}$) does not improve patient survival in the long term.²¹⁹ Therefore, a better means of detecting, following, and treating AAA is required. Identifying circulating markers for AAA has been proposed as a means to improve and/or complement current AAA detection and diagnosis.²²⁰ A better understanding of the various functional influences involved in AAA biology would provide reliable targets that could guide patient management and allow effective diagnostic, prognostic and therapeutic determinants for the disease.

1.2.9 Biomarkers

The United States Food and Drug Administration defines a biomarker as a characteristic which can be independently quantified and appraised as an indicator of normal physiological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.^{221,222} Biomarkers may be lipids, metabolites or proteins such as enzymes which provide functional information about disease that may guide the therapeutic approach. Biomarkers that can reliably predict the risk of contracting a specific ailment, detect the presence and/or severity of a disease, or monitor response to therapy are of considerable medical interest.^{223,224} Although many biomarkers may be expressed within patient tissues, those present within bodily fluids such as serum, plasma or urine are particularly useful as they can be easily sampled in a relatively non-invasive manner.¹⁵

The ideal biomarker would be a highly specific and very sensitive factor found in bodily fluid that is able to detect the presence and/or progress of the disease independently of other established predictors. A number of putative biomarkers have been described for AAA (Table 1.4), nevertheless these biomarkers have not been fully validated and accepted clinically for AAA.

Table 1. 4 Examples of biomarkers associated with abdominal aortic aneurysm

Marker	Tissue Assessed	Association with AAA
OPN ²²⁵	Serum	Inflammation, calcification?
OPG ¹⁹⁷	Serum	Inflammation, calcification?
IFNγ ¹³³	Serum	Inflammation
CRP ⁵⁴	Serum/plasma	Inflammation
PAP ²²⁶	Plasma	Thrombus
IgA ²²⁷	Serum	Infection, inflammation
EP ²²⁶	Serum	ECM turnover
PIIINP ²²⁸⁻²³⁰	Serum	Degradation/turnover of ECM
Cystatin C ²³¹	Serum	Degradation of ECM
MIF ²³²	Serum	Inflammation
MMP-9 ^{29,140-142,233}	Plasma	Degradation of ECM
TNFα ²²⁹	Plasma	Inflammation
D-dimer ²³⁴⁻²⁴⁰	Plasma	Thrombus

Osteopontin (OPN), Osteoprotegerin (OPG), Interferon gamma (IFN γ), C-reactive protein (CRP), P-plasmin antiplasmin complex (PAP), IgA (Immunoglobulin A), Cp (*Chlamydia pneumoniae*), Serum elastin peptide (EP), Pro-collagen type II N-terminal peptide (PIIINP), Macrophage migration inhibitory factor (MIF), and Tumour necrosis factor alpha (TNF α).

The discovery of the urocortins (UCNs) in recent years however has generated a substantial amount of interest in their possible role within the cardiovascular system.⁵ The following sections review the UCNs with particular emphasis on the possibility of them being clinically relevant targets for AAA diagnosis, prognosis and therapy.

1.2.10 The Urocortins

An interesting development in recent years is the identification of a group of peptides known as the UCNs which are members of the corticotrophin-releasing factor (CRF) family of

peptides (Figure 1.5).^{30,31,32} The sequence alignment for this figure was generated using Clustal W,²⁴¹ and the calculation of amino acid identity between proteins created employing BioEdit v.7.0.9.0.²⁴²

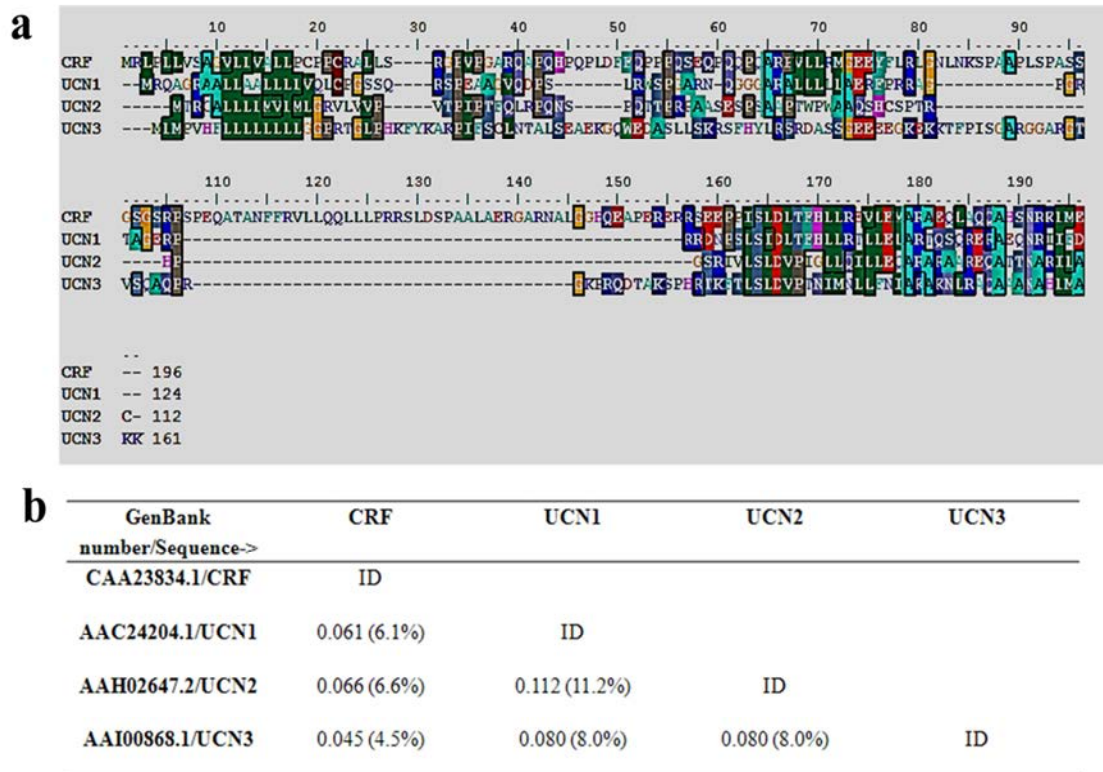


Figure 1. 5 Regions of amino acid similarity between the urocortin related peptides

(a) Clustal W generated multiple sequence alignments of the human CRF peptide family; boxes represent identical regions of amino acid conservation created using BioEdit software v.7.0.9.0.

(b) Calculation of amino acid identity between proteins expressed as decimalised percentages employing BioEdit v.7.0.9.0. Total amino acid identity between sequences is shown in parenthesis.

The UCNs have been demonstrated to be expressed in diverse inflammatory milieu,^{243,244,245} and have been associated with cardiovascular homeostasis.^{244,246-248} They have also been demonstrated to play important modulatory roles in various tissues including the brain, immune system, and gastrointestinal system.^{249,250} The UCNs are implicated in both central and peripheral inflammatory responses.^{243,251} Since their initial identification,³⁰ UCNs are

emerging as putative therapeutic targets for cardiovascular diseases (CVD) based on research using animal models.^{247,252,253} A number of animal and human studies reveal that UCNs may be relevant to AAA and atherosclerosis formation and progression (Table 1.5). UCNs have also been demonstrated to be ligands for cell prosurvival pathways,^{254,255} and inhibitors of inflammation,²⁴⁴ and apoptosis,²⁴³ which are all critical mechanisms in AAA pathology. These data suggest that the UCNs could play a significant protective role in AAA pathogenesis, and may inhibit AAA development.²⁴⁶

The UCNs were first described in 1995, urocortin 1 (UCN1), a 40 amino acid CRF-related peptide was first identified by the Vale group at The Salk Institute, San Diego.³⁰ The name '*urocortin*' was derived from its peptide homology and similar physiological activities to the Teleost (ray-finned fish) hormone urotensin 1 (63%), mammalian/rat CRF (45%), and frog sauvagine (35%).³⁰ Six years after the cloning of UCN1, two UCN-like peptides, UCN2 and UCN3 were cloned and sequenced from mouse and human cDNA libraries.^{31,32,256} Hsu and Hueh identified UCN2 as a 43 amino acid peptide and UCN3 as a 40 amino acid peptide related to CRF and UCN1.²⁵⁶ More recent data suggest that the UCNs may be relevant to the stages of AAA as well as atherosclerosis formation and progression (Table 1.5).^{120,257,258}

27,111,259-261

Table 1.5 Summary of the possible stages of AAA & atherosclerosis progression with relevance to urocortins

CVD Developmental Stage	Cell types Involved	Pathological Traits
AAA initiation	Effector T-cell, macrophages and neutrophils. ²⁶²	Aortic wall inflammation. ^{257,258}
AAA progression	Macrophages, T-cells, mast cells, VSMC, endothelial cells and neutrophils. ^{120,257,258}	VSMC apoptosis, elastin degradation and thinning of aortic media. ^{99,263}
AAA rupture	Macrophages, T-cells, neutrophils, and mast cells. ^{120,257,258}	Extensive inflammatory cell infiltration. ^{102,257,258} ↑MMP1, MMP2, and MMP9 ^{147,148,151}
<hr/>		
Atherosclerosis initiation	Foam cells and macrophages. ^{27,111,259,260}	Tunica intima inflammation, endothelial cell injury and fatty streaks. ^{27,111,259,260}
Atherosclerosis progression	T-cells, mast cells, monocytes, macrophages and VSMC. ²⁷	Vascular remodelling (thickening), VSMC proliferation and migration. ^{27,111,259-261}
Atherosclerosis plaque rupture	Macrophages, T-cells, mast cells, VSMC and endothelial cells. ^{27,110,264-268}	Fibrous cap rupture ¹¹²⁻¹¹⁴ and thrombo-embolism. ^{27,109-111}

1.2.10.1 Synthesis and localisation of UCNs

UCN1 is widely expressed and has been detected within multiple regions of the brain including the Edinger-Westphal nucleus as well as within the heart, and associated with lymphocytes and mast cells.^{30,269-273} Human aortic endothelial cells have been demonstrated to express all three members of the UCN family.³⁴ However, UCN2 expression appears to be limited to specific regions of the brain,³² whereas UCN3 appears to be more ubiquitously expressed and has been identified in the brain, heart, vascular endothelial cells, vascular smooth muscle cells (VSMC), kidneys and plasma.²⁷⁴⁻²⁷⁹ The following cardiovascular cell types have been reported to express the UCNs and their receptors; endothelial cells, smooth

muscle cells, fibroblasts and myocytes.^{33,34} A detailed description of the relative expression of the UCNs by these cell types, has been provided in a review by Davidson and Yellon (Table 1.6).³³ In general, the UCNs appear to be present within the brain and the peripheral tissues.

Table 1. 6 Expression of the UCNs in the heart and vasculature

Cell/Organ	Peptide		
	UCN1	UCN2	UCN3
Endothelial cells	Rat protein		
VSMC	Rat protein		
Fibroblast	Rat neonatal mRNA	Mouse neonatal mRNA	
Myocytes	Rat neonatal mRNA	Rat and mouse neonatal mRNA	Rat mRNA
Heart	Human mRNA and protein	Mouse mRNA, human mRNA and protein	Mouse mRNA, human mRNA (weak expression) and human protein

Table adapted from Davidson and Yellon, 2009. Messenger Ribonucleic acid (mRNA), Urocortin 1 (UCN1), Urocortin 2 (UCN2), Urocortin 3 (UCN3)

1.2.10.2 Urocortin receptors

CRF and its related peptides including the UCNs bind to two distinct G-protein coupled receptors (GPCRs), CRF receptor type 1 (CRFR1) and CRFR type 2 (CRFR2).²⁸⁰ Both CRFR1 and CRFR2 are expressed in the brain, the heart and the blood vessels,²⁷⁸ but the predominant receptor type in the vasculature is CRFR2.^{280,273} CRFR2 and UCN1 messenger ribonucleic acids (mRNAs) have been reported to be present in all four chambers of the human heart.²⁷⁸ Evidence from Tu *et al.* showed that CRFR1 binds UCNs and CRF with similar affinity, whereas CRFR2 binds UCNs with a 40-fold higher affinity than CRF.²⁸¹ Collectively, these reports demonstrate that CRFR1 and CRFR2 are widely expressed in brain and vascular tissues and exhibit selective affinity for the UCNs. Figure 1.5 above, shows the protein sequence alignment of the UCNs and CRF.

1.2.10.3 Urocortin signalling

In general, UCN signalling seems predisposed to maintaining a cardiovascular homeostasis. On binding to CRFR1 and CRFR2, the UCNs activate GPCR signal transduction leading to adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production.²⁸¹⁻²⁸³ By binding to CRFR1 and CRFR2, UCNs induce conformational changes that result in the activation of various intracellular signalling pathways (Figure 1.6). For instance, the UCNs have been reported to initiate an intricate signalling cascade involving activation of phosphoinositide-3-kinase (PI3K), serine threonine specific protein kinase (Akt), mitogen-activated protein kinases (MAPKs), protein kinase A (PKA) along with other less clearly defined signalling pathways.²⁵⁵ The UCNs have been demonstrated to increase survival of murine cardiomyocytes exposed to ischaemia/reperfusion injury by activating the PI3K and Akt pathways.^{254,255} On activating the MAPKs and PKA pathways, UCNs have been known to cause vasodilatation of the aorta,²⁸² whereas by activating protein kinase C epsilon (PKC ϵ), UCNs alter the activity of multiple channels including the mitochondrial permeability transition pore (mPTP), that is directly involved in the induction of apoptosis (Figure 1.6).^{253,284} The mPTP has been reported to be involved in UCN1 induced protection against ischaemia/reperfusion injury in rats.²⁵³ UCNs have been demonstrated to enhance expression of the mitochondrial adenosine triphosphate (ATP) sensitive potassium channel (K_{ATP}),²⁸⁵ and decrease the activity of the calcium insensitive phospholipase A₂ (iPLA2) channels,²⁸⁶ both of which are involved in cardioprotective signalling.²⁸⁵⁻²⁸⁸ By decreasing iPLA2 expression, the UCNs inhibit the expression of lysophosphatidylcholine (LPC), a toxic metabolite of phospholipase A₂ implicated in the reactive oxygen species (ROS) damage to cardiac and aortic cells.^{289,290}

In addition, the PI3K/Akt signalling pathway is involved in migration, growth and cellular survival (Figure 1.6).^{291,292} Phosphorylation of the PI3K/Akt signalling pathway has been proposed as a means by which UCNs exert their cardiovascular protection.^{206,244,247,248} However, UCNs-induced cardiovascular protection conferred through this signalling pathway was reportedly blocked *in vitro* by wortmannin and LY294002 in cultured neonatal and adult cardiomyocytes that suggest the importance of these pathways in UCN mediated effects.²⁹³

Several *in vivo* studies of ischemia/reperfusion models demonstrate increased cell survival following UCNs activation of the anti-apoptotic extracellular signal regulated kinases 1 and 2 (ERK1/2) pathway.^{252,254,294} Proof of principle studies demonstrated that blocking the ERK1/2 pathway with PD98059 and U0126 resulted in a significant decrease in UCNs-induced cellular survival suggesting a cardiovascular protective role of the UCNs through these signalling pathways.^{252,254,294} Similarly, inhibition of the ERK1/2 pathway by PD98059 was also reported to diminish UCN-associated decrease in infarct size during ischemia/reperfusion.²⁵² Furthermore, in a recent study to examine the mechanism underlying the cardiovascular protective effects of UCNs, UCN1 was shown to provoke the expression of heat shock protein 90 (hsp90),²⁹⁵ which helps maintain cellular homeostasis.²⁹⁶ Astressin2-B (Ast-2B), a selective CRFR2 antagonist, and PD98059 abolished the increased cell survival induced by UCN2 and UCN3 in cultured cardiomyocytes,²⁵⁵ suggesting the importance of this signalling molecule in UCNs-mediated effects. PD98059 was also demonstrated to inhibit the expression of hsp90 induced by UCN1.

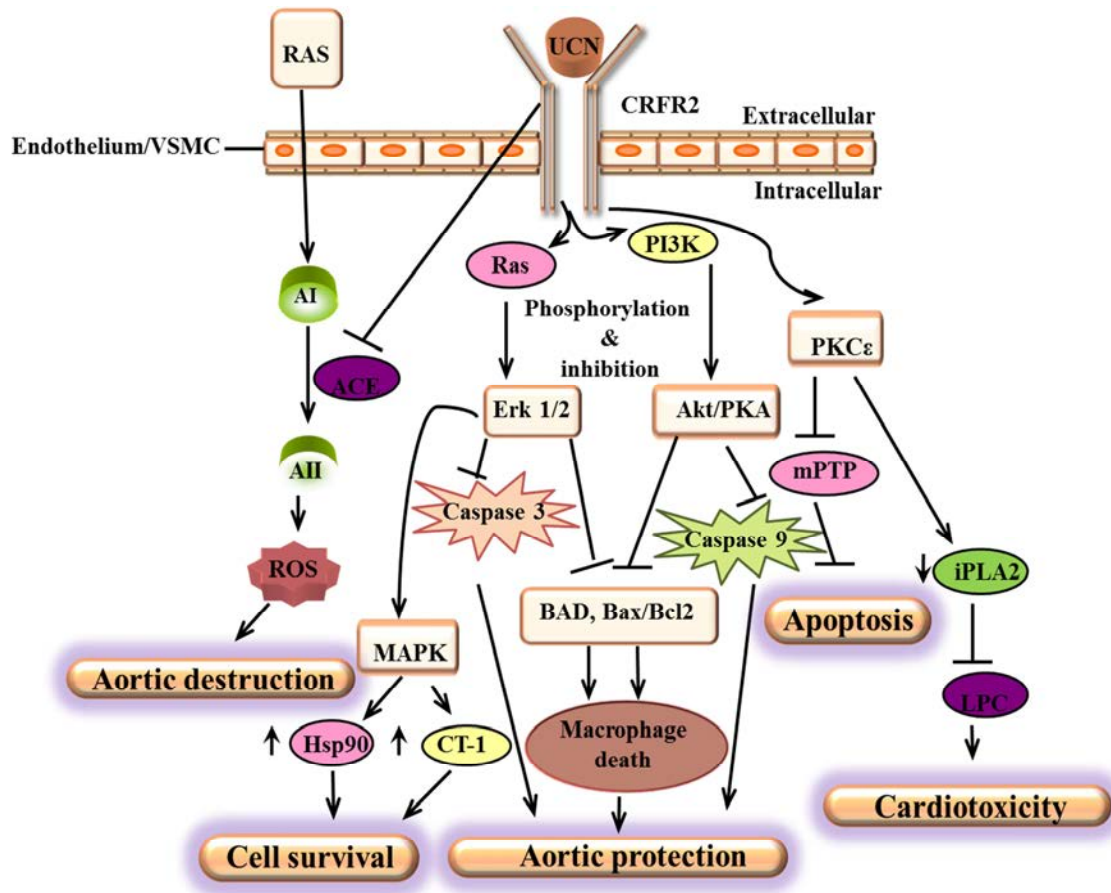


Figure 1. 6 Proposed signalling mechanism of urocortins as relevant to cardiovascular disease

UCNs signalling via CRFR2 are reported to inhibit ACE; preventing conversion of AI to AII thereby decreasing the destructive action of AII induced ROS. UCNs activate PKCε which inhibits the apoptosis inducing mPTP and the toxic LPC. PKCε = Protein kinase epsilon; Raf-Ras-ERK1/2 = extracellular signal regulated kinases 1 and 2; BAD = Bcl-2-associated death promoter; Bax = Bcl-2-associated X protein; Bcl2 = B-cell lymphoma 2; CT-1 = Cardiostrophin-1; and AI = Angiotensin I.

1.2.10.4 Urocortins: Protective or destructive force in the cardiovascular system?

It is widely accepted that UCNs have potential to modify pathological processes critical to CVD in the cardiovascular system, although it is unclear whether the actions of the UCNs promote or impede CVD progression.^{247,255,294,295} Despite this, the specific role of UCNs in cardiovascular disease, especially AAA, has not been clearly demonstrated. Below, a summary of available literature describing the putative relationship between the UCNs and the cardiovascular system is given.

1.2.10.5 Studies in animal models

UCNs have been linked to several roles within the cardiovascular system including anti-inflammatory cardiovascular protection,^{297,244} and vasodilatation.²⁹⁸ For instance, using a mouse model, Agnello *et al.* demonstrated significant reduction in lipopolysaccharide-induced serum TNF α and IL-1 β levels *in vivo* with no effect on serum IL-6 following subcutaneous injection of UCN1 (1 μ g/mouse).²⁹⁹ TNF α has been implicated in the development and progression of CVDs,^{300,301} including atherosclerosis,³⁰² therefore inhibition of TNF α and IL-1 β secretion may be a means by which UCNs could exert an anti-inflammatory protective effect on the cardiovascular system. Also, knockout studies using CRFR2 deficient (CRFR2^{-/-}) mice showed that, CRFR2^{-/-} mice lost the improved cardiac performance or decreased blood pressure associated with systemic UCN1 administration in contrast to wild-type mice, demonstrating that CRFR2 and its agonist UCN1 participate in UCN-induced cardiovascular homeostasis.³⁰³ An extensive interest in the therapeutic potential of UCNs was generated by reports that UCNs can improve cardiac function in heart failure.^{33,304} Studies by Rademaker *et al.* employing a sheep model of heart failure demonstrated consistent positive dose-dependent diuretic, natriuretic, and cardiac effects of the UCNs.^{274,304-306} In sheep in which heart failure was induced, administration of the UCNs induced a range of beneficial effects, including increased urine volume; increased sodium excretion; increased creatinine excretion; increased cardiac output; decreased plasma concentrations of vasopressin, renin, aldosterone, endothelin-1, and atria natriuretic peptide.^{274,304-306} Of interest, the decrease in renin stimulated by the UCNs could potentially have beneficial effects in limiting atherosclerosis and AAA,^{134,196,197} since renin initiates the cascade of events leading to AII production.³⁰⁷ Altogether, these reports suggest a beneficial role of UCNs in the cardiovascular system.

Deleterious effects of the UCNs have been reported in animal studies that demonstrate the presence of immunogenic UCNs. For example, a recent study by Xu *et al.* employing an experimental rat model of thromboangitis obliterans (TAO) determined that exogenous administration of UCN1 exacerbated the vasculitis and the hypercoagulable state induced by sodium laurate via CRFR1.³⁰⁸ Studies in rats suggest that UCN1 stimulates mast cell degranulation,²⁷² and increased vascular permeability and influx of inflammatory cells. Mast cells have been implicated in AAA progression,^{309,310} AAA pathogenesis,³¹¹ atheroma rupture,²⁶⁴ and atherosclerosis progression.^{266,311} Furthermore, given the reports that sustained UCN1 administration promotes angiotensin converting enzyme (ACE) secretion with consequent AII production,^{312,313} and that AII infusion induces atherosclerosis and AAA formation,^{134,196,197} it is possible that the UCNs could promote AII induced pathology such as atherosclerosis and AAA. These reports suggest that the UCNs may not be as favourable to the vasculature as widely accepted. However bearing in mind that Rademaker *et al.* have systematically demonstrated sustained decrease in renin and AII by the UCNs in the circulation,^{274,304-306} and the majority of animal studies suggesting a beneficial effect of UCNs in CVD (Table 1.7), more studies are needed to define the specific roles of these peptides in CVD pathogenesis.

Table 1. 7 Animal studies assessing the role of urocortins in cardiovascular diseases

Animal Model	Intervention	Outcome
Sheep experimental heart failure ²⁷⁴	UCN1 i.v. (10,50 & 100 mg at 2h intervals)	↑ Cardiac output ↓ Peripheral resistance in heart failure sheep compared to controls
Sheep experimental heart failure ³⁰⁶	UCN3 i.v. (10,50 & 100 mg at 2h intervals)	↑ Cardiac output ↓ Peripheral resistance ↓ Renin, aldosterone, vasopressin & endothelin-1 in heart failure sheep compared to controls
Rat experimental blood pressure ³¹⁴	UCN2 i.v. (0.001 -1 mg/kg) at 20 min intervals	↓ Mean arterial pressure & heart rate.

1.2.10.6 *In vitro* investigations of the UCNs

In a recent experiment to determine the effects of UCN1 on human blood vessels, Sanz *et al.*, 2009 reported that UCN1 at concentrations between 1 picomolar (pM) and 10 nanomolar (nM) increases vascular relaxation independent of nitric oxide release by the vascular wall,³¹⁵ which could be beneficial in CVD. In another study examining the mechanism involved in the protective effect of UCNs in myocardial infarction and heart failure,²⁵⁵ UCN2 and UCN3 were reported to protect neonatal and adult mouse ventricular cardiomyocytes from ischaemia/hypoxia and reduce the percentage of infarct size in an *ex-vivo* Langendorff perfused rat heart.²⁵⁵ These protective effects were attributed to the ability of the UCNs to activate the anti-apoptotic ERK1/2 pathway by binding to CRFR2.^{252,254,255,294} UCN1 was also demonstrated to have strong anti-oxidant properties by potently inhibiting the production

of AII-induced ROS in human umbilical cord vascular endothelial cells (HUVECs).²⁴⁴ UCN1 inhibition of ROS production may be a mechanism by which UCNs could protect from CVDs by significantly reducing free radical damage following myocardial infarction (see Table 1.8).³¹⁶ Honjo *et al.* showed that treatment of HUVECs with proinflammatory cytokines like TNF α and interferon gamma (IFN γ) upregulated endothelial UCN1 production with further suppression of ROS.²⁴⁴ Since IL-6 and TNF α are implicated in the pathogenesis of AAA^{317,318} and atherosclerosis,^{302,319,320} inhibition of these cytokines may be one of the mechanisms involved in the purported UCN-mediated cardiovascular protection. Conversely, Huang *et al.* demonstrated that in rat ventricular cardiomyocytes,³²¹ UCN1 induces the synthesis and release of IL-6, a widely accepted independent risk factor for coronary artery disease.³²² This result is also supported by another study demonstrating that in the A7r5 aortic smooth muscle cell line, both UCN1 and UCN2 stimulated IL-6 gene transcription and secretion via CRFR2.³⁹ Overall, available *in vitro* data favours a vascular protective role of the UCNs, although care should be taken in classifying these peptides as exclusively protective.

Table 1. 8 *In vitro* studies assessing the role of urocortins in cardiovascular diseases

Cell type	Intervention	Outcome
Rat cardiac myocytes ³²³	UCN1 treated (10 ⁻⁸ M)	↑ Cell survival post hypoxia
Rat cardiac myocytes ²⁵²	UCN1 treated (10 ⁻⁸ M)	↓ Apoptosis & necrosis ↑ ERK1/2 signalling Pathway
Rat cardiac myocytes ²⁵⁴	UCN1 treated (10 ⁻⁸ M at 10min, 1h,6h &24h)	↓ Apoptosis ↑ Cell survival ↑ ERK1/2 signalling Pathway
Mouse & rat cardiomyocytes ²⁵⁵	UCN2 and UCN3 treated (0,1,10,100nM for 2h)	↑ Cell survival ↑ ERK1/2 signalling Pathway
Human venous smooth muscle cells ³¹⁵	UCN1 treated (1pM-10nM)	↑ Vascular relaxation
Rat cardiac myocytes ²⁵²	UCN1 treated (100nM)	↓ Infarct size of <i>in vitro</i> perfused rat heart ↑ ERK1/2 signalling Pathway

Increase (↑), Decrease (↓), nanomolar(nM), molar (M), hour (h), subcutaneous (s.c)

1.2.10.7 Circulating UCNs concentration in CVD

Four clinical investigations have been described detailing the association of UCNs with CVDs (Table 1.9). In a study investigating the circulating levels of UCN1 in 119 patients with heart failure and 212 age and sex matched normal controls, the plasma levels of UCN1 were shown to be significantly increased especially in the early stages of heart failure in both males ($p < 0.0005$) and females ($p < 0.001$).²⁷⁵ The investigators also reported higher UCN1 expression in males than females, and a gradual decrease in circulating UCN1 levels with

increasing age, and with rising New York Heart Association (NYHA) class.²⁷⁵ The decline in UCN1 with increasing severity of heart failure and increasing age was suggested to be due to the breakdown of the UCN1 response mechanism.²⁷⁵ Contrary to this, in a recent study comparing 42 subjects with heart failure and 20 healthy sex and gender matched controls, Gruson *et al.* reported higher plasma UCN1 concentrations in all stages of heart failure (NYHA class I-IV).³²⁴ Furthermore, in a study looking at the circulating levels of UCN1 in acute myocardial infarction (AMI), blood samples were collected at five time points: days 0 (onset), 1, 3 and 5 and at 3 and 6 months. Plasma UCN1 was increased on days 0, 1, 3 and 5 in 66 AMI patients compared to 21 controls.³²⁵ Also, UCN1 levels were reported to predict heart failure independent of age, hypertension, history of previous myocardial infarction or diabetes.³²⁶ As a whole, these reports suggest that circulating UCN1 concentrations are increased in patients with CHD, particularly in association with heart failure. Although it remains to be verified whether the increase in UCN1 in these cases is due to cardiac injury or as a result of some inherent modulatory mechanism in response to disease, whereby the body releases these peptides to down regulate disease-initiating factors.^{251,327}

Table 1. 9 Studies assessing the association of UCNs with cardiovascular diseases in patients

Subject type	Subject Characteristics	Controls	Findings
Heart failure (HF) ³²⁶	NYHA classes I-IV (n=299)	Age & sex matched (n=98)	↑ UCN1 in HF
Systolic heart failure ²⁷⁵	NYHA classes I-IV (n=119)	Age & sex matched (n=212)	↑ UCN1 in HF Male UCN1 level > female UCN1 ↑UCN1 with increasing age
Heart failure ³²⁴	NYHA classes I-IV (n=42)	Age & sex matched (n=20)	↑ UCN1 in HF
Acute myocardial infarction (AMI) ³²⁵	AMI (n=66)	Age & sex matched (n=21)	↑ UCN1 in AMI

New York Heart Association (NYHA) functional classes, increase (↑), acute myocardial infarction (AMI), number (n), heart failure (HF)

1.2.10.8 The effect of UCN administration in human subjects

A number of studies investigating the effects of administering UCNs in human subjects have been published (Table 1.10).^{315,328-331} Two studies examining the effect of administering UCNs in healthy men have been reported.^{328,329} In the first case, a 50 µg/mL UCN1 bolus was given i.v. to eight healthy males, aged 24–45 years in two instances, 2 weeks apart. The UCN1 bolus was found to increase the circulating levels of atria natriuretic peptide (ANP), cortisol and corticotrophin releasing hormone (CRH).³²⁸ ANP has been demonstrated to be the precursor protein for isolated atria amyloid,^{332,333} and its levels have been shown to increase in isolated atrial amyloidosis.³³²⁻³³⁴ These studies suggest that the UCN1-induced

increase in ANP may be important in the pathogenesis of atrial amyloidosis. However studies need to be done to clearly define the relationship between UCN1 and this disease.

Davis and colleagues investigated the effect of administering UCN2 on cardiovascular function by infusing eight healthy men with UCN2 (Table 1.10).³²⁹ UCN2 was shown to decrease the systemic vascular resistance, but increase cardiac output, heart rate, plasma renin activity, AII and noradrenalin.³²⁹ The increase in cardiac output and heart rate could be favourable in congestive heart failure, but considering the fact that AII may enhance ROS secretion, caution should be applied in classifying UCN2 as beneficial.

Two other studies have demonstrated the effect of administering UCN2 in heart failure.^{330,331} One study reported significant decrease in mean arterial pressure and systemic vascular resistance upon administering 25 µg/mL to 100 µg/mL UCN2 doses i.v. at 3 separate time points two to five weeks apart over the course of 1 h in heart failure patients.³³⁰ The investigators also showed that UCN2 infusion led to an increase in the cardiac output and left ventricular ejection fraction (LVEF).³³⁰ In the second study, incubation of human coronary arteries obtained from seventeen patients with heart failure (NYHA III–IV) with 5-20 nM UCN2 was shown to increase vasodilatation.³³¹ Collectively, these studies demonstrate that UCN1 and UCN2 administration could alter cardiovascular physiology but the overall beneficial or detrimental effects of this are not currently clear.

Table 1.10 Studies assessing the effect of administering urocortins in subjects with or without cardiovascular diseases

CVD type	Intervention	Outcome
None (Healthy men) ³²⁸	UCN1 i.v (50 µg/mL twice 2wk apart)	↑Atria natriuretic peptide ↑Cortocotropin & cortisol
None (Healthy un- medicated men) ³²⁹	UCN2 i.v (25 µg/mL – 100 µg/mL over 1h twice between 2-5 wk)	↑Cardiac output ↑Heart rate ↑Plasma renin activity ↑AII & noradrenalin ↓Systemic vascular resistance
Heart failure ³³⁰	UCN2 i.v (25 µg/mL – 100 µg/mL over 1h thrice between 2-5 wk)	↑Cardiac output ↑LVEF ↓Mean arterial pressure ↓Systemic vascular resistance
Heart failure ³³¹	UCN2 treated (5–20 nM)	↑Vasodilation of coronary Arteries

Increase (↑), Decrease (↓), intravenous (i.v), picomolar (pM), nanomolar (nM), microgram (µg), week (wk), millilitre (mL), hour (h), angiotensin II (AII), left ventricular ejection fraction (LVEF)

1.2.11 Summary

AAA accounts for thousands of deaths annually in Western countries,^{8,16-20} and is estimated to be a leading cause of death in elderly men and women.^{9,14,18,22,165,166,335-337} AAA is rapidly becoming a significant socio-economic ailment as disease-associated morbidity rises in parallel with the ageing global population.¹⁵ Currently, AAAs are often only diagnosed incidentally when imaging to assess other health complaints and due to a lack of approved medication with which to reverse AAA pathology, they are conservatively managed by

surgery.²³ Imaging-based screening of patients worldwide while showing a degree of success is however hindered by a myriad of problems,²¹⁶⁻²¹⁸ including the inability to predict AAA at risk of rupture. Although studies have been carried out to elaborate the processes involved in AAA pathogenesis, none of these studies have been able to define an effective and specific therapeutic target for AAA. As such there are yet to be any available non-surgical pharmacological intervention strategies aimed at preventing disease formation or slowing disease progression. As a result, there are momentous shortfalls in current AAA management procedures, and there is a need to more clearly understand AAA pathogenesis so as to define effective diagnostic, prognostic and therapeutic targets for the disease. This study attempts to fill in this gap in AAA research.

Due to the emergence of the UCNs as putative therapeutic targets for cardiovascular diseases, there has been increasing interest on their pharmacokinetics and pharmacodynamics. UCN2 and UCN3 were shown to demonstrate a more rapid pharmacokinetics and haemodynamic responses relative to UCN1 by Patel and colleagues in their detailed analysis of the metabolism and half-life of these peptides in a healthy sheep.³³⁸ The UCNs have been demonstrated to influence the cardiovascular system; however their specific roles in AAA formation and progression remain to be elucidated in detail. They may exert beneficial and detrimental effects on the heart and vasculature as described in the review above. However, current published data is limited and in particular there have been few studies of the relevance of UCNs in the peripheral circulation. The UCNs have also been demonstrated to be important modulators of glucose homeostasis, metabolic functions,³³⁹ and to stimulate the production of steroids including dehydroepiandrosterone in human foetal cells via CRFR1,³⁴⁰ which may have important clinical implication in AAA management. However, this study

focused mainly on the role of these proteins on AAA development and progression. Available evidence suggests that UCN1 and UCN2 administration improves cardiac output and thus their therapeutic value have been assessed on pilot studies within patients with heart failure. The influence of UCNs in other CVD including AAA is less clear. In addition, the current discrepancies shown in the effect of UCNs in the cardiovascular system (Table 1.11) raise a number of questions: In what context do the UCNs adopt a cardiovascular protective role? In what context do the UCNs exert a deleterious effect in the cardiovascular system? Specifically, do the UCNs promote an aneurysm phenotype? The answers to these questions with respect to AAA, and in particular establishing the role of UCN2 in AAA pathogenesis is the major focus of the research presented here as part of a continuing project to discover and define specific therapeutic targets for AAA.

Table 1. 11 Potential cardiovascular protective and cardiovascular destructive effects of urocortins

Protective	Destructive
↑Nitric oxide ²⁸⁵	↑ UCN1 in various human inflammatory diseases ²⁴⁵
↓Superoxide dismutase (SOD) ²⁸⁵	
↓Oxidative stress by UCN1 ²⁵³	↑Proinflammatory IL-1 β and IL-6 by UCN1 ²⁴⁵
↓ IL-1 β & TNF by UCN1 ²⁹⁹	
↑Phosphorylation & activation of the pro-survival ERK 1/2 pathway by UCN2 & UCN3 ²⁵⁵	↑ Exacerbation of vasculitis in an experimental rat model of thromboangitis obliterans (TAO) by UCN1 ³⁰⁸
↑ Vasodilatation ³³¹	↓Angiogenesis by UCN1 ³⁴¹
↓Myocardial infarct size ²⁵⁴	↓Proliferation & ↑apoptosis of HUVECs by UCN1 ³⁴¹
	↓Expression of vascular endothelial growth factor in vivo by UCN1 ³⁴¹
↑ Anti stress hsp90 by UCN1 ²⁹⁵	↑Vascular permeability& mast cell degranulation by UCN1 ²⁷²
↓Renin & aldosterone activities in heart-failure by UCN1 ²⁴⁷	

Increase (↑), decrease (↓), heat shock protein (hsp), and human umbilical cord vascular endothelial cells (HUVECs)

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

Male ApoE^{-/-} mice were obtained from the Animal Resources Centre (Canning Vale, Australia). These mice were originally developed in the laboratory of Dr. Nobuyo Maeda at The University of North Carolina, Chapel Hill and obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The C57BL/6 strain was produced by backcrossing 10 generations into a C57BL/6J background.

2.1.2 Western blot and immunoprecipitation

Electrophoresis materials and reagents including acrylamide, electrophoresis set, gel casting and western blot apparatus (blot papers and filter papers) were obtained from Bio-Rad (Gladesville, Australia). ECL-Western blot detection reagent was from Amersham International/GE Healthcare, Amersham, UK. Pierce® Classic Immunoprecipitation (IP) kit was from Thermo Fischer Scientific (Scoresby, Australia), antibodies (all rabbit polyclonal) to UCN3 and CRFR2, and goat-rabbit HRP conjugated IgG were purchased from Abcam (Waterloo, Australia). ECL- western blot detection reagent was from Amersham International/GE Healthcare, Amersham, UK.

2.1.3 Real time PCR

RNAlater® solution was obtained from Ambion (Australia), RNeasy Mini extraction kit, DNase, Qiazol and QuantiTect SYBR Green RT-PCR kit, 0.1 ml PCR tubes from Qiagen Pty Ltd (Doncaster, Australia), SABiosciences RT² PCR Primer sets for human UCN3, and mouse GAPDH, human CRFR2 (Sense primer CCATGTCTTCAGTGATTG and anti-sense

AGAGAGCAGAGATGTTTC), CRFR1 (Sense primer AAGTCAGGTGTCATCATCAG and anti-sense ACAGTGCCAGTAAGGTTC), UCN2 (Sense primer GAGTCTTGCTATGTCTGG and anti-sense GAGGCTCCTGAAAGTAAC), UCN1 (Sense primer AACCGCATATTCGACTC and anti-sense TGGGGGAAAGGGGTCAAC) primers from Sigma-Aldrich (Castle Hill, Australia).

2.1.4 Histology and immunohistochemistry

Goat polyclonal antibody to UCN2 was obtained from Santa Cruz Biotechnology (Sapphire Biosciences, Australia); rabbit anti-goat IgG (BA-5000), goat IgG (I-5000), rabbit IgG (I-1000), ImmPACT DAB (Sk-4105), avidin and biotin block (SP-2001) were obtained from Vector labs (Australia), Monoclonal mouse anti-human neutrophil elastase (M0752), monoclonal mouse anti-human smooth muscle actin (M0851), Monoclonal mouse anti-human CD3 (M7254) from Dako (Australia); Streptavidin conjugated horseradish peroxidase (HRP) from Perkin Elmer (Melbourne, Australia), OCT™ and Mayer's haematoxylin were purchased from ProScitech (Townsville, Australia).

2.1.5 ELISA

Mouse UCN3 ELISA kits were obtained from ALPCO (USA), UCN2 ELISA kits purchased from USCN life Sciences (China), Proinflammatory cytokine IL-6 *DuoSet*®, and Cell based Akt phosphorylation ELISA kits obtained from R&D systems (Australia).

2.1.6 Tissue culture

Materials and reagents including serological pipettes (5, 10 and 25 mL), transfer pipettes (3.5mL) and centrifuge tubes (15 and 50mL) were purchased from Sarstedt (Ingle Farm, Australia); Nunc cell culture plates (6, 12, 24 and 96 well) from Thermo Fischer Scientific (Scoresby, Australia); Human aortic smooth muscle cells (HASMC) were obtained from Lonza (Waverly, Australia); DMEM from TropBio (Townsville, Australia), Foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, Dulbecco's Phosphate Buffered Saline (DPBS), trypsin from Ambion (Australia); UCN2 recombinant protein, Ast-2B, trypan blue, ethanol and formaldehyde were from Sigma-Aldrich (Castle Hill, Australia).

2.1.7 Proliferation/viability, apoptosis and cell cycle assays

Apo-ONE Homogeneous Caspase-3/7 assay kits were obtained from Promega (Alexandria, Australia), Bcl-2 Human ELISA kits from Abcam (Australia), AlamarBlue[®] cell viability reagent from Invitrogen (Mulgrave, Australia); Propidium iodide was obtained from Invitrogen (Australia); recombinant UCN2/stresscopin related peptide and Ast-2B from Sigma-Aldrich (Castle Hill, Australia).

2.2 Mouse Studies - The role of urocortin 3 (UCN3) in a mouse model of AAA

See Chapter 3.

2.3 Human studies

2.3.1 Study Design

A schematic of the study design used in the human investigations is shown below.

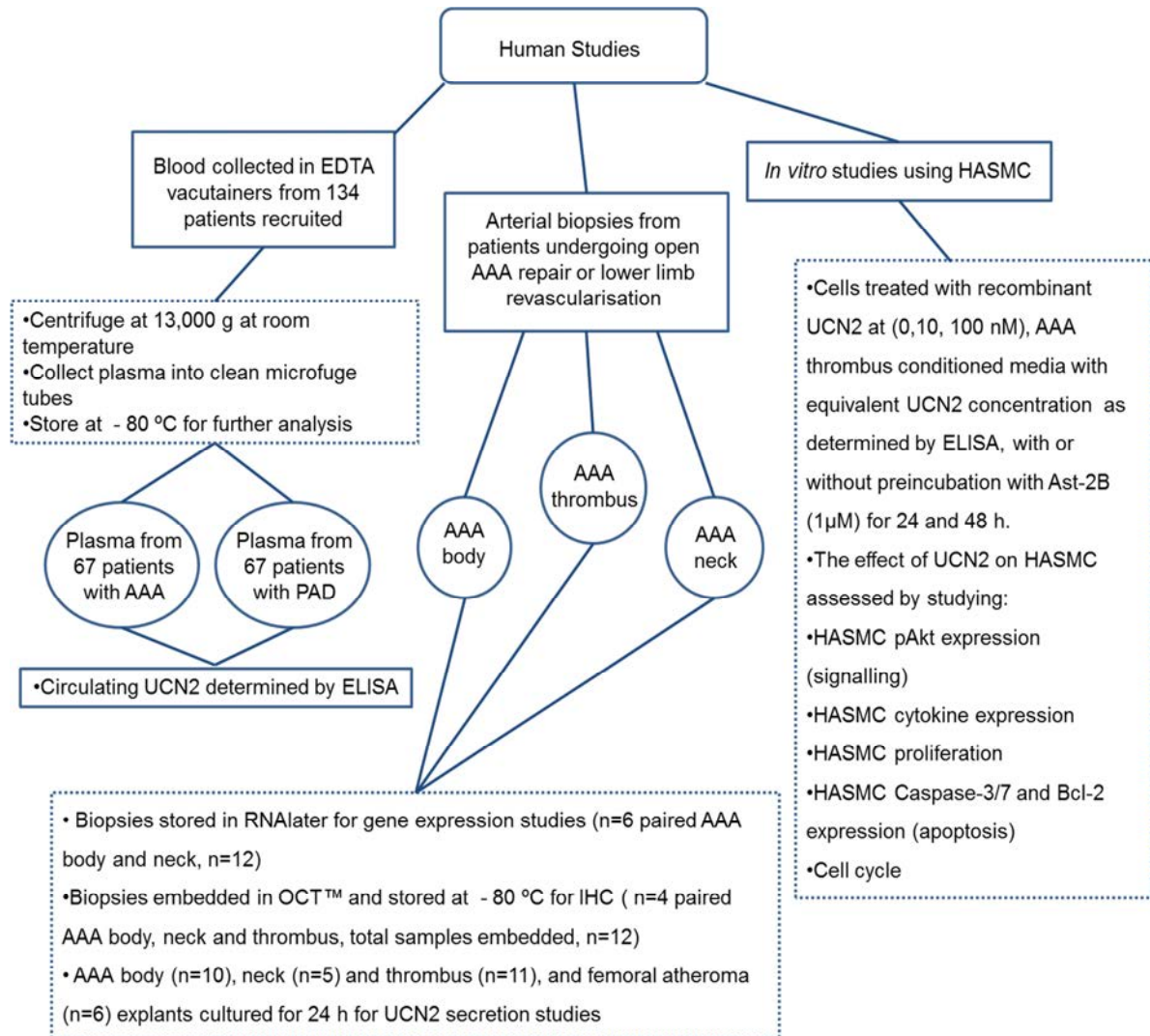


Figure 2. 1 Schematic diagram showing the study design applied in the human studies for the association of UCN2 with human AAA.

Plasma and aortic biopsies obtained from AAA and PAD patients undergoing surgery were used to determine the association of the UCNs with AAA presence. HASMC cells were used to determine the functional effect of UCN2 *in vitro* by investigating the effect of UCN2 on the key mechanism involved in AAA pathogenesis.

2.3.2 Patients

Patients referred to the vascular surgery clinics at The Townsville Hospital and The Royal Brisbane and Women's Hospital, Queensland, Australia for investigation of peripheral artery disease (PAD) or AAA were recruited. The inclusion criteria into the study are detailed in the table below:

Table 2. 1 Patients recruitment criteria applied for the human studies

Inclusion Criteria	Exclusion Criteria
Verbal and written consent	Refusal to participate
PAD or AAA diagnosed by a consultant vascular surgeon and confirmed with imaging	Previous surgical repair of the abdominal aorta Contra-indication to computed topographic angiography (CTA, for example abnormal serum creatinine, contrast allergy and inability to lie flat)

PAD was diagnosed based on an appropriate history; examination and imaging findings by a consultant vascular physician as previously reported.^{342,343} AAA was defined by maximum axial infrarenal aortic diameter of ≥ 30 mm measured from CTA. Risk factors and medication details were collected at entry as previously described.^{342,343} Diabetes, hypertension, dyslipidaemia and coronary heart disease (CHD) were defined by a past history of diagnosis or treatment for these risk factors by the consulting physician. Patient's smoking status was defined as current smoker, past smoker (not smoked in the past month) or non-smoker; and

ultimately presented as an ever or a never smoker. For the part of the study in which plasma UCN2 concentrations were compared in AAA cases and PAD controls, patients were selected based on matching for age and gender on a 1:1 basis. Approval was obtained from the relevant ethics committees (# H3197), and conformed to the guidelines of Declaration of Helsinki.

2.3.3 Plasma samples

Whole blood samples were collected in EDTA vacutainers (BD Bioscience, Australia) from 134 patients recruited into the study. All recruited subjects underwent CTA. Sixty seven (67) patients had AAAs (aortic diameter ≥ 30 mm) and 67 patients had PAD but no AAA (aortic diameter < 30 mm). Plasma was obtained from blood samples within 2 h of collection by centrifugation at 15,000 *g* for 10 min at room temperature. Plasma was removed carefully with aid of 5 mL serological pipettes (Sarstedt, Australia), and aliquoted into clean, dated and labelled microfuge tubes and stored at -80 °C for future analysis.

2.3.4 Aortic and atheroma samples

Arterial biopsies were obtained from subjects undergoing open surgical AAA repair and lower limb revascularisation to correct PAD. Samples collected from the AAA included full thickness biopsies from the anterior wall of the infrarenal aorta (AAA body, Figure 2.2) at the site of maximum dilation near its centre, macroscopically healthy tissue (AAA neck, Figure 2.2) from the proximal neck (site immediately distal to the renal arteries where the aortic diameter appears relatively normal) and intra-luminal thrombus (AAA thrombus). Samples collected included four randomly selected paired AAA body, neck and thrombus biopsies for

the UCN2 protein expression studies as previously reported.³⁴⁴ Arterial biopsies from PAD patients were collected from the main site of atheroma removed from the femoral artery during endarterectomy, as previously described.³⁴⁴ Aortic body (n=10), AAA thrombus (n=11) and femoral atheroma (n=6) biopsies were collected for the explant culture studies. Biopsies were placed in cold sterile Dulbecco's modified eagle medium (DMEM) containing [4.5 g/L glucose, 10% heat inactivated foetal bovine serum [FBS, (Australian origin; Interpath, Australia)], 4 mM L-glutamine, 2.5 µg/mL amphotericin, 100 U/mL penicillin G, 100 U/mL streptomycin, 0.05 mg/mL gentamicin, 100 mL of 100x non-essential amino acids, 20 mM HEPES (pH 7.5), 10 µM β-mercaptoethanol, 2.4 µg/mL sodium bicarbonate, 10 nM sodium pyruvate] obtained from TropBio, Australia and used immediately for the explant culture studies or stored at -80 °C for later cryostat sectioning and immunohistochemistry. Matched and paired AAA body and neck biopsies were collected from six patients randomly selected for the gene expression studies. Biopsies were placed in cold sterile RNeasy[®] solution (Ambion, Australia) and stored at -80 °C for the gene expression studies.

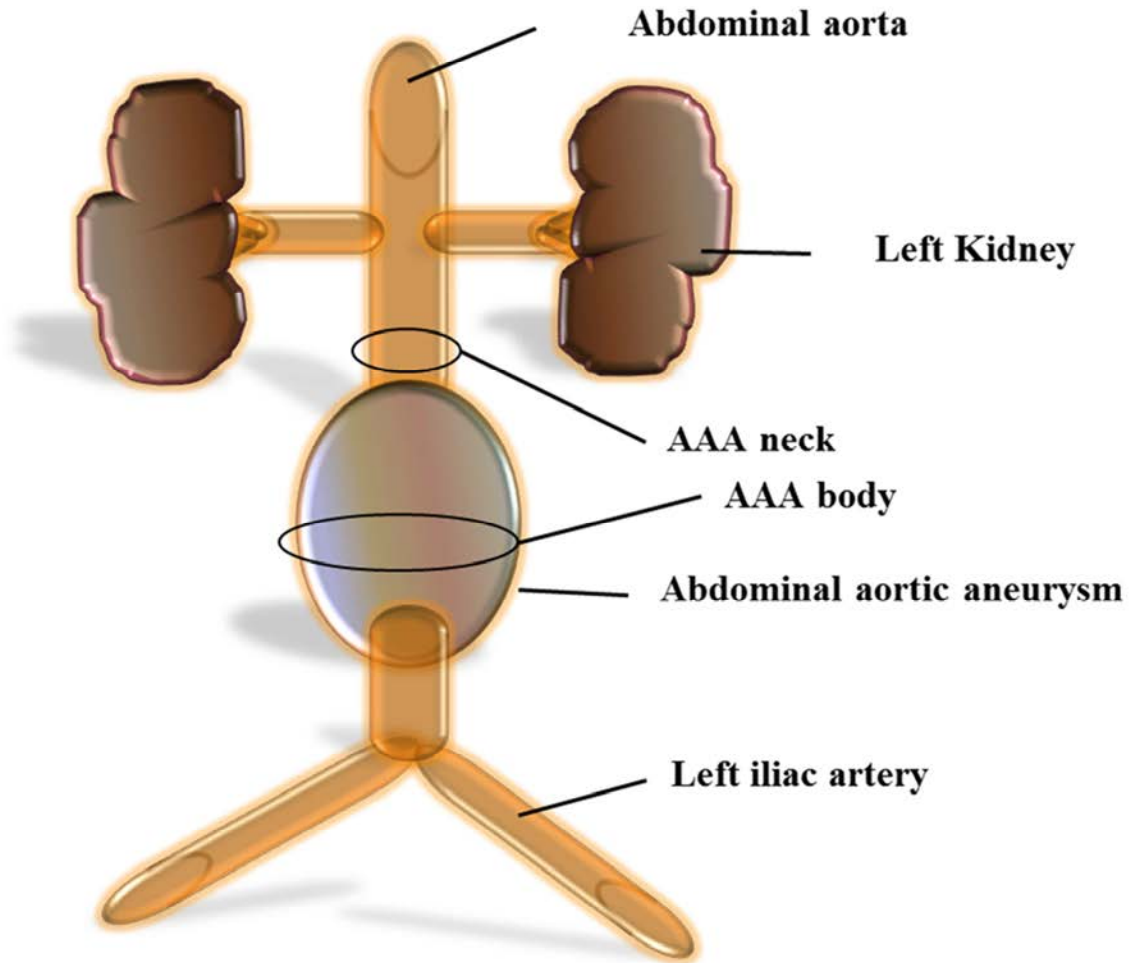


Figure 2. 2 Diagram of the aorta showing an abdominal aortic aneurysm and regions from which aortic biopsies were collected

2.3.5 Gene expression studies

Total RNA was extracted from paired AAA body and neck biopsies collected as described above using RNeasy[®] Mini Kit (Qiagen, Australia) according to manufacturer's instructions. Briefly, samples were thawed to room temperature after which, 200 μ L of Qiazol was added and the sample vortexed. Eight hundred (800) μ L of Qiazol was then added, samples vortexed and incubated at room temperature for 5 min. Two hundred (200) μ L of chloroform

was added, samples shaken vigorously for 10 – 15 s, and then incubated at room temperature for 2 - 3 min. Samples were centrifuged at 12,000 g for 15 min (4°C), the clear aqueous layer was carefully removed. An equal amount of 70% ethanol (EtOH) was added, mixed well, and samples transferred to a new spin column and centrifuged immediately at 8000 g for 25 s. The filtrate was discarded and columns were washed with 350 µL RW1 buffer twice. After this, the column was washed with 250 µL of 100% EtOH leaving the column to dry before DNase treatment (10 µL + 70 µL RDD buffer) for 30 min at 37°C. Columns were washed twice with RW1 and RPE buffer (8000 g, 25 s). The columns were then centrifuged for 2 min at 16,000 g to remove any residual RPE. Columns were placed in new collection tubes and 80 µL of RNase-free water was added (40µL, twice) and centrifuged for 1 min at 8000 g. The resultant eluant contained RNA.

RNA was quantified using a Nanodrop spectrophotometer (Thermoscientific, Australia). The samples were checked for any DNA contamination by running a no RT (reverse transcriptase) reaction on the PCR. Quantitative real-time qPCR was performed on a Corbett Rotor gene 6000 (Qiagen Pty Ltd, Doncaster, Australia) acquired at the SYBR green channel. The reactions were performed in duplicate in a volume of 15 µL containing 40-100 ng/µL RNA, 7.5µl SYBR green master mix, 0.15 µL RT mix and 0.6 µL of appropriate primer in a 0.1 mL RNase-DNase free PCR tubes. Samples were normalised and quantified against the reference gene (internal control), glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Thermal cycling program used was 50 °C for 30 min for cDNA synthesis, 95 °C for 10 min (enzyme activation), 40 cycles of 94 °C, 15 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (extension) for UCN2, UCN3, and CRFR2. The cycling program for UCN1 and CRFR1 was a two-step cycling of 95 °C for 15 seconds and 60 °C for 60 s.

SYBR Green PCR primers were designed using the Biosoft's AlleleID[®] software (PREMIER Biosoft, Palo Alto, USA; CRFR2: 5'-CCATGTCTTCAGTGATTG-3' and 5'-AGAGAGCAGAGATGTTTC-3', CRFR1: 5'-AAGTCAGGTGTCATCATCAG-3' and 5'-ACAGTGCCAGTAAGGTTC-3', UCN2: 5'-GAGTCTTGCTATGTCTGG-3' and 5'-GAGGCTCCTGAAAGTAAC-3', UCN1: 5'-AACCGCATATTCGACTC-3' and 5'-TGGGGGAAAGGGGTCAAC-3'). UCN3 and GAPDH primers were obtained commercially from Qiagen, Australia. Quantitative real-time PCR (qPCR) was performed for UCN1, UCN2, UCN3, CRFR1, and CRFR2 genes using 100 ng of total RNA using the commercially available QuantiTect SYBR Green one-step qPCR assay (Qiagen, Australia). Relative expression of the genes of interest in each sample was calculated by using the concentration-Ct-standard curve method and normalised by the average expression of GAPDH. GAPDH was chosen as the housekeeping gene as previous analyses have shown its expression to be unaffected by AAA biopsy site.³⁴⁵

2.3.6 Histology

Six µm thick serial cryostat sections in Optimal cutting temperature [OCT[™], (ProSciTech, Townsville, Australia)], were cut from AAA biopsies obtained from patients undergoing open surgical aneurysm repair. Biopsies were taken from the paired AAA body and neck prior to staining for UCN2. Roche haematoxylin and eosin stain was carried out to determine the histological morphology of biopsies. Briefly, serial frozen sections were air-dried and fixed in acetone for 20 min at -20 °C. OCT[™] was gently removed by washing for 2 min twice in PBS. Sections were rehydrated by two times five min washes in tap water prior to staining in Mayer' haematoxylin (ProSciTech, Townsville, Australia) for 5 min. A further 2 min wash in tap water was applied. Sections were differentiated by 1 min incubation in 2%

ferric chloride and a 2 min wash in tap water. Slides were washed extensively (5 – 10 min) in running tap water, then stained for 1 min in eosin (ProSciTech, Townsville, Australia), then dehydrated as per standard protocols (2 x 5 min changes in 100% ethanol). Slides were cleared in xylene for 2 x 5 min, and mounted in Depex mounting medium (ProSciTech, Townsville, Australia) for examination and photographed using a Nikon Eclipse 50i microscope, Digital Sight camera and NIS-elements software.

2.3.7 Immunohistochemistry

Immunostaining of serial cryostat sections in OCT™ was performed using standard protocols. Briefly, 6 µm thick sections from AAA biopsies were air-dried and fixed in acetone for 20 min at -20°C. OCT™ was gently removed by washing for 2 min twice in PBS. Sections were incubated in 3% H₂O₂/0.1% sodium azide/PBS to block endogenous peroxidases for 20 min. Sections were blocked in 10% horse serum in PBS for 30 min followed by a 15 min avidin block and a 15 min biotin block. Sections were washed twice in PBS as above and then incubated in primary antibody [anti-human goat polyclonal UCN2 antibody (Santa Cruz, c-16, sc-54449, 1:100 dilution), monoclonal mouse anti-human neutrophil elastase (M0752, 1:100 dilution), monoclonal mouse anti-human smooth muscle actin (M0851, 1:50 dilution), or monoclonal mouse anti-human CD3 (M7254, 1:25 dilution) from Dako (Campbellfield, Australia)] for 18h at 4°C. Sections were rinsed twice in PBS as above then incubated in secondary antibody [biotinylated rabbit anti-goat IgG (BA-5000, 1:200 dilution), or biotinylated goat anti-mouse IgG (BA-9200, 1:200 dilution)] for 60 min. Goat IgG (Vector, I-5000, 1:200 dilution) and mouse IgG (Vector, I-2000, 1:200 dilution) were used as isotype controls. Sections were washed twice in PBS as above and incubated in peroxidase conjugated streptavidin-biotin (Vector, PK-6200) for 30 min. Slides were rinsed

twice in PBS and incubated in the peroxidase substrate 3, 3'-diamminobenzidine (ImmPACT DAB, Vector). Slides were washed twice in PBS as above, counterstained in Mayer's Haematoxylin for 15 min, and rinsed in tap water for 5 min. Slides were dehydrated in 100% ethanol for 2 x 5 min, cleared in xylene for 2 x 5 min, and mounted in Depex mounting medium (ProSciTech, Australia). Sections were photographed using a Nikon Eclipse 50i microscope, Digital Sight camera and NIS-elements software.

2.3.8 Assessment of UCN2 by ELISA

UCN2 levels in conditioned media and plasma were measured by ELISA (USCN life Sciences, China) as per manufacturer's instructions. Briefly, samples and reagents were brought to room temperature. Standards, samples and diluent (100 μ L/well) were added into the wells of a 96 well plate with pre-coated strips in duplicate. The ELISA plate was covered with a plate sealer and incubated for 2 h at 37 °C. The liquid content of each was discarded, plate tapped on adsorbent paper, and 100 μ L/well of detection reagent A added immediately. Plate was incubated for 1 h at 37 °C after covering with a plate sealer. The solution was aspirated and ELISA plate washed three times with 1x wash solution (0.35 mL/ well). After the last wash, ELISA plate was inverted and blotted against absorbent paper. Detection reagent B solution was added into wells (100 μ L/ well), with a further 30 min incubation at 37 °C after covering with a plate sealer. The aspiration-wash step above was repeated 5 times, before adding 90 μ L/ well substrate solution. ELISA plate was covered with a plate sealer and incubated for 15- 25 min at 37 °C in the dark. Stop solution (50 μ L/ well) was added and absorbance read at 450 nm in a multi-mode microplate reader (BMG Labtech, Germany). For the explant culture assessment, weight of the arterial biopsies taken after culture and used to calculate the UCN2 concentration in conditioned medium, and expressed as nanogram per

gram of tissue per 24 h (ng/g/24h). The UCN2 concentration in plasma is expressed as nanogram per milliliter (ng/mL). Mean inter-assay coefficient of variation determined from the standard curves of three separate assays was 7.46%.

2.4 *In vitro* investigations

2.4.1 *Explant culture*

AAA body, AAA thrombus and femoral atheroma control biopsies collected as described above were dissected into ~ 5 mm³ pieces and incubated in DMEM with additives as described in section 2.3.4 above. The AAA body and femoral atheroma samples were cultured in 2 mL culture medium in 12 well plates and AAA thrombus in 6 to 8 mL culture medium in 6 well plates (Nunc, Australia). Explants were cultured at 37°C in a humidified 5 % CO₂ atmosphere. Conditioned media was collected at 24 h and centrifuged at 30,000 g for 30 min at 4 °C to remove debris. Wet weight of biopsies were taken after culture and recorded for use in later analysis. Supernatants were stored at -80°C for later analysis of the thrombus conditioned medium used in the proliferation assays.

2.4.2 *Cell culture*

Human aortic smooth muscle cells (HASMC; Lonza, Australia) were maintained in DMEM (TropBio, Australia) in T75 cm² flasks (Nunc, Australia) at 37°C in a humidified 5% CO₂ atmosphere. HASMC were maintained at a density of 2×10⁵ - 1×10⁶ cells/mL for 4 to 6 passages. The cell line was regularly maintained at a density of 2×10⁵ to 1×10⁶ cells/mL and culture medium changed every 48 h. When cells reached roughly 70 – 80 % confluency, they were passaged by trypsinisation as detailed below.

The culture medium was removed and cells washed with ~ 5 mL sterile PBS which was discarded. Two millilitres (2 mL) of 0.05 % trypsin EDTA (Invitrogen, Mulgrave, Australia) was added into the flask and flask tilted to ensure all cells were covered. The flask was placed in the incubator for 2 – 5 min and monitored every min under the microscope to see when cells had rounded. Flask was tapped to lift off any cells still adhering and 5 mL of trypsin neutralising agent was added to stop the reaction. Cell suspension was transferred into suitable tubes and centrifuged at 500 g for 5 min. The supernatant was discarded and cells resuspended in fresh media.

For storage, adherent cells were trypsinised as described above. The supernatant was removed and cell pellet resuspended in appropriate volume of media and distributed in 1ml aliquots of 5×10^6 cells/mL per cryovials each. DMSO was added to give a final concentration of 10%. Cells were transferred to Mr Frosty units (Sigma-Aldrich, Castle Hill, Australia). The polyethylene vial holder and foam insert were removed and 100 % isopropyl alcohol (250 mL) added just to the fill line. Cryovials containing cells were placed in the vial holder and inserted in the unit. The foam insert was replaced and the unit placed in -80 °C freezer. Samples were left undisturbed for a minimum of 24 h, then vials removed and stored in liquid nitrogen. All cell culture work was carried out under sterile conditions.

2.4.3 Determination of human aortic smooth muscle cell counts

Trypan blue exclusion and cell counting were performed before setting up each experiment as follows: 10 µL of cell suspension was added to 10 µL of trypan blue, placed in the haemocytometer chamber (Hausser Scientific, Horsham, PA, USA), visualised and counted under a microscope to determine which cells take up the dye. This test is based on the

principle that live cells possess intact membranes that exclude certain dyes, such as trypan blue, eosin, and propidium iodide, whereas dead cells do not. Viable cells are present with clear cytoplasm whereas dead cells take up the dye. The total cell count was calculated using the formula:

Total count = cell count of 10 squares \times dilution factor \times 1000 cells/mL \times volume of media.

2.4.4 Akt signalling assessment

Total and phosphorylated Akt were measured with a commercially available cell-based ELISA (R&D systems, Australia) following manufacturer's protocol. Briefly, 2×10^4 HASMC (Lonza, Waverly, Australia) were seeded into each well of a black 96-well plate with clear bottom (Thermo Fischer Scientific, Scoresby, Australia) and incubated until 90 % confluent at 37 °C. Subconfluent HASMC were then growth arrested for 24 h in DMEM containing 1% (v/v) FBS before incubation in experimental medium containing 10% FBS. HASMC were treated with recombinant UCN2 at 0 or 100 nM for the following time periods; 5 min, 10 min, 1 h, 6 h, 12 h or 24 h (six replicates for each experimental condition and three whole experiment repeats). In a further experiment the involvement of the UCN2 receptor CRFR2 was assessed. HASMC were cultured as above, then incubated in experimental medium containing recombinant UCN2 (0 or 100 nM) and AAA thrombus conditioned medium prepared as described in section 2.4.1 (0 or 100 nM UCN2); with and without 10 min pretreatment with 1 μ M Astressin 2B (Ast-2B, Sigma, Australia) a selective CRFR2 antagonist,³⁴⁶ for 24 h (six replicates for each experimental condition and three whole experiment repeats). Determination of the optimum number of cells used for cell culture, the UCN2 and the Ast-2B doses are presented in Chapter 4. Cells were fixed with 100 μ L of 4 % formaldehyde in 1 x PBS for 20 min at room temperature. Formaldehyde was removed and

cells washed three times with 200 μ L of 1 x wash buffer with gentle shaking for 5 min at each wash step. Cells were further incubated in quenching buffer (0.6% H_2O_2 in wash buffer) for 20 min at room temperature; with a further three washes before incubating in 100 μ L buffer at room temperature for 1 h. Cells were washed three times, then the primary antibodies [rabbit anti-phospho-Akt (S473) and mouse anti-total Akt] were added and cells incubated at 2-8 $^{\circ}C$ for 18 h. This was followed by three washes as above then incubation in secondary antibodies (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG) at room temperature for 2 h. Two more washes in wash buffer were performed followed by two washes in 1 x PBS with gentle shaking as described. PBS was removed and substrates F1 added and cell incubated in the dark for 1 h followed by the addition of substrate F2 for a further 40 min. Fluorescence was read with excitation at 540 nm and emission at 600 nm (phosphorylated Akt) and with excitation at 360 nm and emission at 450 nm (total Akt) with a multi-mode microplate reader (BMG Labtech, Germany).

2.4.5 Examination of cytokine secretion

Subconfluent HASMC were growth arrested prior to incubation in experimental medium as described in section 2.4.4. HASMC were treated with recombinant UCN2 at 0 or 100 nM for 24 h (6 replicates for each experimental condition and three whole experiment repeats). The involvement of CRFR2 was also investigated. The Q-PlexTM multiplex ELISA assay (lot number HCEM1107124, Quansys Bioscience, USA) was used to determine the secretion of proinflammatory cytokines IL-1 α and TNF- α in pre-treated HASMC supernatants. In brief, standards and samples were added into each well of a 96 well plate pre-spotted with antibodies to the targeted cytokines. After a series of wash steps to remove unbound material, biotinylated detection antibodies were used to detect specific cytokines. Unbound detection

antibodies were removed by washing and Streptavidin-HRP was added. After another wash, chemiluminescent substrate reagents were added producing signals equivalent to the proportionate amount of each cytokine bound in the initial step. Plates were read using a digital imaging system (Chemidoc™ System, Bio-Rad, Australia) and pixel intensity measured employing analytical software (Q-View™, Quansys Bioscience, USA).

The secretion of the proinflammatory cytokine IL-6 was assessed in pre-treated HASMC supernatants using the human IL-6 *DuoSet*® ELISA Development System (R&D systems, Australia) according to manufacturer's protocol. Briefly, a 96-well microplate was coated with 100 µL per well of diluted capture antibody, sealed and incubated overnight at room temperature. Plate was aspirated and washed three times with wash buffer (0.05% Tween® 20 in PBS, pH 7.2 -7.4). An hour blocking step in reagent diluents was performed to limit nonspecific binding. Wash step was repeated as above and 100 µL of standards or samples in reagent diluent added into each well and incubated at room temperature for 2 h. Plates were washed as above and 100 µL detection antibody added into each well and incubated for a further 2 h at room temperature. Aspiration/wash step was repeated and 100 µL of Streptavidin-HRP added to each well for 20 min incubation at room temperature. A final aspiration/wash step was carried out and 100 µL of substrate solution [1:1 mixture of colour reagent A (H₂O₂) and colour reagent B (Tetramethylbenzidine)] added producing signals equivalent to proportionate amount of IL-6 bound. The reaction was stopped by adding 50 µL stop solution (2N H₂SO₄). Colourimetric analysis (optical density) of each well was performed at 450/595 nm utilising a *Sunrise*™ microplate reader (Tecan property, Australia). Mean inter-assay coefficient of variation determined from the standard curves of three separate assays was 2.50%.

2.4.6 Determination of HASMC proliferation

Subconfluent HASMC were growth arrested for 24 h in DMEM containing 1 % (v/v) FBS before incubation in experimental medium containing 10 % FBS and recombinant UCN2 (stresscopin-related peptide, Sigma, Australia) at 0, 10 or 100 nM or AAA thrombus conditioned medium containing UCN2 at 0, 10 or 100 nM for 24 h (six replicates for each experimental condition and three whole experiment repeats). In a further experiment, the involvement of CRFR2 was also assessed as described in section 2.4.5. Cell proliferation was determined by AlamarBlue® assay (Invitrogen, Australia) as previously described.^{347,348} Briefly AlamarBlue® (10 µL/well i.e 10 % of sample volume) was added to the treated and untreated HASMC 24 h after experimental set up. Cell proliferation was measured after 4 h with a multi-mode microplate reader (BMG Labtech, Germany) at 570/585 nm. Mean inter-assay coefficient of variation determined from the standard curves of three separate assays was 4.32 %.

2.4.7 Apoptosis investigations

UCN2 induced apoptosis was investigated by investigating two pathways with which UCNs have been associated (caspase-3/7 and the Bcl-2 pathways). The Apo-One Homogenous caspase-3/7 assay (Promega, Australia) and the Bcl-2 human ELISA kit (Abcam, Australia) were employed for this analysis. In both cases, subconfluent HASMC were growth arrested prior to incubation in experimental medium as previously described (section 2.4.4). HASMC were treated with recombinant UCN2 at 0 or 100 nM for 24 h (six replicates for each experimental condition and three whole experiment repeats). The involvement of CRFR2 was also assessed by making cells take on Ast-2B, the CRFR2 blocker. The Apo-ONE Homogeneous caspase-3/7 assay is based on the presence of caspase 3/7 activity and the

resulting fluorescence due to cleavage of pro fluorescent reagent rhodamine-110 (bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) by caspase, releasing DEVD peptides. The rhodamine group released is intensely fluorescent. This was measured at an excitation wavelength of 499 nm and emission wavelength of 521 nm. Briefly, as per the manufacturer's recommended protocol for each assay, an equal amount of assay reagent (a homogeneous mixture of substrate and buffer) was added to the cell culture after UCN2 treatment and incubated for 30 min at room temperature on an orbital shaker (350 rpm). The plate was pre-equilibrated to room temperature for 30 min for the viability assay after which reaction reagent was added and incubated. Fluorescence (apoptosis) was then measured using a multi-mode microplate reader (BMG Labtech, Germany). An additional step for including shake and settling of 10 s each was included before the measurements were taken.

The Bcl-2 human ELISA kit (Abcam, Australia) was used to analyse Bcl-2 expression. Briefly, a 96 well plate pre-coated with an anti-human Bcl-2 antibody was washed twice in 400 μ L wash buffer. Pretreated HASMC lysate and standards (100 μ L) were added into each well in duplicate, followed by the addition of 50 μ L biotin-conjugated anti-human Bcl2 antibody which binds to human Bcl-2 captured by the antibody coating. Cells were incubated at room temperature for 2 h on a microplate shaker with gentle shaking at 100 rpm. Cells were washed three times in 400 μ L wash buffer, and 100 μ L streptavidin-HRP added into each well and ELISA plate incubated for 1 h with gentle shaking at 100 rpm. Cells were washed 3 times with 400 μ L wash buffer as above, and 100 μ L TMB substrate added. ELISA plate was incubated in the dark at room temperature for 10 min. Substrate development was stopped after 10 min and absorbance read at 450 nm with reference wavelength of 620 nm with a *Sunrise*TM microplate reader (Tecan property, Australia).

2.4.8 Cell cycle investigations using flow cytometry

UCN2 induced changes in the cell cycle phases were investigated by measuring the cellular DNA content using the propidium iodide (PI) incorporation assay (Invitrogen, Australia) as previously published.^{349,350} Subconfluent HASMC grown in six well Nunc cell culture plates (Thermo Fischer Scientific, Australia) were incubated in experimental medium as previously described (section 2.4.5). HASMC were treated with recombinant UCN2 at 0 or 100 nM for 24 h (six replicates for each experimental condition and three whole experiment repeats). The involvement of CRFR2 was also assessed using Ast-2B to block the CRFR2 on HASMC. Experimental cells were harvested after 24 h by trypsinisation as previously described and centrifuged at 200 g for 6 min. The supernatant was discarded and cells re-suspended in DMEM with 10 % FBS. Cells were centrifuged again at 200 g for 6 min and the supernatant discarded. Cells were counted as previously described and re-suspended at 1×10^6 in 0.5 mL PBS in pre-labeled 5 mL polystyrene round bottom tubes (BD, Australia). Samples were centrifuged at 200 g for 5 min to pellet. Cells were again re-suspended in 0.5 mL PBS and centrifuged to pellet as above. One (1) mL of hypotonic PI staining buffer for DNA (0.1% sodium citrate, 0.1 % Triton-x 100, 40 μ g/mL PI, 100 μ g/mL Ribonuclease A, distilled H₂O to 250 mL) was added to pellets drop wise whilst gentling vortexing to ensure thorough mixing. Samples were kept on ice in the dark for 30 min to 1h (prolonged exposure to hypotonic buffer results in increase debris). Cells were acquired by flow cytometry (CyAn ADP, Beckman Coulter, Australia) and DNA content analysed with the Summit® flow analysis software, version 4.3 (Beckman Coulter, Australia).

2.5 Statistical analysis

Data were analysed using the SPSS statistical package v.19.0 software (IBM, Australia). All the experiments were performed at least three times and the representative experiment has been shown. In some cases pooling of data was considered and appropriately presented. In some experiments, data were normalised with the control expression as maximum expression and data from other groups represented as percentage of control. Results are expressed as median and inter-quartile range unless otherwise stated.

The effect of recombinant UCN2 and/or AAA thrombus media on HASMC cytokine secretion, Akt phosphorylation, caspase-3/7 activity, Bcl-2 expression and the cell cycle were presented as median and inter-quartile range and evaluated by the Kruskal Wallis test. The Mann Whitney U test was applied to compare two groups when appropriate. Statistical significance was assumed at $P \leq 0.05$.

For the plasma and conditioned media level of UCN2, quantitative data was presented as median and inter-quartile range and compared between patient populations using the Mann Whitney U test. Conditioned media from paired AAA thrombus and AAA body biopsies were compared by Wilcoxon signed ranks test. Nominal data was presented as number and percentages and compared by Chi-square test. The association of UCN2 with AAA presence was assessed employing multiple regression adjusting for age, CHD, smoking, hypertension, dyslipidaemia, and diabetes. Receiver operator characteristic (ROC) curve were generated to assess the ability of UCN2 to predict AAA presence.

For qPCR, samples were run in duplicate, results from samples and duplicates were exported to Microsoft excel. The calculated concentration data for each of the genes were exported from the Corbett Rotor gene run-analysis software. Two columns were prepared with

reference gene on one and the gene of interest on the other. The relative expression was obtained by using the formula:

$$\text{Relative concentration} = \frac{\text{gene of interest (calculated concentration)}}{\text{reference gene (calculated concentration)}}$$

Wilcoxon's signed ranks test was performed to identify differences between UCN1, UCN2, UCN3, CRFR1, and CRFR2 gene expression levels in human AAA. Statistical significance was defined at the conventional $P \leq 0.05$.

CHAPTER 3

THE ASSOCIATION OF UROCORTIN 3 (UCN3) WITH ABDOMINAL AORTIC ANEURYSM

3.1 INTRODUCTION

Animal models of abdominal aortic aneurysm (AAA) are increasingly being utilised to investigate mechanisms underpinning aneurysm development and progression in order to develop new non-surgical therapies and more efficient means of monitoring disease progression.¹³⁴ The infusion of angiotensin II (AII) into the hyperlipidaemic apolipoprotein E deficient (ApoE^{-/-}) mouse model is the most commonly employed mouse model of AAA.^{178,196,200} This mouse model has a number of similarities to human AAA such as the prevalence of aneurysm formation in males and the focal nature of aortic dilatation.^{178,196} Aneurysm formation in this model is restricted to the suprarenal aorta (SRA), with no incidence of infrarenal (IRA) aneurysm reported.^{134,197,246} In contrast, AAA is frequently restricted to the infrarenal aorta in humans. It is thus suggested that the IRA in this mice model is resistant to aneurysm formation.^{134,246}

In a recent study utilising the ApoE^{-/-} mouse model of AAA, Rush *et al.* employed microarrays to compare the relative gene expression of SRA and IRA segments of ApoE^{-/-} mice to identify genes and pathways associated with aneurysm formation. A number of genes, including urocortin 3 (UCN3), differentially expressed between the SRA and IRA were identified.²⁴⁶ The study by Rush and colleagues identified UCN3 to be downregulated in the SRA compared to the IRA of 14 week old ApoE^{-/-} mice prior to AII infusion, suggesting that UCN3 protein may act to protect the IRA from the aneurysm inducing effects of AII.²⁴⁶ Notably, UCN3 has been demonstrated to antagonise AII induced production of oxygen derived free radicals,²⁴⁴ and play a cardiovascular protective role in animal models.³⁰⁶ Other studies have suggested a role for the urocortins (UCNs) including UCN3 in blood pressure regulation and in the pathophysiology of cardiovascular diseases.^{304,305,351,352} For example,

Rademaker and colleagues suggest that intravenous infusion of UCN3 in sheep with pacing-induced heart failure is associated with beneficial haemodynamic, endocrine and renal effects,³⁰⁶ implying that UCN3 may have therapeutic potential in patients with heart failure. In the latter study, UCN3 was shown to improve cardiac output and decrease arterial pressure and plasma renin levels.³⁰⁶ Agnello *et al.* reported that subcutaneous injection of UCN1 significantly decrease lipopolysaccharide-induced serum proinflammatory TNF and IL-1 β in rodents,²⁹⁹ and *in vitro* UCN3 was shown to increase the secretion of the anti-inflammatory cytokine IL-4 from endometrial cells.³⁵³ It is possible that the anti-oxidant, anti-inflammatory, and beneficial blood pressure effects of UCN3 may in part explain the putative relative protection of the IRA from AII induced AAA in the ApoE^{-/-} mouse model.

UCN3 is known to bind to the G-protein coupled receptors, corticotrophin releasing factor receptor 1 and 2 (CRFR1 and CRFR2),³⁵⁴ but exhibits a higher affinity for CRFR2.³⁵⁵ Both UCN3 and CRFR2 have been reported to be expressed within the vasculature.^{33,34} Interestingly, mice deficient in CRFR2 have been reported to have increased UCN3 mRNA expression,³⁵⁶ implying that CRFR2 may modulate the role of UCN3. Others have shown that UCN3 acting on CRFR2 increased insulin-like growth factor 1 (IGF-1) in UCN3 transgenic mice with a consequent favourable metabolic phenotype including protection against obesity and hyperglycaemia.³⁵⁷ UCN3 has also been shown to be protective against ischaemic and reperfusion injury via its binding to CRFR2 in murine species.²⁵⁵

In contrast, others have reported that the UCNs including UCN3 stimulate mast cell degranulation,^{272,358} resulting in an increase in vascular permeability and influx of

inflammatory cells. Mast cells are implicated in AAA pathogenesis,^{309,310,311,264} suggesting that UCN3 may not play a favourable role in AAA.

In view of the association between the UCNs and inflammatory diseases and the influence of these peptides in modulating the cardiovascular environment,⁵ this Chapter investigates the association of UCN3 with AAA in mice. It is hypothesised that UCN3 protein is decreased within the aorta of ApoE^{-/-} mice that develop AAA following 4 week AII infusion. Specifically this Chapter aims to:

1. Define the relationship between aortic UCN3 protein expression and the plasma UCN3 concentrations with aneurysm formation
2. Determine the relationship between aortic expression of CRFR2 and the presence of AAA

3.2 MATERIALS AND METHODS

3.2.1 Effects of angiotensin II (AII) infusion on ApoE^{-/-} mouse aortic diameter and blood pressure

3.2.1.1 Mice

This investigation conformed to the “*Australian code of practice for the care and use of animals for scientific purposes*” see publication (Australian Government National Health and Medical Research Council, 7th Edition, 2004). Ethics approval was obtained from the James Cook University animal ethics committee prior to commencement of the study (# A1538, Appendix 1). Forty male ApoE^{-/-} mice were obtained from the Animal Resources Centre, Canning Vale, Western Australia. The number of mice used in this study is typical of the sample size employed in similar studies.^{196,197,246,359} Mice were aged ~ 3 months and maintained on normal mouse chow and water until age 6 months. Mice were housed in a purpose-built mouse facility at James Cook University, Townsville, Australia for the duration of the experiments.

3.2.1.2 Angiotensin II treatment to induce AAA

At 6 months of age, ApoE^{-/-} mice (n=40) were anaesthetised via intra-peritoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg), and subcutaneously implanted with osmotic minipumps (Model 1004, Alzet, Durect Corporation). Briefly, a small incision was made lateral to the superior-dorsal midline of each mouse for placement of pumps in the subcutaneous space. All incisions were stitched closed and a topical antibiotic (Betadine[®]; Purdue Products L.P, USA) applied. All incisions healed rapidly without further need for

medication. Pumps delivered either angiotensin II (1.44 µg/ kg/ min, n=30) or saline (vehicle controls, n=6) over 28 days, as previously described.^{246,360,361}

3.2.1.3 Blood pressure and heart rate determination

Blood pressure; systolic (SBP), diastolic (DBP) and mean (MBP), and heart rate (HR) were obtained at days 0, 14 and 27 post AII or vehicle infusion. Mice were immobilised with intraperitoneal injections of ketamine (40 mg/kg) and xylazine (4 mg/kg). Blood pressure and heart rate were monitored using a CODA Monitor non-invasive small animal blood pressure apparatus (Kent Scientific). A minimum of three repeats measurements were obtained from mice infused with AII and mice infused with vehicle. Mice then underwent abdominal aortic diameter ultrasound measurement.

3.2.1.4 Ultrasound and plasma assessment of aneurysm progression

Aneurysm progression was monitored by ultrasound assessment of the abdominal aortic diameter immediately prior to AII infusion (day 0), and on days 14 and 27 of the experimental period. Mice were immobilised with intraperitoneal injections of ketamine (40 mg/kg) and xylazine (4 mg/kg). Ultrasound was performed in B-mode using a MyLab™ 70 VETXV platform (Esaote, Italy) with a LA435 linear transducer (Easote, Italy) at an operating frequency of 10 MHz,³⁶² to generate sagittal images of the suprarenal (SRA) and infrarenal (IRA) aortas. Maximum SRA diameter was measured at peak systole using the calliper measurement feature. We have previously shown good inter-observer reproducibility (coefficient of repeatability 0.92, 95% confidence intervals 0.883-0.946; average coefficient of variation 9.5%).³⁵⁹ All measurements were collected by two observers blinded to the

treatment groups. The average of two independent readings was used for future analysis. Whilst under anaesthesia, blood samples were collected in EDTA Microtainers (BD Bioscience, Australia) on days 0, 14 and 27 by tail bleeds as previously described.^{363,364} Briefly, a local anaesthetic cream (EMLA[®], AstraZeneca, Australia) is applied on the tail 15 min before placing mice in the warming chamber at 39 °C for 10 to 15 min. Mice were carefully monitored to avoid dehydration. Mice were then placed in a tube restrainer, and a small incision/prick made at lateral tail vein which is approximately a third of the way down from the tail tip with a sterile scalpel or 25G needle, and blood (50-100 µL) collected in the microtainers. Blood flow was stopped by applying finger pressure with a soft tissue placed at the sampling site for about 30 s. Plasma was separated from whole blood by centrifuging at 1500 g for 10 min, supernatant collected and transferred into freshly labeled 1.5 mL microfuge tubes. Plasma samples were stored at -80 °C for future assessment of putative circulating biomarkers.

3.2.1.5 Aorta morphometry

After the 28-day infusion period, mice were euthanised by carbon dioxide asphyxiation. Mouse aortas were perfused with phosphate buffered saline (pH 7.4) via cannulas placed in the left ventricle and atrium. Aortas were harvested from their origin at the left ventricle from arch to iliac bifurcation inferiorly. Aortas were placed on a calibrated black background, digitally photographed (Coolpix 4500, Nikon), and divided into four regions: arch (originating as the ascending aorta from the left ventricle turning into the aortic arch proper and ending at the level of the left subclavian artery), thoracic (also called descending aorta, left subclavian artery to the diaphragm), suprarenal (runs from just below the diaphragm to just above the renal arteries) and infrarenal (runs from just below the renal arteries to the

bifurcation into the right and left common iliac arteries) aortas (Figure 3.1), and stored at -80 °C for further analysis.

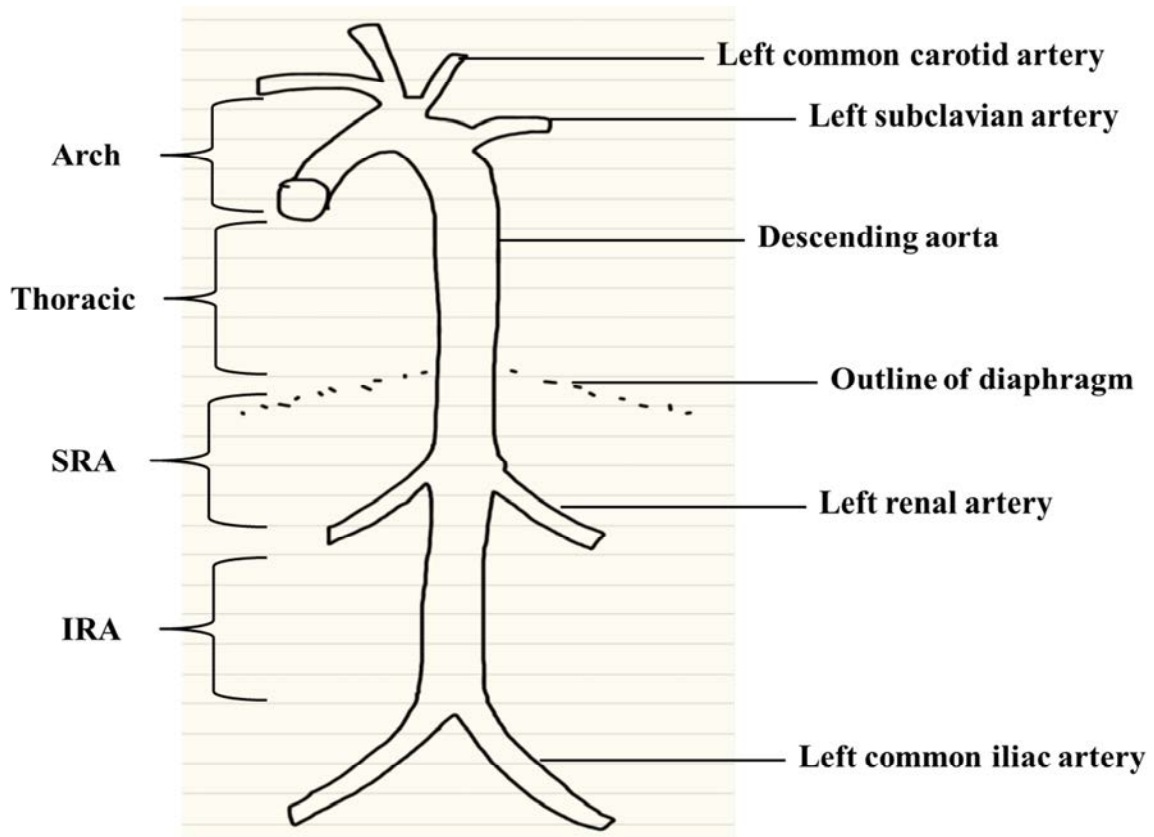


Figure 3. 1 Diagram of the mouse aorta showing the different regions used in morphometric analysis

Maximum diameters of the aortic arch, thoracic, SRA and IRA were determined from the images using computer-aided analysis (Adobe CS5 extended). It was established in a previous study (n=27), that these measurements could be repeated with good intra-observer reproducibility (Coefficient of repeatability 0.98, 95% confidence intervals 0.975-0.982, and coefficient of variation 4%).²⁴⁶ AAA was defined as a morphologically visible enlargement of the SRA diameter $\geq 50\%$ of that before AII infusion,³⁶³ with a mean maximum SRA diameter (MMD) \geq median for the group as used in this study.

3.2.2 Comparison of urocortin 3 (UCN3) levels in aneurysmal versus non-aneurysmal mice

3.2.2.1 Extraction of aortic proteins

Mouse suprarenal aortas were removed from -80 °C storage, thawed and stored on ice. Aortas were rinsed in 200 µL lysis buffer E developed in house (1 % Triton X 100, 0.1 % SDS, 10 mM Cacodylic acid, 40 mM Tris.HCl, pH 7.4, Roche complete protease inhibitor cocktail tablet) and finely sliced. Chopped aortas were twice snap-frozen in liquid nitrogen for ~30 s and thawed to break cell membranes. Samples were then homogenised in lysis buffer E using 0.5 mm Zirconium oxide (ZrO₂) beads in a Bullet Blender™ (Next Advance). Homogenates were centrifuged at 18,000 g for 30 min and supernatants containing extracted proteins removed and stored. Protein concentration were determined using Reagent 500-0006 (Bio-Rad) as directed by the manufacturer according to the method of Bradford.³⁶⁵ Briefly, a standard curve containing 0, 1, 2, 3, 4, 5, 6, 7, 8 µg of protein was prepared from a stock solution of 1 mg/mL IgG (Sigma, Australia). A 1:10 dilution of samples was made (5 µL samples in 45 µL dH₂O) and kept on ice. Five (5) µL of sample and standards were transferred to a 96 well microplate and made up to a total volume of 160 µL with distilled water (dH₂O). Bradford reagent (40 µL) was added to each well and plate incubated for 30 min at room temperature. Optical density (OD) was measured at 595 nm in a microplate reader (Tecan, Tecan Group, Switzerland) and samples refrozen at -80 °C for later UCN3 analysis.

3.2.2.2 Mouse UCN3 ELISA

Aortic (suprarenal and infrarenal) and plasma UCN3 concentration were measured in samples collected from mice infused with AII at the end of the 28 day experimental period using a commercially available mouse UCN3 competitive ELISA (ALPCO, USA). Circulating UCN3 was measured in plasma samples from 8 mice with macroscopically visible AAA (mean maximum aortic diameter for this study ≥ 1.66 mm), and 6 mice without AAA (mean maximum aortic diameter < 1.66 mm). Only mice that were successfully bled at the three time points excluding mice that died of aortic rupture (n=9) were used for this study. SRA protein lysate (n=9, mice with AAA; n=5 mice without AAA), and IRA protein lysate (n=7, mice with AAA, n=5 mice without AAA) were used to determine UCN3 expression in mouse aortas. Briefly, samples were thawed and reagents brought to room temperature. Following three times washes with 350 μ L wash buffer (1 x PBS), plates were inverted and blotted on absorbent paper. The standards and samples were added in duplicate to the 96 well ELISA plate according to manufacturer's instruction. The ELISA plate was sealed and incubated at 4 $^{\circ}$ C for 18 h. The ELISA plate was then brought to room temperature for 40 min. The plate seal was removed, samples aspirated and wells washed with 350 μ L wash buffer. The wash step was repeated to give a total of 4 washes. Plates were inverted and blotted on absorbent paper as before, 100 μ L of streptavidin-horse radish peroxidase (SA-HRP) solution provided in the ELISA kit was added into each well, plates sealed and incubated at room temperature for 2 h on an orbital shaker at 100 rpm. The plate seal was removed and the plates washed 4 times as above. One hundred (100) μ L of substrate [o-Phenylenediamine dihydrochloride, (OPD)] was added to each well and plates were incubated at room temperature for a further 20 min. The reaction was stopped with stop solution (1 M H₂SO₄) and absorbance measured at 490 nm in a Tecan microplate reader (Tecan Group, Switzerland). Assay sensitivity as given by manufacturer was $\sim 0.41 - 100$ ng/mL.

3.2.3 Expression of the UCN3 receptor CRFR2 expression in aortas from aneurysmal and non-aneurysmal mice

3.2.3.1 Extraction of aortic proteins

Mouse suprarenal aortas were removed from -80 degree freezer and thawed on ice. Aortic protein extraction and quantification was carried out as described above (Chapter 3, section 3.2.2.1).

3.2.3.2 Immunoprecipitation

The Thermo Scientific Pierce Classic Immunoprecipitation (IP) kit was used to concentrate CRFR2 in mouse SRA protein lysate according to manufacturer's protocol. This was carried out at 4 °C in 3 major steps unless otherwise stated:

I. Preparation of immune complexes: 2 µg of affinity purified rabbit polyclonal CRFR2 antibody (Abcam, Australia) was combined with 300 µg of mouse aorta protein in a microcentrifuge tube. The antibody/lysate solution was diluted to 500 µL with IP lysis/wash buffer containing (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4) and incubated overnight at 4 °C.

II) Capture of immune complexes: Pierce protein A/G plus agarose was gently swirled to obtain an even suspension. 20 µL of the resin slurry was added into a Pierce spin column using a cut pipette tip and centrifuged at 1000 g for 1 min. Flow through was discarded and the resin washed twice with 100 µL lysis/wash buffer with the flow through being discarded

after each wash step. The column was gently tapped on filter paper to remove excess fluid after the final wash. The antibody/lysate sample was then added onto the protein A/G plus agarose in the spin column. The screw cap was attached and samples incubated with gentle end-over-end mixing or shaking for 1 h at room temperature. Columns were placed in a collection tube and centrifuged at 1000 g for 1 min after removing the bottom plug and loosening the screw cap. The flow through was labelled and saved and the column placed in a new collection tube. 200 μ L IP lysis buffer was added to resin and columns centrifuged at 1000 g for 1 min with flow through discarded for a total of 4 times. 100 μ L of 1 x conditioning buffer (pH 7.0) was added and column centrifuged at 1000 g for 1 min.

III) Elution of immune complexes: A low pH elution method was used. Briefly, column-containing resin was placed into a new collection tube and 50 μ L of elution buffer (pH 2.8, containing primary amine) was added and incubated for 10 min at room temperature. Columns were then centrifuged at 1000 g for 1 min and flow through collected. Five (5) μ L of 1 M Tris, pH 9.5 was added into the flow through to neutralise the pH and samples were stored at -80 °C for further western blot analysis.

3.2.3.3 Western blotting

Thirty microgram (30 μ g) in 0.5 μ L of immunoprecipitated protein mixed with an equal volume of Laemmli buffer (0.125 M Tris.HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, 0.004% bromophenol blue; pH 6.8) and boiled for 5 min at 95 °C. Proteins were loaded into the wells of a 7 cm 12% homogenous polyacrylamide gel (Bio-Rad, Australia) and electrophoresed at a constant voltage of 120 V for 60 min. Gels were

equilibrated in western blotting transfer buffer (3 g Tris-base, 14.4 g glycine, 20% (v/v) methanol, made up to 1 L in deionized H₂O) for 5 min, and transferred to PVDF membranes at 15 V for 30 min using a Trans-Blot[®] SD semi-dry transfer cell (Bio-Rad). Membranes were blocked overnight at 4 °C in blocking buffer [2% (w/v) FBS, 50 mM Tris.HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6] with gentle agitation. The blocking solution was removed and the membranes incubated in rabbit polyclonal anti-CRFR2 antibody (1:2000, Abcam, Australia) in blocking buffer for 2 h at room temperature on an orbital shaker. Membranes were washed twice in TBST buffer (50 mM Tris.HCl, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6) for 15 min and incubated in goat anti-rabbit HRP (1:1000, Abcam) for 2 h at room temperature. After washing in TBST as above; membranes were further washed twice for 5 min with TBS (50 mM Tris.HCl, 150 mM NaCl, pH 7.6). Antibody binding was visualised using the enhanced chemiluminescence kit (ECL *Advance*[™]; Amersham, Australia) employing a ChemiDoc[™] imaging system supported by QuantityOne[™] analysis software (Bio-Rad, Australia). Stain intensity of bands was quantified using the densitometry function of the QuantityOne[™] software package. All reported densitometry data are expressed as relative density units (RDU) per µg protein.

3.2.4 Statistics

Data were analysed for normal distribution by the Shapiro-Wilk normality test. Aortic and circulating concentrations of UCN3, and blood pressure parameters were not normally distributed and thus compared using box plots and the Mann Whitney U test. Blood pressure parameters and ultrasound data were compared between mice infused with AII and mice infused with vehicle. Morphometric comparison of AAA formation was between mice infused with AII with and without AAA, and vehicle infused mice without AAA. CRFR2

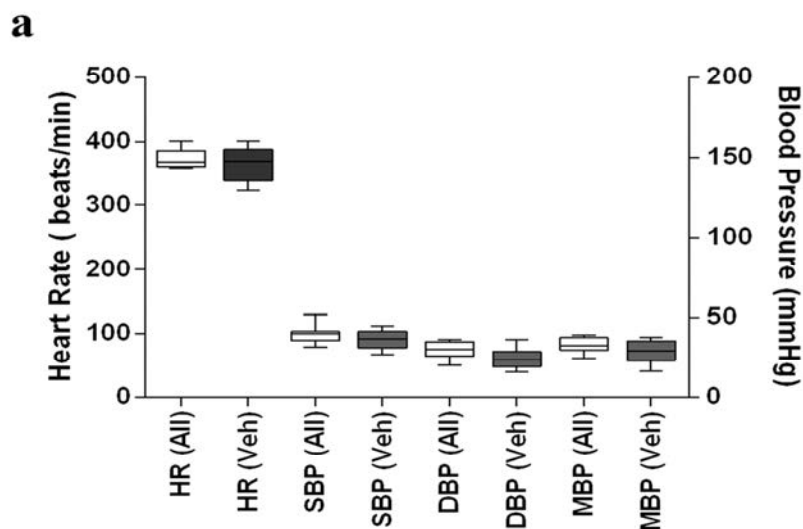
expression was compared between mice infused with AII with AAA and those without AAA. Survival rate between mice infused with AII and mice infused with vehicle were compared by the Kaplan Meier survival test. Statistical significance was assumed at $P \leq 0.05$.

3.3 RESULTS

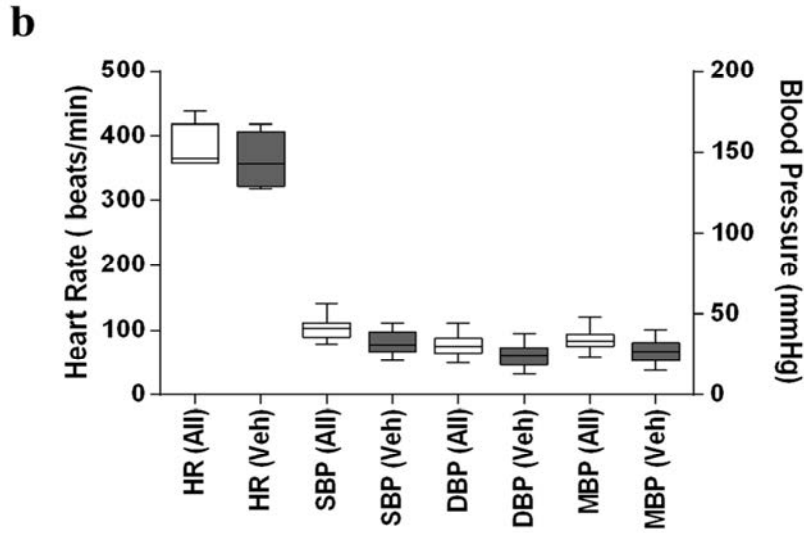
3.3.1 Blood pressure is increased in ApoE^{-/-} mice following AII infusion

There was no significant difference in blood pressure parameters observed between mice infused with AII and mice infused vehicle control at baseline (day 0) and at day 14 post pump insertion (Figures 3.2a and b). However, after 27 days AII infusion, median systolic blood pressure (SBP) was significantly higher in AII infused mice (98.5 mmHg, inter-quartile range 80.0 – 120.5) compared to mice receiving vehicle control (74.8 mmHg, inter-quartile range 61.9 – 92.6, $P=0.050$, Figure 3.2c). Similarly median diastolic blood pressure (DBP) was significantly increased in the mice receiving AII for 27 days (80.5 mmHg, inter-quartile range 67.0 – 102.5) compared to vehicle control infused mice (54.5 mmHg, inter-quartile range 41.1 – 68.9, $P=0.039$, Figures 3.2c). Heart rate and mean arterial pressure (MBP) did not differ significantly between the two groups (Figure 3.2).

Day 0 (Baseline)



Day 14



Day 27

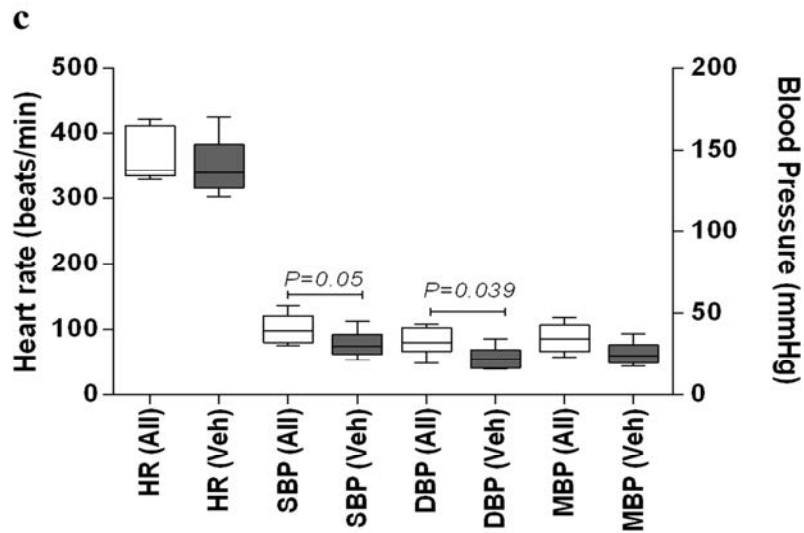


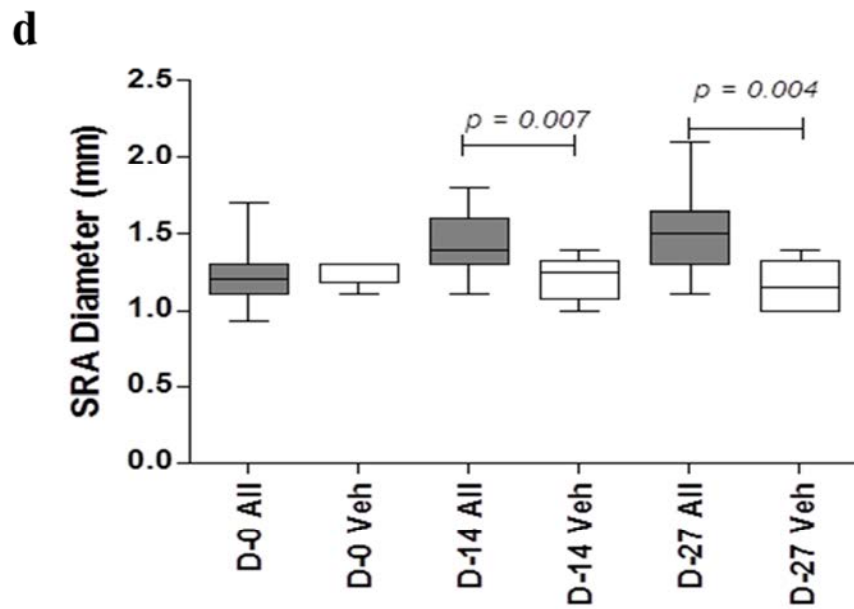
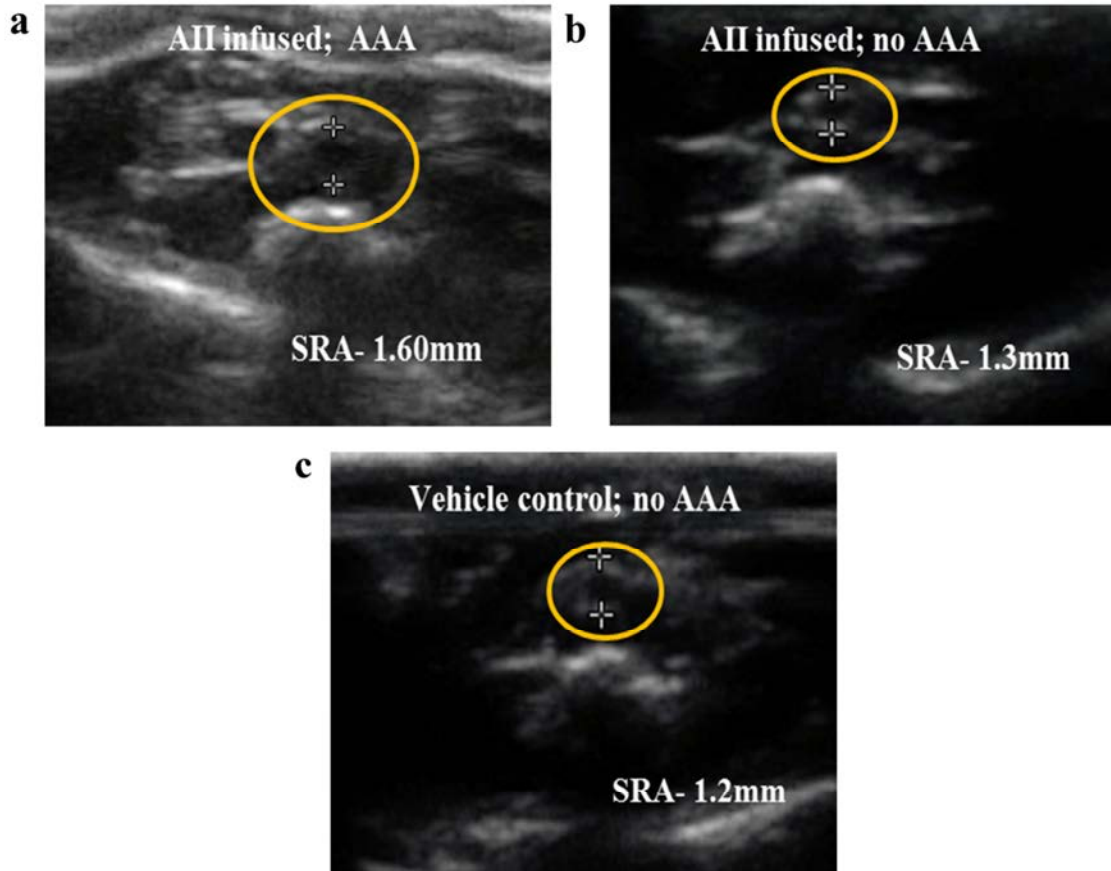
Figure 3. 2 Blood pressure and heart rate measured at days 0, 14 and 27 in ApoE^{-/-} mice that received AII or vehicle

(a and b) Effect of AII on blood pressure and heart rate at days 0 and 14 was not significant, $p>0.05$.

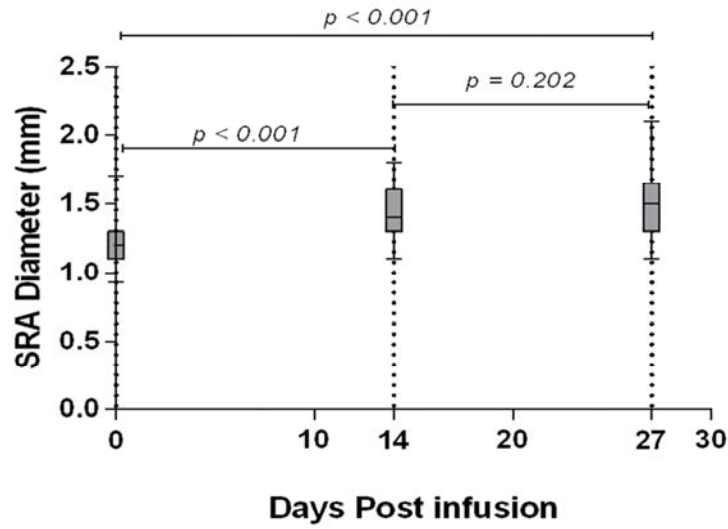
(c) Both SBP and DBP were significantly higher in AII infused mice compared to vehicle control infused mice at day 27 post infusion, $p=0.050$ and $p=0.039$, respectively. Data is presented as median and inter-quartile in box plots and 2 groups compared by the Mann Whitney U test. Angiotensin II (AII), diastolic blood pressure (DBP), heart rate (HR), mean arterial blood pressure (MBP), systolic blood pressure (SBP), vehicle control (Veh).

3.3.2 Aneurysm progression in angiotensin II (AII) infused ApoE^{-/-} mice

At baseline (day 0), there was no significant difference in SRA diameter of mice infused with AII and those infused with vehicle control (Figures 3.3 d to f). A significant difference in aortic diameter as measured by ultrasound was observed between mice infused with AII and control mice on days 14 and 27. Mice infused with AII showed progressive enlargement in aortic diameter (Figure 3.3d). Median SRA diameter at day 14 was 1.40 mm in AII infused mice (inter-quartile range 1.30 – 1.60) and 1.24 mm in vehicle control infused mice (inter-quartile range 1.10 – 1.33, $P=0.007$, Figure 3.3d). Median SRA diameter at day 27 was 1.50 mm in AII infused mice (inter-quartile range 1.30 – 1.65) and 1.20 mm in vehicle control infused mice (inter-quartile range 1.00 – 1.33, $P=0.004$, Figure 3.3d). Comparison of aortic diameter growth in mice infused with AII showed significant differences between SRA diameter at day 14 and 27 compared to day 0, $P<0.001$ (Figure 3.3e), but no significant difference between days 14 and day 27, $P=0.2015$ (Figure 3.3e). Comparison of the SRA diameters in vehicle infused mice between days 0, 14 and 27 revealed no significant difference, $P=0.686$ (Figure 3.3f).



e



f

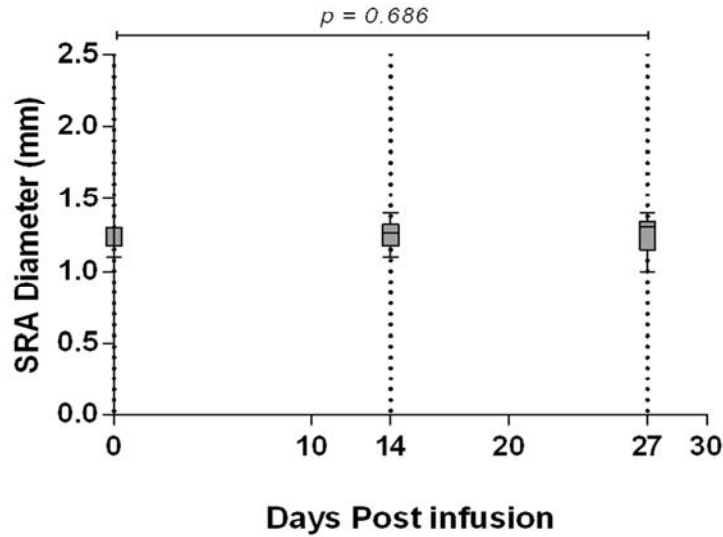


Figure 3. 3 Ultrasound comparison of aneurysm growth in mice that received AII or vehicle.

Representative ultrasound images of mice infused with AII that develop aneurysm (a), mice infused with AII without aneurysm (b) and vehicle infused mice (c). Box plots showing quantitative analysis of suprarenal aortic diameter between AII infused mice and controls at days 0, 14 and 27 (d) Boxplots showing increasing suprarenal aortic diameter in AII infused mice at days 0, 14 and 27 (e) Boxplots showing no difference in SRA diameter growth in vehicle control infused mice (f, Kruskal-wallis test).

3.3.3 Aortic diameter morphometry in the aneurysmal and non-aneurysmal mice

Dissection revealed that 67% of mice infused with AII developed AAA and/or died of aortic rupture (SRA diameter ≥ 1.66 mm, Figure 3.4). Subsequent data analysis excluded mice that died of aortic rupture (n=9) in the AII infused group within the experimental period as shown by Kaplan Meir Log-rank test ($P=0.014$, Figure 3.5).

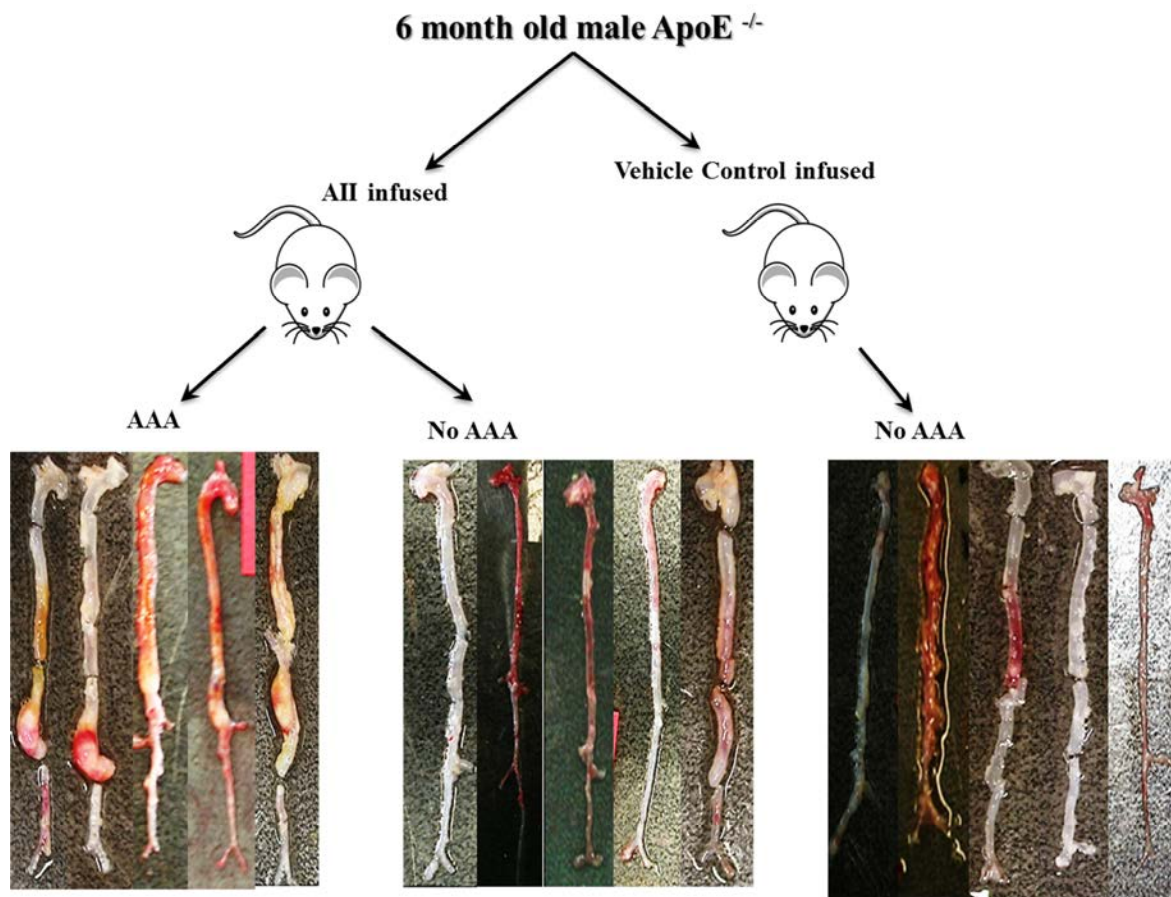


Figure 3. 4 Study design showing the experimental groups and dissected aortas from mice that received AII with and without aneurysm, and mice that received vehicle at necropsy

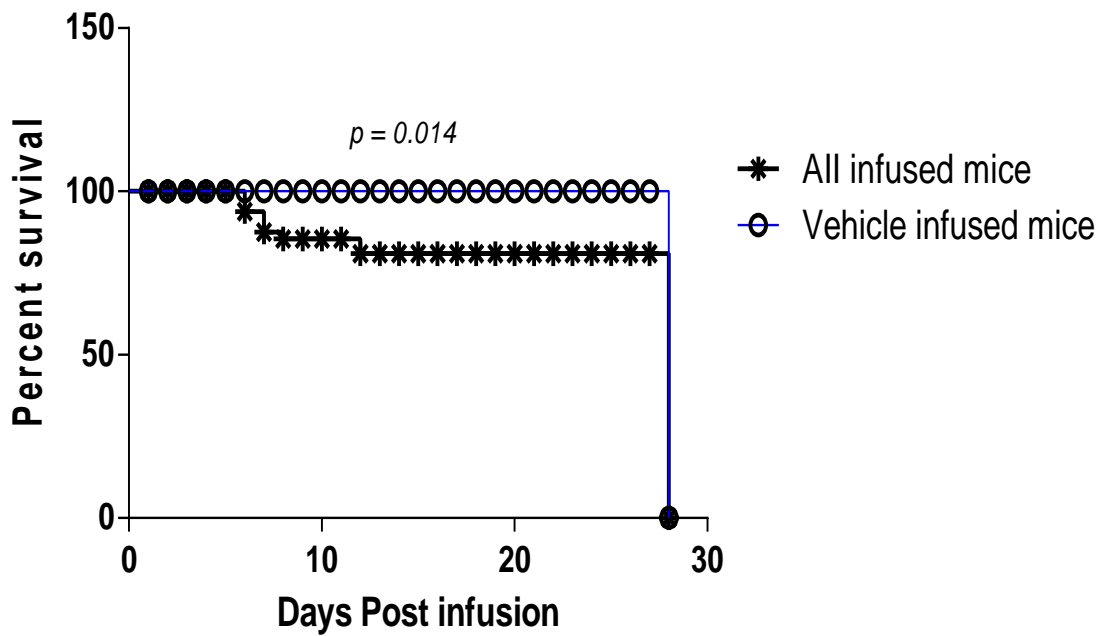
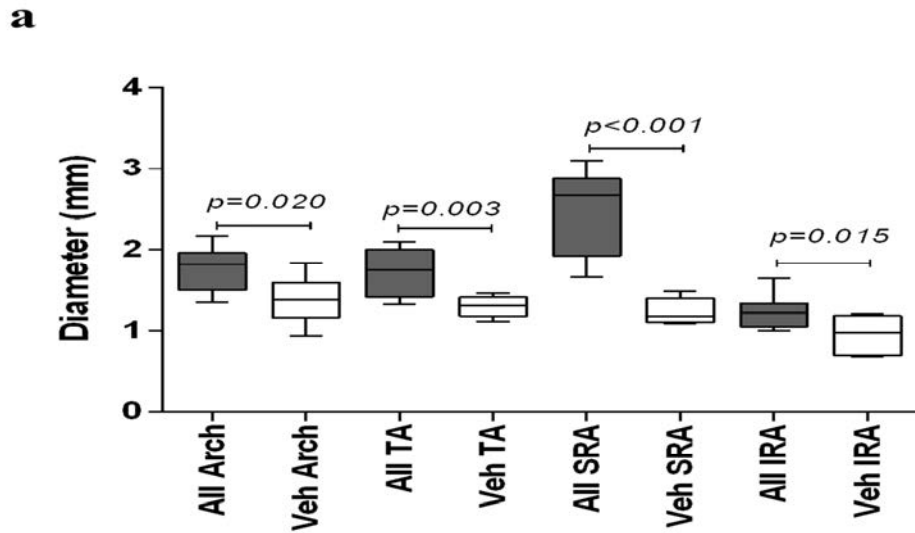


Figure 3. 5 Kaplan Meier plot showing overall survival curves according to study treatment

Aortic morphometric comparisons at necropsy revealed that all aortic regions (arch, thoracic, SRA and IRA) had significantly higher diameter in mice that develop AAA compared to mice without AAA or vehicle control group (Figures 3.6a and b). This was consistent with the ultrasound data presented in section 3.3.2 above.

Regional aortic diameter



SRA diameter

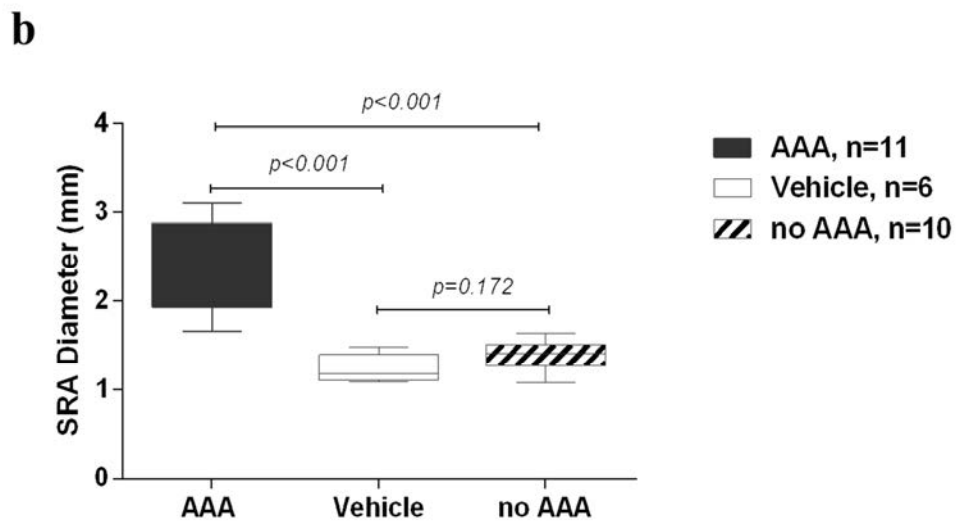


Figure 3. 6 Comparison of maximum aortic diameter measured from 4 regions of aortas (arch, thoracic, suprarenal and infrarenal) at necropsy in mice that received AII and vehicle

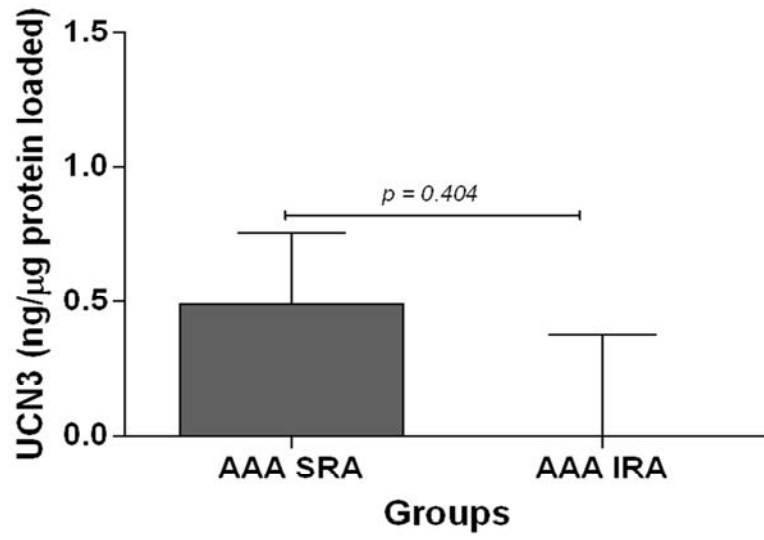
(a) Regional comparisons of aortic diameter between mice infused with AII with or without AAA and vehicle control-infused mice;

(b) SRA diameter comparison of AII infused mice with or without AAA and vehicle control-infused mice without AAA. Suprarenal aorta (SRA), infrarenal aorta (IRA).

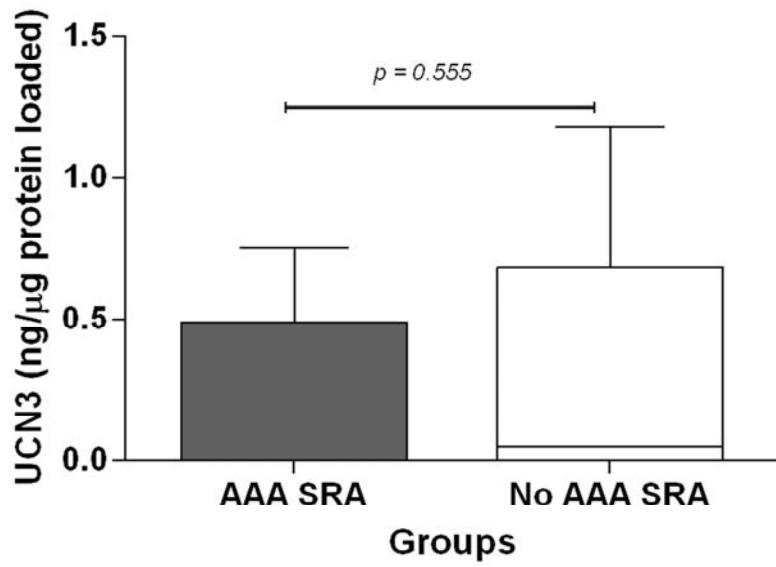
3.3.4 UCN3 protein is not upregulated in mice with AAA

For the UCN3 protein analysis, mice were grouped into (SRA diameter ≥ 1.66 mm = AAA, SRA diameter < 1.66 mm = no AAA) as previously stated. Aortic and plasma concentrations of UCN3 as determined by ELISA were not significantly different in AII-infused ApoE^{-/-} mice with or without AAA. UCN3 was not differentially expressed between the aneurysm prone SRA and aneurysm resistant IRA in this mouse model. Median aortic UCN3 protein expression was 0 ng in AII infused mice with AAA SRA (n=9, inter-quartile range 0 – 0.49) and 0 ng in AAA IRA (n=7, inter-quartile range 0 – 0.0, $P=0.404$, Figure 3.7a). Consistent with this result, median UCN3 protein expression was 0 ng in AII infused mice with AAA SRA (n=9, inter-quartile range 0 – 0.49) and 0 ng in the SRA of mice without AAA (n=5, inter-quartile range 0 – 0.68, $P=0.555$, Figure 3.7b). Working on the hypothesis that UCN3 levels in the aorta in AAA affected mice may have been depleted by constant secretion into blood, the plasma concentrations of UCN3 were analysed. There was no significant difference in plasma UCN3 concentrations between AII infused mice that developed AAA and mice that did not. Median plasma UCN3 was 0.03 ng/mL in AII infused mice with AAA (inter-quartile range 0 – 0.24) and 0.03 ng/mL in AII infused mice without AAA (inter-quartile range 0 – 0.28, $P=0.945$, Figure 3.7c).

a



b



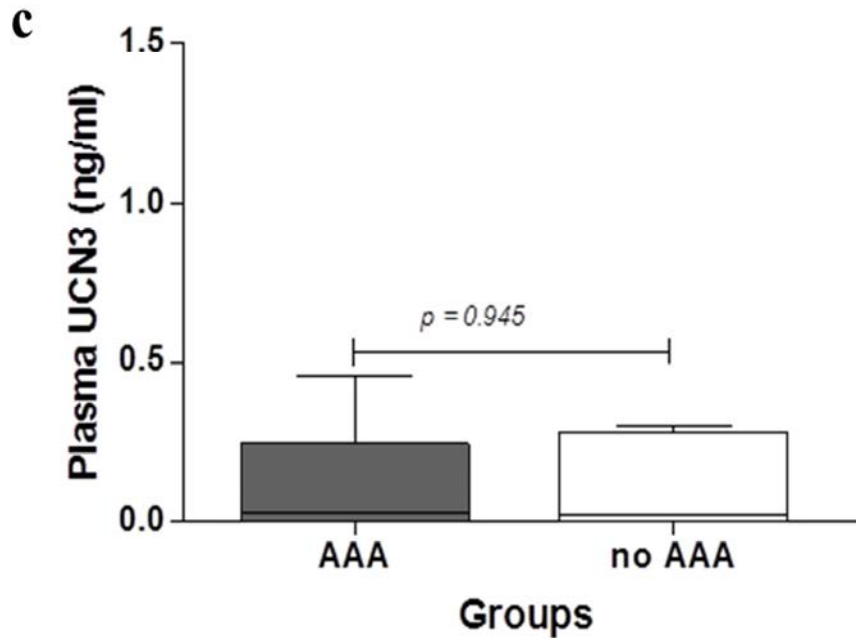


Figure 3. 7 Comparison of aortic and plasma UCN3 protein concentrations in mice that received AII with and without AAA

(a) Boxplot showing UCN3 protein expression within the SRA and IRA of mice with AAA (b) UCN3 protein expression is similar within the SRA of mice with and without AAA (c) Boxplot showing plasma UCN3 in mice with and without AAA. Suprarenal aorta (SRA), infrarenal aorta (IRA)

3.3.5 Expression of CRFR2 in mouse aorta

UCN3 is reported to bind to CRFR2 in the periphery, and it is suggested that UCN3 exerts its physiological effect through this receptor.^{5,354} Based on this, the expression of CRFR2 in the SRA of ApoE^{-/-} mice infused with AII (n=5) was investigated. Initial western blot analysis was unable to clearly define single bands for CRFR2 at the expected site of 48 kDa, with multiple bands present (Figure 3.8a).

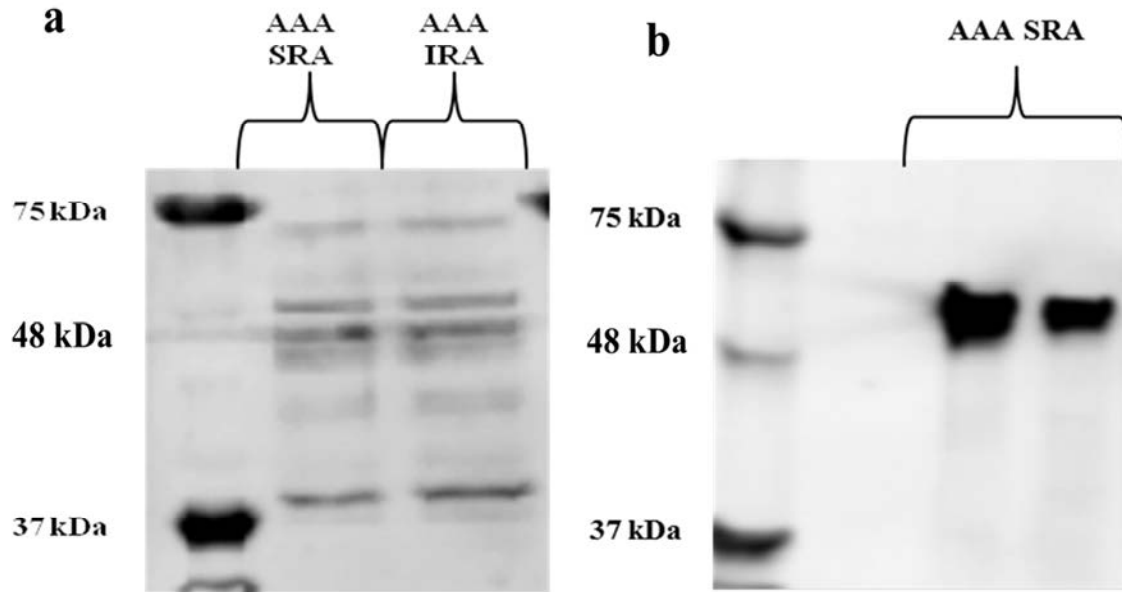


Figure 3. 8 Representative images of Western blot comparison of CRFR2 protein expression within the SRA and IRA of ApoE^{-/-} mice that received AII

(a) Western blot of mouse aortic CRFR2 before immunoprecipitation;

(b) Western blot of mouse SRA CRFR2 following immunoprecipitation. Angiotensin II (AII), corticotrophin releasing factor receptor 2 (CRFR2), kilodaltons (kDa), infrarenal aorta (IRA), suprarenal aorta (SRA).

A further optimisation following immunoprecipitation (IP) to purify and concentrate CRFR2 protein revealed clear single bands for CRFR2 (Figure 3.8b). However no significant differential expression of CRFR2 between the aneurysm prone SRA and aneurysm resistant IRA of AII infused mice with AAA (Figure 3.9) was seen after optimisation, although slightly increased in IRA. Median CRFR2 protein expression was 3.39 RDU in AAA SRA (n=6, inter-quartile range 1.80 – 6.01) and 5.74 RDU in AAA IRA (n=5, inter-quartile range 1.80 – 8.13, $P=0.537$, Figure 3.9).

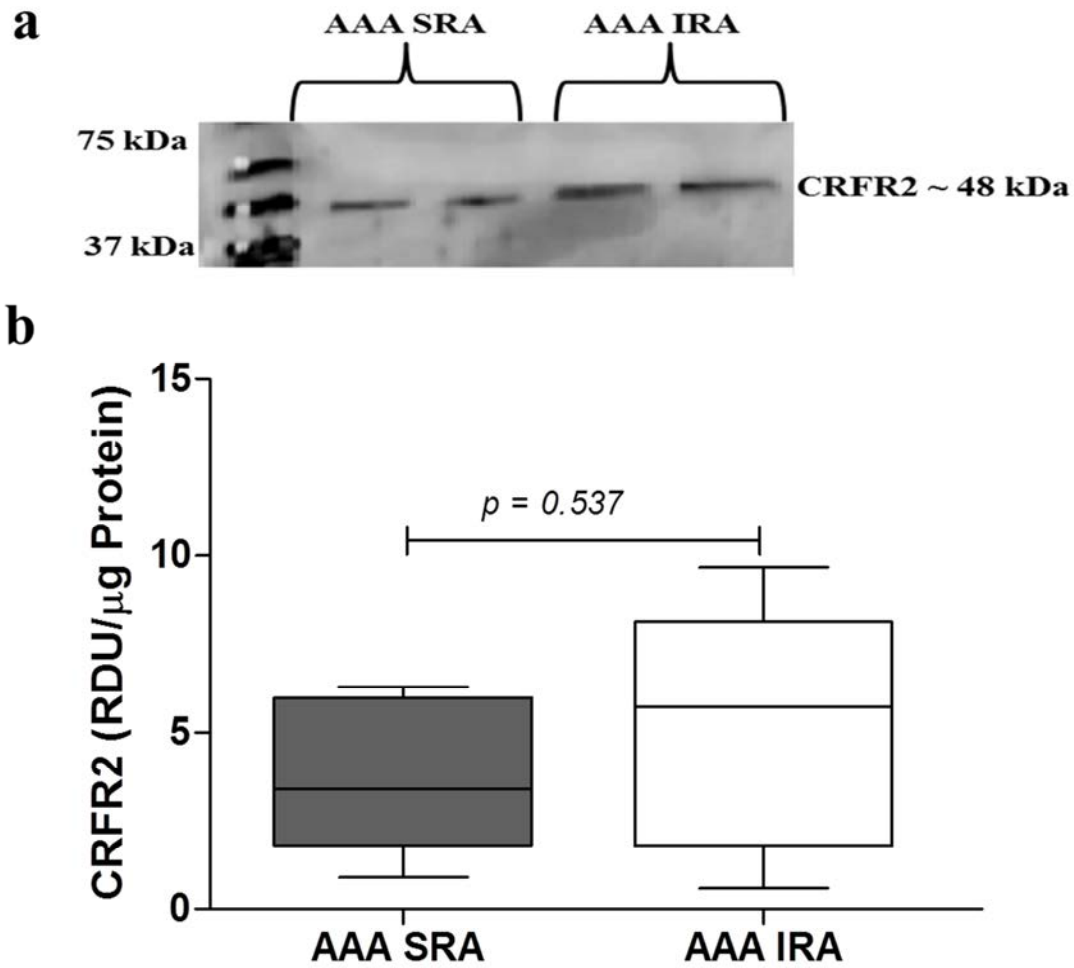


Figure 3. 9 CRFR2 protein distribution within the SRA and IRA of ApoE^{-/-} mice that received AII with AAA

(a) Representative Western blot analysis following immunoprecipitation of CRFR2 protein distribution in the SRA and IRA;

(b) Box plot shows result of relative densitometry analysis of CRFR2 protein distribution in the SRA and IRA. Results are median and inter-quartile range; (n=6&5). Corticotrophin-releasing factor receptor 2 (CRFR2), kilodaltons (kDa).

The protein expression of CRFR2 within the SRA of mice with and without AAA was similar. Median CRFR2 protein expression was 3.39 RDU in mice with AAA SRA (n=6, inter-quartile range 1.80 – 6.01) and 2.10 RDU in mice without AAA SRA (n=5, inter-

quartile range 0.84 – 8.04, $P=0.409$, Figure 3.10). However, mice without AAA appear to have slightly higher distribution of CRFR2 in the SRA.

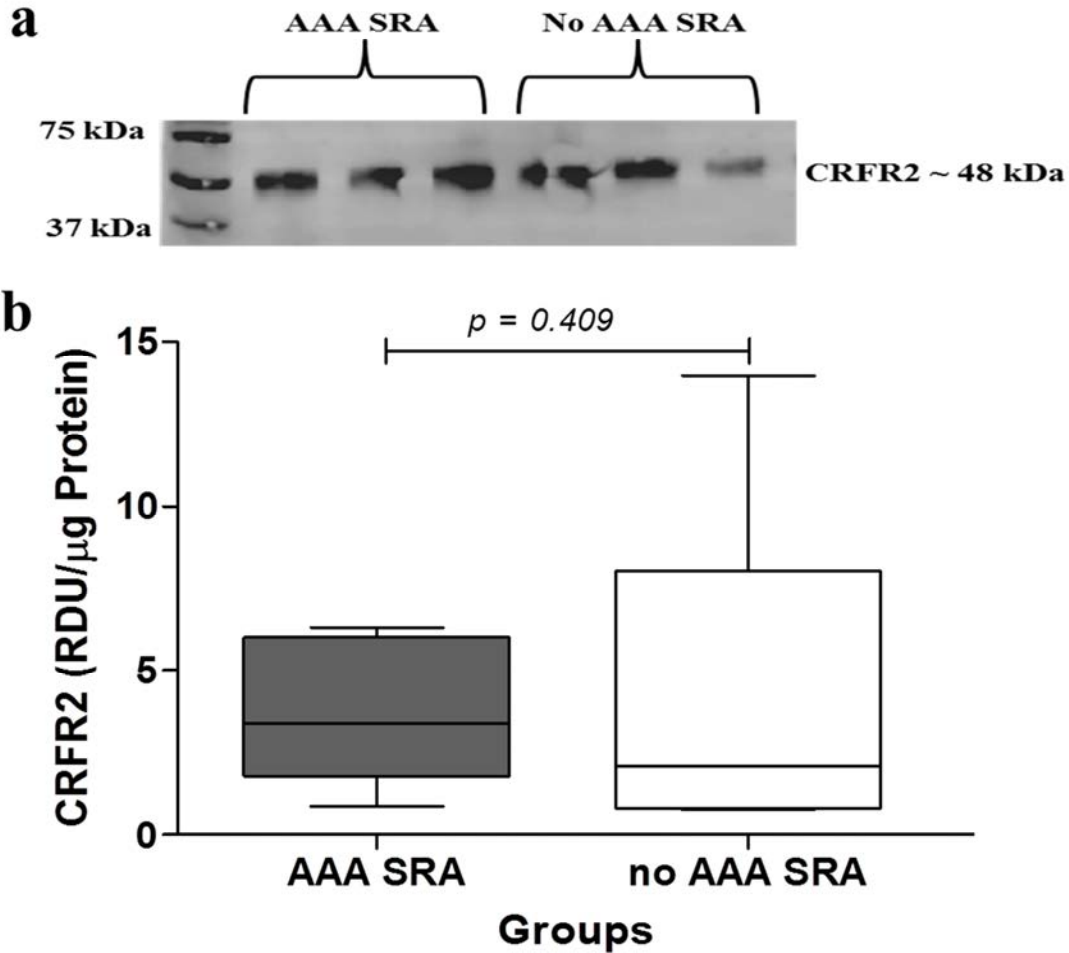


Figure 3. 10 CRFR2 protein expression within the SRA of mice that received AII with and without AAA

(a) Representative Western blot analysis following immunoprecipitation of CRFR2 protein expression in the SRA;

(b) Box plot shows result of relative densitometry analysis of CRFR2 protein expression in the SRA. Results are median and inter-quartile range; (n=6&5). Corticotrophin-releasing factor receptor 2 (CRFR2), kilodaltons (kDa).

Comparison of CRFR2 protein expression in pooled aortas (SRA + IRA) of ApoE^{-/-} mice infused with AII that develop AAA and those without AAA also demonstrate no significant

difference. Median CRFR2 protein expression was 4.39 RDU in AII infused mice with AAA (n=9, inter-quartile range 2.25 – 6.44) and 3.93 RDU in AII infused mice without AAA (n=6, inter-quartile range 1.65 – 12.56, $P=1.000$, Figure 3.11).

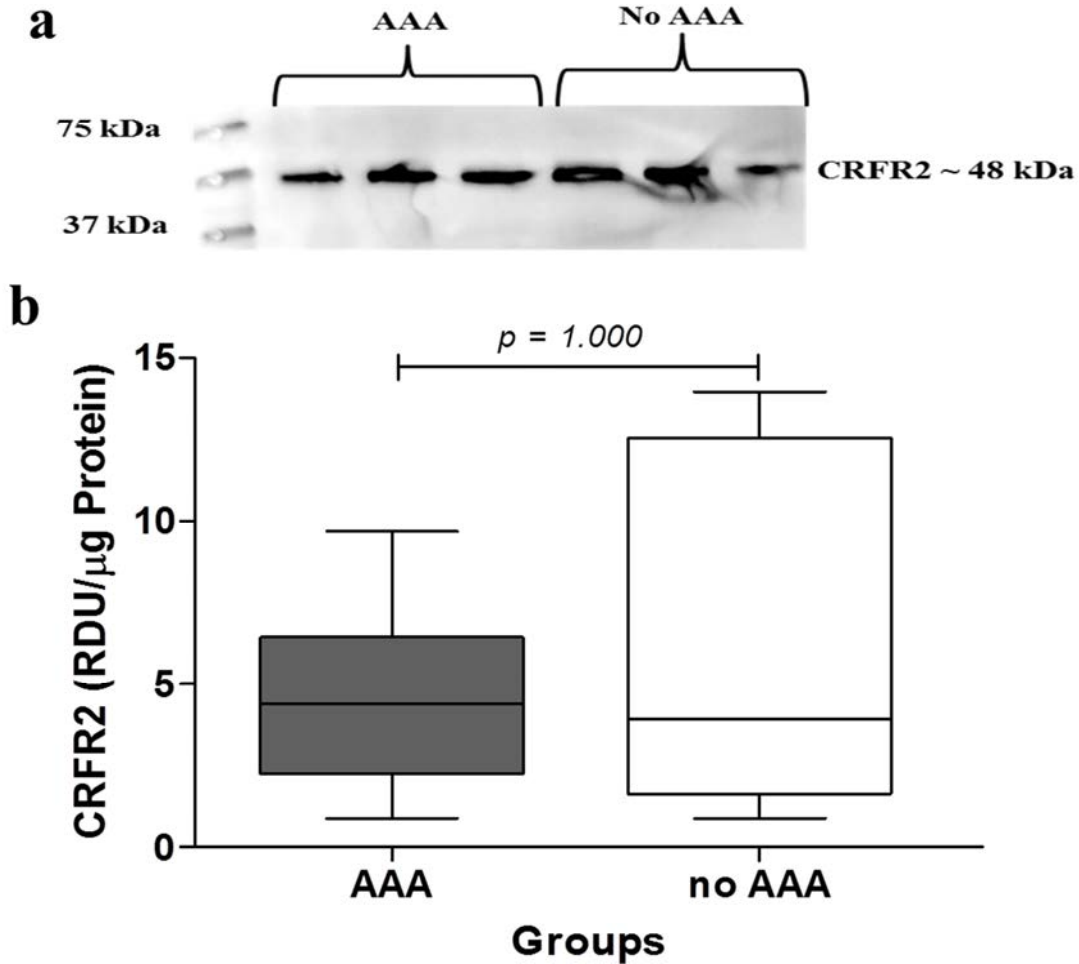


Figure 3. 11 CRFR2 protein expression within the aortas (SRA + IRA) of ApoE^{-/-} mice that received AII with and without AAA

(a) Representative Western blot analysis following immunoprecipitation of CRFR2 protein distribution;

(b) Box plot shows result of relative densitometry analysis of CRFR2 protein distribution in the aortas. Results are median and inter-quartile range; (n=9&6). Corticotrophin-releasing factor receptor 2 (CRFR2), kilodaltons (kDa).

3.4 DISCUSSION

The aim of this chapter was to investigate the association of UCN3 with AAA presence in AII infused Apo E^{-/-} mice, and whether UCN3 and CRFR2 levels were implicated in the pathogenesis of AAA. The data presented here demonstrate that over 60% of AII infused mice developed AAA. This result is consistent with previous data from this group,²⁴⁶ but different from those obtained elsewhere.¹⁹⁶ Daugherty *et al.* reported that 33% of ApoE^{-/-} mice infused with AII developed AAA in their study.¹⁹⁶ The consistent finding in all these studies is that not all mice infused with AII develop aneurysm, and the restriction of aneurysm to the aortic region above the renal arteries. Thus, it is of interest to study the molecules and mechanisms involved in the site-specificity and resistance to aneurysm formation in the ApoE^{-/-} mouse model following AII infusion.

Recently, UCN3 mRNA was shown to be upregulated in the aneurysm resistant IRA of untreated ApoE^{-/-} mice,²⁴⁶ suggesting that this peptide could play a role in the site specific predilection to aneurysm formation in this mouse model. However in this study, there was no significant difference in protein level expression of UCN3 between the IRA and the SRA of AII infused mice that developed AAA. Similarly, no significant difference in SRA UCN3 protein expression was observed between mice that developed AAA in response to AII and mice that did not. Analysis of circulating UCN3 in plasma also revealed no significant difference in mice with and without AAA, thereby refuting the hypothesis that UCN3 levels in the aorta may have been depleted by constant secretion into blood. It should be noted that these results may have been limited by the inability to detect UCN3 protein in mouse aortas and plasma by ELISA. However, based on the findings, it is feasible that AII infusion alters UCN3 expression. In addition, Rademaker and colleagues have previously published that

infusion of UCN3 into sheep with experimental heart failure decreased plasma renin.³⁰⁶ Renin has been reported to initiate the cascade of events leading to AII production,³⁰⁷ therefore a change in renin levels may alter the expression levels of UCN3 since an overall increase in systemic AII will negatively regulate renin synthesis. However bearing in mind that the study by Rush *et al.*²⁴⁶ only reported the differences in UCN3 expression prior to AII infusion in ApoE^{-/-} mice without AAA, it is likely that a comparison of UCN3 gene expression in AII infused ApoE^{-/-} mice would show no significant difference. It should be noted that although DNA microarrays have proven to be powerful tools for multiplexed comparative analysis of gene expression yielding important insights into gene expression patterns associated with disease states,³⁶⁶ a crucial limitation is that changes in gene expression profiles do not always reflect what happens at the proteome and cellular levels,³⁶⁷ with no correlation between gene profiles and protein synthesis and final cellular protein levels.⁷¹ Furthermore, studies have shown that transcriptional and post-translational modifications can alter the effects of changes in transcript abundance.^{71,366} In this regards, it is speculated that the absence of a significant UCN3 protein expression observed here may be due to alteration of the transcriptional process. However, these results may have been limited by the inability to detect UCN3 protein in mouse aortas and plasma by ELISA.

On analysing the expression of CRFR2, the receptor for UCN3, it was observed that although highly expressed within the aorta, there was no significant difference between the groups. It was expected that CRFR2 would be significantly higher in the region of the aorta where UCN3 is exerting its biological effect. The CRFR2 data presented here suggest that UCN3 may not be playing any role in aneurysm development in this mouse model. It is possible that other analogues of UCN3, for example UCN2, may play a more significant role via this receptor in this mouse model since evidence suggests a higher affinity of UCN2 for CRFR2.⁵

Abundant evidence in the literature demonstrates that the cardiomodulatory effects of UCN2 and UCN3 are mainly via this receptor in the pathogenesis of cardiovascular diseases.⁵ There is ample evidence suggesting the UCNs play an important role in CVD pathogenesis.^{254,330,352} Nevertheless, based on the low amount of tissue available for this study, it was not feasible to investigate the role of UCN1 and UCN2 in this model.

An interesting observation in the course of this investigation was the hypertensive effect of AII in ApoE^{-/-}. Blood pressure was demonstrated to be elevated in mice receiving AII compared to vehicle with no significant effect on the heart rate. This result is similar to that published by Arsenescu and colleagues demonstrating elevated systolic blood pressure in AII infused hyperlipidaemic mice administered with polychlorinated biphenyls,³⁶⁸ and that by Cassis *et al.* showing increased blood pressure in AII infused ApoE^{-/-} mice.³⁶⁹ In contrast the report by Daugherty and company showed no significant effect of AII infusion on blood pressure in ApoE^{-/-} mice.¹⁹⁶ This study used older ApoE^{-/-} mice (6 months) which could have contributed to the different results obtained, since enhanced sensitivity to AII and increased blood pressure in aged ApoE^{-/-} mice have been previously reported.³⁷⁰ Cassis *et al.* suggested that the elevation of blood pressure following AII infusion is simply due to vasoconstriction independent of AAA,³⁶⁹ however recent data suggest that AII-induced blood pressure elevation may play a more significant role in AAA pathogenesis.³⁷¹ It would be of interest to investigate in a larger cohort whether AII-infused mice that developed AAA have significantly higher blood pressure compared to those resistant to aneurysm formation, especially since the UCNs including UCN3 have been reported to exert antihypertensive effects.^{304,306,352}

The present study has a number of limitations. First, although sample size is typical of this kind of study,^{196,197,246,359} it is limited and may have been underpowered to detect an association between UCN3 and CRFR2 with AAA development. Validation of findings in larger studies will be required. Secondly, UCN3 protein expression in mice was very low and in most cases was below the assay limit of detection. This may have affected the results presented here. Validation of findings using a more sensitive technique like the recently described Nano-ELISA will be required.³⁷² The Nano-ELISA is a protein detection method which employs micro-magnetic beads modified with monoclonal antibody of the target protein. Gold nanoparticles (AuNPs) are modified with a detection monoclonal antibody and HRP for signal amplification. The method increases the sensitivity of a normal ELISA by a 100 fold.³⁷³

In conclusion, the evidence presented above does not support the hypothesis that UCN3 protein is decreased within the aorta and plasma of ApoE^{-/-} mice that develop AAA. It could be stated that UCN3 plays no role in AAA development in ApoE^{-/-}. However, this does not necessarily reflect the significance of its role in human AAA pathogenesis. With this in mind, and the fact that the ultimate aim of this study is to define a therapeutic target for human AAA, it was decided to appraise the role of the UCNs in human AAA as presented in the following chapters.

CHAPTER 4

UROCORTIN 2 AND THE PRESENCE OF HUMAN ABDOMINAL AORTIC ANEURYSM

4.1 INTRODUCTION

In the previous Chapter (Association of UCN3 with AAA), it was demonstrated that urocortin 3 (UCN3) has no association with abdominal aortic aneurysm (AAA) in mice. As previously mentioned in the literature review, AAA is amongst the top leading causes of death in the elderly in the western world.^{2,4,374} AAA associated mortality is typically due to rupture and surgical complications or related cardiovascular disease (CVD) events in the elderly.³⁷⁴ In addition, emerging evidence suggests a putative role for the urocortins (UCNs) in various cardiovascular pathologies in humans,⁵ including ischaemic heart,^{254,323,375} heart failure,^{35,274,305,306,327} and hypertension.^{274,329,330} To date however, there has been no validated reports associating the UCNs with human AAA.

The UCNs are members of the corticotrophin-releasing factor (CRF) family of peptides,³⁵⁴ comprising the three isoforms (UCN1, UCN2, and UCN3).^{354,376} UCNs are ligands for two G-protein coupled receptors, corticotrophin releasing factor receptor 1 and 2 (CRFR1 and CRFR2).³⁵⁴ UCN1 binds to CRFR1 and CRFR2, while UCN2 and UCN3 are selective ligands for CRFR2.³⁵⁵ UCNs have been implicated in CVD pathogenesis based on the range of their effects on vascular cell survival,²⁵⁴ apoptosis,²⁴³ and inflammation,^{40,244} within the cardiovascular system which are all processes involved in AAA pathology. There are conflicting reports on the beneficial and/or detrimental effect of the UCNs in the vasculature,⁵ with overall evidence favouring a more beneficial role for these peptides. UCNs are widely expressed within the heart and vasculature, and have been detected in vascular smooth muscle cells, endothelial cells, myocytes and fibroblasts.^{33,34} Although UCNs have been reported to have a beneficial effect in heart failure for example,^{243,247,331} there is no current

data on the expression of the UCNs in the human adult aorta, or the relationship between the UCNs and human AAA.

In order to detect AAA early, ultrasound screening programs have been introduced in some countries and appear to be effective in reducing mortality due to aortic rupture in some countries, although initial appraisals indicate that screening program detection of AAA incidence is lower than expected.^{214,215} Identifying circulating markers for AAA has been proposed as a means to improve and/or complement current AAA detection and diagnosis.²²⁰ However this is hindered by incomplete understanding of AAA pathogenesis reflected by significant shortfalls in patient management, such as the current lack of medications that effectively slow AAA progression.³⁷⁷

Examination of human aortic biopsies suggests that impaired proliferation and apoptosis of vascular smooth muscle cells are important mechanisms in AAA development and progression.²⁵ Other characteristic pathological findings of human AAA biopsies include degradation of aortic elastin and collagen, and high concentrations of proinflammatory cytokines.⁷⁷ Despite the above observed characteristic pathology of AAA, a suitable diagnostic, prognostic or therapeutic target for the disease is yet to be validated.⁷⁷ Circulating blood borne markers for AAA could putatively contribute to AAA screening in at-risk populations whilst providing a possible therapeutic target. The emergence of the UCNs provide an exciting avenue of study into AAA pathogenesis especially since these peptides have been associated with a range of cardiovascular diseases such as heart failure, coronary heart disease (CHD) and acute myocardial infarction, although it is currently unclear whether these peptides exert pathogenic or protective effects.^{5,35,119} Furthermore, increasing

circulating concentrations of UCN1 and UCN2 have been reported in patients with heart failure.^{35,378} These increases in UCN1 and 2 have been suggested by the authors to be a beneficial effect in the disease.^{35,378} However, the relationship between the UCNs and AAA is yet to be clearly elucidated.

In this Chapter, the following hypotheses are tested:

1. The UCNs and their receptors are differentially expressed between AAA body and AAA neck in human AAA biopsies taken during aneurysm repair.
2. UCN2 is upregulated in the plasma of patients with human AAA.

Specifically, the aims of this Chapter include:

1. Determining the aortic concentration of the UCN1-3 in human AAA biopsies;
2. Elucidating the aortic distribution of the UCNs receptors (CRFR1 and 2) within human AAA tissue;
3. Determining the protein expression, and cell types expressing UCN2 in human AAA tissue;
4. Determining whether UCN2 is secreted by human AAA explant tissues in culture;
5. Investigating the relationship between plasma concentration of UCN2 and the presence of human AAA;
6. Investigating the potential of UCN2 as a blood borne marker for human AAA.

4.2 MATERIALS AND METHODS

4.2.1 Study design and patients

Initially one main screening study to determine if the UCNs and their receptors are differentially expressed in human AAA was performed. UCN1, UCN2, UCN3, CRFR1, CRFR1, and CRFR2 gene expression were compared in paired AAA body and AAA neck biopsies (n=6) by quantitative real-time polymerase chain reaction (qPCR, study 1). Body biopsies were obtained from the visually most dilated part of the AAA while neck biopsies were obtained from the macroscopically non-dilated proximal neck of the AAA just below the site the aorta was clamped (Chapter 2, Figure 2.1). In study 2, the expression of UCN2 in matched AAA body and AAA neck biopsies (n=4) by immunohistochemistry (IHC) was assessed. In study 3, the release of UCN2 from biopsies of AAA body (n=10), AAA neck (n=5), AAA thrombus (n=11), and femoral atheroma (n=6) was compared by enzyme-linked immunosorbent assay (ELISA). In study 4, patients selected based on matching for age and gender on a 1:1 basis were assessed for the association between the circulating levels of UCN2 and AAA presence (n=134). In Chapter 5, the functional significance of UCN2 in AAA was assessed by determining its effect on human aortic smooth muscle cells (HASMC) *in vitro*. Patients referred to the vascular surgery clinics at The Townsville Hospital and The Royal Brisbane and Women's Hospital, Queensland, Australia for investigation of peripheral artery disease (PAD) or AAA were recruited. The inclusion and exclusion criteria for patients' recruitment are described in Chapter 2, section 2.3.1. The method of diagnosing AAA and PAD and the criteria for risk factors has previously been published.^{342,343}

4.2.2 Aortic and atheroma samples:

Sample details and collection methodology are described in full in Chapter 2, section 2.3.3. Briefly, paired AAA body and AAA neck biopsies (n=6) were collected from 6 patients, placed in cold sterile RNAlater[®] solution (Ambion, Australia) and stored at -80 °C for the gene expression studies. Paired AAA body and AAA neck samples (n=4) were stored at -80 °C for later immunohistochemistry. AAA body (n=10), AAA neck (n=5), and femoral atheroma (n=6) biopsies were collected, rinsed in PBS and used in explant culture studies.

4.2.3 Expression of the UCNs and CRFR1/ 2 genes in human AAA biopsies

Total RNA was extracted from paired AAA body and neck biopsies (n=6) using RNeasy[®] Mini Kit (Qiagen, Australia) according to the manufacturer's instructions (Chapter 2, section 2.3.4). Patients' risk factors are detailed in Table 4.2. SYBR Green PCR primers were designed for CRFR1, CRFR2, UCN1 and UCN2, whilst UCN3 and GAPDH primers were commercially sourced as previously described (Chapter 2, section 2.3.5). Quantitative real-time PCR (qPCR) was performed for UCN1, UCN2, UCN3, CRFR1, and CRFR2 genes as outlined in Chapter 2, section 2.3.5.

4.2.4 Immunohistochemical staining for UCN2, lymphocytes and neutrophils

Risk factors of the patients analysed are detailed in Table 4.1. Briefly, 6 µm thick sections from AAA biopsies embedded in OCT[™] (ProScitech, Townsville, Australia) were immunostained with the following antibodies; primary antibodies include, goat anti-human UCN2 IgG (c-16, sc-54449, Santa Cruz), monoclonal mouse anti-human neutrophil elastase IgG (M0752, Dako), or monoclonal mouse anti-human CD3 (M7254, Dako). Secondary

antibodies include, biotinylated anti-goat IgG (BA-5000, Vector), or biotinylated anti-mouse IgG (BA-9200, Vector). Isotype control antibodies include Goat IgG (I-5000, Vector) and mouse IgG (I-2000, Vector). The chromogenic diaminobenzidine (DAB) substrate (ImmPACT, SK-4105, Vector) was used to perform IHC as previously described (Chapter 2, section 2.3.6 to 2.3.7). Following a series of rinses in phosphate buffer saline (PBS), slides were counterstained, rinsed, dehydrated, cleared, mounted and visualised on a Nikon Eclipse 50i microscope. T-lymphocytes and neutrophils were appraised as they have been previously suggested to be a possible source of endogenous UCN2.^{33,379}

Table 4. 1 Risk factors of the patients providing AAA biopsies used to assess expression of UCNs and their receptors in qPCR and immunohistochemistry

Characteristics	Paired AAA body and neck biopsies in gene expression study	Paired AAA body, thrombus, and neck biopsies in protein expression study
*Number	6	4
Aortic diameter (mm)	58 (47 - 68)	58 (47 – 68)
Male	4 (67 %)	3 (75%)
Age (years)	70 (64 - 77)	76 (64 – 77)
Hypertension	4 (67 %)	3 (75 %)
Diabetes mellitus	2 (33 %)	1 (25 %)
Ever smoker	4 (67 %)	4 (100 %)
CHD	4 (67 %)	2 (50 %)
Aspirin prescription	5 (83 %)	2 (50 %)
Statin prescription	5 (83 %)	2 (50 %)
ACE inhibitor prescription	3 (50 %)	1 (25 %)
ARB prescription	1 (17 %)	0
Beta blockers	1 (17 %)	3 (75 %)

Nominal variables are presented as numbers (%). Continuous variables are presented as median (inter-quartile range). AAA = abdominal aortic aneurysm; ACE = Angiotensin converting enzyme; ARB = Angiotensin receptor blocker; CHD = Coronary heart disease; * only four paired AAA body, neck and thrombus sample were available.

4.2.5 UCN2 secretion from AAA biopsies

As previously described (Chapter 2, section 2.4.1), AAA body (n=10), AAA neck (n=5), AAA thrombus (n=11), and femoral atheroma (n=6) biopsies were collected for the explant

culture studies. Three patients provided paired AAA body, AAA neck, and AAA thrombus biopsies, 7 patients provided paired AAA body and AAA thrombus biopsies, 4 patients provided paired AAA body and AAA neck biopsies, 1 patient provided paired AAA thrombus and AAA neck biopsies, and 4 patients provided AAA thrombus biopsies only (n=14 patients for AAA explants tissues in total). This variation in samples is due to the fact not all AAA are associated with the thrombus. In some cases, due to the limited amount of biopsies collected, the AAA neck samples were exhausted in other studies. Biopsies collected as described were dissected into ~ 5 mm³ pieces and cultured in 2 mL of media in 12 well plates (Nunc, Australia) at 37°C in a humidified 5% CO₂ atmosphere. Conditioned media were collected at 24 h and centrifuged at 30,000 g for 30 minutes at 4 °C to remove debris. Wet weight of biopsies were taken after culture and recorded for use in later analysis. Supernatants were stored at -80°C for later analysis. Patient characteristics are presented in Table 4.2.

Table 4. 2 Risk factors of the patients providing aortic and femoral atheroma biopsies used to assess expression of UCN2 in explant cultures

Characteristics	Paired AAA body, thrombus, and neck biopsies in protein expression study	*Explant Studies			
		AAA body biopsies	AAA neck biopsies	AAA thrombus biopsies	Femoral atheroma biopsies
Number	4	10	5	11	6
Aortic diameter (mm)	58 (47 – 68)	69 (51 - 80)	51 (34 – 53)	73 (51 - 88)	35 (24 - 37) [‡]
Male	3 (75 %)	8 (80 %)	3 (60 %)	10 (91 %)	5 (83 %)
Age (years)	76 (64 – 77)	68 (65 - 75)	73 (66 – 81)	69 (68 – 77)	65 (54 -77)
Hypertension	3 (75 %)	6 (60 %)	3 (60 %)	8 (73 %)	6 (100 %)
Diabetes mellitus	1 (25 %)	1 (10 %)	1 (20 %)	0	1 (17 %)
Ever smoker	4 (100 %)	10 (100 %)	5 (100 %)	9 (82 %)	4 (67 %)
CHD	2 (50 %)	1 (10 %)	1 (20 %)	5 (46 %)	5 (83 %)
Aspirin prescription	2 (50 %)	4 (40 %)	3 (60 %)	6 (55 %)	4 (67 %)
Statin prescription	2 (50 %)	4 (40 %)	3 (60 %)	8 (73 %)	5 (83 %)
ACE inhibitor prescription	1 (25 %)	3 (30 %)	2 (40 %)	3 (27 %)	3 (50 %)
ARB prescription	0	1 (10 %)	0	2 (18 %)	0
Beta blockers	3 (75 %)	2 (20 %)	2 (40 %)	5 (46 %)	3 (50 %)

Nominal variables are presented as numbers (%). Continuous variables are presented as median (inter-quartile range). AAA = abdominal aortic aneurysm; ACE = Angiotensin converting enzyme; ARB = Angiotensin receptor blocker; CHD = Coronary heart disease; * 3 patients provided paired AAA body, AAA neck, and AAA thrombus, 7 patients provided paired AAA body and AAA thrombus, 4 patients provided paired AAA body and AAA neck, 1 patient provided paired AAA thrombus and AAA neck, and 4 patients provided AAA thrombus only (n = 14 patients for AAA explants tissues in total). [‡] No aortic diameter data available for one patient.

4.2.6 Circulating concentration of UCN2 in AAA

In brief, plasma was obtained from blood samples collected in EDTA vacutainers (BD Bioscience, Australia, n=134) by centrifugation within 2h of collection and stored at -80 °C prior to analysis as previously described (Study 2, Chapter 2, sections 2.3.2 and 2.3.3). All recruited subjects underwent CTA. Sixty seven (67) patients had AAAs (aortic diameter \geq 30 mm) and 67 patients had PAD but no AAA (aortic diameter $<$ 30 mm). The number of patients recruited into the study was much higher than numbers employed by similar studies, for example Davis *et al.* administered UCN2 into 8 subjects to investigate its effect on cardiovascular function,³²⁹ Smani and colleagues investigated the effect of UCN2 on human coronary arteries vasodilatation in 17 patients with heart failure,³³¹ and Gruson *et al.* investigated plasma UCN1 concentrations from 42 subjects with heart failure and 20 healthy sex and gender matched controls.³²⁴

4.2.7 Determination of plasma UCN2 by ELISA

UCN2 levels in conditioned media and plasma were measured by ELISA (USCN life Sciences, China) as per manufacturer's instructions. UCN2 concentration in conditioned medium was expressed as nanogram per gram of tissue per 24 h (ng/g/24h), and in plasma as nanogram per millilitre (ng/mL). Mean inter-assay coefficient of variation was 7.46%.

4.2.7 Statistics

UCN1, UCN2, UCN3, CRFR1, and CRFR2 gene expression was compared in paired biopsies from AAA body and neck using Wilcoxon signed ranks test and presented as arbitrary units (arb. unit). Risk nominal data from the patient characteristics were presented as number and percentages and compared by Chi-square test. The conditioned media concentrations and plasma concentrations of UCN2 were not normally distributed and thus compared using box plots and the Mann Whitney U test. UCN2 levels were compared between AAA body and neck, AAA body and femoral atheroma, AAA neck and femoral atheroma samples. UCN2 levels were also compared between AAA thrombus and AAA neck, and between AAA thrombus and femoral atheroma. Conditioned media from paired AAA thrombus and AAA body biopsies were compared by Wilcoxon signed ranks test. Plasma UCN2 from Study 2 were compared between 67 age and gender matched patients with AAA and 67 patients with non-aneurysmal PAD. Nominal data were presented as number and percentages and compared by Chi-square test. The association of UCN2 with AAA presence was assessed using multiple logistic regression adjusting for CHD, smoking, hypertension, dyslipidaemia, and diabetes. Receiver operated characteristic (ROC) curve were generated to assess the ability of UCN2 to predict AAA presence. The relationship between UCN2 and increasing aortic diameter was assessed by Spearman rho correlation. Statistical significance was taken as $P \leq 0.05$.

4.3 RESULTS

4.3.1 Primer design and real time PCR optimisation

Quantitative real time PCR (qPCR) requires the use of primers that are specific for the gene of interest and free of internal secondary elements. In general, primers are between 18 – 24 nucleotides in length and should avoid stretches of polybase sequences e.g repeating motifs that can hybridise to the template. PCR primers were designed using Biosoft AlleleID[®] software (PREMIER Biosoft, Palo Alto, USA). Quantitative real-time PCR (qPCR) was performed to test primers for UCN1, UCN2, UCN3, CRFR1, and CRFR2 genes using 100 ng of total RNA by the SYBR Green assay (Chapter 2, section 2.3.5). A two-step thermal cycling program was used initially to test all 5 primers. CRFR1 and UCN1 primers had distinct products with no primer-dimers formed (Figures 4.1a and b). UCN2, UCN3 and CRFR2 did not form any products (Figures 4.1c – e). The thermal cycling was modified into a three- step run and UCN2, UCN3 and CRFR2 primers assessed. Self-designed UCN2 and CRFR2 primers formed products with no primer-dimers seen (Figures 4.2a and b). Self-designed UCN3 primers did not form any product, and commercially sourced UCN3 primers (Qiagen, Australia) were employed for this study. Details of the thermal cycling program used are detailed in table 4.3 below:

Table 4. 3 Details of the thermal cycling steps used in the gene expression studies

Two-Step qPCR	Three-step qPCR
I. 50 °C for 30 min for cDNA synthesis	50 °C for 30 min for cDNA synthesis
II. 95 °C for 10 min (enzyme activation)	95 °C for 10 min (enzyme activation)
III. 40 cycles of 95 °C, 15 s (denaturation)	40 cycles of 94 °C, 15 s (denaturation)
IV. 60 °C for 30 s (annealing and extension)	55 °C for 30 s (annealing)
V.	72 °C for 30 s (extension)

In summary, two-step thermal cycling program was used for UCN1 and CRFR1, whilst a three-step program was used for UCN2, UCN3 and CRFR2.

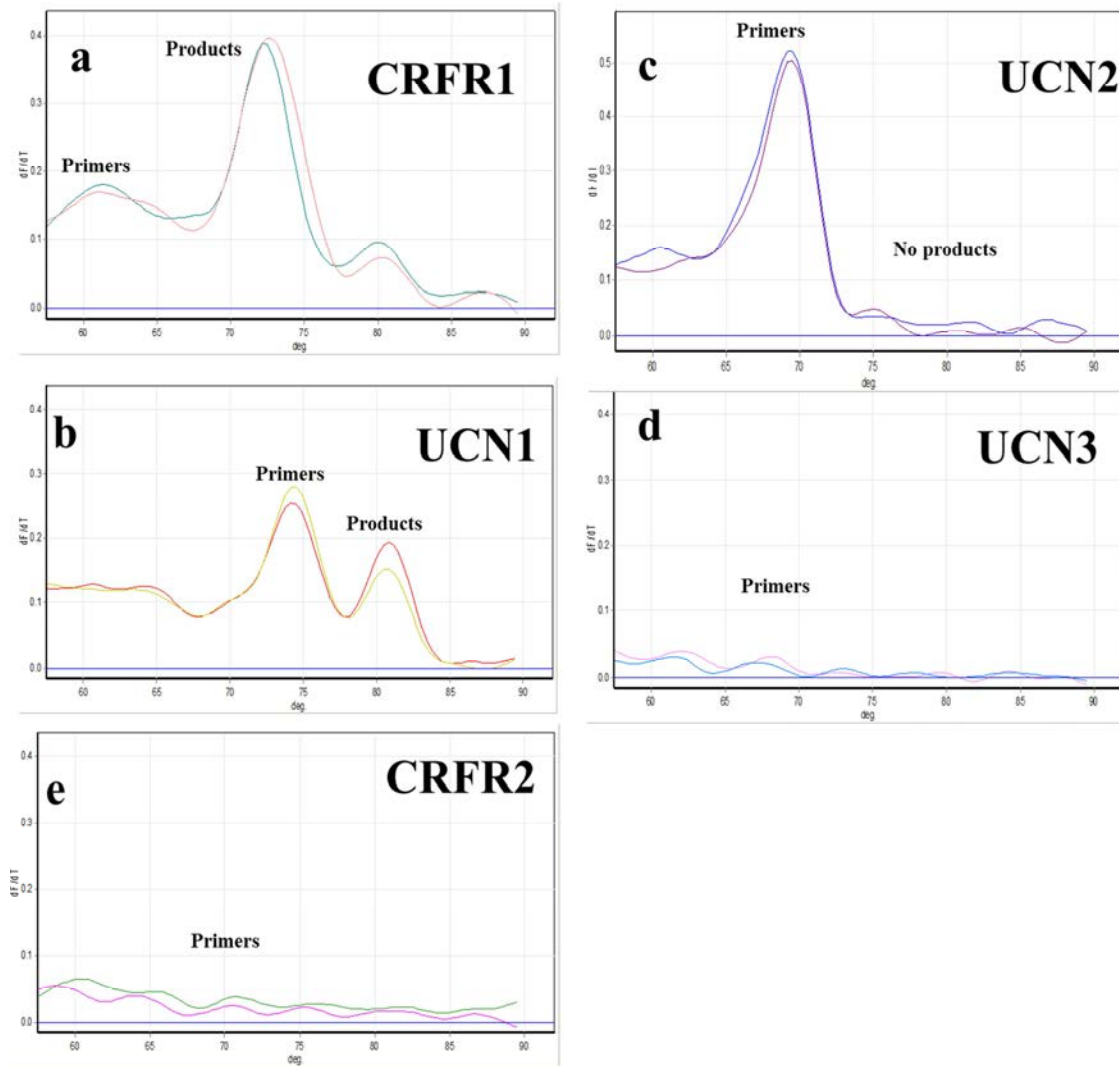


Figure 4. 1 Melt curves showing primer optimisation for the UCNs and their receptors on a two-step qPCR thermal cycling program

CRFR1 and UCN1 primers had distinct products (4.1a and b). UCN2, UCN3 and CRFR2 did not (4.1c –e)

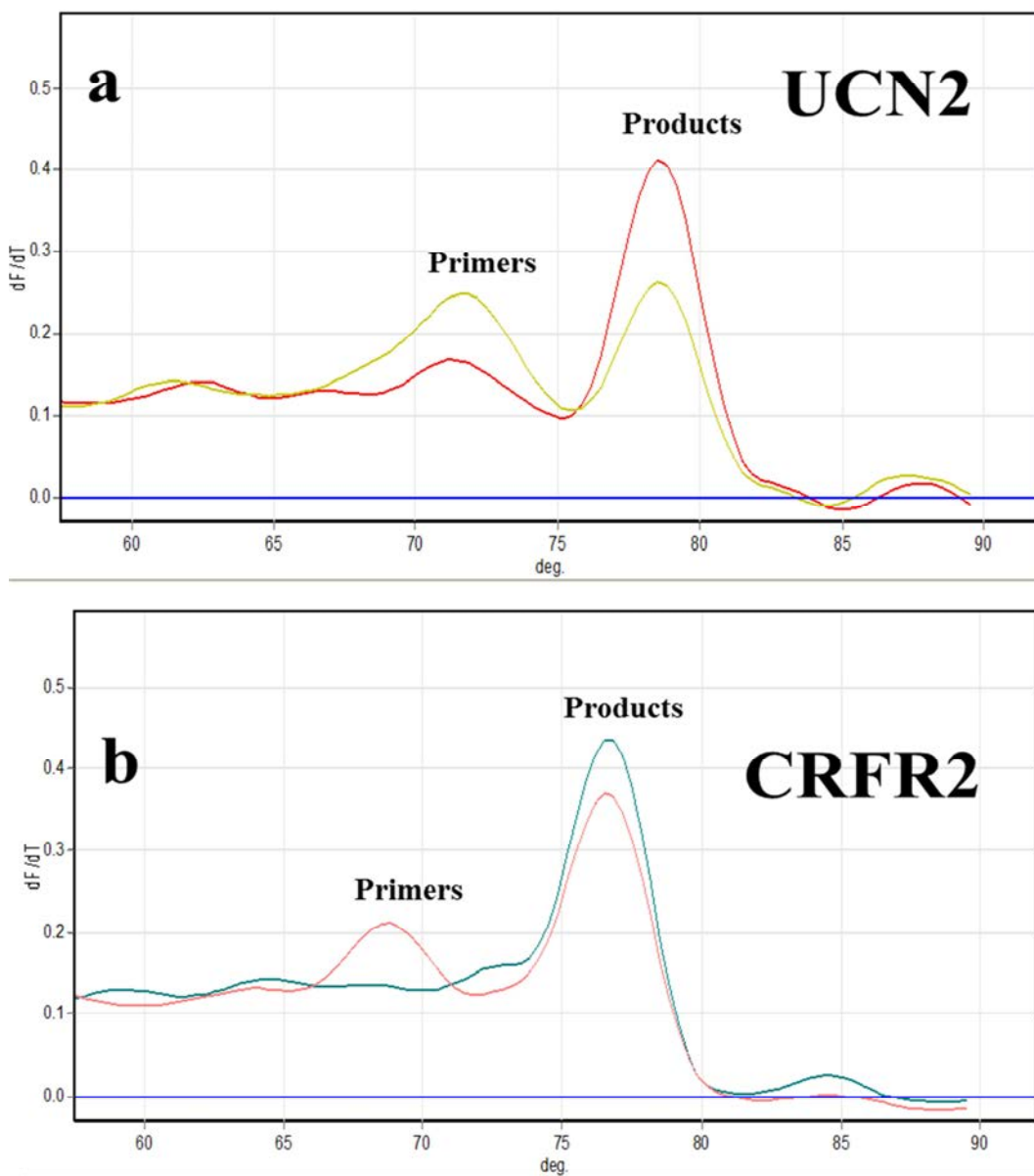


Figure 4. 2 Melt curves showing primer optimisation for the UCN2 and CRFR2 on a three-step qPCR thermal cycling program

UCN2 and CRFR2 primers had distinct products (4.2a and b).

4.3.2 UCN2 and its receptor, CRFR2 expression are upregulated in human AAA

Relative gene expression levels of the UCNs and their receptors were compared between AAA body and neck samples using qPCR. Risk factors of patients from which AAA biopsies for gene expression studies were obtained are shown in Table 4.1. After normalising to GAPDH, the relative expression of both UCN2 and CRFR2 were significantly greater in AAA body biopsies compared to paired AAA neck (representative fluorescence vs cycle curve for UCN2 shown in Figure 4.3a). Median UCN2 expression was 5.31 arb unit in human AAA body (inter-quartile range 1.28 – 8.96) and 0.38 arb unit in paired AAA neck controls (inter-quartile range 0.04 – 0.66, $P=0.028$, Figure 4.3b, Table 4.4). Median CRFR2 expression was 0.49 arb unit in human AAA body (inter-quartile range 0.18 – 3.80) and 0.04 arb unit in relatively healthy human AAA neck controls (inter-quartile range 0.00 – 0.12, $P=0.046$, Figure 4.3b, Table 4.4). The relative expression of UCN1, UCN3 and CRFR1 in AAA body and AAA neck biopsies did not differ significantly (Table 4.4).

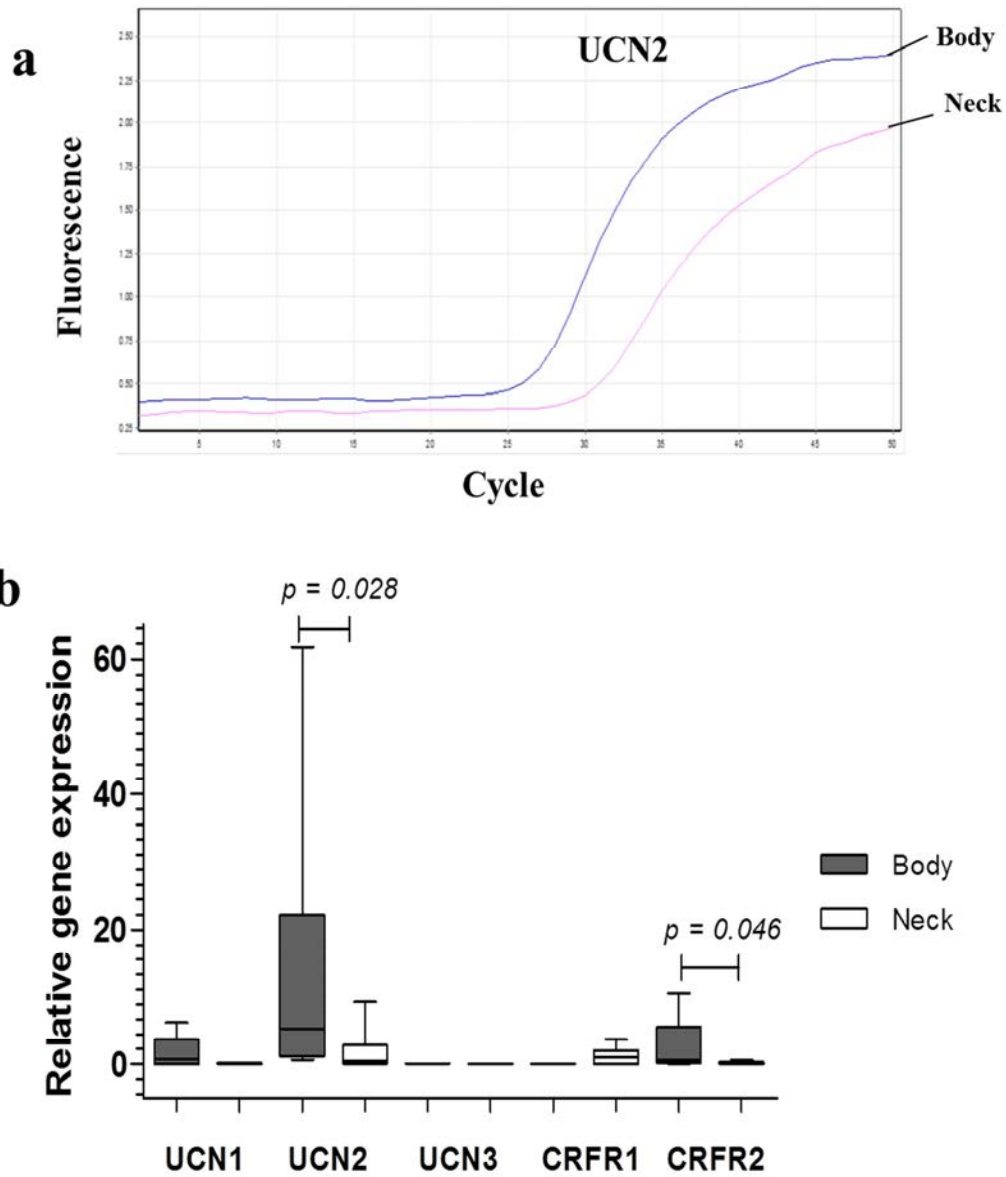


Figure 4. 3 Representative fluorescence versus cycle number showing amplified peaks for UCN2 in AAA neck and AAA body on a three-step qPCR thermal cycling program

(a) Box plot analysis of the UCNs and their receptors in human AAA tissue biopsies;

(b) Relative gene expression calculated by using the concentration-Ct-standard curve method and normalised by the average expression of GAPDH.

Table 4. 4 Relative expressions of the urocortins and their receptors in six matched AAA body and neck control biopsies

Gene	Sample	N	Median (arb unit)	IQR	P value
UCN1	AAA body	6	0.664	0.000-2.706	0.052
	AAA neck	6	0.000	0.000-0.084	
UCN2	AAA body	6	5.312	1.283-8.955	0.028
	AAA neck	6	0.377	0.039-0.662	
UCN3	AAA body	6	0.029	0.023-0.045	0.345
	AAA neck	6	0.000	0.000-0.036	
CRFR1	AAA body	6	0.032	0.011-0.050	0.116
	AAA neck	6	0.940	0.012-1.471	
CRFR2	AAA body	6	0.488	0.180-3.814	0.046
	AAA neck	6	0.044	0.000-0.118	

Results were compared with the Wilcoxon signed ranks test. AAA = abdominal aortic aneurysm; arb unit = arbitrary units; CRFR = corticotrophin releasing factor receptor; N = number; UCN = Urocortin; Results are expressed as relative expression of gene compared to GAPDH; IQR = inter-quartile range

4.3.3 Assessment of UCN2 protein expression in AAA biopsies

Patients and controls were well matched for age, gender, risk factors, and medication history, as described in (Chapter 2, section 2.3.2) and by virtue of using control biopsies from the AAA neck of the same patient (internal control). Risk factors for these patients are shown in Table 4.2 above. Examples of the histological characteristics of the AAA biopsies are shown in Figure 4.4. The distribution of UCN2 in AAA affected tissues was assessed via immunohistochemical analyses on matched AAA body, neck and thrombus biopsies obtained from patients undergoing aneurysm repair (n=4). UCN2 protein expression was relatively abundant in AAA body (Figures 4.5b and c), moderate in AAA neck (Figures 4.5d and e) and

sparse in AAA thrombus (Figures 4.5f and g). Expression of UCN2 within the aortic wall was concentrated around inflamed regions and co-located with infiltrating T-lymphocytes (Figures 4.6b and c) and neutrophils (Figures 4.6d and e).

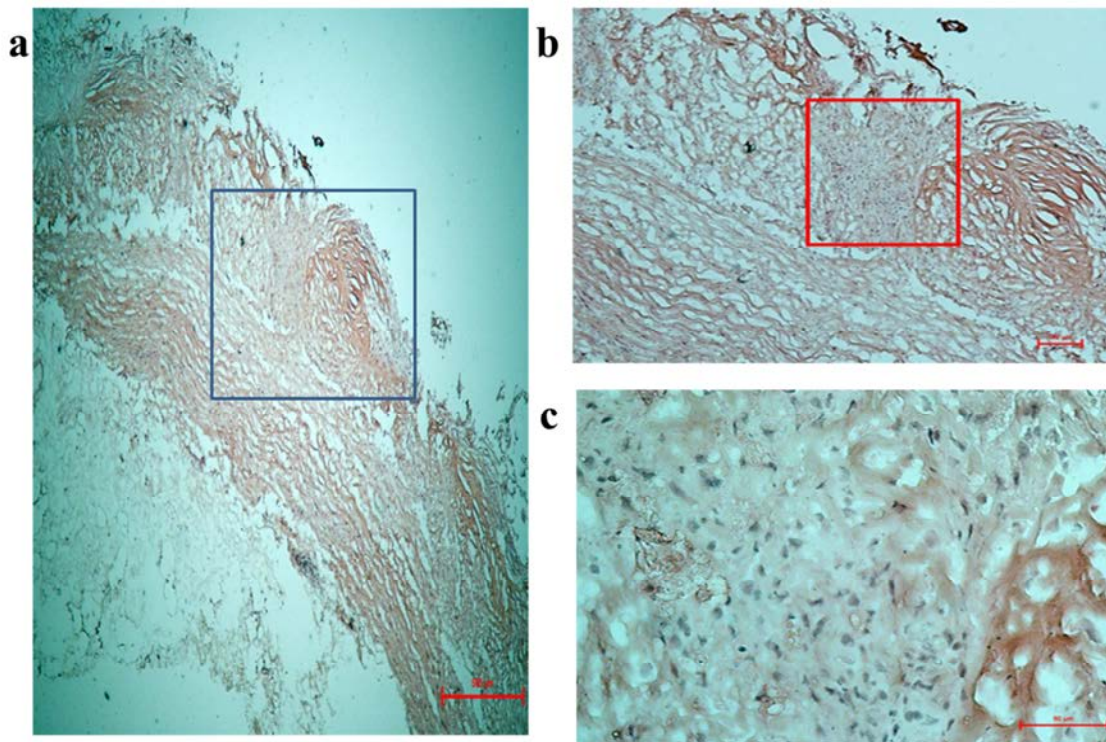


Figure 4. 4 Examples of histological findings in human AAAs.

AAA body sections were stained with haematoxylin and eosin (H&E)
Haematoxylin stain nuclei blue and eosin stains other structures a shade of pink, red or orange.
Photomicrographs are at x4 (**a**), x10 (inset of media highlighted with blue square showing infiltrating cells from the adventitia, **b**) and (**c**) at x40 (inset of region highlighted with red rectangle in **b**). AAA biopsy exhibits marked medial elastin degradation (**a**) infiltration of inflammatory cells.

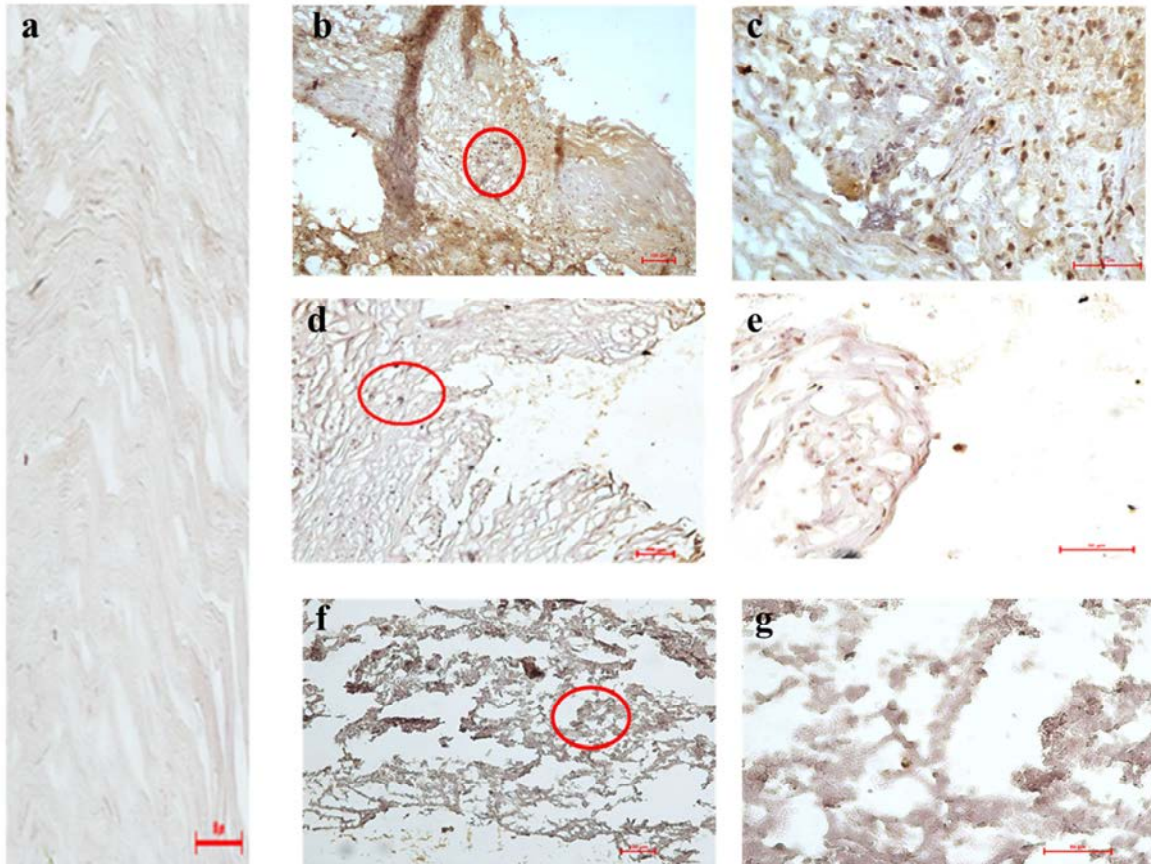


Figure 4. 5 Examples of UCN2 expression in human AAA

Cryostat sections of paired AAA body, AAA neck and AAA thrombus (n=4) were incubated with goat anti-human UCN2. Sections were incubated with rabbit biotinylated anti-goat IgG. Colour was developed with DAB. Nuclei were counterstained with haematoxylin. AAA biopsy incubated with goat IgG Isotype control (a) at higher magnification x40. AAA body stained for UCN2: (b) low magnification x10 and (c) higher magnification x40 of region indicated in b. AAA neck stained for UCN2: (d) low magnification x10 and (e) higher power magnification of region indicated in d. AAA thrombus stained for UCN2: low magnification x10 (f) and higher magnification x 40 (g) Staining for UCN2 was marked in the adventitia and media in AAA body, sparse in the AAA neck and negligible in AAA thrombus.

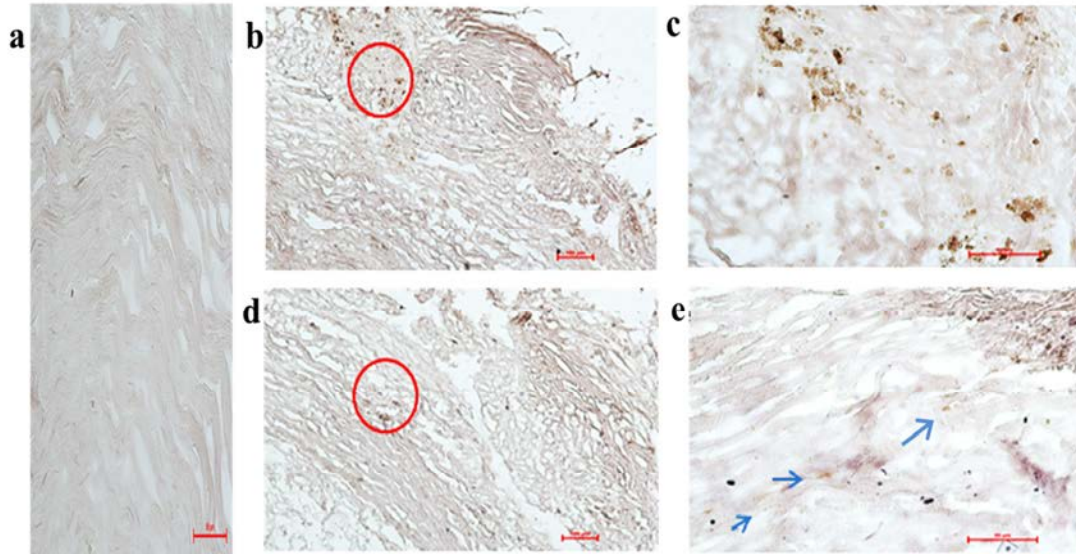


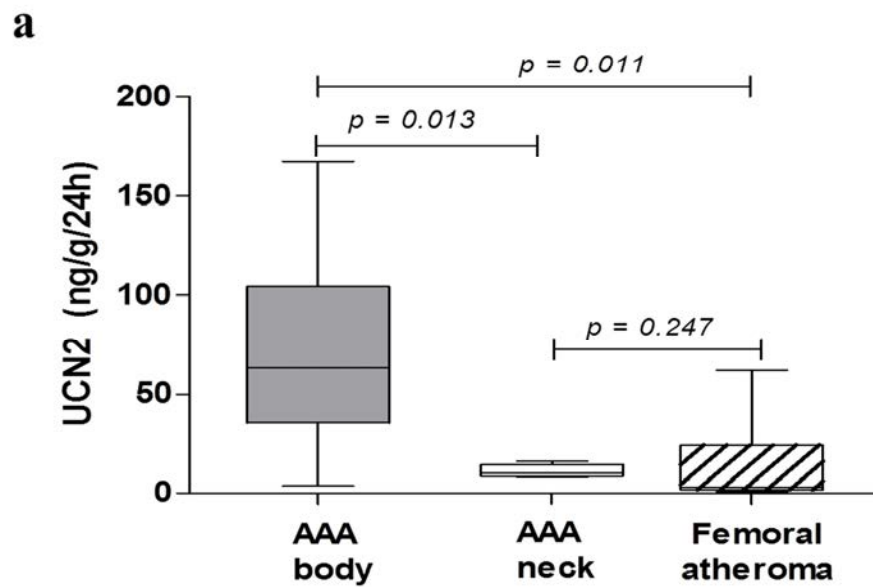
Figure 4. 6 T lymphocyte and neutrophil populations in inflammatory infiltrates of human AAA body biopsies

Cryostat sections of human AAA body biopsies (n = 4) were incubated with control murine IgG: **a**, monoclonal mouse anti-human CD3: **b** and **c**, and neutrophil elastase: **d** and **e**. Blue arrow show neutrophil stains. After washing in PBS, sections were incubated with rabbit biotinylated anti-mouse IgG. Colour was developed with DAB. Nuclei were counterstained with haematoxylin. Magnification for a, b and d = x10 objective, for c and e = x40 objective.

4.3.4 UCN2 is secreted by AAA explant biopsies

To determine whether UCN2 was secreted from AAAs, conditioned media from AAA body (n=10), AAA neck (n=5), AAA thrombus (n=11) and femoral atheroma tissue explants (n=6) were analysed by ELISA. Risk factors of the patients from which AAA and femoral atheroma biopsies were obtained are shown in Table 4.2. Significantly higher concentrations of UCN2 were secreted from AAA body compared to AAA neck or femoral atheroma (representative of PAD) controls ($P=0.013$ and $P=0.011$ respectively, Figure 4.7a). UCN2 secretion was not significantly different between AAA neck and femoral atheroma controls ($P=0.247$, Figure 4.7a). Interestingly, UCN2 secretion from AAA thrombus explants was significantly higher than AAA neck or femoral atheroma samples ($P=0.013$ and $P=0.004$ respectively, Figure

4.7b). A comparison of matched AAA thrombus and AAA body samples (n=7) reveal a significantly higher secretion of UCN2 from the thrombus ($P=0.016$, Figure 4.7c). The median level of UCN2 released from AAA thrombus and AAA body were 315 ng/g/24h and 63.08 ng/g/24h (67.3 nM/24h and 13.4 nM/24h equivalent concentrations) respectively. The amount of UCN2 released in culture was corrected for wet weight of tissue, and data expressed as ng/g/24h. This value was converted to nM/24 h in order to calculate the concentration of UCN2 used in the *in vitro* studies.



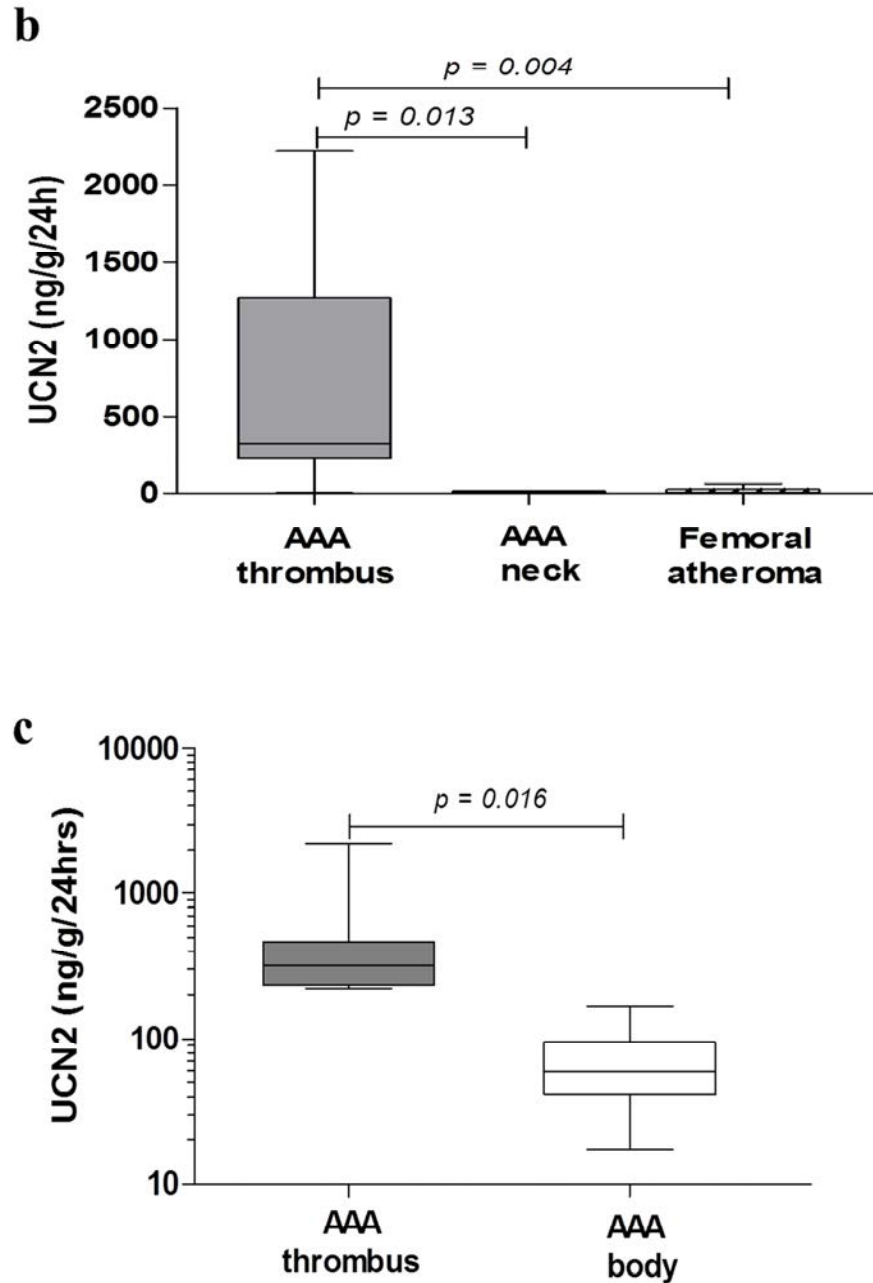


Figure 4. 7 Comparison of UCN2 secretion from aortic aneurysm and femoral atheroma explants over 24 hours

(a) AAA body explants secreted higher amounts of UCN2 compared to AAA neck or femoral atheroma control biopsies ($p=0.013$ and $p=0.011$ respectively, Mann Whitney U test). UCN2 secretion was not significantly different between AAA neck and femoral atheroma controls ($p \geq 0.05$). Median AAA body UCN2 = 63.08 ng/g/24h (b) AAA thrombus secreted greater amounts of UCN2 compared to AAA neck or femoral atheroma controls ($p = 0.013$ and $p = 0.004$ respectively, Mann Whitney U test) Median AAA thrombus UCN2 = 315 ng/g/24h; g = gram; h = hour; ng = nanogram. (c) AAA thrombus explants secreted higher amounts of UCN2 compared to matched AAA body biopsies ($p = 0.016$, Wilcoxon matched paired test. Note y- axis is Log10).

4.3.5 Circulating UCN2 is significantly elevated in AAA and correlates with aortic diameter

UCN2 concentration was analysed in plasma samples from 134 patients in relation to AAA presence. Plasma samples collected from 67 patients with AAA (infrarenal aortic diameter \geq 30 mm) and 67 patients with non-aneurysmal PAD (infrarenal aortic diameter $<$ 30 mm) were included in this analysis. Traditional cardiovascular risk factors (Table 4.5) were similar in both patient groups, although the incidence of diabetes was higher in patients with non-aneurysmal PAD than those with AAA. Median plasma UCN2 was 2.20 ng/mL in AAA patients (inter-quartile range 1.14-4.55) and 1.11 ng/mL in non-aneurysmal PAD patients (inter-quartile range 0.76-2.25), $P=0.001$ (Table 4.5).

Table 4. 5 Comparison of subjects with and without AAA used for plasma UCN2 measurement

Characteristic	AAA	PAD but no AAA	P value
Number	67	67	
Aortic diameter (mm)	55 (52 - 65)	22 (19 - 25)	<0.001
Male	58 (87 %)	58 (87 %)	1.000
Age (years)	72 (68-78)	72 (68-78)	1.000
Hypertension	50 (75 %)	52 (78 %)	0.685
Diabetes mellitus	9 (13 %)	19 (28 %)	0.034
Ever smoker	58 (87 %)	57 (85 %)	0.804
CHD	34 (51 %)	29 (43 %)	0.387
Aspirin prescription	38 (57 %)	40 (60 %)	0.726
Statin prescription	40 (60 %)	40 (60 %)	0.915
ACE inhibitor prescription	25 (37 %)	29 (43 %)	0.481
ARB prescription	12 (18 %)	15 (22 %)	0.518
Beta blockers	28 (42 %)	18 (27 %)	0.069
Plasma UCN2 (ng/mL)	2.20 (1.14 - 4.55)	1.11 (0.76 - 2.25)	0.001

Nominal variables are presented as numbers (%) and compared by Chi-square test. Continuous variables are presented as median (inter-quartile range) and compared by Mann Whitney U test. AAA = Abdominal aortic aneurysm; PAD = Peripheral artery disease; CHD = Coronary heart disease; UCN2 = Urocortin 2; ACE = Angiotensin converting enzyme; ARB = Angiotensin receptor blocker; mm = Millimeter; ng/mL = Nanogram/milliliter

The area under the ROC curve for UCN2 concentration in predicting AAA presence was 0.661 (95 % CI 0.57 – 0.75, $P=0.001$, Figure 4.8a). UCN2 was positively associated with AAA presence after adjusting for other risk factors (see Table 4.6). Patients with UCN2 in the highest quartile had a 4.12-fold greater prevalence of AAA than those with UCN2 in the lowest quartile (95 % CI 1.37 -12.40, $P=0.012$). Plasma UCN2 was very weakly correlated with aortic diameter (n=134, $r=0.280$, $P=0.001$; Figure 4.8b).

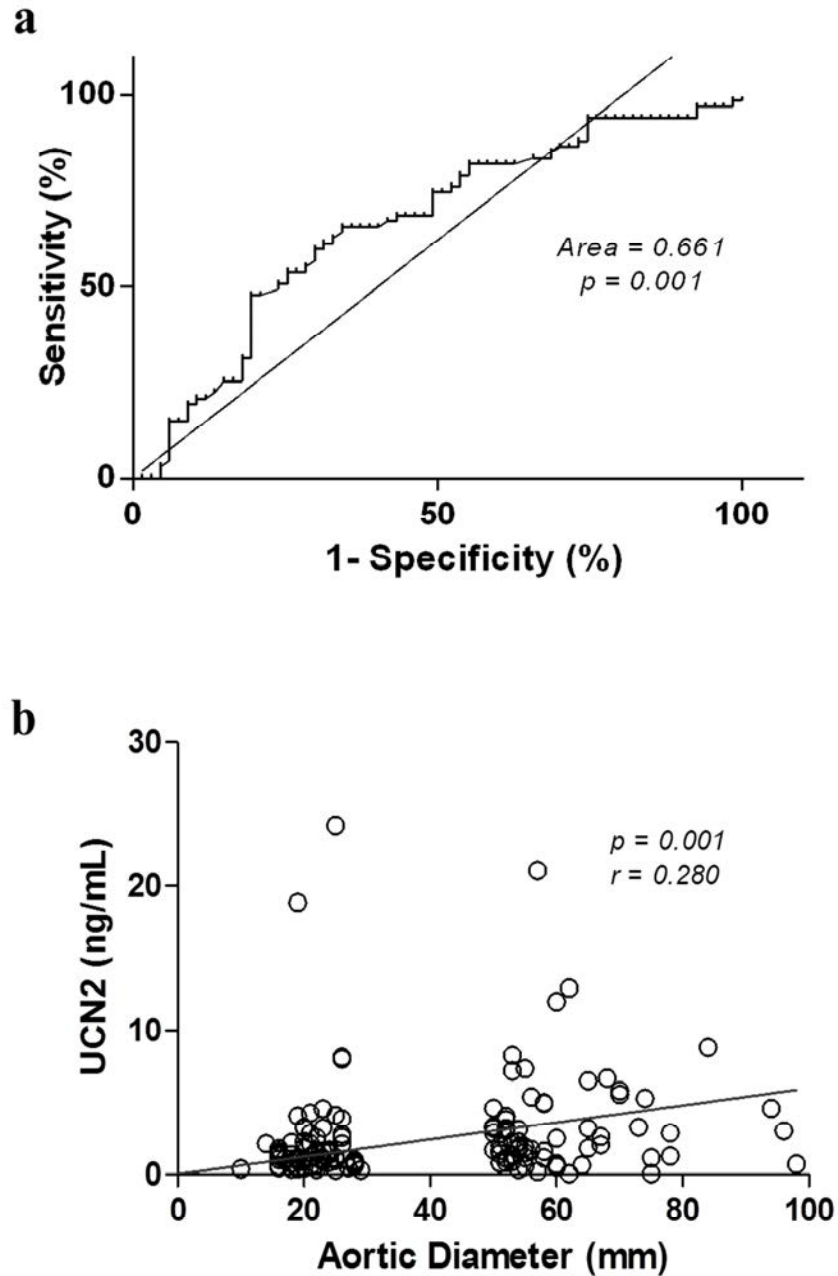


Figure 4. 8 Correlation between plasma UCN2 concentration and aortic diameter and ROC curve for UCN2 in predicting AAA presence

- (a) There was a weak positive correlation between plasma UCN2 and aortic diameter in 134 patients ($r=0.280$, $p=0.001$);
- (b) ROC curve showing the sensitivity and specificity of UCN2 in predicting AAA presence.

Table 4. 6 Independent association of plasma UCN2 with AAA in 134 patients

Characteristic	Odds ratio	95% CI	P value
Plasma UCN2 in ng/mL*			
< 0.89	1.00	Reference	
0.89 - 1.64	0.85	0.28 - 2.60	0.779
1.64 - 3.17	3.66	1.19 - 11.22	0.023
>3.17	4.12	1.37 - 12.40	0.012
Beta blockers	1.65	0.70 - 3.89	0.256
CHD	1.43	0.63 - 3.25	0.388
Hypertension	1.24	0.38 - 4.03	0.719
Statin	0.97	0.43 - 2.16	0.931
ARB	0.82	0.26 - 2.60	0.731
ACE inhibitor	0.67	0.24 - 1.87	0.448
Ever smoker	0.66	0.20 - 2.17	0.493
Aspirin	0.65	0.29 - 1.44	0.288
Diabetes mellitus	0.32	0.11 - 0.94	0.039

AAA = Abdominal aortic aneurysm. CHD = Coronary heart disease; UCN2 = Urocortin-2; ng/mL = nanogram/millilitre. For nominal variables the comparisons are to subjects without the risk factor. * Subjects with plasma UCN2 concentrations in the top, third and second quartiles were compared with subjects who had plasma UCN2 in the lowest quartile.

4.4 DISCUSSION

The aim of this chapter was to investigate the association of the UCNs and their receptors with human AAA. The expression of the UCN1, 2, 3 and CRFR1/ 2 distribution in AAA biopsies was examined by qPCR. UCN2 protein expression was also localised in AAA biopsies by immunohistochemistry. Furthermore, AAA biopsies were analysed *in vitro* for UCN2 release, and the circulating concentration of UCN2 in AAA patients determined by ELISA. The data presented here to my knowledge is the first to directly appraise the association of UCNs with human AAA. The UCNs are known to be expressed in smooth muscle cells, endothelial cells and immune cells which are all important in AAA pathogenesis.²⁹⁸ UCN2 was reported to modulate cell survival, apoptosis, and inflammation; mechanisms implicated in AAA pathogenesis.^{40,243,254}

The main finding of this Chapter is the novel association of UCN2 with AAA. Expression of UCN2 was significantly increased at the main site of AAA dilatation both at the gene and protein levels. The upregulation of UCN2, as demonstrated here may be particularly relevant to AAA pathogenesis. Given the dearth of endothelial and smooth muscle cells within the aortic wall, and the dense inflammatory infiltrate in end stage human AAA, it was hypothesised that T-lymphocytes and neutrophils were the primary source of UCN2. Immunohistochemistry studies suggested that T-lymphocytes and neutrophils may be co-locating with UCN2 within the fragmented arterial media in human AAA biopsies. The UCNs are suggested to be upregulated by immune cells,³³ and have been demonstrated to promote the secretion of proinflammatory cytokines including IL-1 β .²⁴⁵ In addition, IL-1 β has been reported to enhance the secretion of the potent proinflammatory cytokine IL-32,³⁸⁰

which has been shown to elicit the stimulation of an array of proinflammatory cytokines including IL-1 α , IL-1 β , IL-6 and TNF α through the activation of NF- κ B.³⁸⁰

The main receptor for UCN2, CRFR2 was found to be significantly upregulated in the AAA body compared to the relatively healthy paired AAA neck. UCN2 is known to selectively bind to CRFR2 and exerts its physiological effect through this receptor.^{5,256} Hence, the significant upregulation of UCN2 and its receptor in AAA body suggest a plausible physiological role of this peptide via its receptor in AAA. The expression of the other UCN genes and their receptors in human AAA biopsies were also examined. UCN1 expression was also found to be relatively high in AAA body biopsies compared to the macroscopically healthy AAA neck samples. In contrast, minimal expression of UCN3 was found in human AAA biopsies, whilst AAA thrombus expressed low amounts of UCN2 protein by IHC. This is most likely due to poor consistency of the thrombus tissue and technical problems in detecting UCN2 due to thrombus characteristics, as AAA thrombus was shown to release vast quantities of UCN2 in culture. The negligible expression of UCN3 in human AAA corroborates the earlier findings from the mouse studies (Chapter 3) demonstrating that UCN3 plays no significant role in AAA pathogenesis.

Examination of AAA explants demonstrated that significant amounts of UCN2 was released by AAA thrombus and body biopsies, while relatively low amounts of UCN2 was released from AAA neck and femoral atheroma samples. UCN2 secretion was six fold greater in AAA body than AAA neck biopsies; and twenty fold greater in AAA body than femoral atheroma biopsies. Likewise UCN2 secretion was thirty fold greater in AAA thrombus than AAA neck; and one hundred fold greater in AAA thrombus than femoral atheroma biopsies.

Interestingly, there was fivefold more secretion of UCN2 in AAA thrombus than AAA body biopsies. The reason for this as previously demonstrated may be due to sequestration of circulating markers by the thrombus as they migrate from the aortic wall into the blood.³⁸¹ In addition, the thrombus has also been reported to comprise of immune cells including T-lymphocytes and neutrophils,³⁸² which have been suggested to be a good source of UCN2.³³ However, the immunohistochemical staining for UCN2 did not corroborate this finding in this study. This may be due to the technical difficulty in preparing AAA thrombus for immunohistochemistry due to its gel-like consistency. The increased secretion of UCN2 by AAA associated tissues found here led to the hypothesis that high levels of UCN2 from AAA tissue may be reflected by increased concentrations of circulating UCN2 in the plasma of patients with AAA.

Plasma UCN2 concentration was significantly higher in patients with AAA than in patients with non-aneurysmal PAD. Allowing for other known cardiovascular risk factors on multiple regression analysis, a significant association between plasma UCN2 and AAA presence was found, although a low area under the ROC curve (0.661) suggests that UCN2 is unlikely to be sufficiently robust as a stand-alone marker for AAA diagnosis. It will more likely complement established diagnostic imaging techniques. AAA development has been reported to occur in stages,³⁸³ consequently the risk factors and mechanisms involved in mid to end point disease pathology may be different from those at disease initiation.³⁸⁴ A weak positive correlation between plasma UCN2 and AAA diameter was demonstrated. This could indicate that UCN2 may play a role in AAA progression. However, the weak correlation may be due to the small sample size for this assessment which may not provide sufficient power to detect a stronger association between UCN2 and AAA growth.

The data presented here is only from a small number of samples, but this preliminary work has found that UCN2 and its receptor, CRFR2 are up regulated in AAA and may be relevant to AAA pathogenesis. The data suggest that UCN2 is not only two fold increased in the circulation in human AAA but actively secreted by diseased tissue. It is possible that upregulation of UCN2 within AAA biopsies as demonstrated in this study may trigger a cascade of inflammatory events that promote AAA progression. Alternatively, it may be that the observed upregulation of UCN2 shown in this study was simply a consequence of the inflammatory process rather than a cause. UCN2 is known to be upregulated in CHD and heart failure, and has been reported to promote proinflammatory cytokine secretion which may be relevant in AAA.^{35,39,378} In addition, UCN2 was reported to modulate cell survival, apoptosis, and inflammation; mechanisms implicated in AAA pathogenesis.^{40,243,254} The data presented here together with available data in the literature suggest that the upregulation of UCN2 may be particularly relevant to AAA pathogenesis. UCN2 may promote AAA pathogenesis.

The present study has a number of limitations. First, although reasonable for a study of this type, the sample size for this investigation was limited. Validation of findings in larger studies will be required. Both UCN2 and UCN1 have been suggested as putative biomarkers for heart failure.²⁹⁸ UCN1 was identified to be increased within AAA biopsies although not significantly, it is possible that the study may have been under powered to detect an association between UCN1 and AAA. Secondly, though patients included in the study were generally well matched, there were some differences observed between cohorts which may have altered UCN2 expression. For example, the incidence of diabetes mellitus was significantly higher in patients with non-aneurysmal PAD than those with AAA. A negative association between AAA and diabetes had previously been reported,^{385,386} therefore this

finding is not unexpected, and was adjusted for in the multivariate analyses. Therefore the increased plasma UCN2 level shown in this study was independent of diabetes mellitus.

In summary, the present study demonstrates for the first time an association of UCN2 with AAA. UCN2 was shown to be abundantly secreted from AAA associated tissues and elevated within the plasma of patients with AAA, although weak correlation with aortic diameter and poor discriminatory ability indicate that UCN2 is unlikely to be a stand-alone marker for human AAA. In multifactorial diseases such as AAA, it is unlikely that diagnosis will be made on the basis of a single circulating factor; rather it is more likely that next generation diagnostic tools will rely on the analysis of multiple indicators.⁷⁷ Future studies investigating the potential diagnostic value of UCN2 combined with other putative biomarkers would be needed to meticulously evaluate any clinical value of these findings. In Chapter 5, data on the physiological action of UCN2 on aortic smooth muscle cells key to AAA pathogenesis is studied and presented. To conclude, data from this chapter for the first time suggest the following:

1. UCN2 is significantly increased in human AAA body compared to neck
2. The UCN2 receptor- CRFR2 is also upregulated in AAA body compared to neck
3. UCN1 expression was not significantly different between AAA body and neck
4. UCN3 expression was sparse in both AAA body and neck
5. UCN2 protein expression may be co-locating with T- lymphocytes and neutrophils
6. UCN2 is significantly increased in plasma in human AAA
7. UCN2 may be significantly secreted into the circulation by human AAA associated tissues
8. UCN2 may serve as a prognostic factor for AAA

CHAPTER 5

UROCORTIN 2 (UCN2) FUNCTIONAL EFFECT ON SMOOTH MUSCLE CELLS *IN VITRO* IN HUMAN ABDOMINAL AORTIC ANEURYSM

5.1 INTRODUCTION

Urocortin 2 (UCN2) was shown to be upregulated within the aorta and in plasma of patients with abdominal aortic aneurysm (AAA) in Chapter 4. Within the vascular wall, both smooth muscle cells and inflammatory cells may be a source of UCN2. Endothelial cells and myocytes have been demonstrated to express UCN2.^{33,34} UCN2 was expressed in the aneurysmal aorta wall and released *in vitro* from cultured aortic wall explants. Vascular smooth muscle cells are the principal cell type in the normal blood vessel and contribute to the elastic structure of the aortic wall via their production of elastin and other extracellular matrix proteins. Histological examination of AAA tissue demonstrates infiltration of inflammatory cells including T-lymphocytes, macrophages and granulocytes that are major sources of proteolytic enzymes responsible for degradation of the aortic wall and the death of vascular smooth muscle cells. Apoptosis of smooth muscle cells is a characteristic feature of AAA pathology.²⁵⁻²⁷

Emerging evidence suggests an important role for the UCN2 in regulating cardiovascular homeostasis.⁵ UCN2 is known to selectively bind to CRFR2,³⁵⁵ and exert its physiological effect mainly through this receptor.^{5,256} On binding to CRFR2, UCN2 has been reported to initiate an intricate signalling cascade involving phosphoinositide-3-kinase (PI3K) and serine threonine specific protein kinase (Akt),²⁵⁵ to influence cellular migration, growth, cell cycle, cell survival, and cytokine secretion via effects on nuclear factor kappa beta (NFκB).^{291,292,387-389} Phosphorylation of Akt on various cell types has been reported to be associated with increased proliferation and survival.^{291,292} A number of studies have reported that Akt regulates NFκB activation and inflammatory cytokine production depending on the type of stimulus.³⁸⁸⁻³⁹¹ Available data suggest that the urocortins (UCNs) including UCN2 stimulate

the production of proinflammatory cytokines from human peripheral blood mononuclear cells.²⁴⁵ Increased secretion of IL-1 β is reported to set off a cascade of events involving IL-32,³⁸⁰ which elicits the induction of an array of proinflammatory cytokines including IL-1 α , IL-1 β , IL-6 and TNF α via activation of NF κ B, p38MAPK and AP-1.³⁸⁰ Interleukin-6 and TNF α are both implicated in the pathogenesis of AAA^{317,318} and atherosclerosis.^{302,319,320} In addition, plasma TNF α levels have been associated with severity of early atherosclerosis and correlates with both cellular and metabolic alterations key to development of cardiovascular disease (CVD).³²⁰ However the effect of UCN2 on Akt phosphorylation in AAA has not yet been defined.

Data from the previous chapter demonstrated that UCN2 was significantly upregulated in AAA, and the UCN2 selective peripheral receptor-CRFR2 was expressed in AAA tissue. UCN2 has previously been reported to modulate cell survival, apoptosis, and inflammation; mechanisms implicated in AAA pathogenesis.^{40,243,254} It is also possible that elevated UCN2 expression in patients with AAA as demonstrated in the previous chapter simply reflects a response to disease formation. In order to describe the functional significance of increased UCN2 in relation to aneurysm development and progression, it is hypothesised that UCN2 alters human aortic smooth muscle cells (HASMC) to promote an aneurysmal phenotype. In this chapter, the physiological consequence of elevated UCN2 in AAA on HASMC is assessed *in vitro* by:

1. Investigating the effect of UCN2 on HASMC Akt phosphorylation *in vitro*
2. Determining the effect of UCN2 on HASMC cytokine secretion *in vitro*
3. Investigating the effect of UCN2 on HASMC proliferation *in vitro*

4. Elucidating the effect of blocking CRFR2 on UCN2 mediated effects on HASMC *in vitro*
5. Determining the effect of AAA thrombus explant culture conditioned media on HASMC proliferation *in vitro*
6. Investigating the effect of UCN2 on HASMC apoptosis *in vitro*
7. Elucidating the effect of UCN2 on HASMC cell cycle progression *in vitro*

5.2 MATERIALS AND METHODS

5.2.1 Determination of UCN2 secretion from AAA thrombus explant cultures and concentration used in the *in vitro* investigations

A physiologically relevant UCN2 concentration to use in the cell culture studies was determined by measuring the amount of UCN2 released from AAA thrombus explant. AAA thrombus biopsies (n=5) were dissected into ~ 5 mm³ pieces and incubated in DMEM with additives as described (Chapter 2, section 2.3.4). Explants were cultured in 2 mL media in 12 well plates (Nunc, Australia) at 37°C in a humidified 5% CO₂ atmosphere. Conditioned media was collected at 24 h, pooled and centrifuged at 30,000 g for 30 min at 4 °C to remove debris. UCN2 level in pooled AAA thrombus conditioned media was determined by UCN2 ELISA as previously described (Chapter 2, section 2.3.8). The amount of UCN2 in AAA thrombus used in the *in vitro* studies was arrived at by calculating the assayed UCN2 concentration from pooled AAA thrombus conditioned media using the formula [amount of UCN2 in AAA thrombus conditioned media (nM) = concentration of UCN2 in AAA thrombus media/ Molecular weight of UCN2 x 1000].

5.2.2 Cell culture

HASMC were maintained at a density of 2×10^5 - 1×10^6 cells/mL for 4 to 6 passages in DMEM plus additives (TropBio, Townsville, Australia) in T75 cm² flasks (Nunc, Australia) at 37 °C in a humidified 5 % CO₂ atmosphere as previously described (Chapter 2, section 2.4.2). Trypan blue exclusion and cell counting were performed before setting up each experiment.

5.2.3 Determination of the efficacy of the AlamarBlue[®] cell proliferation and viability assay

Actively proliferating HASMC were harvested by trypsinisation as per passaging (Chapter 2, section 2.4.2). Cells were washed by centrifugation in PBS, supernatant discarded and pellets resuspended in CSL-DMEM with 10 % FBS (TropBio, Australia). HASMC were counted as described using the Haemocytometer (Chapter 2, section 2.4.3) and plated in flat bottom 96 well plates (Nunc, Australia) in DMEM with 10 % FBS. HASMC were divided into three groups:

- Group 1 (20,000 cells/well)
- Group 2 (50,000 cells/well)
- Group 3 (100,000 cells/well)

HASMC were cultured for 24 h at 37 °C, 5 % CO₂ and the fluorescence intensity determined after 1 h (1:1 dilution) incubation with AlamarBlue[®] assay reagent (Invitrogen, Australia) as previously described (Chapter 2, section 2.4.3). A total number of six replicates per group of cells and three experiments were performed.

5.2.4 Determination of the optimal concentration range of UCN2 and Ast-2B for *in vitro* studies

Previous *in vitro* studies examining the biological effect of UCN2 on various cell types employed UCN2 concentrations ranging from 0.1 nM to 100 nM.^{38,39,243,331,392} Based on these studies and on the median UCN2 concentrations determined from the explants study in Chapter 4 (67.3 nM and 13.4 nM for AAA thrombus and AAA body biopsies respectively) which is a better reflection of the amount of UCN2 at the physiological level, HASMC were

incubated with 0, 5, 10, 50, 100, and 200 nM concentrations of UCN2 (six replicates per group and a total of three experiments). The effect of UCN2 doses on HASMC proliferation was determined by the AlamarBlue[®] assay as previously described (Chapter 2, section 2.4.6).

In a different experiment, the optimal concentration of Ast-2B that could block the effect of UCN2 via CRFR2 was determined. In a previous study, Grossini *et al.* used Ast-2B at 1 μ M concentration to block the effect of UCN2 on nitric oxide production on binding to CRFR2 in porcine aortic endothelial cells *in vitro*.³⁴⁶ In this study, the efficacy of Ast-2B to inhibit UCN2 effect on HASMC via CRFR2 was examined using 0.5, 1 and 2 μ M Ast-2B concentrations (six replicates per group and a total of three experiments). HASMC were cultured as described (Chapter 2, section 2.4.2). Cells were harvested and grouped as follows:

Group 1 (Untreated control)

Group 2 (100 nM UCN2 + 0.5 μ M Ast-2B)

Group 3 (100 nM UCN2 + 1 μ M Ast-2B)

Group 4 (100 nM UCN2 + 2 μ M Ast-2B)

The efficiency of Ast-2B doses to block UCN2 binding to CRFR2 was determined by measuring UCN2 inhibition of HASMC proliferation as previously described (Chapter 2, section 2.4.6).

5.2.5 HASMC proliferation assay

Subconfluent HASMC were growth arrested for 24 h in DMEM containing 1% (v/v) FBS before incubation in experimental medium containing 10 % FBS and recombinant UCN2 at

0, 10 or 100 nM or AAA thrombus conditioned medium (UCN2 concentration 0, 10 or 100 nM) for 24 and 48 h time points (six replicates for each experimental condition and three whole experiment repeats) as described in Chapter 2, section 2.4.6. As previously described, the involvement of the receptor CRFR2 was investigated by incubating cells with 1 μ M Ast-2B prior to addition of UCN2 or thrombus conditioned medium. HASMC were cultured and treated as above but with AAA thrombus conditioned medium (0 or 100 nM UCN2); with and without 10 min pretreatment with 1 μ M Astressin 2B (Ast-2B) for 24 h (six replicates for each experimental condition and three whole experiment repeats). Cell proliferation was determined by AlamarBlue® assay (Invitrogen, Australia) as previously described (Chapter 2, section 2.4.6). Mean inter-assay coefficient of variation determined from the standard curves of three separate assays was 4.32 %.

5.2.6 Akt signaling in HASMC

Subconfluent HASMC seeded in clear bottom black 96 well plates were growth arrested prior to incubation in experimental medium as described above (Chapter 2, section 2.4.4). HASMC were treated with recombinant UCN2 at 0 or 100 nM for the following time points; 5 min, 6 h and 24 h time points (six replicates for each experimental condition and three whole experiment repeats). 100 nM concentration of UCN2 was chosen because it was similar to the physiological concentration of UCN2 released from AAA thrombus biopsies (Chapter 4, section 4.3.4). The involvement of CRFR2 was also assessed by incubating cells with 1 μ M Ast-2B prior to addition of UCN2. The effect of UCN2 on Akt phosphorylation was measured by a commercially available cell-based human Akt immunoassay (RnD systems; Chapter 2, section 2.4.4).

5.2.7 Cytokine assays

Subconfluent HASMC were growth arrested prior to incubation in experimental medium as described above (Section 5.2.6). HASMC were treated with recombinant UCN2 at 0 or 100 nM for 24 h (six replicates for each experimental condition and three whole experiment repeats). The involvement of CRFR2 was also investigated as described in section 5.2.4. The effect of UCN2 on inflammatory cytokine (IL-1 α and TNF- α) secretion was determined by Q-Plex™ multiplex ELISA assay (Quansys Bioscience, USA) and analysed with a digital camera imaging system (Chemidoc™ System, Bio-Rad, Australia). The human IL-6 DuoSet® ELISA Development kit (catalogue number DY206, R&D Systems, Australia) was used to measure the concentration of the proinflammatory cytokine IL-6 in culture supernatants according to manufacturer's protocol. Mean inter-assay coefficient of variation for IL-6 determined from the standard curves of three separate assays was 2.50 %. Full description of protocols is given in Chapter 2, section 2.4.5.

5.2.8 Apoptosis investigations

UCN2-induced apoptosis was investigated by two different assays measuring different molecules associated with apoptosis pathways; the Apo-One Homogenous caspase-3/7 assay (Promega, Australia) and by the Bcl-2 human ELISA kit (Abcam, Australia). In both cases, subconfluent HASMC were growth arrested prior to incubation in experimental medium and treated as in section 5.2.4 above. The effect of UCN2 on HASMC caspase 3/7 activity was determined by the Apo-ONE Homogeneous caspase-3/7 Assay as previously described (Chapter 2, section 2.4.7).

In a second experiment, HASMC were treated as above. Cells were lysed and the effect of UCN2 on HASMC Bcl-2 expression determined by the Bcl-2 ELISA kit (Abcam, Australia) as described (Chapter 2, section 2.4.7)

5.2.9 Cell cycle progression analysis

UCN2-induced changes in HASMC were investigated as described (Chapter 2, section 2.4.8). Briefly, HASMC were cultured in complete DMEM (TropBio, Townsville, Australia) to 80 % confluence. After treatment with or without UCN2 (0, 100 nM) and/or 1 μ M Ast-2B for 24 h, cells were harvested by trypsinisation. Harvested cells were centrifuged at 200 g for 5 min to pellet cells, counted and resuspended at 1×10^6 in 0.5 mL PBS in pre-labeled 5 mL polystyrene round bottom tubes (BD, Australia). Samples were resuspended in 0.5 mL PBS and centrifuged to pellet as above. Samples were incubated in 1 mL hypotonic PI DNA staining buffer in the dark for 30 min on ice as previously described (Chapter 2, section 2.4.8). Cells were acquired by flow cytometry (CyAn ADP, Beckman Coulter, Australia) and DNA content analysed with the Summit[®] flow analysis software, version 4.3 (Beckman Coulter, Australia). Propidium iodide incorporated into DNA was excited at 488 nm and detected in the FL3 channel (613/20 nm) as previously described.³⁹³

5.2.10 Statistics

The effects of recombinant UCN2 on HASMC proliferation, Akt phosphorylation, cytokine secretion, caspase-3/7, Bcl-2 expression, and cell cycle phases are presented as median and inter-quartile range and evaluated by the Kruskal Wallis test. When relevant, the Mann Whitney U test was applied for comparison between two groups. The effect of aortic thrombus conditioned medium on HASMC proliferation is presented as median and inter-quartile range and evaluated by the Kruskal Wallis test. When appropriate, the Mann Whitney U test was applied for comparison between two groups. Statistical significance was assumed at $P \leq 0.05$.

5.3 RESULTS

5.3.1 Efficacy of the AlamarBlue® cell proliferation and viability assay

There was a linear relationship between the number of cells incubated (20,000; 50,000; 100,000) and fluorescence increased with increasing number of cells (Fig 5.1). Fifty thousand cells was chosen as the number of cells to be plated for subsequent experiments to avoid apoptosis as a result of overcrowding when cells are 100% confluent, but to provide strong enough signals for accurate data acquisition.

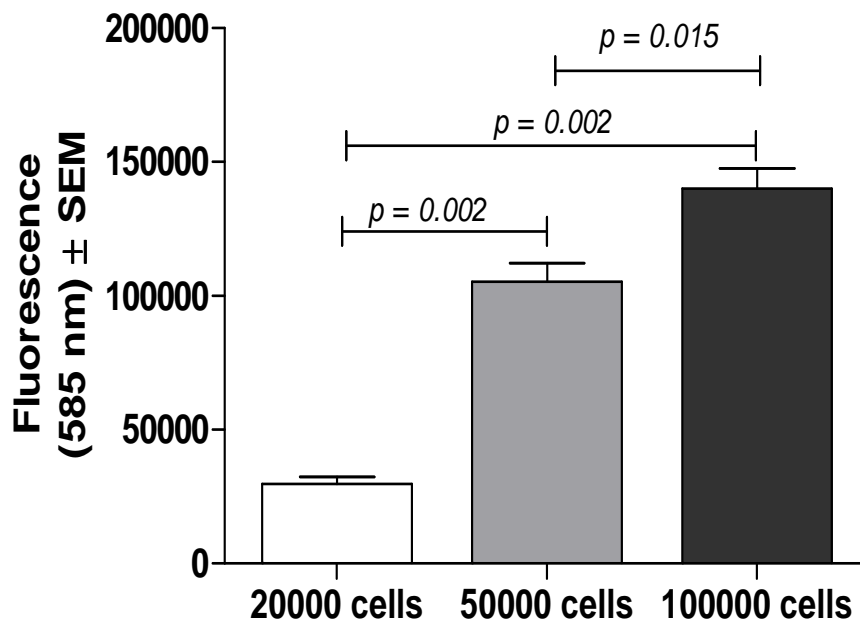


Figure 5. 1 Validation of the AlamarBlue® assay *in vitro*

Fluorescence intensity emitted by varying amounts of HASMC after 24 h culture were measured and compared by the AlamarBlue® test after 1h incubation period. Data are presented as mean ± SEM. Fluorescent intensity was seen to increase with increasing number of cells ($p < 0.05$, Mann Whitney U test). SEM = standard error of mean.

5.3.2 Optimal concentration range of UCN2 and Ast-2B for *in vitro* studies

UCN2 decreased HASMC proliferation dose dependently and peaked at 100 nM concentrations ($P < 0.0001$, Kruskal Wallis test, Figure 5.2). There was no significant difference in HASMC proliferation on incubating with 100 nM or 200 nM UCN2 ($P = 0.937$, Mann Whitney U test, Figure 5.2).

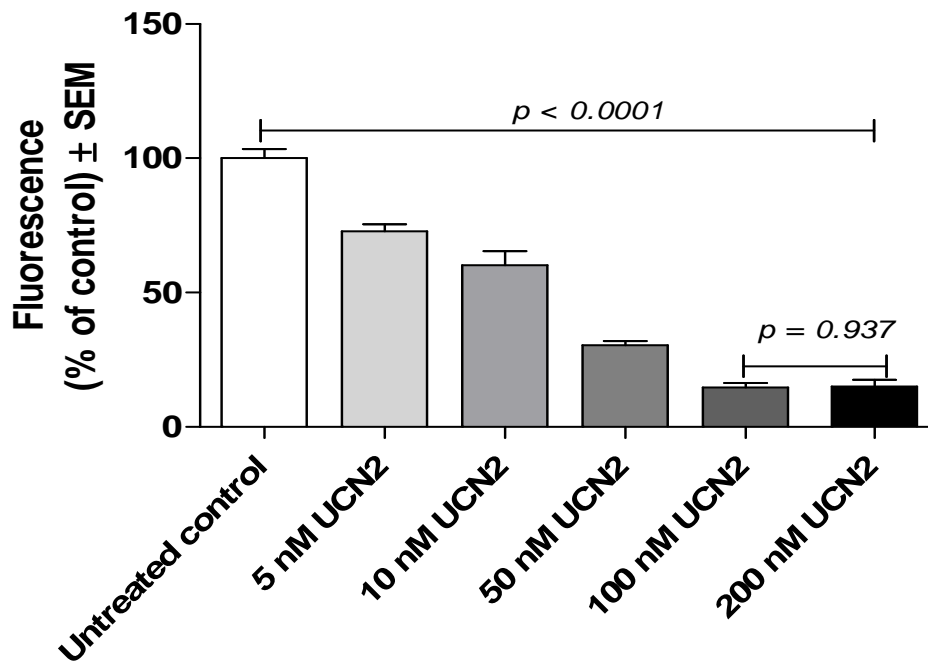


Figure 5. 2 Determination of UCN2 dose used in the *in vitro* studies

Actively proliferating HASMC were treated with increasing doses of UCN2 over 24 h. UCN2 dose dependently inhibited HASMC proliferation. Median and inter-quartile range of triplicate cultures, $P < 0.0001$ (Kruskal-Wallis test).

The data presented here demonstrated that Ast-2B did not abrogate the effect of UCN2 on HASMC proliferation at a dose of 0.5 μM ($P = 0.002$, Figure 5.3), Ast-2B abrogated the effect of UCN2 on HASMC proliferation at 1 μM and 2 μM doses ($P = 0.065$ and $P = 0.132$ respectively, Figure 5.3). There was no significant difference between the 1 μM and 2 μM

doses of Ast-2B ($P=0.810$, Figure 5.3). Based on this data, 1 μM dose of Ast-2B was used to inhibit UCN2 binding to CRFR2 in subsequent experiments.

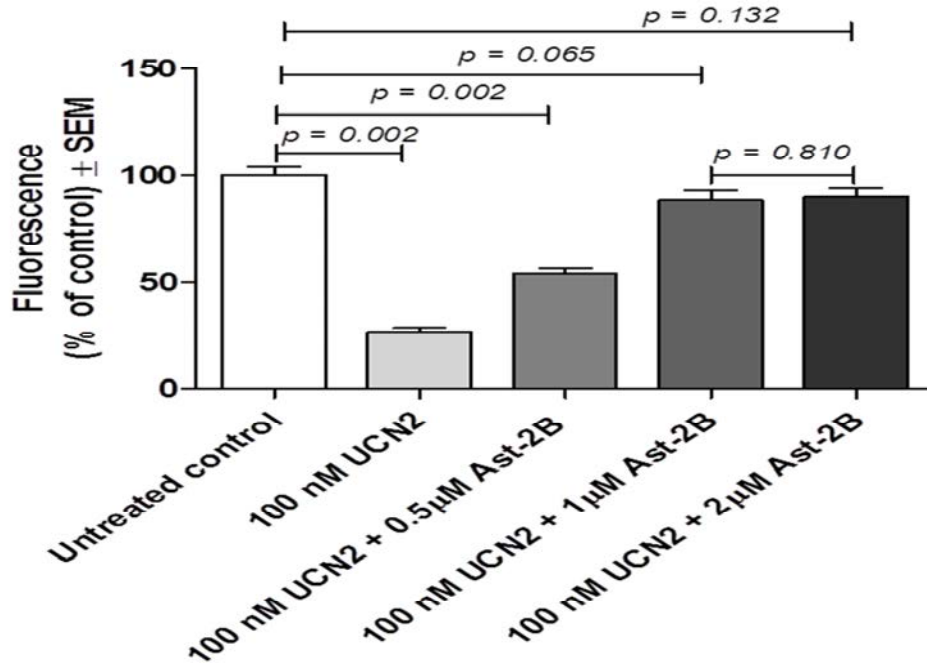


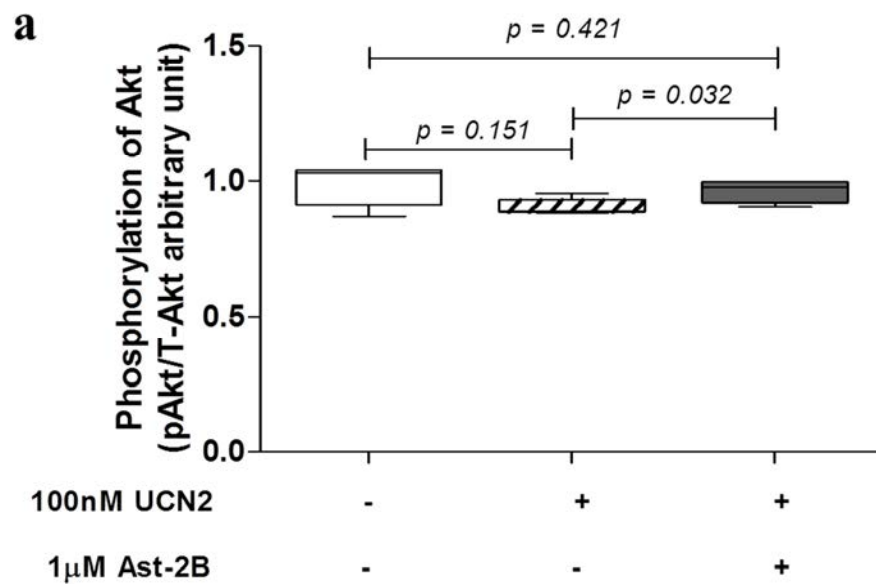
Figure 5. 3 Determination of Astressin 2B (Ast-2B) dose used in the *in vitro* studies

HASMC were treated with 0 or 100 nM UCN2 and the effect of pre-incubating with increasing doses of Ast-2B assessed after 24 h. Ast-2B abrogated the effect of UCN2 on HASMC proliferation at 1 and 2 μM doses ($p < 0.05$)

5.3.3 UCN2 inhibited Akt phosphorylation in HASMC

In chapter 4, experimental evidence demonstrated that UCN2 was significantly increased within AAA biopsies and in the plasma of patients with AAA. In order to further elaborate the functional significance of increased UCN2 in human AAA, the effect of UCN2 on Akt phosphorylation in HASMC was examined. UCN2 was shown to inhibit Akt phosphorylation in a time dependent manner (Figure 5.4). The effect of UCN2 on HASMC Akt phosphorylation was not significant at 5 min incubation ($P=0.151$, Figure 5.4a). Ast-2B significantly inhibited the effect of UCN2 on HASMC after 5 min ($P=0.032$, Figure 5.4a).

UCN2 significantly inhibited Akt phosphorylation in HASMC at 6 h and sustained this inhibition for 24 h ($P=0.008$, Figure 5.4b and $P=0.002$, Figure 5.4c respectively). Camptothecin (CPT), a topoisomerase I inhibitor implicated in apoptosis and Akt activation,³⁹⁴ used as a positive control significantly inhibited Akt phosphorylation at 6 and 24 h time points ($P=0.008$, Figure 5.4b and $P=0.002$, Figure 5.4c respectively). The effect of UCN2 on Akt phosphorylation was significantly abrogated by prior incubation of HASMC with the CRFR2 antagonist, Ast-2B at 6 h and 24 h ($P=0.008$, Figure 5.4b and $P=0.002$, Figure 5.4c).



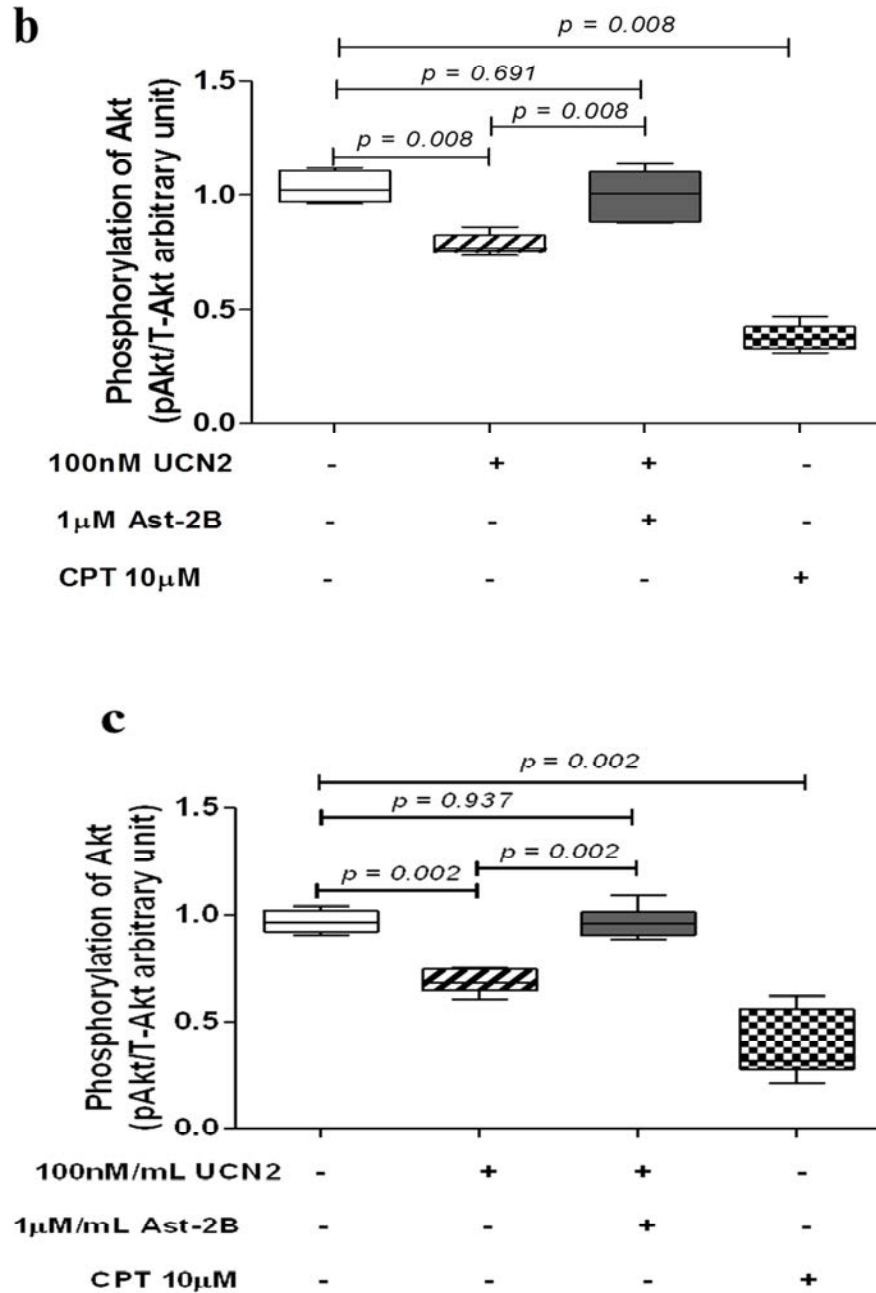


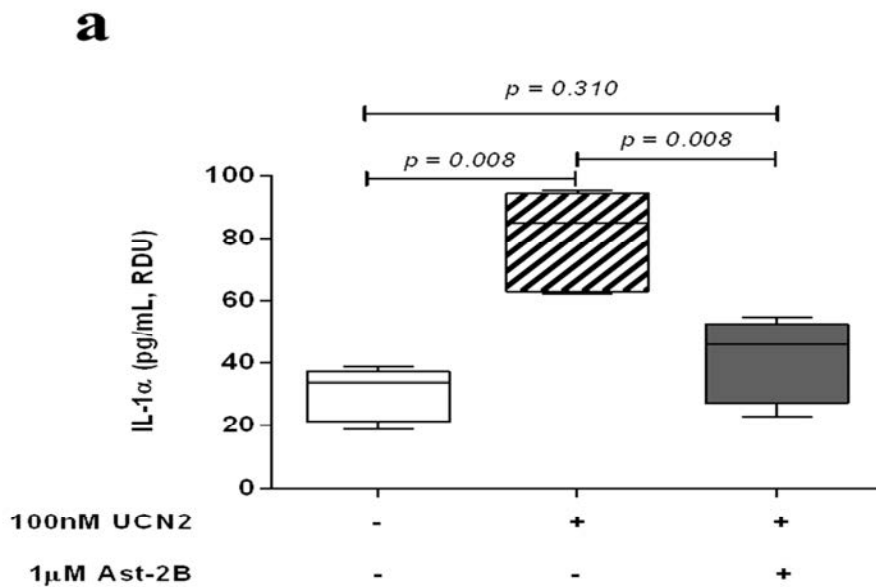
Figure 5. 4 Effect of UCN2 on healthy HASMC Akt phosphorylation *in vitro*

Akt phosphorylation on HASMC was inhibited by UCN2 over 24 h.

(a) Effect of UCN2 on HASMC Akt phosphorylation at 5 min. (b) UCN2 and CPT inhibited HASMC pAkt at 6 h. (c) UCN2 and CPT sustained HASMC pAkt inhibition after 24 h. Median and inter-quartile range of triplicate cultures (Mann Whitney U test) Ast-2B = Astressin 2B, CPT = Camptothecin (Positive control), pAkt = phosphorylated Akt, and UCN2 = Urocortin 2.

5.3.4 Effect of UCN2 on HASMC cytokine secretion

Enhanced secretion and presence of proinflammatory cytokines has been generally accepted as one of the key features in AAA pathogenesis. Inhibition of Akt phosphorylation has been linked with decreased cell proliferation,²⁹² and increased proinflammatory cytokine secretion.^{388,389} In the previous experiment (Section 5.3.3), UCN2 was shown to inhibit HASMC Akt phosphorylation upon binding to CRFR2. Here the effect of UCN2 *in vitro* on HASMC proinflammatory cytokine secretion was examined. UCN2 significantly upregulated the secretion of IL-1 α ($P=0.008$, Figure 5.5a) and IL-6 ($P=0.008$, Figure 5.5b) after 24 h. These effects on HASMC secretion of IL-1 α and IL-6 were significantly abrogated by prior incubation with Ast-2B ($P=0.006$ and $P=0.008$ respectively, Figures 5.5a and b). UCN2 had no significant effect on HASMC TNF- α secretion (Figure 5.5c) after 24 h.



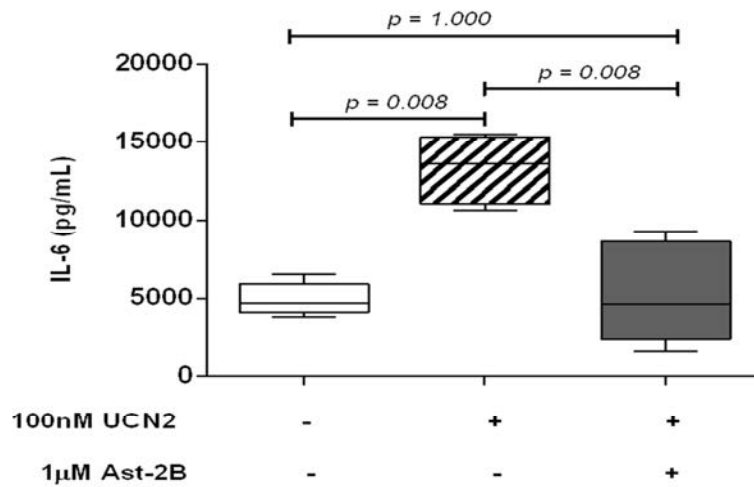
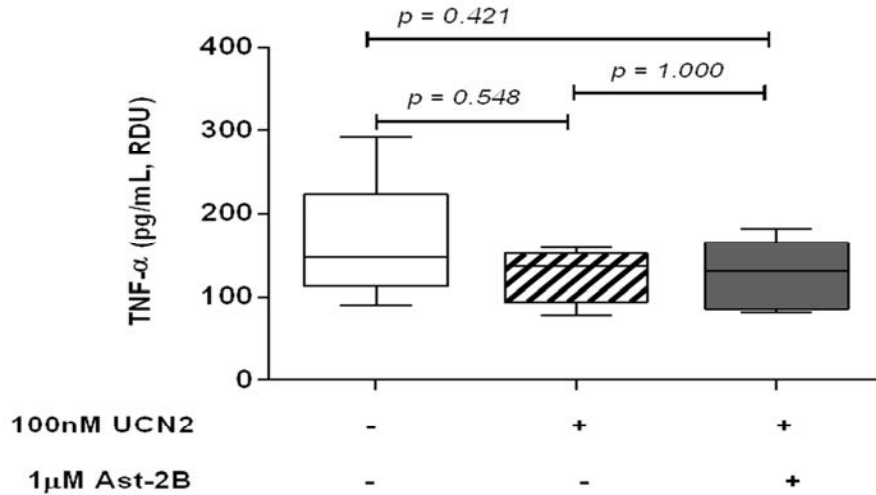
b**c**

Figure 5. 5 Effect of UCN2 on HASMC cytokine secretion *in vitro*

- (a) UCN2 significantly increased HASMC IL-1 α secretion over 24 h. This effect was significantly abrogated by prior incubation with Ast-2B. Median and inter-quartile range of triplicate cultures (Mann Whitney U test).
- (b) UCN2 significantly increased HASMC IL-6 secretion over 24 h. This effect was abrogated by prior incubation with Ast-2B. Median and inter-quartile range of triplicate cultures (Mann Whitney U test).
- (c) UCN2 has no significant effect on HASMC TNF- α secretion over 24 h. Median and inter-quartile range of triplicate cultures (Mann Whitney U test. Ast-2B = Astressin 2B, IL-1 α = interleukin one alpha, IL-6 = interleukin six, TNF- α = Tumour necrosis factor alpha, and UCN2 = Urocortin 2).

5.3.5 UCN2 inhibited HASMC proliferation

After demonstrating that UCN2 inhibits HASMC Akt phosphorylation (section 6.3.1) leading to increased IL-1 α and IL-6 secretion (section 5.3.4), the effect of UCN2 on HASMC proliferation was assessed. Increasing concentrations of UCN2 induced a significant dose-dependent decrease in HASMC proliferation after 24 h ($P=0.002$, Figure 5.6). Similar results were obtained when incubating for 48 h ($P=0.006$, Figure 5.6).

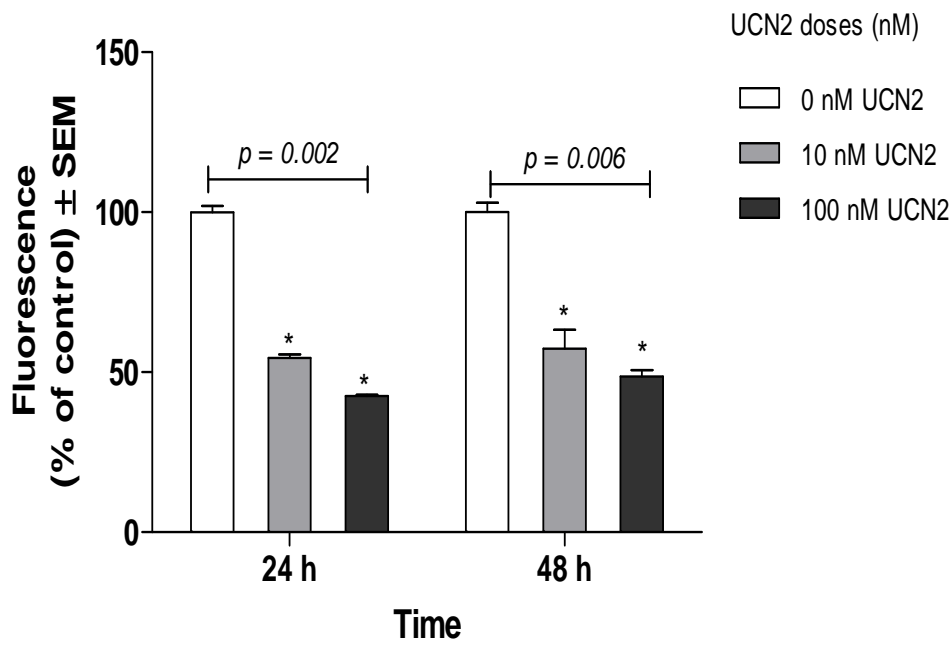


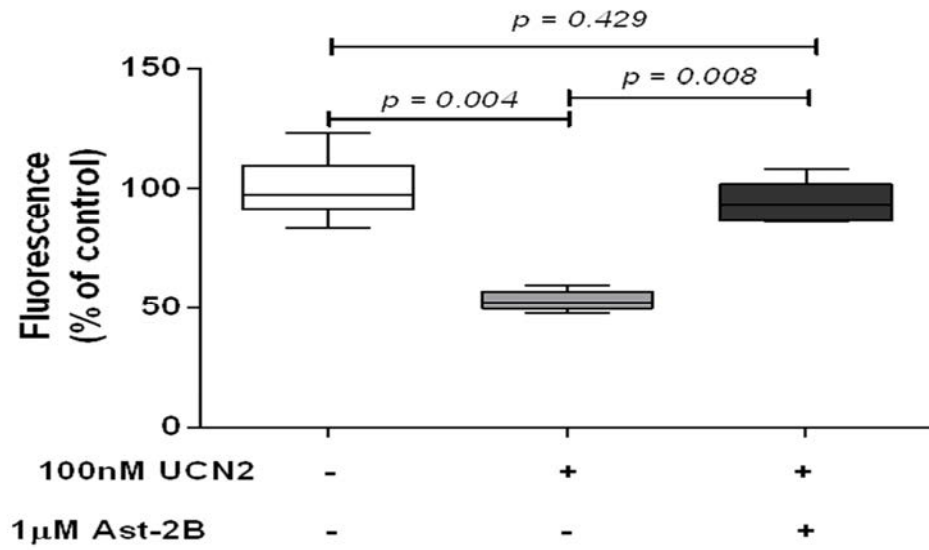
Figure 5. 6 Effect of UCN2 on HASMC proliferation *in vitro*

Proliferation of HASMC was inhibited by UCN2 over 24 and 48 h. Data presented as bar graphs \pm SEM of triplicate cultures, $P = 0.002$ at 24 h and $P = 0.006$ at 48 h (Kruskal-Wallis test). UCN2 inhibited HASMC at 10 and 100 nM doses ($*P < 0.05$, Mann Whitney U test)

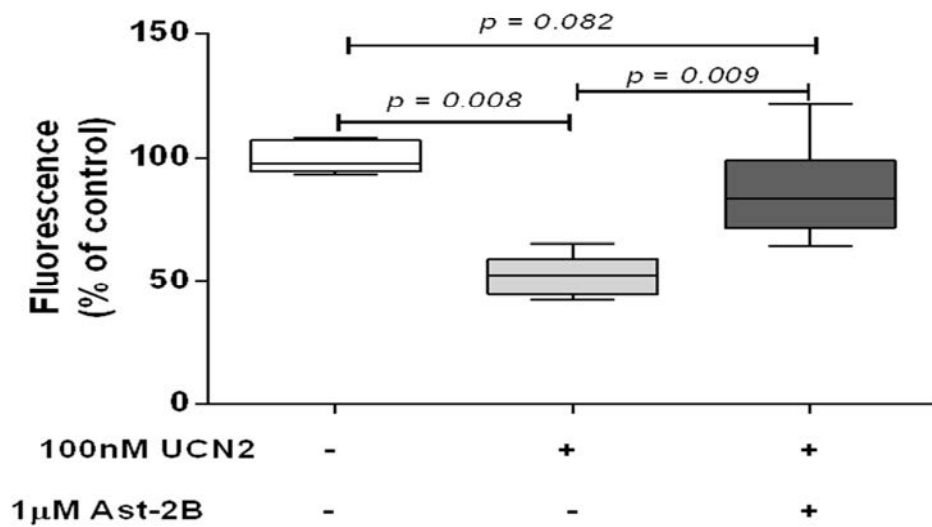
To determine whether the inhibitory effect of UCN2 on HASMC proliferation was via its receptor, HASMC were incubated with Ast-2B prior to exposing them to 100 nM UCN2 *in vitro*. Pre-treatment of HASMC with Ast-2B significantly abrogated the effect of UCN2 on

HASMC after 24 h ($P=0.008$, Figure 5.7a) and 48 h ($P=0.009$, Figure 5.7b). There was no significant effect of Ast-2B alone on HASMC proliferation ($P=0.132$, Figure 5.7c).

a



b



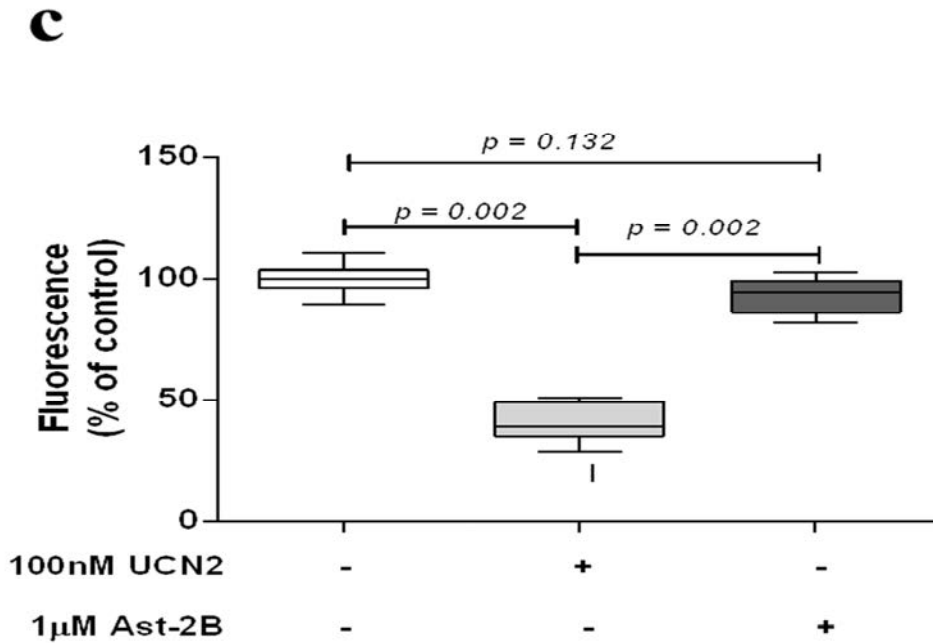


Figure 5. 7 Effect of CRFR2 blockade on UCN2 induced inhibition of HASMC proliferation *in vitro*

- (a) Ast-2B significantly abrogated UCN2 induced inhibition of HASMC proliferation over 24 h ($P=0.008$).
- (b) Similar effect was seen at 48 h ($P=0.009$).
- (c) No significant effect of Ast-2B on HASMC proliferation. Median and inter-quartile range of triplicate cultures (Mann Whitney U test).

5.3.6 AAA thrombus conditioned media inhibits HASMC proliferation

In Chapter 4, AAA thrombus explants were shown to secrete fivefold more UCN2 in culture than AAA body explants. Based on this and the report that AAA thrombus sequesters circulating proteins that may alter AAA progression, the effect of AAA thrombus media was examined after determining the amount of UCN2 in AAA thrombus media as described above (Section 5.2.1). Proliferation of HASMC incubated in AAA thrombus conditioned media was significantly inhibited after 24 h ($P=0.002$, Figure 5.8). Inhibition of HASMC proliferation was also sustained after 48 h incubation ($P=0.003$, Figure 5.8).

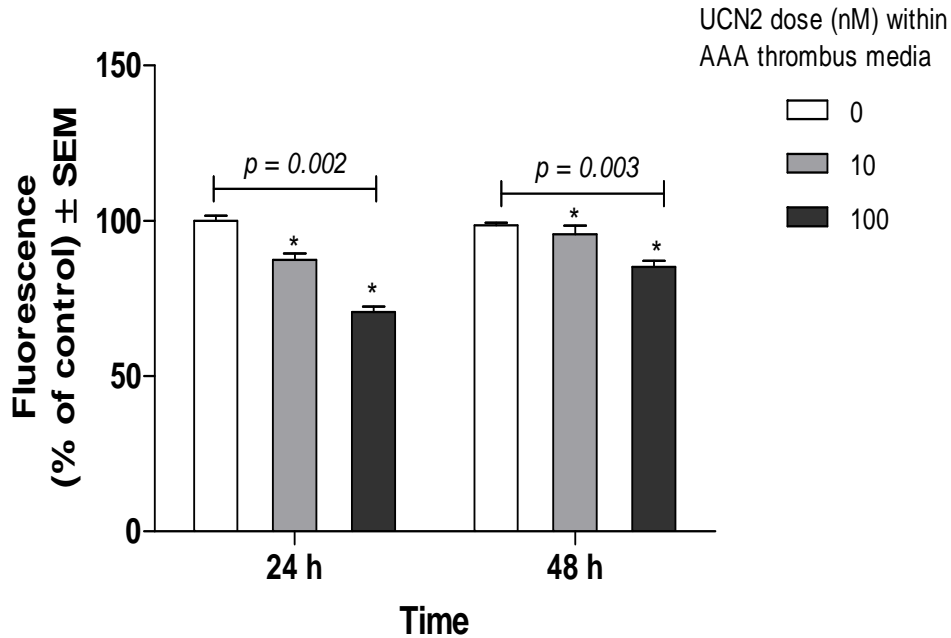


Figure 5. 8 Effect of AAA thrombus conditioned media on HASMC proliferation *in vitro*

Proliferation of HASMC was inhibited by UCN2 over 24 and 48 h. Data presented as bar graphs \pm SEM of triplicate cultures, $P=0.002$ at 24 h and $P = 0.003$ at 48 h (Kruskal-Wallis test). UCN2 inhibited HASMC at 10 and 100 nM doses ($*P<0.05$, Mann Whitney U test)

Pre-incubation of HASMC with Ast-2B before treating with UCN2 was shown to significantly inhibit the effect of UCN2 on HASMC proliferation (Section 5.3.6). To determine whether Ast-2B exerts a similar effect on the ability of AAA thrombus conditioned media to inhibit HASMC proliferation. HASMC were incubated with Ast-2B prior to exposing them to AAA thrombus conditioned medium containing UCN2 at a calculated concentration of 100 nM UCN2 *in vitro*. Ast-2B significantly abrogated the inhibitory effect of AAA thrombus media on HASMC proliferation at 24 h ($P=0.036$, Figure 5.9a), but not at 48 h ($P=0.277$, Figure 5.9b).

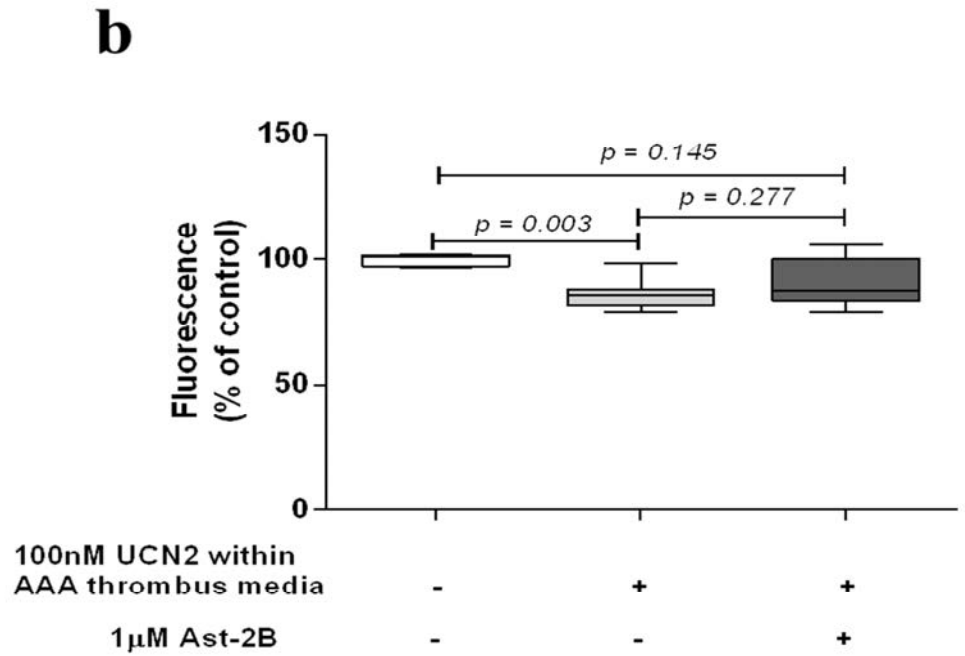
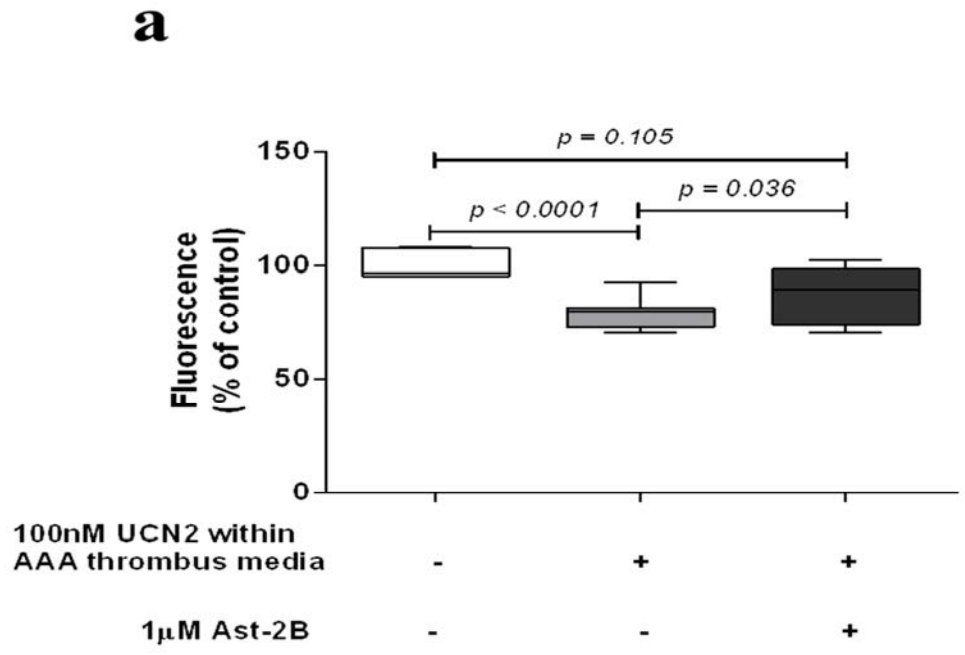


Figure 5.9 Effect of CRFR2 blockade on AAA thrombus conditioned media induced inhibition of HASMC proliferation *in vitro*

- (a) Ast-2B significantly reduced AAA thrombus conditioned media inhibition of HASMC proliferation over 24 h with no significant effect after 48 h;
- (b) Median and inter-quartile range of triplicate cultures, $P = 0.036$ and $P = 0.277$ respectively (Mann Whitney U test).

5.3.8 UCN2 had no significant effect on HASMC apoptosis

A decrease in the anti-apoptotic Bcl-2 levels and an increase in caspase-3/7 activity have been associated with increased programmed cell death.^{395,396} The previous experiments demonstrated that UCN2 inhibited HASMC Akt phosphorylation, leading to increased cytokine secretion and decreased proliferation. These effects by UCN2 were shown to be significantly inhibited by prior incubation of HASMC with the selective CRFR2 antagonist, Ast-2B. In order to determine whether UCN2 also causes HASMC apoptosis, the effect of UCN2 treatment on HASMC caspase activity and Bcl-2 expression *in vitro* was examined. There was no significant difference in caspase-3/7 activity between untreated controls and HASMC incubated with 100 nM UCN2 at 24 h ($P=0.095$, Figure 5.10a). Ast-2B reversed the slight inhibition of caspase-3/7 by UCN2 (Figure 5.10a). Camptothecin (CPT), a topoisomerase I inhibitor,³⁹⁴ used as positive control significantly increased HASMC apoptosis ($P=0.008$, Figure 5.10a). To further examine the effect of UCN2 on HASMC apoptosis, the effect of UCN2 on HASMC Bcl-2 expression was analysed. There was no significant effect on Bcl-2 expression following incubation of HASMC with UCN2 ($P=0.405$, Figure 5.10b) after 24 h.

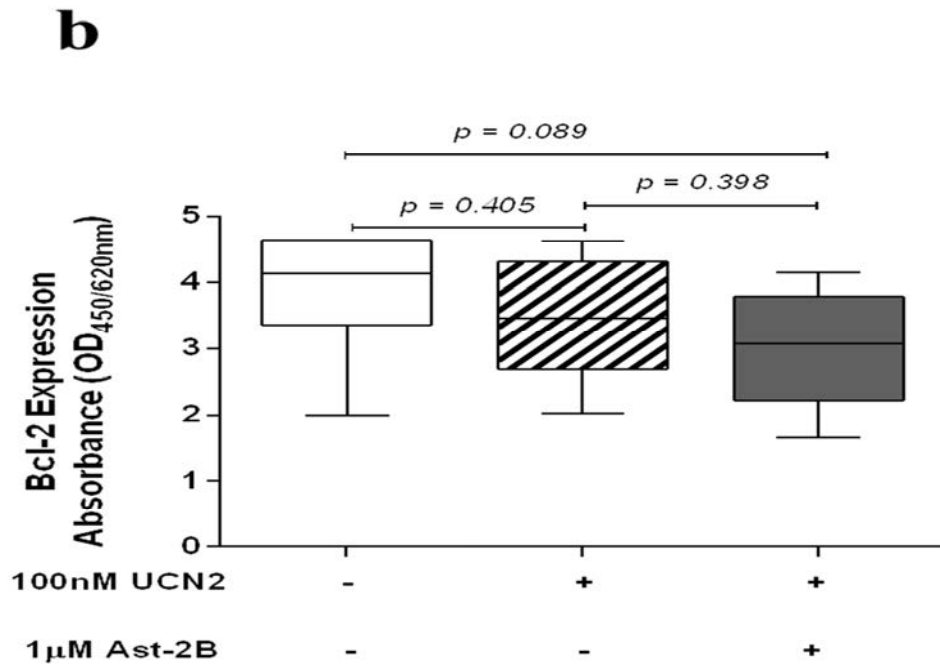
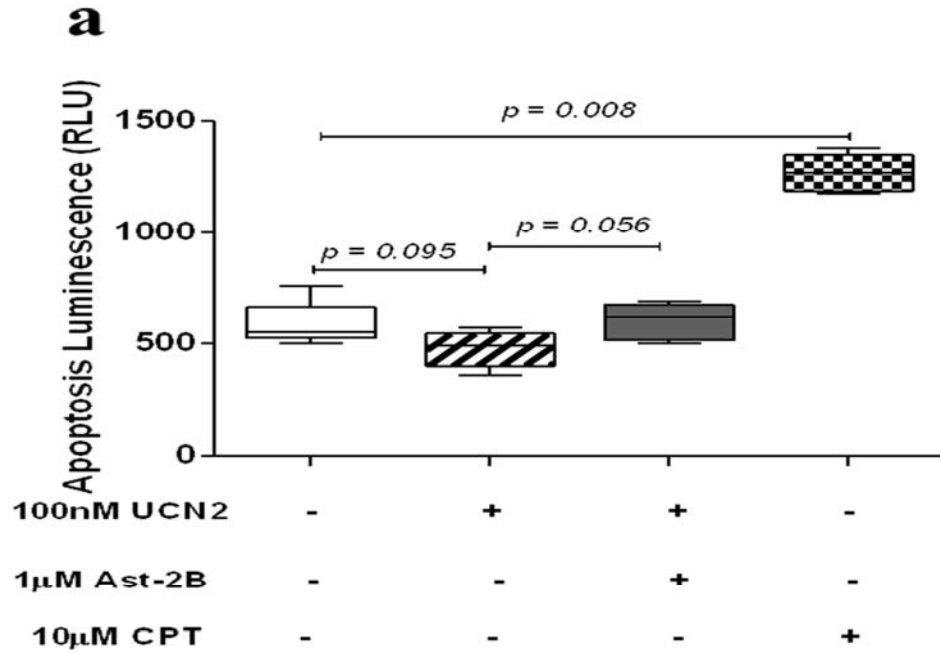


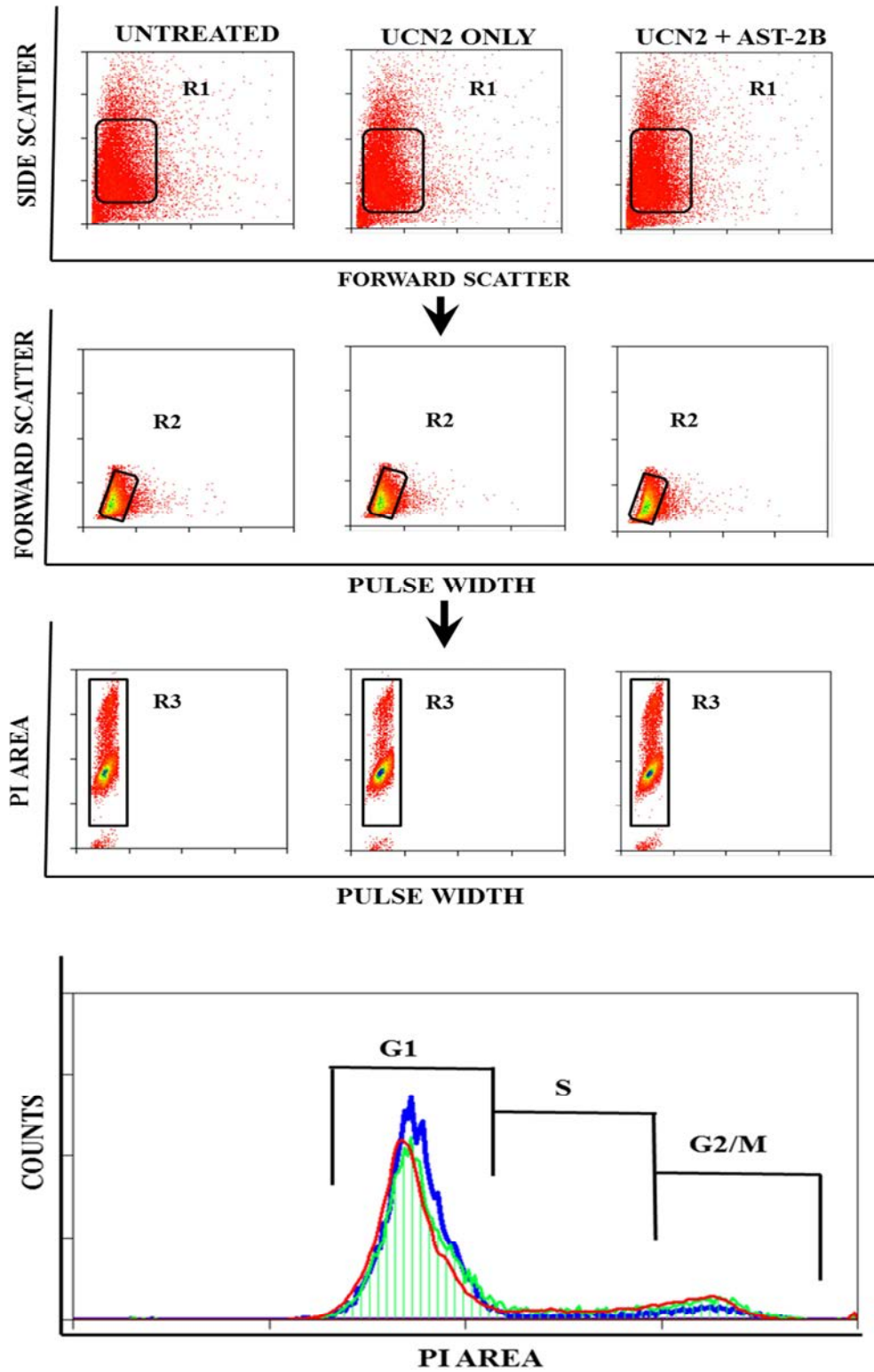
Figure 5.10 Effect of UCN2 on HASMC apoptosis *in vitro*

- (a) Determination of UCN2 on HASMC apoptosis by the Apo-One Caspase-3/7 assay. UCN2 appears to slightly reduce HASMC apoptosis ($P=0.095$). Although not significant, the effect was abrogated by blocking CRFR2 on these cells. Camptothecin (CPT) significantly increased HASMC apoptosis.
- (b) UCN2 has no significant effect on HASMC Bcl-2 expression, $P= 0.405$. Median and inter-quartile range of triplicate cultures (Mann Whitney U test).

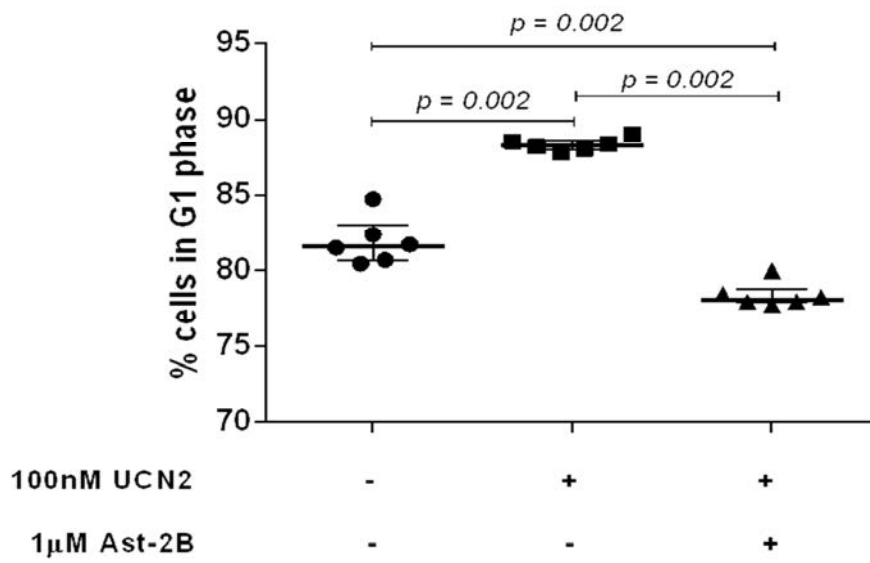
5.3.10 UCN2 induces a G1 arrest in HASMC

Data from above demonstrated that UCN2 inhibited HASMC proliferation but had no significant effect on apoptosis. Next, to investigate whether UCN2 influences HASMC cell cycle progression, HASMC incubated with UCN2 for 24 h were analysed by measuring DNA content based on propidium iodide incorporation. There were marked and consistent changes in the cell cycle at 24 h as shown by cell cycle distribution analysis using flow cytometry (Figure 5.11). UCN2 significantly increased the percentage of cells in the G1 phase ($P=0.002$, Figure 5.11b) with a concomitant decrease in the S phase ($P=0.002$, Figure 5.11c) compared to controls. UCN2 decreased the number of cells in the G2/M phase, however this effect was not significant ($P=0.065$, Figure 5.11d) compared to controls after 24 h. Ast-2B significantly abrogated the effect of UCN2 on HASMC cell cycle phases after 24 h ($P<0.05$, Figures 5.11b-d)

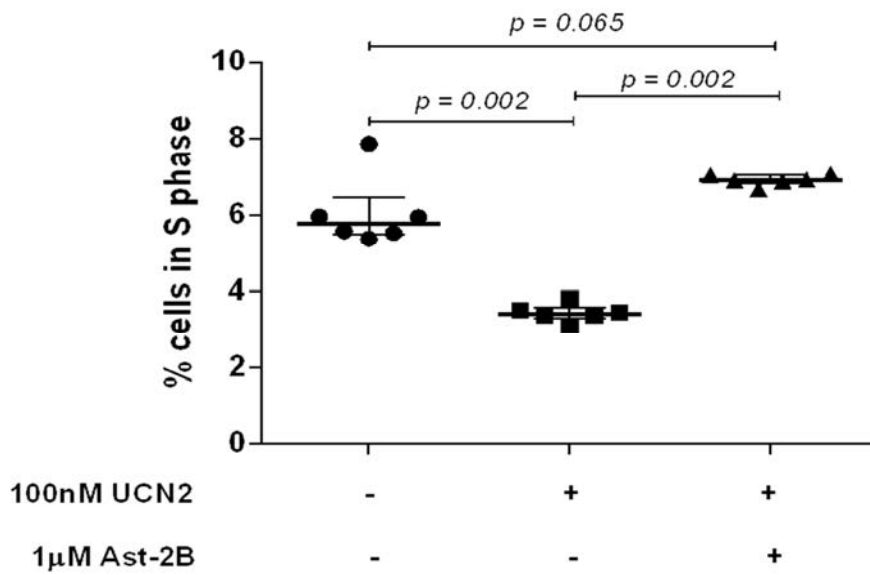
a



b



c



d

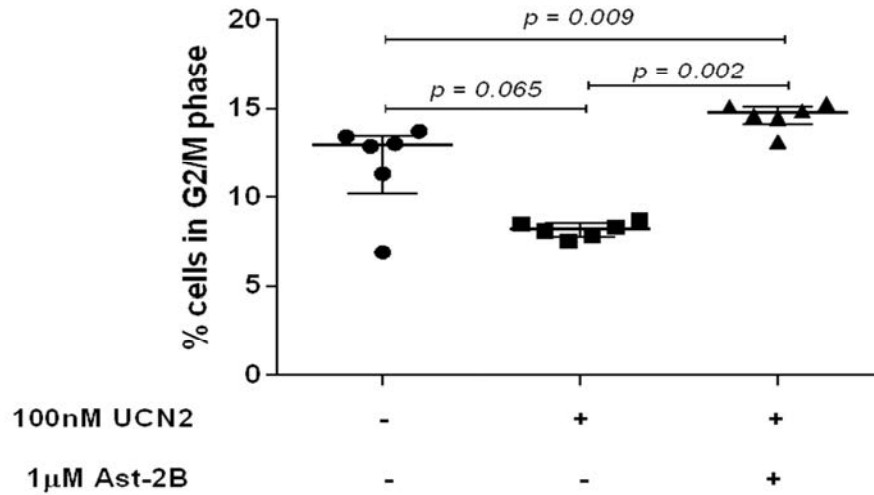


Figure 5. 11 Cell cycle distribution analysis of HASMC exposed to UCN2 by flow cytometry

(a) Cell cycle distribution analysis by flow cytometry. HASMC were exposed to UCN2 (0 or 100 nM and/or Ast-2B (1µM) over 24 h, washed and harvested. Cells were fixed and stained with propidium iodide and DNA content analysed by flow cytometry. Representative histogram shown, green histogram = untreated cells, blue histogram = 100nM UCN2, and red histogram = 100nM UCN2 + 1µM Ast-2B (b) UCN2 significantly increased percentage of cells in G1 phase, $p=0.002$. (c and d) Following G1 cell cycle phase arrest, there was a decrease in percentage of UCN2 treated cells in S phase, $p=0.002$ and G2/M phase, $p=0.065$. Ast-2B significantly abrogated the effects of UCN2 on the cell cycle, $p<0.05$ (b-d). Triplicate cultures are shown. Ast-2B = Astressin 2B and UCN2 = Urocortin 2. Values represent median and inter-quartile range of triplicate cultures.

5.4 DISCUSSION

The ability of HASMC to alter their phenotype in response to environmental stimuli plays a vital role in cardiovascular homeostasis.³⁹⁷ UCNs are known to be expressed in smooth muscle cells, endothelial cells and immune cells, and to regulate cardiovascular homeostasis.^{5,298} Data from Chapter 4 suggest that UCN2 is significantly upregulated in AAA. UCN2 is reported to play a role in the activation and phosphorylation of key substrates in HASMC which regulate cellular morphology, migration, growth, cell cycle, cell survival, and cytokine secretion.^{291,292,375,387-389} UCN2 has also been reported to influence other cellular processes associated with AAA pathogenesis, such as survival, apoptosis, and inflammation.^{40,243,254} Therefore, this chapter investigates the functional significance of increased UCN2 in relation to aneurysm development and progression by assessing the effect of UCN2 via CRFR2 on HASMC phenotype *in vitro*.^{35,39,378}

UCN2 concentrations employed in previous studies investigating the biological effect of the protein varied between 1 nM and 100 nM.^{39,392} Based on these and the UCN2 concentrations obtained from the explants studies in Chapter 4 (i.e the median concentrations of UCN2 in conditioned media of AAA body and AAA thrombus explants collected over 24 h; 13.4 and 67.3 nM, respectively), a range of concentrations of UCN2 were used in cell culture studies to select the optimum and most physiologically relevant. For the *in vitro* studies, HASMC was exposed to a concentration range of 0 to 100 nM UCN2 or AAA thrombus conditioned medium (0 to 100 nM UCN2) over 24 or 48 h. The upper end of the range was selected by considering the plausible accumulation of UCN2 within AAA wall and thrombus over the course of AAA development.¹⁹⁷

This study showed that incubation of HASMC with UCN2 markedly inhibited Akt phosphorylation via CRFR2. This effect was significantly abrogated upon prior incubation of HASMC with Ast-2B. Phosphorylation of Akt on various cell types has been reported to be associated with increased proliferation and survival.^{291,292} Others have reported that activation of CRFR2 induces phosphorylation and activation of the p38MAPK and Akt signalling pathway.^{255,398,399} The data presented here, although contrary to these reports is supported by evidence from Wang *et al.* that UCN2 inhibits Akt phosphorylation via CRFR2,³⁷ and data from Chen *et al.* demonstrating that UCN2 inhibits insulin induced phosphorylation of Akt and ERK1/2 in muscle cells via CRFR2.⁴⁰⁰ The Akt signalling pathway is known to regulate key processes implicated in AAA development including inflammation, proliferation, apoptosis and the cell cycle.^{291,292,375,387-389} The inhibitory effect of UCN2 on HASMC Akt activation shown here may have downstream implications on some or all of these processes.

The inhibition of Akt activation has been associated with increases in proinflammatory cytokine secretion.^{388,389} A classic feature of AAA is the presence of inflammatory cytokines in both the circulation and in tissues.¹²⁹ Cytokines are believed to be responsible for initiating the tissue damage associated with the disease.^{103,130,131} Examination of proinflammatory cytokine secretion on incubating HASMC with UCN2 demonstrated increased secretion of IL-1 α and IL-6 upon binding to CRFR2. UCN2-mediated increase in IL-1 α and IL-6 secretion was abrogated upon prior incubation of HASMC with Ast-2B. Both IL-1 α and IL-6 have been associated with enhanced infiltration of both Th1 and Th2 cells within AAA homogenates.⁴⁰¹ Recent reports suggest that IL-1 α in association with IL-23 promotes IL-17-production from Th17 cells and $\gamma\delta$ T cells and may have major pathogenic roles in autoimmune diseases.⁴⁰² It is plausible that the deleterious autoimmune effect of IL-1 α is another mechanism by which UCN2 may promote aneurysm progression. There is a strong

association between IL-6 and the pathogenesis of AAA.^{317,318} For instance, circulating concentration of IL-6 have been shown to correlate with poor prognosis of patients with AAA.^{317,318} IL-6 has been reported to induce the upregulation of the angiotensin II type 1 receptor (AT1) expression resulting in angiotensin II-mediated enhanced reactive oxygen species production and endothelial dysfunction,⁴⁰³ which promotes aneurysm formation.⁴⁰⁴ IL-6 is known as a key facilitator of vascular inflammation by promoting the secretion of chemoattractants such as monocyte chemoattractant protein 1 (MCP-1), IL-8 and IL-6, and the expression of the adhesion proteins CD54 and CD106.^{405,406} Elevation of IL-6 by UCN2 demonstrated here would likely exacerbate the inflammatory process and encourage AAA development. Others have also reported increased proinflammatory cytokine secretion on various cell types exposed to UCN2 via CRFR2,³⁹ such as IL-8 and MAPK in human colon cells.³⁶ These reports together with the significant increase in IL-1 α and IL-6 secretion demonstrated here suggest that UCN2 may contribute to the inflammatory process seen in AAA.

Impaired proliferation and loss of medial vascular smooth muscle cells is a hallmark of AAA.²⁵ The data presented suggest that UCN2 inhibited Akt phosphorylation. The inhibition of Akt phosphorylation has been linked with decrease in cell proliferation.²⁹² The data presented here demonstrated that UCN2 dose-dependently inhibited HASMC proliferation both at 24 and 48 h upon binding to CRFR2. Ast-2B significantly abrogated the anti-proliferative effect of recombinant UCN2 on HASMC *in vitro*. Previous studies investigating the action of UCN2 on cell survival and proliferation are conflicting. For example, UCN2 has been suggested to promote macrophage apoptosis,²⁴³ but inhibit mesenteric smooth muscle cell apoptosis.³⁹² UCN2 was reported to encourage cell survival by protecting against ischaemic injury,³⁷⁵ but inhibit cell proliferation in cancer models.⁴⁰⁷ On the contrary, data

from this thesis demonstrated that UCN2 inhibited smooth muscle cell proliferation. Similarly, Wang *et al.* showed that UCN2 inhibited the proliferation of human small lung cancer cell proliferation by blocking Akt phosphorylation and inhibiting VEGF secretion.³⁷ The anti-proliferative effect of UCN2 in other diseases has been suggested to be beneficial.³⁸ For example, Hao *et al.* suggested that by inhibiting the proliferation of Lewis Lung Carcinoma Cell (LLCC) and suppressing neovascularisation, UCN2 may serve as a good therapeutic target in malignancies.³⁸ Although this effect is more likely to be pathological in AAA,²⁵ since paucity of medial smooth muscle cells is associated with aneurysm pathogenesis.²⁵ Additional data presented here showed that AAA thrombus conditioned medium with equivalent UCN2 concentration (0, 10 and 100 nM) dose-dependently inhibited HASMC proliferation, although the effect of AAA thrombus conditioned medium was less marked than that of recombinant UCN2 especially at 48 h. On prior incubation of HASMC with Ast-2B *in vitro*, the anti-proliferative effect of AAA thrombus conditioned medium was significantly abrogated at 24h. However, the action of Ast-2B was less apparent at 48 h incubation, although reasons for this remain unclear. It is possible though that secretion from AAA thrombus conditioned medium contains unidentified proteins, which block the action of Ast-2B, or other anti-proliferative proteins unrelated to UCN2 that exert similar effect on HASMC. Likewise, there may be other proteins present in AAA thrombus conditioned medium acting via other signalling pathways to regulate HASMC proliferation.

A decrease in the anti-apoptotic Bcl-2 levels and an increase in caspase-3/7 activity have been associated with increased programmed cell death.^{395,396} DNA damage frequently activates responses that result in an alteration of the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family.⁴⁰⁸ This increases the permeability of the mitochondrial with consequent release of cytochrome *c* initiating caspase-mediated

apoptosis.⁴⁰⁹ Examination of apoptotic mechanisms on HASMC incubated with UCN2 demonstrated that UCN2 had no significant effect on caspase-3/7 activity or on Bcl-2 expression. These data suggest that UCN2 plays no role in smooth muscle cell apoptosis; another mechanism implicated in the pathogenesis of AAA,⁶¹ and is supported by a previous report by Jin *et al.* demonstrating that UCN2 has no effect on apoptosis *in vitro* in a prostate cancer model.⁴¹⁰ It is possible that UCN2 may be modulating HASMC apoptosis by a caspase-3/7 independent pathway.⁴⁰⁸ The caspase-independent pathway proceeds in a Bcl-2-independent manner and involves changes in redox-sensing mitochondrial membrane proteins such as the flavin adenine dinucleotide, Apoptosis inducing factor (AIF) which translocate to the nucleus disrupting chromatin fragmentation.^{408,411} The present study however did not investigate the involvement of UCN2 in the caspase-independent pathway.

Being that available data demonstrate that UCN2 inhibited HASMC proliferation but had no significant effect on apoptosis. In order to understand the mechanism involved in UCN2-mediated decrease in proliferation, HASMC cell cycle progression on incubating with UCN2 was assessed. Cell cycle analysis revealed that UCN2 increases the population of cells in the G1 phase of the cell cycle and this appears to be via CRFR2 as it was abrogated upon prior incubation of HASMC with Ast-2B. Cell proliferation is known to be regulated by various factors affecting the cell cycle phases.⁴¹² It is proposed that by inducing a G1 phase arrest, UCN2 inhibits HASMC regeneration which is an important process in vascular homeostasis and may in part explain the paucity of medial smooth muscle cells seen in end-stage AAA. Evidence supporting this data include reports that cells expressing activated Akt continue to proliferate in the presence of mutagens²⁹¹ and are not restricted in the G2/M to G1 transition of the cell-cycle induced by DNA damage.⁴¹³ The data presented here demonstrated that UCN2 both inhibited HASMC Akt activation and restricts the HASMC cell cycle progression

to the G1 phase. HASMC have been reported to play a protective role within the arterial wall by exerting a paracrine driven homeostasis against inflammation and proteolysis,⁴¹⁴ and failure of these protective mechanisms is suggested to lead to AAA formation.^{47,414} It is proposed that alteration of HASMC phenotype by UCN2 as demonstrated here may promote AAA pathogenesis. Furthermore, the significant abrogation of UCN2-mediated effects on HASMC *in vitro* by Ast-2B implies that CRFR2 is engaged in the deleterious effects of UCN2, and suggest a plausible role as a therapeutic target for this receptor in AAA.

This study has a number of limitations. Firstly, a dysregulated immune response within the aortic wall is commonly accepted as a key factor contributing to human AAA development. UCN2 was shown here to increase HASMC secretion of the proinflammatory cytokines IL-1 α and IL-6 which may be important in the recruitment of effector immune cells such as T-cells and neutrophils. However, whether UCN2 directly drives the recruitment of immune cells was not investigated. Although, in the previous Chapter, T-cells and neutrophils were shown to congregate in regions of dense UCN2 expression, but it was not definitely proven that UCN2 has a direct role in the recruitment of these cells in AAA. A second limitation of this study is that examination of the functional effects of UCN2 in AAA pathogenesis concentrated on HASMC. Investigation of the effects of UCN2 on other cell types including macrophages and endothelial cells would further strengthen the key findings presented here. Another limitation is that it was not determined whether the effect of UCN2 on the cell cycle was permanent or temporary. It would be of clinical interest to determine if UCN2 mediated G1 phase arrest could be recovered, as this could provide a means of slowing aneurysm progression as medial smooth muscle cells are able to regenerate. These results and previous data from Chapter 4 provide a potential mechanistic pathway by which UCN2 may work to promote an aneurysmal phenotype of smooth muscle cells (Figure 5.12).

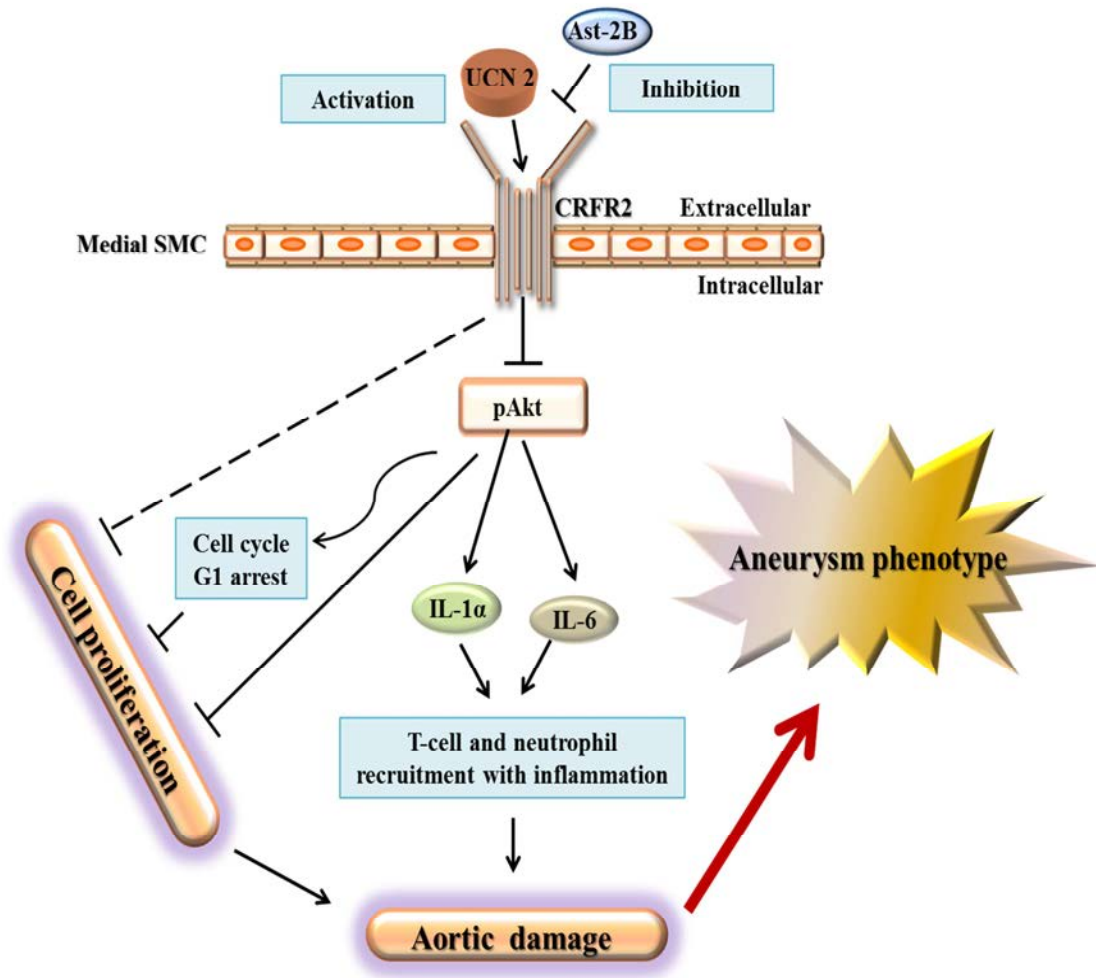


Figure 5. 12 Putative mechanisms by which UCN2 promotes an aneurysm phenotype

Dashed lines = tentative inhibition, solid lines = direct inhibition, dashed arrows = tentative stimulation, solid arrows = stimulation, Ast-2B = astressin 2B, Bcl-2 = B cell lymphoma 2, CRFR2 = corticotrophin releasing factor receptor 2, SMC = smooth muscle cells, IL-6 = interleukin six, pAkt = phosphorylated Akt, and UCN2 = urocortin 2

Taken together, these findings support a possible pathological role for UCN2 in AAA, although further investigations are required. It is proposed that UCN2 may serve as a possible therapeutic target in AAA, although further work is required to validate these findings.

The main findings from this Chapter are:

1. UCN2 inhibits Akt phosphorylation on HASMC
2. UCN2 increases proinflammatory cytokine IL-1 α and IL-6 secretion from human aortic smooth muscle cell
3. UCN2 inhibits human aortic smooth muscle cell proliferation
4. AAA thrombus conditioned media inhibits HASMC proliferation
5. UCN2 exerts no effect on HASMC apoptosis
6. UCN2 induces a G1 phase arrest in HASMC
7. Ast-2B abrogates the effects of UCN2 on HASMC
8. UCN2 may be promoting an aneurysm phenotype by inhibiting the pro-survival Akt signalling pathway, increasing proinflammatory cytokine secretion, and decreasing HASMC proliferation by arresting the cell cycle

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 GENERAL DISCUSSION

Abdominal aortic aneurysm (AAA) is an important cause of sudden death in older adults due to aortic rupture and other cardiovascular complications.³⁷⁴ Unlike other cardiovascular disease (CVD) including coronary heart disease (CHD), cerebrovascular disease, and peripheral artery disease (PAD), AAAs are usually asymptomatic, so diagnosis is often incidental when imaging to assess other health complaints.^{19,208} In addition, AAAs are often detected at a late stage often when the aortic diameters are ≥ 50 mm; consequently therapy is focussed on management of advanced disease pathology,⁵ by surgical intervention since the risk of rupture which is significantly associated with mortality is believed to be more important than perioperative risks for most patients.^{41,42}

Meticulous clinical examination by the attending physician may identify the presence of an aneurysm; however the majority of AAAs are not palpable particularly in overweight and obese individuals. This together with the unavailability of approved prognostic factors often results in a false-negative diagnosis. The advent of imaging technologies has resulted in an increase in the number of patients diagnosed with AAA. Ultrasound screening programs introduced in some countries have been demonstrated to effectively reduce mortality due to aortic rupture, although initial appraisal indicates that the incidence of AAA detected by these programs is lower than expected.^{214,215} There are also other problems associated with these imaging-based screening trials.²¹⁶⁻²¹⁸ One of the main issues is that such imaging based screening, primarily identified small AAAs, but are limited in their ability to identify “at risk” AAA which may expand to a size that may rupture or require intervention.¹⁹ Also repeat imaging assessments are necessary to monitor AAA expansion,⁴¹ particularly in the absence of medications to slow aneurysm progression and subsequent AAA rupture. Besides,

imaging-based screening and surveillance is expensive and not always accurate. Therefore, imaging alone is an inefficient way of detecting and monitoring AAA,^{19,208} and is reflected by significant shortfalls in patient management.³⁷⁷ Limited understanding of the pathophysiology of AAA remains a significant gap to be filled in order to elucidate the mechanisms underlying AAA pathogenesis so as to predict accurate and relevant biomarkers of diagnostic, prognostic and/or therapeutic potential for the disease. It has been proposed that the identification of circulating markers for AAA may serve as a means to improve and/or complement current AAA detection, diagnosis, prognosis and therapy.²²⁰

The precise mechanisms involved in AAA formation and progression is yet to be elucidated. It is generally accepted that AAA pathogenesis appear to be the outcome from interactions between environmental risk factors and genetic predisposition which exacerbate the normal ageing processes.⁷¹ Evidence abounds implicating inflammatory cytokines including interleukin-1 and 6 (IL-1 and IL-6), and tumor necrosis factor-alpha (TNF- α) in AAA pathogenesis.^{61,127,133,415} Some authors have suggested that angiotensin II (AII) is implicated in AAA pathogenesis.^{134,196} AII infusion has been shown to potentiate AAA initiation, formation and progression,^{196,416} and has formed the basis for the AII infused ApoE^{-/-} AAA mouse model. In recent years, the urocortins (UCNs) have been implicated in CVD pathogenesis.^{5,248,417} There appears to be a dichotomy as to whether these peptides play a beneficial role or a deleterious role in the cardiovascular system. The focus of this thesis was to investigate the role of the UCNs in AAA pathogenesis. The aims of the study were to:

1. Assess the relationship between the UCNs and AAA presence (Chapters 3 and 4);
2. Elucidate the possible mechanism by which UCN2 may promote an aneurysm phenotype (Chapter 5);

3. Assess the effect of blocking CRFR2 on UCN2 induced effects in AAA development (Chapter 5).

One of the major limiting factors in the animal model used in this project is the extent to which experimental aneurysm generated in the mouse model represents the human condition. There are no perfect experimental models of AAA; therefore investigations utilising animal models will remain limited and their relevance to the human condition will always be open to criticism. With this in mind, the majority of work in this study focussed mainly on the use of human samples and HASMC in defining the role of the UCNs in AAA. The initial part of this study concentrated on investigating the role of UCN3 in AAA pathogenesis based on previous data suggesting a possible role of UCN3 in the protection of the infrarenal aorta in ApoE^{-/-} mice to aneurysm formation.²⁰⁶ Infusion of ApoE^{-/-} mice with AII results in AAA formation which simulates the key characteristics found in the human condition with a few notable differences. First, a predominant suprarenal aneurysm in the mice compared to an infrarenal aneurysm in humans. Second, unlike in humans, there is wide spread tissue damage not generally limited to *tunica media* in the mouse model. Both animal and human studies presented in Chapters 3 and 4 provided strong evidence that UCN3 has limited implication in AAA pathogenesis. UCN3 protein was weakly expressed in the mouse aorta but not significantly different in mice with AAA compared to non-aneurysmal controls. Plasma UCN3 was below the assay limits of detection and not significantly different between the groups. CRFR2 protein expression was highly expressed in mouse aorta, but was not significantly different in mice with AAA and those without. It is possible that UCN2, the other analogue of UCN3 with a higher affinity for CRFR2, may play a more significant role via this receptor in this mouse model but due to low expression of the UCNs in mice tissue, this was not assessed. In addition, qPCR analysis of UCN3 expression in human AAA

biopsies demonstrated negligible expression of UCN3 in human AAA supporting the hypothesis that UCN3 plays little role in AAA pathogenesis.

Interestingly, both UCN2 and its receptor CRFR2 were demonstrated to be significantly upregulated in the diseased human aorta (AAA body) compared to relatively matched healthy aorta (AAA neck). Expression of UCN2 in human AAA was validated by immunohistochemistry staining of AAA biopsies in which UCN2 expression was high in diseased AAA body biopsies compared to their matched AAA neck biopsies and co-located with the presence of T- lymphocytes and neutrophils. AAA tissue biopsies also secreted high amounts of UCN2 in culture compared to controls which suggest a functional role for UCN2 in AAA pathogenesis. UCN1 gene expression was higher than UCN3 expression but was not significantly different between AAA body and neck biopsies. Possibly, an increase in the sample size may give sufficient power to enable a significant change in UCN1 gene expression levels to be demonstrated.

Examination of the cohort of 67 patients with AAA and 67 patients with non-aneurysmal PAD that were age and gender matched demonstrated a weak but positive correlation of plasma UCN2 with increasing aortic diameter. This data suggested that UCN2 may play a role in AAA progression. A stronger correlation factor however would be more convincing. The weak correlation may be due to the small sample size for this assessment resulting in insufficient power to detect a stronger association between UCN2 and AAA growth. This finding may provide a basis for the use of increased plasma UCN2 as prognostic marker identifying aneurysm growth rate in existing AAA patients under management.

Evidence presented in this thesis (Chapter 4) supports and helps to clarify the disparity between AAA and PAD. The data demonstrated that plasma UCN2 was 4.12 fold greater in patients with AAA compared to age and gender matched patient with non-aneurysmal PAD even after adjusting for other traditional cardiovascular disease risk factors. Although a low area under the ROC curve suggests that UCN2 will likely be more effective as complementary marker to established diagnostic imaging technique in AAA, however this needs to be verified in a larger cohort. As a result, one may speculate that increased circulating UCN2 may play a role in AAA pathogenesis. A recent study by Topal and colleagues suggested that UCN2 concentration was elevated within the serum of patients with mild to moderate systolic dysfunction.³⁵ Others have reported elevated levels of the UCN peptide family in CVD including heart failure and acute myocardial infarction.^{275,324-326} The general consensus from these studies was that elevated circulating UCNs may be related to an anti-inflammatory response denoting cardioprotective roles for these peptides. In contrast, data presented in this thesis suggest that it is more likely that elevated tissue and plasma UCN2 is deleterious with respect to AAA pathogenesis.

In Chapter 5, UCN2 was shown to inhibit Akt phosphorylation upon binding to CRFR2 with resultant decrease in cell proliferation, a G1 cell cycle arrest, increase secretion of proinflammatory IL-1 α and IL-6, but no effect on apoptosis. These effects of UCN2 on HASMC were abrogated by prior incubation of HASMC with Ast-2B the selective CRFR2 antagonist supporting a role for CRFR2 in UCN2 mediated effect on HASMC. UCN2 and AAA thrombus conditioned media dose-dependently inhibit HASMC proliferation at 24 and 48h time points. However, recombinant UCN2 had a more acute inhibitory effect on smooth muscle proliferation compared to AAA thrombus conditioned media, though AAA thrombus was demonstrated to secrete high amounts of UCN2 in culture. It is plausible to suggest that

the lesser effect of AAA thrombus media on HASMC proliferation compared to the recombinant UCN2 effect is due to other factors present in the media competitively vying for CRFR2 on HASMC. These data are supported by a previous study on cancer cells demonstrating that UCN2 inhibits proliferation via CRFR2.^{37,38}

Surprisingly, UCN2 exhibited no significant effect on HASMC apoptosis via the caspase-dependent pathways involving caspase-3/7 activation and Bcl-2 expression. It is possible that UCN2 may be acting through a caspase-independent apoptotic pathway, but this was not investigated. The apoptotic data presented here is in contrast to previous data demonstrating that UCN2 encourages apoptosis of macrophages,²⁴³ and tumour cells *in vitro*.³⁷ Based on the hypothesis that UCN2 may be inhibiting HASMC proliferation via regulation of the cell cycle, the effect of UCN2 on HASMC cell cycle progression was examined. The data presented here clearly demonstrated that UCN2 induced a G1 cell cycle arrest in HASMC. This implies that UCN2 may be preventing the regeneration of HASMC which may explain the scarcity of smooth muscle cell observed in advance stage AAA.²⁵ Allaire *et al.* have reported that a change in smooth muscle cell phenotype results in AAA formation,^{47,414} further strengthening the evidence presented here that UCN2 is pro-aneurysmal.

Abdominal aortic aneurysm has been associated with severe atherosclerotic damage as seen in PAD which historically is denoted as a key aetiological factor in disease formation.^{66,220} It is believed that both AAA and PAD share environmental and genetic risk factors but the mechanism underlying their development is distinct,²²⁰ for example most PAD results in arterial luminal stenosis which may result in compensatory extracellular matrix remodelling,^{418,419} and T-helper type 1/2 (Th1/2) immune responses have been reported to be

predominant in AAA relative to PAD.⁴¹⁵ This study demonstrated that UCN2 was upregulated in AAA and associated with an influx of T-lymphocytes and neutrophils. AAA is characterised by the degradation of extracellular matrix, loss of aortic smooth muscle cells, and infiltration of inflammatory cells including T-lymphocytes, macrophages and granulocytes that are major sources of proteolytic enzymes.²⁵⁻²⁷ Within the aortic wall, both smooth muscle cells and inflammatory cells have been suggested as possible sources of UCN2.^{33,34} UCN2 has been reported in various inflammatory environments,⁵ and was shown in this study to elevate the secretion of proinflammatory IL-1 α and IL-6. Increased IL-1 α and IL-6 has been associated with elevation of Th1/2 cytokines in AAA biopsies homogenates.⁴⁰¹ Elevation of IL-6 secretion within AAA tissue was also reported to correlate with poor prognosis in CVDs.^{317,318} In addition, UCN2 is known to be elevated in response to oxidative stress.⁴²⁰ This suggests that UCN2 is upregulated by aortic smooth muscle cells in response to injury, stress and/or extracellular matrix disruption. The data presented in this study demonstrated that elevated UCN2 may increase the recruitment of inflammatory immune cells, T-lymphocytes and neutrophils which further augment UCN2 expression. This results in increased expression and secretion of inflammatory cytokines (IL-1 α and IL-6) that promotes extracellular matrix degradation, aortic smooth muscle death and aneurysm progression. Data from Chapters 4 and 5 suggest that UCN2 may be detrimental in AAA and provides us with a putative mechanistic pathway by which UCN2 works to promote an aneurysmal phenotype (see Figure 5.12).

6.2 LIMITATIONS AND FUTURE DIRECTIONS

The present study has a number of limitations which have been listed within the relevant experimental chapters. Overall, in the animal study (Chapter 3), sample size may have been

underpowered and UCN3 expression was very low which may have affected the ability to detect an association between UCN3 and CRFR2 with AAA development. This may have affected the results presented here. Validation of findings using a more sensitive technique and a larger sample size will be required.

For the human investigation, the sample size used especially in the biopsy part of the study was limited, whilst in the *in vitro* studies, functional effects of UCN2 were investigated on one cell type (HASMC). The biopsy part of the study is further hindered by the difficulty in obtaining AAA biopsies from the decreasing number of patients opting for open aneurysm repair, as more patients are choosing endovascular aneurysm repair. It may be worthwhile to utilise donor samples in future investigations, however this comes with its own set of problems. The IHC studies focussed on determining the expression of UCN2 in the biopsies part of the human studies. It is possible that there may be a difference in CRFR2 receptor in humans, but this was not determined mainly because initial Western blotting demonstrated no significant distribution in CRFR2 protein between aneurysmal and non-aneurysmal mouse aortas in the animal studies (Chapter 3). The data presented here suggest that UCN2 may be co-locating with T-lymphocytes and neutrophils (Chapter 4), and a dysregulated immune response within the aortic wall is commonly accepted as a key factor contributing to human AAA development. Examination of the functional effects of UCN2 on immune cells including T- and B-lymphocytes, neutrophils, mast cells, macrophages and endothelial cells would further strengthen the findings presented here. It would be of interest to investigate the other functional effects of AAA thrombus conditioned medium on inflammatory cytokine secretion, Akt activation and apoptosis. Experiments incorporating UCN2 incubation with agents that could recover Akt activation would be ideal in determining whether UCN2 could modulate HASMC phenotype via non-Akt pathways.

6.3 SUMMARY

In summary, the present study demonstrated for the first time an association of UCN2 with AAA. UCN2 was demonstrated to be abundantly secreted from AAA biopsies and elevated within the plasma of patients with AAA, although a weak correlation with aortic diameter and a poor discriminatory ability indicate that UCN2 is unlikely to be a stand-alone marker for AAA. In multifactorial diseases such as AAA, it is unlikely that diagnosis will be made on the basis of a single circulating factor; rather it is more likely that next generation diagnostic tools will rely on the analysis of multiple indicators.⁷⁷ Future studies assessing the potential diagnostic value of UCN2 combined with other biomarkers are needed to more fully assess any clinical value of these findings.

In vitro, UCN2 inhibited HASMC Akt phosphorylation, increased IL-1 α and IL-6 secretion, inhibited HASMC proliferation, and induced a G1 cell cycle arrest, but had no significant effect on HASMC apoptosis. Taken together, these observations suggest an active role for UCN2 in AAA pathogenesis. Ultimately, a clinical trial is the final step in appraising the potential of UCN2 as a therapeutic target for AAA since laboratory studies are limited in their ability to create the ideal disease condition even with the use of animal models that closely simulate disease state. Investigations are also hindered by limited availability of aortic biopsies which are often received at end-stage of disease and the ethical quagmire associated with harvesting donor tissues. Nonetheless, to fully evaluate the value of UCN2 as a therapeutic, diagnostic or prognostic target for human AAA, several issues necessitate additional investigation. The following are of particular significance:

1. **Verification of UCN2 as a proaneurysmal agent.** This thesis came to the conclusion that UCN2 is deleterious and significant in human AAA pathogenesis and provides *in vivo*, *in vitro* and human association evidence to support this hypothesis.

Notwithstanding, human data remain mostly correlative. To further elaborate the functional significance of UCN2 in human AAA formation and progression, gene knockout technology can be applied. For instance, *in vivo* investigations utilising AII-infused apolipoprotein E deficient (ApoE^{-/-}) mice model of AAA can be executed with simultaneous ablation of UCN2 gene. Other options include the concomitant daily peritoneal injection of UCN2 or UCN2 antibody in AII infused ApoE^{-/-} mice model of AAA. Outcome would include aortic morphometry to determine aneurysm formation and growth rate, cytokine/chemokine profile at least as far as its importance in AII-induced changes within the aortic wall.

In contrast, similar studies could be performed *in vitro* by utilising *siRNA* to knock down the UCN2 gene expression in HASMC or any other cell type of interest. These cells would then be treated under various conditions including treating with AAA explant conditioned media and determining the effect of UCN2 knock down on processes such as apoptosis, proliferation and signalling.

2. **Investigation of existing UCN2-regulating agents in experimental AAA.** The present study has demonstrated that blocking the peripheral receptor for UCN2, CRFR2 with its antagonist, Ast-2B abrogated UCN2 induced functional changes on

HASMC. This suggests there is a potential benefit in blocking CRFR2 as a therapeutic option for AAA. Although further work needs to be done to clarify the benefits both in experimental models and clinically.

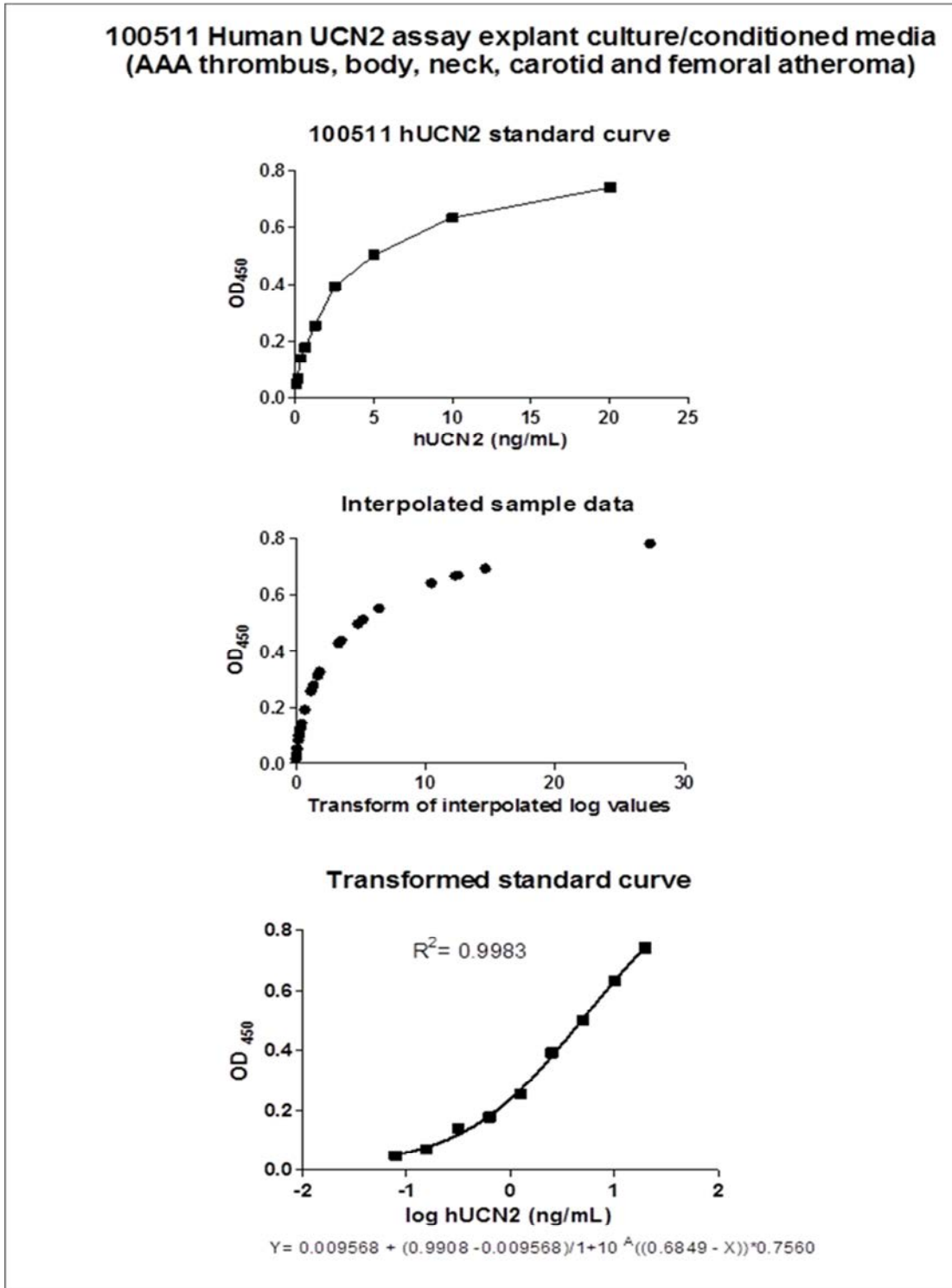
- 3. A better understanding of the molecular mechanisms of UCN2 action in AAA pathogenesis.** A significant effect of UCN2 on HASMC biology was demonstrated *in vitro*. These include inhibition of Akt phosphorylation, increased IL-1 α and IL-6 secretion, decreased HASMC proliferation and cell cycle G1 arrest, but no significant effect on apoptosis. An in-depth examination of UCN2 expression and effect on other cells relevant in AAA pathogenesis such as macrophages, T lymphocytes, neutrophils, mast cells may provide important information relating to the role of UCN2 within the vasculature. In addition, elaborating the UCN2 signalling mechanism could putatively identify additional therapeutic targets for AAA.

APPENDIX 1- ETHICS APPROVAL

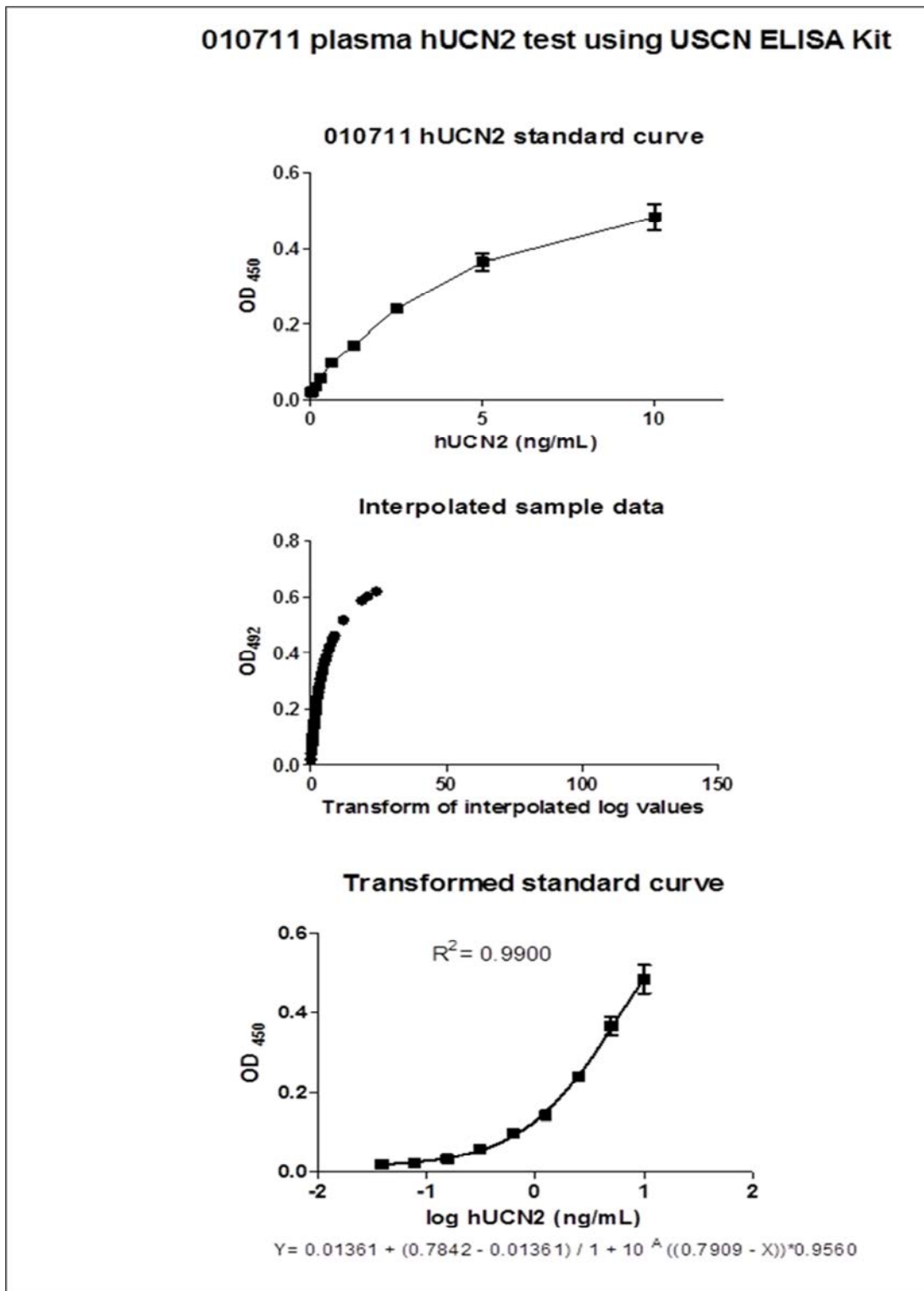
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APPENDIX 2- STANDARD CURVES

1. UCN2 Explant Cultures ELISA standard curve

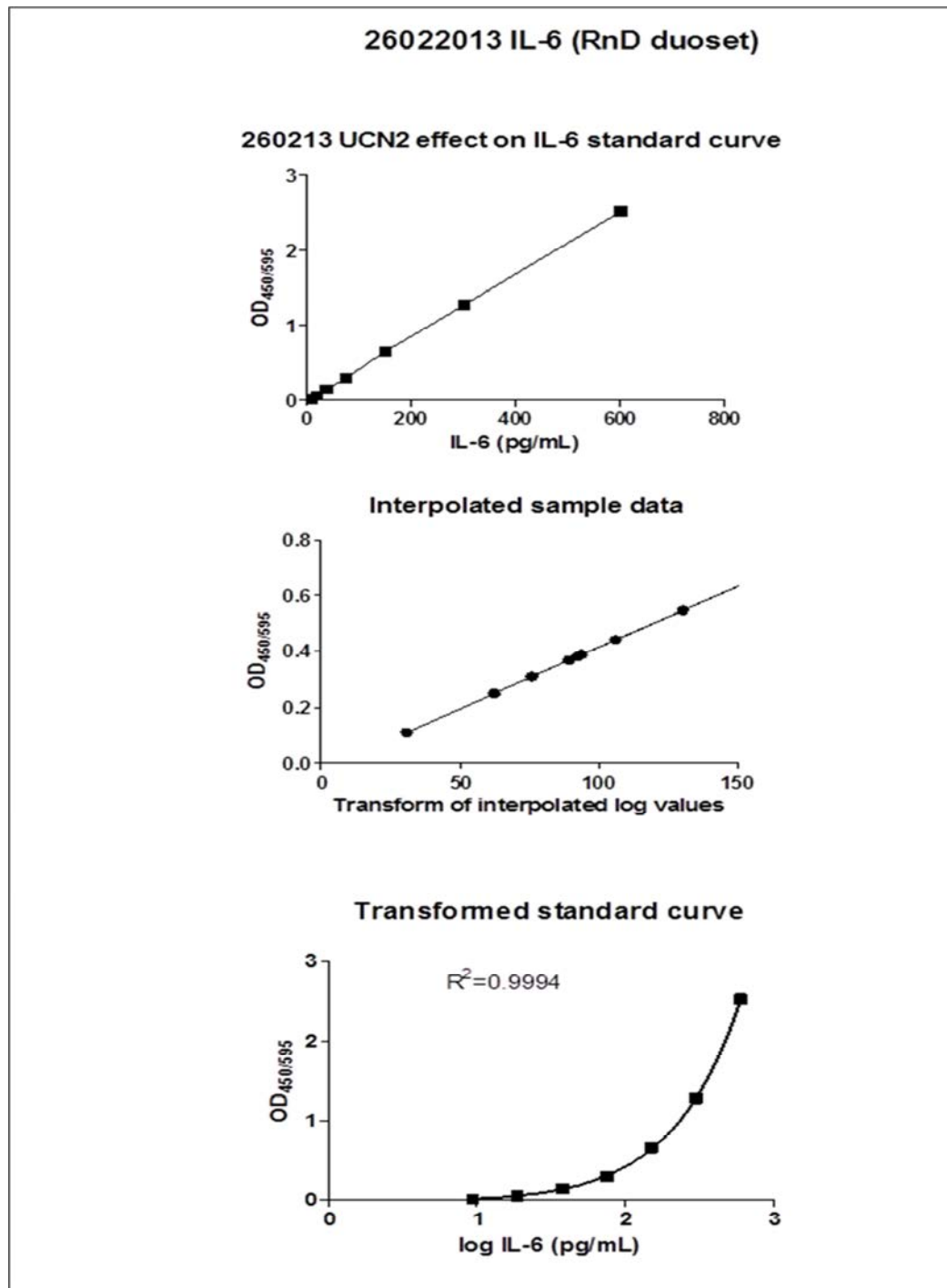


2. UCN2 Plasma ELISA standard curve

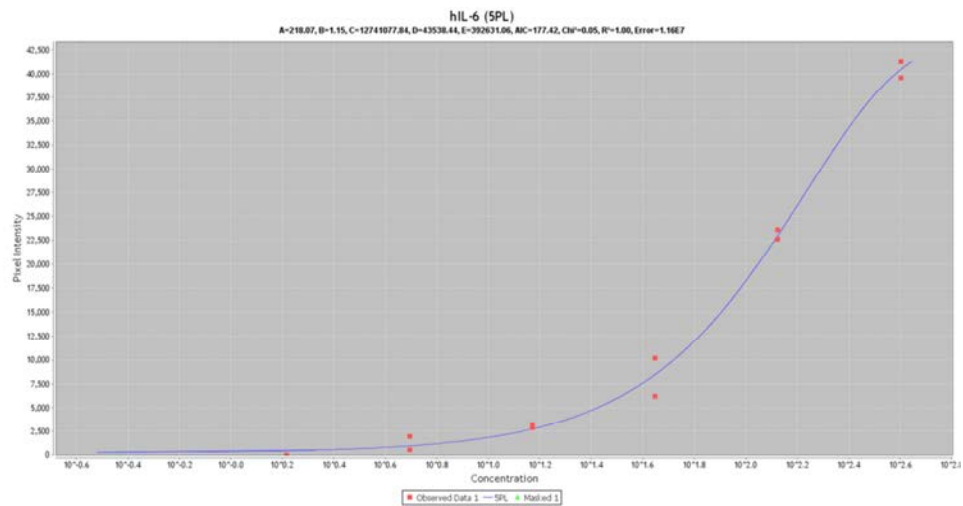
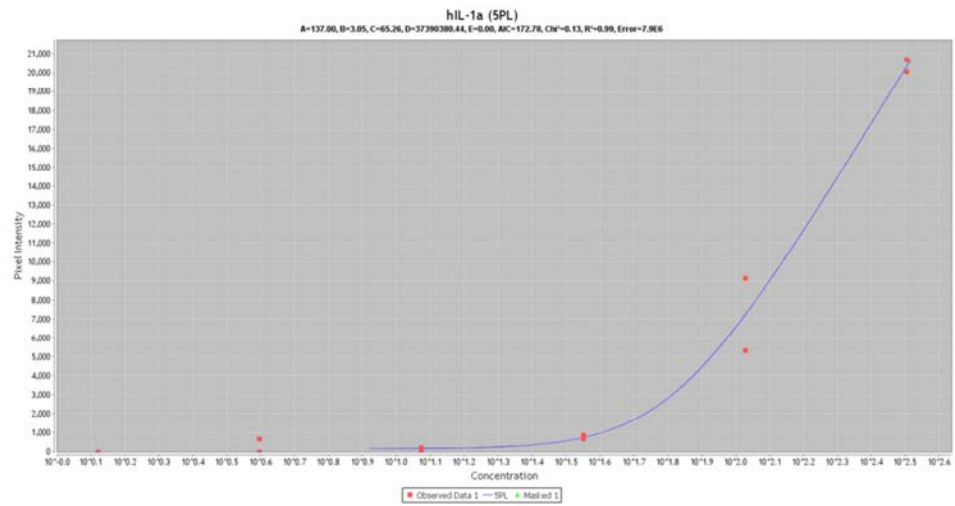
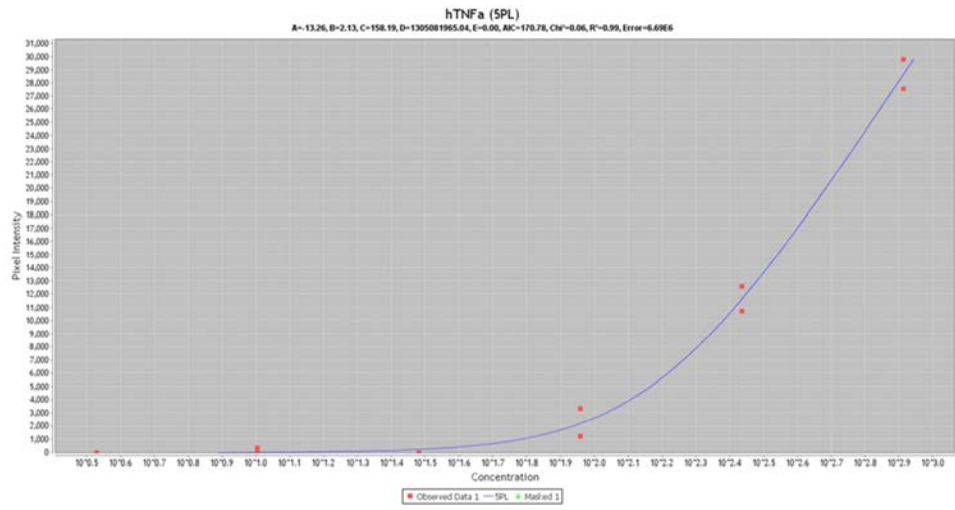


3. Standard curves for the cytokine studies

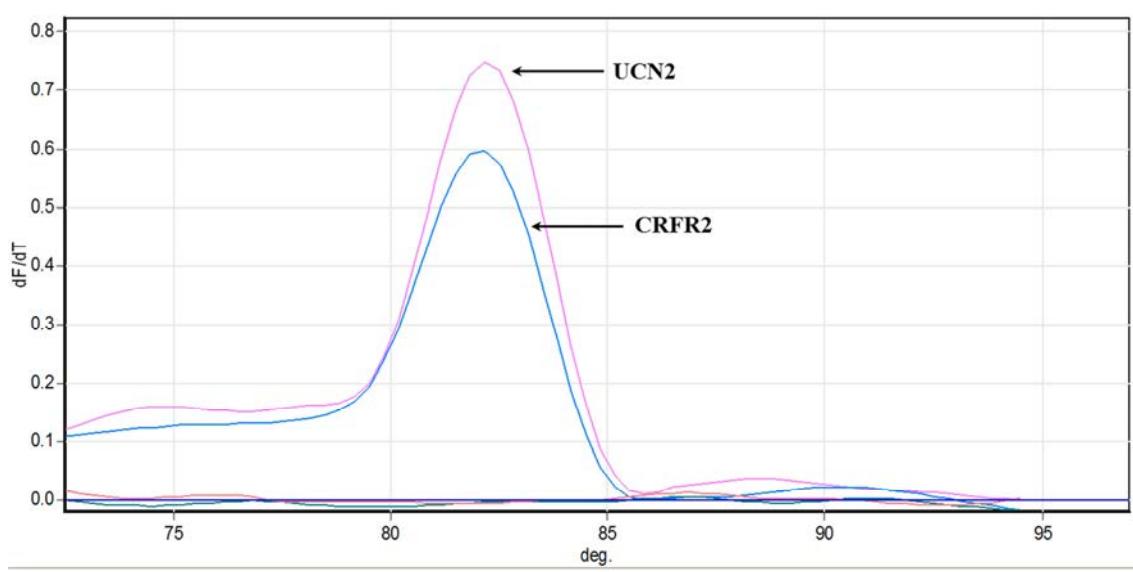
3.1 IL-6 Duoset ELISA standard curve



3.2 Multiplex assay standard curves



4. Representative Melt curve for UCN2 and CRFR2



APPENDIX 3- LIST OF SUPPLIERS

Abcam (Sapphire Biosciences, Redfern, NSW, Australia)

ALPCO diagnostics (Salem, NH, USA)

Amersham bioscience corporation (Piscataway, NJ, USA)

BD biosciences (North Ryde, NSW, Australia)

Beckham Coulter (Gladsville, NSW, Australia)

BIO-RAD (Gladesville, NSW, Australia)

BMG labtech (Allmendgruen, Ortenberg, Germany)

Direct Corporation (Bioscientific Pty. Ltd, Gymea, NSW, Australia)

Esaote S.p.A (Genoa, Italy)

Invitrogen (Mulgrave, VIC, Australia)

Kent Scientific Corporation (Torrington, Connecticut, USA)

Next Advance (Averill Park, NY, USA)

Nunc (Thermo Fischer Scientific, Scoresby, VIC, Australia)

Promega (Alexandria, NSW, Australia)

ProSciTech (Kirwan, QLD, Australia)

Qiagen Pty Ltd (Doncaster, VIC, Australia)

Quansys Bioscience (Logan, Utah, USA)

Roche (Castle Hill, NSW, Australia)

R&D Systems (Sapphire Biosciences, Redfern, NSW, Australia)

SAbiosciences (SA, Australia)

Sarstedt (Ingle Farm, South Australia, Australia)

Sigma-Aldrich (Castle Hill, NSW, Australia)

Thermo Fischer Scientific (Scoresby, VIC, Australia)

USCN Life Sciences (Wuhan, China)

Vector Labs (Abacus ALS, Brisbane, QLD, Australia)

APPENDIX 4- REAGENTS AND SOLUTIONS

DMEM CULTURE MEDIUM

(Working Solution)

4.5g glucose

10% heat inactivated FBS

4 mM L-glutamine

2.5 $\mu\text{g}/\text{mL}$ amphotericin

100 U/mL penicillin G

100 U/mL streptomycin

0.05 mg/mL gentamicin

1 x non-essential amino acids

20 mM HEPES (pH 7.5)

10 μM β -mercaptoethanol

2.4 $\mu\text{g}/\text{mL}$ sodium bicarbonate

10 nM sodium pyruvate

PROTEIN EXTRACTION BUFFER (BUFFER E)

1% Triton X 100

0.1% SDS

10 mM Cacodylic acid

40 mM Tris.HCl, pH 7.4

Cocktail of protease inhibitors (Roche)

LYSIS/WASH BUFFER FOR IMMUNOPRECIPITATION

0.025 M Tris

0.15 M NaCl

0.001 M EDTA

1% NP-40

5% glycerol, pH 7.4

LAEMMLI BUFFER

0.125 M Tris.HCl

4% (w/v) SDS

20% (v/v) glycerol

10% (v/v) β -mercaptoethanol

0.004% bromophenol blue; pH 6.8

TRIS-BUFFERED SALINE (TBS BUFFER)

50 mM Tris.HCl

150 mM NaCl

0.05% (v/v)

TBST BUFFER

50 mM Tris.HCl

150 mM NaCl

0.05% (v/v) Tween-20, pH 7.6

0.25% TRYPSIN SOLUTION

2.5g/L Trypsin

0.38g/L EDTA

A 0.05% solution was prepared by diluting in DMEM media

WESTERN BLOT TRANSFER BUFFER

3 g Tris-base

14.4 g glycine

20% (v/v) methanol

Water up to 1 L

WESTERN BLOT BLOCKING BUFFER

2% (w/v) FBS

50 mM Tris.HCl

150 mM NaCl

0.05% (v/v) Tween-20, pH 7.6

HYPOTONIC STAINING BUFFER FOR DNA

0.25g Sodium Citrate

0.75mL Triton-x 100

0.025g Propidium iodide

0.005g Ribonuclease A

250mL Water

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