# Interleukin-1α as a biomarker of human abdominal aortic aneurysm (AAA) development and progression

By

Miss Mehtab Ahmad BMedSci(Hons), MB ChB, MRCS(Ed)

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From

The University Department of Vascular Surgery, Heart of England NHS Foundation Trust, Birmingham, UK

And

University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK

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## Abstract

Current strategies to treat human abdominal aortic aneurysm (AAA) centre on maximal aortic size, which is at present the best-known predictor of AAA progression and impending rupture. However, the natural history of AAA remains poorly defined and most people with the disease die with their AAA from other co-morbidities, rather than from their aneurysm. An AAA intervention strategy based on size alone most likely leaves some patients with small ('low risk') AAA unprotected from rupture, and others with larger ('high risk') AAA exposed to unnecessary surgery with its associated significant morbidity and mortality. There is thus an urgent need to identify a surrogate marker for AAA growth, progression and need for intervention.

This Thesis presents an analysis of the role of interleukin (IL)-1α (IL-1α) as a potential future surrogate biomarker for AAA. It is the only research work to date to have looked into the role of IL-1α as a biomarker in AAA disease, correlating titres with different anatomical, morphological and patient-related factors. It is the first piece, in over 20 years of published literature, to have performed a robust methodology study on the measurement of IL-1a in serum samples using different techniques. A comparison study of commercially available immunoassays in the context of IL-1α has never been undertaken before, and we are the first to undertake one. Additionally the work on the natural history of AAA is one of the largest single-centre cohort studies to analyse AAA growth in surveillance.

The work covers three main areas: identifying why current strategies for monitoring AAA are ineffective, analysis of different serum processing methodologies and

commercially available immunoassays used to measure IL-1α, and linking IL-1α to different anatomical, morphological and patient-related AAA factors.

## Dedication

I would like to dedicate this work to the memory of my mother, Farida Ahmad.

## Acknowledgements

First and foremost, I would like to thank my supervisors Professor Andrew Bradbury and Professor Rajiv Vohra for supporting me through the long, often tortuous journey that producing this work has been. Without them, I would not have had the opportunity to develop skills in research methodology and further my understanding of aneurysm disease. You have both shown relentless enthusiasm for seeing my work to completion, and I am grateful for it.

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## Collaborators

Through the course of this work, collaborations with the following co-investigators have been formed (in alphabetical order):

- Mr Donald Adam, MD FRCSEd, Consultant Vascular and Endovascular Surgeon, HEFT
- Mr Martin Claridge, MD FRCS, Consultant Vascular and Endovascular Surgeon, HEFT
- Dr Murray Clarke, PhD, Division of Cardiovascular Medicine, Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge
- James Hodson, Statistician, Department of Statistics, Wolfson Computer Laboratory, University of Birmingham, Birmingham, UK
- Dr Sahithi J Kuravi, MSc, MRes, PhD, Postdoctoral Research Fellow, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham
- Dr Souad Messahel, PhD, Human Biomaterials Resource Centre, College of Medical and Dental Sciences, University of Birmingham
- Mr Rakesh Mistry, undergraduate medical student, University of Birmingham
- Professor Gerard Nash, PhD, Professor of Cardiovascular Rheology and Section Lead: Cardiovascular and Respiratory Sciences, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham

- Professor Ed Rainger, PhD, Reader in Chronic Inflammation, Cardiovascular and Respiratory Sciences, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham

- Dr Jane Steele, PhD, Director, Human Biomaterials Resource Centre, College of Medical and Dental Sciences, University of Birmingham

In addition, through the course of this work, contacts have been established with the following institutions:

- Clinical Immunology Service (CIS), School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham

- Human Biomaterials Resource Centre (HBRC), College of Medical and Dental Sciences, University of Birmingham

- Medical Innovation Development Research Unit (MIDRU), Heartlands Hospital, Birmingham

During the course of some of this work, until November 2013, we have also held the patent for IL-1α as a biomarker for AAA through the University of Birmingham, Alta Innovations Alta Innovations Ltd, Birmingham Research Park, B15 2SQ (UK GB2480292; International - WO2011141746].

## Ethical approval

The project was approved by the NRES Committee West Midlands (Solihull) on 24 August 2012 (REC 12/WM/0204)

## Project sponsor

**The University of Birmingham**

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## List of Abbreviations and Acronyms

AAA Abdominal aortic aneurysm

ADAM The Veterans Affairs Aneurysm Detection and Management (ADAM) Study

ARDS Acute respiratory distress syndrome

BMT Best medical therapy

BPH Benign prostatic hypertrophy

CCF Congestive cardiac failure

CIS Clinical Immunology Services

cm Centimetres

COPD Chronic obstructive pulmonary disease

CPEX Cardiopulmonary exercise testing

CRP C-reactive protein

CT Computed tomographic

CTA Computerised tomography angiogram

CTnT Cardiac troponin-T

CVA Cerebrovascular disease

DES Desmosine

DIC Disseminated intravascular coagulation

DM Diabetes mellitus

DPD Deoxypyridinoline

EC Endothelial cell

ECM Extracellular matrix

ELISA Enzyme-linked immunosorbent assay

EPR Electronic patient records

ESRF End-stage renal failure

EVAR Endovascular aneurysm repair

HEFT Heart of England NHS Foundation Trust

HBRC Human Biomaterials Resource Centre

hs-CRP Highly-sensitive CRP

ICAM Intracellular adhesion molecule

IFN Interferon

IGF Insulin-like growth factor

IHC Immunohistochemistry

IHD Ischaemic heart disease

IL- Interleukin

IL-1α Interleukin-1 alpha

IL-1R1 Interleukin-1 receptor type 1

IL-1RA Interleukin-1 receptor antagonist

IL-1RAcP IL-1 Receptor Accessory Protein

ILT Intra-luminal thrombus

IMA Inferior mesenteric artery

IRAK IL-1 receptor associated kinase

isoDES Isodesmosine

Jnk c-Jun kinase

KDa Kilodalton

Lp(a) Lipoprotein(a)

MAPD Maximum anterior-posterior diameter

MAPKs Mitogen-activated protein kinases

MCP Monocyte chemoattractant protein

MDT Multidisciplinary team

mm Millimetres

MMP Matrix metalloproteinase

MODS Multiple organ dysfunction syndrome

MRI Magnetic resonance imaging

MyD88 Myeloid differentiation factor 88

NF-κB Nuclear factor κB

NHS-AAA-SP NHS AAA Screening Programme

NK-cells Natural killer cells

OD Optical density

OR Open aneurysm repair

OOR Out of range

oxLDL oxidised-LDL

PAD Peripheral arterial disease

Pg/ml Picogram/millilitre

PYR Pyridinoline

QIP Quality Improvement Programme

RA Rheumatoid arthritis

RCT Randomised controlled trial

RT Room temperature

SA-PE Streptavidin-phycoerythrin

SD Standard deviation

SIRS Systemic inflammatory response syndrome

SMA Superior mesenteric artery

SMC Smooth muscle cells

SNPs Single nucleotide polymorphisms

SS Systemic sclerosis

SST Serum separating tubes

SWBH Sandwell and West Birmingham Hospitals NHS Trust

TAA Thoracoabdominal aneurysm

TACE Tumour necrosis factor-α converting enzyme

TAT Thrombin antithrombin complex

TGF Transforming growth factor

TIMP Tissue inhibitor of metalloproteinases

TNF Tumour necrosis factor

tPA Tissue plasminogen activator

TRAF6 TNF receptor associated factor 6

UHB University Hospitals Birmingham NHS Foundation Trust

UK-NAAASP UK National AAA Screening Programme

UKSAT UK Small Aneurysm Trial

US Ultrasound scan

UND Undetectable

VCAM-1 Vascular cell adhesion molecule-1

VSGBI Vascular Society of Great Britain and Ireland

WCC White cell count

## Preface

This MD thesis is submitted to the University of Birmingham as fulfilment of the degree of Doctor of Medicine (MD). It has been supervised by Professor Andrew Bradbury and Professor Rajiv Vohra.

This thesis represents my research work between October 2012 and April 2014 at the University Department of Vascular Surgery, Heart of England NHS Foundation Trust and University Hospitals Birmingham NHS Foundation Trust.

# Table of Contents

# Chapter 1: Introduction

The purpose of this research work has been to assess the utility of IL-1α as a potential biomarker for human abdominal aortic aneurysm (AAA) progression. The work covers three main areas: identifying why current strategies for monitoring AAA are ineffective; analysis of different serum processing methodologies and commercially available immunoassays used to measure IL-1α; and the relationship between IL-1α and different AAA anatomical, morphological and patient-related factors.

In order to conduct this work, it has been necessary to firstly ascertain an understanding of the natural history of AAA in our own patient population, and other biomarkers which have already been postulated to correlate with AAA disease. We have then used this knowledge to develop a rationale for why IL-1α may be a suitable candidate to identify those AAA which warrant treatment. This includes analysis of a large database of patients with AAA at Heart of England NHS Foundation Trust (HEFT). Following from this, we have recruited and tested serum samples from patients with AAA for IL-1α, having first undertaken a robust methodology study to validate sample processing and immunoanalysis techniques. Finally we have correlated this with various AAA patient and morphological factors.

The Thesis is presented in segments to illustrate the progress of the research. The first part sets the scene, by identifying the on-going need for biomarker development in AAA through discussion of biomarkers which have been identified before, and their shortcomings. The second part describes why IL-1α should be a focus of interest in AAA disease. Thirdly, it addresses the methodology used to test IL-1α

using commercially available immunoassays The results from this methodology study are then presented, before a final analysis of IL-1α in patient recruits.

**Chapters 2 and 3** are an overview of the anatomy of the human aorta, its structure, composition and the biomechanics of blood flow through it as well as a description of human (AAA), their incidence, pathogenesis and treatment options.

**Chapter 4** describes biomarkers that have already been considered in the literature as potential AAA biomarkers.

**Chapter 5** provides a description of IL-1α and its role to date in the pathogenesis of cardiovascular disease.

**Chapter 6** presents the results from an analysis of the natural history of AAA in our own patient population at HEFT.

**Chapter 7** presents the methodology study from which IL-1α results were attained.

**Chapter 8** presents the results of Study 1, looking at serum IL-1α titres in patients with asymptomatic, infra-renal AAA size, morphology and growth rates.

**Chapter 9** presents the results from Study 2, looking at serum IL-1α as a marker of chronic inflammation in patients with asymptomatic AAA.

**Chapter 10** summarises the work and draws general conclusions from all of the studies, including ideas for potential future areas of research in the field.

**Thesis hypothesis and aims**

We hypothesise that IL-1α can be utilised as a surrogate marker for AAA progression in those with asymptomatic, infra-renal AAA. We speculate that IL-1α titres are elevated in those with AAA, and that these titres correlate with AAA size, morphology and patient-dependent factors such as co-morbidity.

The aims of this Thesis are to:

1. Establish the natural history of AAA growth, identifying growth rates in our own patient population with AAA at HEFT.
2. Investigate how best to measure IL-1α in a reliable, reproducible fashion using commercially available immunoassays.
3. Investigate the relationship between IL-1α in a cohort of patients with asymptomatic, infra-renal AAA.
4. Accumulate data on which to base future studies investigating IL-1α as a biomarker of AAA disease.

**Summary**

The proposed study is, to the best of our knowledge, the first to investigate changes in circulating levels of IL-1α in patients with asymptomatic infra-renal AAA.

## Chapter 2: Anatomy, structure and function of the aorta

### Aims

The aim of this chapter is to provide a basic understanding of the anatomy of the human aorta and its role as a conduit for blood in the body. Additionally, the composition of its wall and natural changes which occur with aging are described.

### Anatomy of the human aorta

The human aorta is the largest artery of the body, arising from the left ventricle of the heart. It supplies oxygenated blood via unidirectional flow to all organs and peripheral tissues thus maintaining perfusion, and blood pressure, through its elastic properties. It has four distinct anatomical segments: the ascending aorta; aortic arch; descending thoracic aorta; and abdominal aorta. The ascending aorta begins at the aortic orifice of the left ventricle, which is the point of attachment of the cusps of the aortic valve. At this point, its normal diameter is approximately 30mm. It ascends approximately 5cm to the sternal angle, at which point it becomes the aortic arch, which then passes posteriorly in a curved fashion, to the left of the trachea and oesophagus. The thoracic aorta is a continuation of the arch arising at the level of the second sterno-costal joint. Its normal diameter is approximately 25mm, and it descends in the posterior mediastinum through the thoracic cavity to the diaphragm to the left of the vertebral column.

The abdominal aorta begins as the level of the diaphragmatic aortic hiatus, at the level of the T12 and L1 vertebral discs and is approximately 13cm in length. The normal diameter for the abdominal aorta is 18mm to 20mm. It is a retroperitoneal structure lying anterior to, and against, the vertebral bodies as it passes just to the left of the midline. It terminates at the level of the L4 vertebra by bifurcating into the

paired common iliac arteries (CIA), which diverge and descend infero-laterally to the pelvic brim where they bifurcate into the internal and external iliac arteries.

The branches of the abdominal aorta include unpaired visceral branches from its anterior surface (the coeliac trunk at T12 vertebra, the superior mesenteric artery (SMA) at L1 and the inferior mesenteric artery (IMA) at L3), paired visceral branches arising from its lateral surface (the renal arteries at L1 and the gonadal arteries at L2) as well as paired and unpaired parietal branches supplying the posterior abdominal wall, vertebral column and spinal cord.(1)

Like all arteries, the aorta is comprised of three histologically distinct layers, the intima, media and adventitia. The intima is composed of a thin layer of endothelial cells (EC), sub-endothelial connective tissue and an internal elastic lamina. The media consists of smooth muscle cells (SMC) and extracellular matrix (ECM). It is the most prominent layer in large vessels such as the aorta, and is responsible for the pressurised transmission of blood from the heart. The outermost layer, the adventitia, is made of connective tissue which enfolds its own vascular supply (the vasa vasorum), as well as autonomic nerves (nervi vascularis) which supply and control the smooth muscle of the media (**Figure 1**).(2)

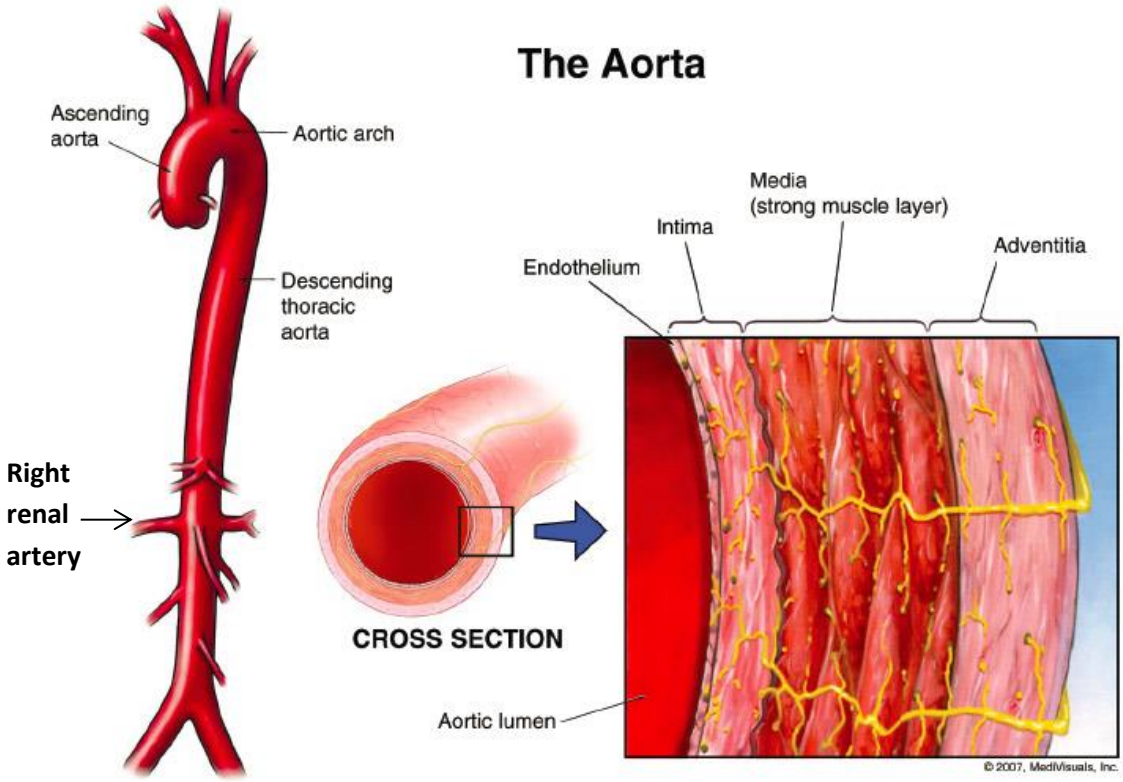


**Figure 1. Anatomy of the human aorta, including cross sectional representation of distinct histological layers.**(3)

**Structure and function of the human abdominal aorta**

The aorta is the body's largest elastic artery, serving as a conduit for the passage of oxygenated blood from the heart, through the entire arterial tree to supply distal tissues as well as an elastic reservoir maintaining blood pressure throughout the cardiac cycle.

The structure of the aortic wall is dependent on the relative proportions of two major proteins which form the ECM: elastin and collagen, both of which are vital in determining aortic tensile strength and wall stiffness. In health, the aortic wall contains concentric layers of smooth muscle, elastin and collagen with the number of

medial elastin layers decreasing from the thoracic aorta distally into the infra-renal aorta, where there is medial thinning in exchange for intimal thickening.(4)

Elastin acts as the primary load-bearing component structure by forming organised cross-linked filaments using desmosine (DES) and isodesmosine (isoDES). These cross-links enable aortic stretch and recoil. Collagen within the aorta acts as the connective component, providing support and form through its abundance within the aortic ECM. Its fibres are stabilised by cross-links formed primarily by pyridinoline (PYR) and also deoxypyridinoline (DPD).(4) Although at least 29 different types of collagen are known to exist within the human body, the predominant aortic types are fibrillar types I and III, which account for >90% of all collagenous content, and then lesser amounts of types IV, V, VI and VIII.(5)

During the natural aging process, particularly after the age of 50, there is a decrease in the total aortic collagen and elastin content, however their overall concentrations increase due to a global degeneration of aortic components which outweighs the degeneration of elastin and collagen. Relative to each other, elastin degradation exceeds that of collagen, so there is a relative increase in the collagenous content of the aorta, which contributes to increased arterial wall stiffness. Increased stiffness reduces compliance and induces an increase in pulse-wave velocity within the systemic circulation which alters normal pulse wave reflections in the cardiac cycle, with early return in late systole resulting in eventual ventricular hypertrophy by placing greater load on myocardial muscle as well as increasing central pulse load.(6)

**Summary**

The aorta is the human body's main conduit for blood, providing the entire systemic circulation with oxygenated blood. Its structure allows for elastic recoil, and the

content of elastin and collagen within the aortic ECM account for its strength and form. The natural aging process alters the relative concentrations of these component proteins, causing increased aortic wall stiffness.

# Chapter 3: Human abdominal aortic aneurysms (AAA)

## Aims

The aim of this chapter is to provide an understanding of the pathophysiology of AAA, including its epidemiology, the rationale for disease surveillance and screening, and interventions used to treat the condition.

## Incidence, pathogenesis and options for treatment

The origins of the word aneurysm come from the Greek word ‘aneurusma’ meaning dilatation, or widening. Aneurysms can occur along the path of any vessel, but an abdominal aortic aneurysm (AAA) can be defined as a focal, irreversible dilatation of all three layers of the aortic wall exceeding 50% of the normal vessel diameter.(7) Although there is individual variability in vessel diameters, and ‘normal’ size can vary with age, gender and body habitus, the upper limit of normal in the human infra-renal aorta is <30mm, and slightly larger for the supra-renal segment.(8,9) By definition, therefore, the abdominal aorta is said to be aneurysmal once it has reached at least 30mm in diameter. The majority of AAAs develop in the segment below the renal arteries, up to the aortic bifurcation into the paired common iliac vessels. Aneurysms at this point are known as infra-renal aneurysms. It has been postulated that the reasons for the predominance of infra-renal AAA over juxta- or supra-renal AAA, are due to changes in the composition of the aortic wall below the renal arteries, particularly the destruction of elastic lamellae in its medial segment.(10)

Population-based screening studies suggest that the prevalence of infra-renal AAA is between 4.1 to 11.5% in European men ≥65 years, and 1.3% in women, and that the global incidence has continued to rise for the past four decades peaking in the eighth decade of life.(11–16)

A multitude of factors are implicated in the pathogenesis of AAA with increasing age, male gender, smoking and a positive family history for the disease as the strongest risk factors. Other associations include hypertension and possibly hypercholesterolaemia as well as a poorly defined genetic predisposition, with certain races (particularly Caucasians) being more susceptible to AAA development.(17–21) Women are less likely to develop AAA, but once they do, have an increased risk of aneurysm rupture and aneurysm-associated mortality.(22)

In recent years there has been increasing interest in the biological properties of AAA, their composition and localised structural changes within the aortic wall which lead to expansion and rupture. Histopathologically, AAA are characterised by the loss of elastin and collagen from the media resulting in reduced vessel wall compliance and tensile strength. The proportional losses of elastin are greater than that of collagen however, and studies suggest that there is an increase in cross-link formation in old collagen in aneurysmal disease, with a defective collagen biosynthesis.(4) It is widely accepted that AAA represents a chronic inflammatory condition separate from atherosclerosis with which it nevertheless often co-exists due to shared risk factors.[6] Microscopically, nearly all AAA are characterised by some degree of inflammation, with the degree of inflammatory infiltrate, containing large numbers of activated macrophages and T-cells within the media and fragmentation of the elastic lamellae, correlating with AAA size.(23)

**Atherosclerosis as a causative risk of AAA**

Atherosclerosis is thought to result as a ‘response to injury’ from substances such as oxidised low-density lipoprotein (LDL), by-products of cigarette smoking, diabetes and hypertension which elicit localised inflammatory reactions in vessel walls.(24,25) The initial lesions are fatty streaks, which are focal increases of

lipoproteins within the intima.(26) These fatty streaks are clinically asymptomatic, and can either involute or progress to fibrous plaque formation. They originate from blood-born monocytes which then differentiate into macrophages, engulfing sub-intimal LDL, particularly small density LDL, remnant lipoproteins and lipoprotein(a). In doing so, they take on an engorged, foamy appearance, giving rise to the name 'foam-cells'. Accumulation and progression of foam cells results in plaque formation (**Figure 2**).(27)

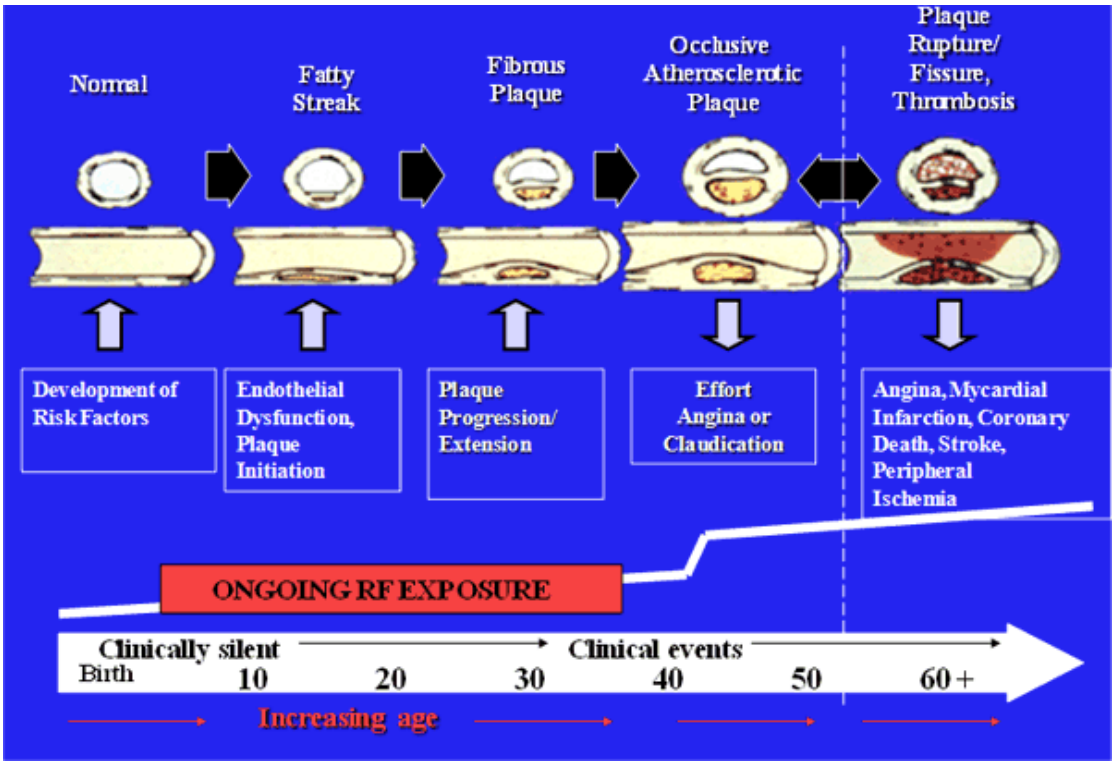


**Figure 2. Pathological progression of atherosclerosis.**(28)

There are two predominant schools of thought as to why atherosclerosis is important in the pathophysiology of AAA. One theory is that environmental and genetic factors result in aortic atherosclerosis. Resultant remodelling, intimal thrombosis, and pro-inflammatory cytokine release stimulates secondary matrix degradation and adventitial inflammation to promote AAA development. The second is that environmental and genetic stimuli directly affect the vessel intima leading to

adventitial inflammation which directly causes AAA formation. Atherosclerosis formation then occurs as a secondary local event.

**The vascular inflammatory response**

In normal circumstances, vascular endothelial tissue is resistant to leukocyte adhesion. In the presence of pro-inflammatory stimuli however, there is endothelial expression of adhesion molecules resulting in attachment of monocytes and circulating lymphocytes. The mediators involved in cell-adhesion include P-selectin, E-selectin, intracellular adhesion molecule- (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are present in endothelial cell walls.(26,29) Chemoattractants such as oxidised-LDL (oxLDL), Lp(a), monocyte chemoattractant protein (MCP-1), IL-1 and TNF-α allow sub endothelial migration of monocytes and T-lymphocytes. Studies have demonstrated that deficiency in MCP-1, or its receptor CCR-2, in mice significantly reduces atherosclerotic lesion development, suggesting that MCP-1 may be the most important chemoattractant factor in the disease process. Within the intima, monocytes mature into macrophages under stimulus from macrophage colony-stimulating factor.(30,31)

Although the natural history of AAA remains poorly understood, in broad terms it is of gradual aortic expansion over time until rupture occurs. Unfortunately, most AAAs remain asymptomatic until rupture, which is fatal in up to 85% of cases.(32) Mortality from ruptured AAA remains a significant cause of death, accounting for approximately 2500 deaths in men aged ≥60 in England and Wales per year, or 2.5% of all deaths in males in that age group.(33,34) These figures are expected to reduce though, due to the advent of a national UK-wide NHS AAA Screening Programme (NHS-AAA-SP) in 2013, which should mean that patients with the disease are detected early and monitored closely. At present, the best-known predictor of AAA

rupture is considered to be maximum anterior-posterior diameter (MAPD), however as noted above, other factors may be equally or even more important in certain patients. Studies suggest that the 5-year risk of rupture in AAA < 50mm is 5%, 50 to 60mm is 25%, 60 to 70mm is 35% and in AAA>70mm is 75%. The UK Small Aneurysm Trial (UKSAT) and The Veterans Affairs Aneurysm Detection and Management (ADAM) Study have shown that the risk of rupture in AAA less than 55mm MAPD is small at approximately 1% per year and that routine surgical intervention in asymptomatic AAAs less than 55mm confers no benefit.(35–38)

UKSAT: recruited 1090 patients with small (40 to 55mm) AAA and randomised them into early open aneurysm repair (OR) or an ultrasound (US)-based surveillance programme. There was a short term survival disadvantage in those undergoing OR but no significant difference in mortality between groups at 2, 4 and 6 years of follow-up.(36)

ADAM: recruited 1136 patients with small (40 to 55mm) AAA and also randomised them into early OR or an US-based surveillance programme, with OR being offered when the AAA reached >55mm. ADAM concluded that early OR of small AAA did not increase long-term survival and that deferring OR of small AAA until they had reached 55mm did not increase operative mortality.(37)

**The (changing) epidemiology of AAA**

In its 2008 publication ‘Fact file: Abdominal Aortic Aneurysms’, the British Heart Foundation stated that ‘‘Ruptured aortic aneurysms are the 13th commonest cause of death in the UK, responsible for 12,000 deaths per year……The incidence of both AAA and ruptured AAA continues to increase year on year.’’(39)

In fact, the incidence of ruptured AAA has reduced significantly in the UK in recent years and continues to do so.(40) Although this reduction pre-dates the NHS-AAA-SP it still seems sensible to detect AAA as early as possible in the hope that lifestyle advice and medical treatment will not only prevent AAA disease progression, so reducing the numbers of patients requiring intervention, but also reduce the high rates of non-AAA related cardiovascular morbidity and mortality observed in this patient population.

One problem with the NHS-AAA-SP is that on reflection, given the changing epidemiology, the decision to screen at 65 years (which was based on historical data) may have been incorrect. In fact, at this relatively young age far fewer AAAs are being detected than was anticipated, raising questions of the cost-effectiveness of the programme as it currently operates. In addition to formal screening, the increasing use of radiological investigations for other pathologies means that AAA are being increasingly identified as incidental findings.(40,41)

It can therefore be argued that the current problem with AAA is not primarily that they rupture (in fact the observed rupture risk is quite low, and through early detection and intervention is likely to reduce further still in at-risk patients), but the excess non-AAA related cardiovascular morbidity and mortality seen in this patient group, irrespective of whether they have intervention or not.

With the advent of endovascular aneurysm repair (EVAR) it was suggested that the MAPD intervention threshold for AAA repair, which (rightly or wrongly) currently stands at 55mm, might be reduced on the grounds that:

1. EVAR is associated with less 30-day morbidity and mortality than OR

2. By waiting until the AAA were ‘large’ (MAPD >55mm), the ‘window of opportunity’ for EVAR might close through the loss of anatomical suitability

In order to test this hypothesis, the Comparison of Surveillance versus Aortic Endografting for Small Aneurysm Repair (CAESAR) Trial recruited 360 patients with small (MAPD 41 to 54mm) AAA and randomised them to early EVAR or US-based surveillance.(42) The investigators reported no significant advantage to early EVAR and suggested that, in fact, less than one in six patients managed with surveillance might subsequently become unsuitable for EVAR.

With advances in stent-graft design this number will most likely fall further still. More recent studies have also suggested that although small AAA (MAPD <54mm) may be anatomically better suited to EVAR, early repair does not translate into improved clinical outcomes.(43)

At the present time, therefore, there is no indication to routinely repair asymptomatic AAA less than 55mm in MAPD either by OR or EVAR.

Importantly, although it is widely assumed that patients with AAAs exceeding 55mm benefit from routine OR or EVAR, and although that is likely to be true for selected patients, there is at present no data from randomised controlled trials (RCTs) (level 1 evidence) to support such a contention. Unfortunately, it seems unlikely now that such RCTs will ever be performed, and many would view that as a missed opportunity.

As the majority of AAAs remain asymptomatic, at present, AAA size (specifically MAPD) is by far the most commonly used surrogate for disease progression and rupture risk. However, the natural history of AAA remains poorly defined, and most

people with AAA die with their aneurysm from other co-morbidities, rather than from their aneurysm.

**AAA surveillance: rationale**

In the UK, the great majority of asymptomatic AAA less than 55mm in MAPD are entered into an US-based surveillance programme and offered, so-called, best medical therapy (BMT) comprising advice and help on smoking cessation; statin therapy to lower cholesterol and for their anti-inflammatory properties; control of hypertension; and anti-platelet agents to reduce the risk of thrombotic complications.(44)

Data from UKSAT suggest that small AAA (MAPD <40mm) can safely be offered US at 24-monthly intervals and those with AAA with MAPD between 41-45mm an annual US. Patients with AAA approaching 55mm in MAPD who are being considered for OR or EVAR should probably be offered 3-6 monthly US scans as the larger the AAA, in general, the more likely it is to expand.(36,45)

HEFT utilises the following MAPD-driven US-based surveillance protocol: up to 39mm - annual scan, 40-49mm - 6 monthly scan; and 50-55mm - 3 monthly scan. Once the AAA exceeds 50mm MAPD on US, the patient is usually referred onto a vascular surgeon for further investigations, including CT angiogram (CTA) and cardiopulmonary exercise testing (CPEX)) pending an MDT decision, collaboratively made by vascular surgeons, interventional radiologists and anaesthetists, on whether to proceed with OR or EVAR.

**Evidence for AAA screening**

Several RCTs have suggested that population US-screening for AAA for men aged 65 years and above is a cost-effective means of reducing AAA-related deaths although a reduction in all-cause mortality has been less convincing.

The Multicentre Aneurysm Screening Study (MASS): recruited 67,800 men aged 65-74 years and randomly allocated them to an offer, or no offer, of US-based screening.(46) In those offered screening, 80% attended and in those that attended, AAA (defined as MAPD > 30mm) were detected in 4.9%. In the group offered screening and then considered for open surgery (OR) when the AAA exceed 55mm, or became symptomatic or had a growth rate >10mm/year, there was a significant reduction in AAA-related mortality, although all-cause mortality was not significantly different between the two groups.

The Viborg Vascular (VIVA) Screening Trial: was of similar design to MASS and also reported an AAA detection rate of about 4% together with a significant reduction in AAA-related deaths, but no significant decrease in all-cause mortality, in those offered screening.(47)

The NHS-AAA-SP was first established in 2009 and by 2013 it was the intention to be able to offer all men in the UK an abdominal US to confirm or exclude a diagnosis of AAA as they reach their 65$^{th}$ birthday.

Those found to have small AAA (MAPD 30-44mm) are offered an annual repeat US; those with AAA of MAPD 45-54mm 3-monthly US; and those with AAA of MAPD >55mm are referred to specialist vascular services for consideration of repair.(48)

A Markov model, based on UK MASS trial data, has been used to predict an anticipated 1749 saved ruptures and 1236 AAA-related deaths prevented after 30 years of the screening programme being implemented.(48)

However, it is apparent that these estimates, and so the screening protocol itself, may need to be reconsidered in the light of the lower than expected screening acceptance and AAA detection rates.

Intuitively, population screening for AAA seems a good idea. However, until such time as we have a better understanding of the (changing) epidemiology and natural history of the condition and can, therefore, better identify those patients who would benefit from OR or EVAR, there is a real risk that national AAA screening in its present form may afford no overall (cost)-benefit, even cause net harm, and represent a sub-optimal use of already stretched healthcare resources.(36,49,50)

**Intervention for AAA: Choosing between open aneurysm repair (OR) and endovascular aneurysm repair (EVAR)**

At present patients are offered treatment if they are symptomatic, usually due to pressure effects of the AAA on adjacent structures, have complications such as distal limb ischaemia from embolisation of the intra-luminal thrombus (ILT), a rapidly expanding AAA ( often defined as an increase in MAPD >5mm per year) or present with rupture. However, the most common reason for intervention is that an AAA has reached the threshold size of 55mm. Patients with AAA deemed to require surgical intervention can be offered OR or EVAR.

OR involves laparotomy, aortic exposure and cross-clamping above and below the AAA. The AAA is then opened and a prosthetic graft sutured in place to replace the diseased segment. As noted above, the operation is associated with a significant risk

of mortality and major morbidity (5-10%). However, if patients survive the surgery, protection against future AAA-related complications is verging on complete.

EVAR involves insertion of a stent-graft into the abdominal aorta via the femoral arteries and avoids laparotomy and aortic cross-clamping. Not surprisingly, therefore, the EVAR-1 and EVAR-2 RCTs showed that EVAR is associated with a highly significant reduction in short-term (30-day) morbidity and mortality. However, for reasons that are not entirely clear, as soon as 6 months after intervention, the apparent survival advantage for EVAR has disappeared. Furthermore, the durability of EVAR has been called into question as a significant proportion of patients require further interventions for complications such as endoleaks and graft occlusion.(51,52)

So, whereas a patient who has undergone successful OR can be discharged from follow-up, those who have undergone EVAR need to be re-entered into a life-long US-based surveillance programme aimed at detecting and correcting graft related problems before they become symptomatic. This adds significantly to the costs of EVAR and means that the long-term safety and effectiveness of EVAR depends heavily on patients' compliance with a well-resourced, life-long post-operative surveillance programme. One suspects that neither of these conditions is universally met for all patients in hospitals offering EVAR.

**Endoleaks: biomechanical and biological significance**

Endoleak is a complication of surgery unique to EVAR and refers to persistent flow within the AAA sac despite graft deployment; five types are described:(53)

- Type 1: loss of haemostatic seal between the graft and the vessel wall
- Type 2: residual filling from feeding collateral vessels around the graft; for example, IMA or lumbar arteries

- Type 3: mechanical defect in the graft such as fabric tear or limb dislocation
- Type 4: graft material porosity
- Type 5: continued expansion of the AAA sac despite the absence of radiological evidence for endoleaks types 1 to 4

The EVAR-1 RCT reported an endoleak incidence of 22%, of which 35% required secondary interventions to exclude aneurysm perfusion within 3 years of EVAR, whilst a systematic review reported that 17.5% of all EVARs showed evidence of endoleak at 30 days, which increased to 21.3% by 12 months.(51,54)

All endoleaks except Type 2 and Type 4 are considered to usually require further (urgent) intervention which is often endovascular but may require conversion to open surgery. Type 4 endoleaks often subside over time without intervention. Many Type 2 endoleaks also spontaneously resolve due to eventual thrombosis of the feeding vessels. However, such endoleaks should be actively monitored and if the AAA sac continues to expand, many surgeons would advocate intervention.(53)

As well as re-exerting biomechanical stress on the AAA sac, the presence of an endoleak reconnects potentially biologically active ILT with the systemic circulation. Both seem likely to affect the outcome of treatment, not only in terms of local AAA-related complications, but through the re-emergence of a systemic, pro-inflammatory diathesis.

To date the literature has reported increasing interest in better understanding changes in circulating levels of biomarkers as an alternative to US-based surveillance of endovascular stent-graft failure after EVAR, with serum D-dimer levels reported as being elevated in those with unstable type-1 endoleaks.(55)

**Operative mortality: a cause for concern**

It perhaps goes without saying that for the repair of asymptomatic AAA (especially those detected through screening) to be a net saver of lives, and so an ethically and clinically appropriate treatment strategy, the morbidity and mortality associated with OR and EVAR must be kept as near to zero as possible. Worryingly, recent data suggest that the mortality associated with AAA repair in the UK is not as low as it could, or should, be.

The first VASCUNET data report (2007) compared AAA surgical outcome data from several European countries and identified the UK as having one of the highest mortality rates (7.9%, compared to a European average at 3.5%).(56)

While this disparity might be related at least in part to the fact that in the UK AAA surgery is usually reserved for larger AAA, as well as differences in the accuracy of reporting between different countries, the report raised sufficient concern for the Vascular Society of Great Britain and Ireland (VSGBI) to introduce an AAA Quality Improvement Programme (QIP).

This has further fuelled the debate over whether AAA surgery should be further concentrated into a smaller number of high-volume centres; encouraged participation in the National Vascular Database (now Registry)(57); and has driven QIP strategies to try to reduce the mortality associated with elective infra-renal AAA repair to 3.5% by 2013.(58)

With the increasing use of EVAR one could argue that this reduction in mortality would have occurred anyway and many believe that a mortality of 3.5% is still too high given that we are operating on an asymptomatic, increasingly screen-detected condition of uncertain natural history.

The observation that a growing number of repairs are being performed in octogenarians raises yet further concerns about the clinical and cost-effectiveness.

# Chapter 4: Biomarkers and their role in AAA disease

AAAs typically remain asymptomatic and undetected until complications arise; most commonly rupture, which is associated with a high risk of mortality. Several RCTs have suggested the risks of surgical intervention outweigh the benefits in patients with AAA <55mm MAPD. Although at present, MAPD is probably the best predictor of rupture risk as it reflects degeneration of the aortic wall and as such acts as a surrogate marker of impending rupture. An AAA intervention strategy based on MAPD alone most likely leaves some 'at risk' patients with small AAA unprotected from rupture and subjects others with larger AAA to unnecessary surgery which is associated with significant morbidity and mortality.

Improved understanding of the molecular mechanisms underpinning aneurysmal disease is vital to better clarify the pathophysiology of the disease process and target at-risk patients in a clinical and cost-effective manner. Although a number of potential biomarkers have been identified and investigated, to date, none can be considered sufficiently sensitive or specific to AAA as to be considered clinically useful. In this chapter, surrogate markers for AAA disease studied in the literature are discussed.

**Aims**

The aim of this chapter is to review the various biomarkers that have been investigated in the pathogenesis and progression of AAA disease as reported in the English-language scientific literature at the time of starting this work.

**Methods**

We performed a MEDLINE/PubMed database search looking for English-language articles using search terms 'abdominal aortic aneurysm', 'AAA', and 'biomarker'

and ‘inflammatory response’. The search was restricted at July 2015. From this, a more targeted search was performed where identified biomarkers and terms such as human AAA, abdominal aortic aneurysm, and EVAR were included amongst others. Further literature sources were identified following PubMed links and by cross-referencing from the citations of major articles or by following those used in source studies. Articles relating purely to thoracic aortic aneurysms were excluded.

**Biomarkers in disease**

Biological markers, or ‘biomarkers’ can be defined as a ‘‘cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids’’.(59) Increasingly however, the term is also used to refer to biological characteristics of processes which can be measured either as predictors of disease development, activity, natural history and progression, or response to therapy.(60) It has been suggested that biomarkers can be categorised into two groups: those which predict the onset of disease or can identify patients likely to become affected (so called ‘biomarkers of exposure’) and those which can be used to quantify, asses or monitor disease progression and response to therapeutic interventions.

In order for a biomarker to be of any practical use it should ideally be easy to measure with commercially available assays and exhibit low variability in terms of when it is measured. Additionally, the following criteria should also be met:(61)

- It must be central to the pathophysiological process implicated in the disease
- It must therefore reflect or be a very clear surrogate of that disease process
- It must be stable and only vary with events known to relate to disease progression

- Those at risk with a higher value at baseline must become more prevalent as disease severity increases
- The biomarker must predict progression
- The biomarker must also be sensitive to intervention factors that are known to be effective

Identifying biomarkers for a variety of disease processes has become an increasingly studied area in the field of translational research. In the context of AAA, to date MAPD has been used almost exclusively in clinical practice as the preferred surrogate marker of disease progression, even though it is well known that small AAAs are still capable of rupture and some very large AAAs remain asymptomatic until the patient dies of some other cause. Thus finding alternatives to MAPD remains an important area of AAA research, not only in terms of influencing clinical practice, but also as a means to identifying modifiable pathophysiological disease pathways.

With respect to AAA, studied biomarkers include genetic polymorphisms and markers of proteolysis, inflammation, immune response, and thrombosis and fibrinolysis.(62) Although a variety of potential biomarker candidates have been evaluated, few have been validated as clinically viable tests of AAA disease progression or response to surgery.

**The urgent need for an AAA biomarker**

From the discussion in Chapter 3, it is apparent that there exists an urgent need for a biomarker that can help determine which AAA are mostly likely to rupture so that pre-intervention screening and surveillance, intervention (OR and EVAR) and post-intervention surveillance can all be conducted in the most clinically and cost-

effective manner. With respect to AAA, potential biomarkers might include markers of proteolysis, or inflammation, but for widespread clinical application, ideally should be measurable in easily-collectable samples e.g.: serum or plasma, or calculated from radiological imaging techniques.

**Markers of proteolysis**

Proteolytic enzymes play an important role in the regulation and remodelling of the extra-cellular matrix, and hence also in the degradation of the aortic wall. This in turn promotes AAA formation, and subsequent rupture, by destabilising the aortic wall and its tensile strength. Matrix metalloproteinases (MMPs) are a group of such enzymes which have been shown to play a role in AAA disease, with histological studies of aneurysmal aortic tissue demonstrating elevated levels of MMP and decreased levels of tissue-inhibitor of metalloproteinases (TIMP) activity. MMPs that are considered important in AAA include MMP-1,-2, -3 and -9, cathepsins and serine proteases, but other enzymes include; chymase; tryptase; plasmin and tissue plasminogen activator (tPA).(63–68)

Animal studies have shown that decreased levels of MMPs, particularly MMP-2,-9 and-12, and increased TIMP preserves aortic wall elastin content thereby inhibiting, or at least controlling, AAA formation.(69) The source of the elevated levels of MMPs and other associated proteases remains, as yet, undetermined, although there is some early evidence to suggest that AAA intra-luminal thrombus (ILT) may be a potential source.(70) One small study has shown that ILT-load correlates with MMP activity and others suggest MMP-9 may have some potential in post-EVAR monitoring as levels have been shown to normalise following surgical repair. MMPs are unlikely however to be of value in diagnostics, as levels have not been found to correlate with AAA expansion pre-operatively.(71–74)

## Genetic markers

Although poorly defined, it is widely accepted that there is a strong genetic component to AAA disease, with studies suggesting that up to 15% of AAA patients have a positive family history. The literature suggests that in the past few decades much work has been undertaken on genetic association studies in AAA using single nucleotide polymorphisms (SNPs) in biologically important genes of the ECM, cardiovascular and immune systems. Widespread population generalizability of results from much of this work has been limited however, due to small study sample sizes and a lack of reproducibility or replication across sample sets.(75)

The more recent approaches of identifying the genetic basis of AAA are family-based DNA-linkage studies and genome-wide genetic association studies, which have the potential of identifying the genetic basis of AAA. SNPs associated with AAA have already been identified in other large, multicentre trials and whole-genome scans have suggested loci on chromosomes 19q13 and 4q31. Although there has been interest in the role of genetics in the development of AAA, the inherent problems of linking genetic susceptibility with the actual development of AAA in patients limits the role of genetic markers.(76–78)

## Markers of inflammation

There is a body of evidence in the literature suggesting that patients with AAA have abnormalities within their inflammatory and coagulation profiles. As such, patients with the disease can be considered to be in a ‘pro-thrombotic, hypercoagulable, pro-inflammatory’ state.(79–82) In addition, it has previously been shown that patients with AAA have abnormal baseline markers of coagulation and inflammation, and that these are attenuated in the immediate post-operative period, and normalise in

some (but not all) cases. Even when there is a reduction of such biomarkers to pre-operative levels, they remain elevated when compared to controls.(83)

Several groups have hypothesised that changes in serum markers of inflammation, coagulation and fibrinolysis play a significant role in the short and long-term mortality and morbidity observed after both OR and EVAR. Specifically, an association has been observed between abnormalities in the coagulation and fibrinolytic systems and adverse clinical outcomes, such as the systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS) and acute respiratory distress syndrome (ARDS), all of which are expressions of an inappropriate generalised response to inflammatory stimuli and are associated with a significant (up to 80%) mortality.(84,85)

In the elective setting, AAA repair is associated with a mortality and major complication rate of up to 5-10%. However, in cases of rupture mortality is disproportionately high relative to peri-operative haemodynamic instability. In modern practice, mortality and morbidity is more often the result of (prothrombotic) complications such as myocardial infarction (MI) and MODS, rather than from intra- and post-operative haemorrhage.

It is hypothesised that this may be because patients with AAA are already in a 'primed' hypercoagulable state, a form of low-grade disseminated intravascular coagulation (DIC), which then escalates rapidly in response to the physiological changes (low blood pressure, hypoxia) associated with rupture. Although patients undergoing elective OR and EVAR may also exhibit such changes, the peri-operative physiological disturbance (and so the clinical consequences) are less severe.(85,86) This idea is supported by one study which demonstrated an association between elevated pre-operative serum thrombin antithrombin complex (TAT) and a post-

operative rise in cardiac troponin-T (cTnT), yet most of the patients had no clinical cardiac symptoms or signs.(87)

The proteolytic generation of elastin and collagen degradation products attracts circulating inflammatory cells such as macrophages and mononuclear lymphocytes, which then produce pro-inflammatory cytokines, chemokines and immunoglobulins to further perpetuate the remodelling process.(62,88–90) In normal circumstances, vascular endothelial tissue is resistant to leukocyte adhesion. In the presence of pro-inflammatory stimuli however, there is endothelial expression of adhesion molecules resulting in attachment of monocytes and circulating lymphocytes. The mediators involved in cell-adhesion include P-selectin, E-selectin, intracellular adhesion molecule- (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are present in endothelial cell walls.(26,29,91) Chemo-attractants such as oxidised-LDL (oxLDL), lipoprotein(a) [Lp(a)], MCP-1, IL-1 and TNF-α allow sub endothelial migration of monocytes and T-lymphocytes. Studies have demonstrated that deficiency in MCP-1 or its receptor CCR-2 in mice significantly reduces atherosclerotic lesion development, suggesting that MCP-1 may be the most important chemoattractant factor in the disease process.(30,31)

Work on the pathogenesis in AAA disease suggests that important markers of the inflammatory response include pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin (IL-) 6 and 8, acute-phase proteins such as C-reactive protein (CRP) as well as anti-inflammatory cytokines such as transforming growth factor (TGF)-β. Histopathological analysis of diseased aortas has shown focal abundance of such inflammatory cells compared to normal specimens. The difficulty in using such inflammatory markers as biomarkers of AAA progression is, of course, that they are general, non-AAA specific markers of inflammation.(81,88,92)

**IL-6**

IL-6 is produced by many cells, including macrophages. On binding to its target receptors it acts on activated B cells, plasma cells, and T cells amongst others to exert proliferation, pro-atherogenic, stem cell differentiation and acute phase responses.(93)

IL-6 has been described as one of the most important pro-inflammatory cytokines in the initiation and progression of the inflammatory cascade, and its role as a predictor of future cardiovascular events in clinically asymptomatic patients is well documented. In the context of AAA however, an association with IL-6 is less clearly established, although it is the cytokine most consistently described in AAA disease. In vitro studies have suggested that AAA may be a source of IL-6 with circulating levels higher in patients with the disease than in controls.(94,95) A prospective study looking at 360 patients with AAA found elevated IL-6 levels compared to a non-diseased control population and found a correlation between increasing AAA size and elevated levels. Another smaller study found levels to be elevated in patients with AAA and a possible correlation with size.(82,96–100) Additionally, IL-6 levels have been found to correlate with aortic diameters in healthy subjects.(95,101,102)

**IL-8**

IL-8 is produced by macrophages, endothelial cells, monocytes and T-cells. On binding to its receptor, present on neutrophils, T-cells and monocytes it acts to promote pro-atherogenic, pro-inflammatory effects. Its over-expression in AAA has been reported, and there is also evidence to suggest that its source may be the ILT. Some studies also describe a significant correlation between plasma IL-8 and maximal AAA diameter; however results in the literature are conflicting. Some report IL-8 levels to be significantly low in patients with large AAA and so its role

as a diagnostic tool, or as a reliable predictor of AAA progression has not yet been determined.(93,95,103–105)

**CRP**

C-reactive protein (CRP) is an acute-phase protein detectable in only trace amounts in the serum of healthy subjects, which rises rapidly in response to inflammation or physiological insult. In response to cytokine stimulus, particularly by IL-6, its role in AAA may be linked to its ability to activate monocytes, mediate endothelial dysfunction, induce a pro-thrombotic state and increase cytokine release.(106–109)

Several studies investigating the role of CRP and highly-sensitive CRP (hs-CRP) in AAA disease have provided mixed results: animal studies have shown significantly increased CRP and hs-CRP levels in AAA models when compared to controls and in men with small AAA compared to sex and age or race-matched controls. Others have suggested a correlation between CRP and the extent of AAA disease, with levels being higher in patients with symptomatic or ruptured cases. A few small studies have also shown some correlation between hs-CRP or CRP and aneurysm size, however others have found levels to be within normal limits, with no such correlation. Although CRP, or even hs-CRP, would make a potentially attractive biomarker of AAA disease, being commercially readily-available, relatively cheap tests that can produce reliable, reproducible results their inherent disadvantage is again the lack of sensitivity or specificity to AAA disease.(74,80,99,110–112)

**TGF-β**

TGF-β consists of a group of more than 30 proteins, and as such forms a ‘superfamily’ group of cytokines which all play a role in cellular growth, differentiation, adhesion and apoptosis. It has been shown to down regulate inflammation and inhibit MMP-dependent proteolysis and animal studies have

suggested over-expression can slow the expansion of AAA, suggesting there may be a role for it in future pharmacological control of AAA progression.(113–115)

**TNF-α**

Tumour necrosis factor (TNF)-α is predominantly produced by macrophages and binds to its receptor, present on many cell-types, to act as a mediator within the inflammatory response by increasing neutrophil endothelial adhesion and inhibiting anticoagulatory mechanisms. It has been identified as an important mediator in AAA formation, with studies suggesting significantly elevated levels in AAA compared to matched-controls, increased expression of its converting enzyme, tumour necrosis factor-α converting enzyme (TACE), in human aneurysmal aortic segments and prevention of AAA development in animal studies where TACE was deleted, or blocked. The concern in using TNF-α as anything other than an identifier of active inflammation within patients with AAA is, of course, as with many of the above named markers of inflammation, that it is a general marker, that can be elevated or suppressed in many different pathologies, which limits its value as a tool to monitor the progression of AAA in patients.(100,116,117)

**Intraluminal thrombus as a biologically-active component of AAA**

Most AAAs contain intraluminal thrombus (ILT) as a result of endothelial injury, platelet activation, a prothrombotic diathesis and turbulent flow within the sac.(118) Thrombus contains high concentrations of MMPs, elastase and plasmin, which are all proteolytic enzymes capable of altering the structure, and so the biomechanical properties, of the aortic wall.(63,68) The proteolytic breakdown of elastin and collagen attracts circulating inflammatory cells such as macrophages and mononuclear lymphocytes which then produce pro-inflammatory cytokines, chemokines and immunoglobulins, thereby perpetuating the remodelling

process.(88,90) Thrombus volume has been shown to correlate with AAA growth and some authorities believe that thrombus burden may be a better predictor of rupture than diameter.(119–121) There is growing evidence to show intraluminal thrombus (ILT) is biologically active and contributes to AAA progression and the development of complications; it may also influence the response to treatment and could, as such, be measured to possibly predict those AAA that are more 'biologically active' and therefore likely to result in clinical complications.(122,123)

**Conclusions**

A number of potential biomarkers have been proposed but to date none can be considered sufficiently sensitive or specific to AAA disease to be clinically useful. However, there remains a strong argument to identify surrogate factors which could be used to quantify those with the greatest clinical need for operative intervention as size alone is a poor predictor of AAA complications in asymptomatic patients.

## Chapter 5: IL-1α and its role to date in the pathogenesis of disease

Cytokines are small (less than 20kDa), soluble proteins involved in the inter- and intra-cellular transmission of cellular information. They are key molecular mediators of inflammation, and as such are cell-signalling molecules, released in response to a stimulus by a variety of cells to exert a diverse range of effects.(124)

IL-1α is a member of the IL-1 'super-family' of pro-inflammatory cytokines. IL-1 was first discovered in the 1940s, when it was referred to as 'endogenous or leukocytic pyrogen', linking infection and inflammation to fever. It has since been found to have at least 11 members (IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37, IL-38, IL-1β, IL-1RA and IL-18). This broad group of polypeptides can be sub-divided into 3 sub-families (IL-1, IL-18 and IL-36) according to the length of each member's individual precursor.(125) Members of the IL-1 sub-group function by causing pro-inflammatory effects, induction of other cytokines, and promote cell differentiation, proliferation and apoptosis.

The members of the IL-1 sub-group include IL-1α, IL-1β, IL-33 and a naturally occurring IL-1 receptor antagonist (IL-1RA). IL-1α and IL-1β are produced by different genes but demonstrate high sequence homology, having 22% identical amino-acids.(126) Nevertheless, they remain distinct entities and are thought to exert different biological effects. The IL-1α precursor is cleaved predominantly by the membrane-bound protease calpain into its mature form, but some transformation can also take place via extra-cellular neutrophil proteases.(125)

IL-1 is primarily produced by peripheral blood monocytes as well as specialised tissue cells such as macrophages in response to infection or tissue injury, although epidermal, epithelial, lymphoid and vascular tissues are also able to produce it.(126)

In health, interleukin-1 alpha (IL-1α) is not detectable within the circulation, being primarily an intra-cellular entity, found in the cytosol of cells, and on plasma membranes, but it may become so in certain diseased states.(127) Production of IL-1 is regulated at several stages including transcription, translation pro-IL-1 cleavage as well as release. IL-1α is present in two isoforms: a precursor (pro-IL-1α), which has a molecular weight of 31-33kDa and a 'mature' 17kDa molecule. Once activated, mature IL-1α can be secreted from cells to trigger an inflammatory cascade after binding to IL-1 receptor type 1 (IL-1R1).(127–130) There is evidence to suggest that pro-IL1α acts as an alarmin molecule by initiating inflammatory pathways and, as such, is biologically active.(131) The wall of human aneurysmal aortas have been found to contain elevated levels of IL-1α, however it is as yet not known which isoform is biologically active in the AAA disease process.(104,128,132,133)

Activation of pro-IL-1α occurs through membrane-associated calpain-mediated cleavage. Calpeptin is a synthetic calpain-inhibitor which acts to inhibit activation of pro-IL-1α into its mature isoform.(129,134–137) IL-1α acts by binding to the IL-1 receptor type 1 (IL-1R1) to induce subsequent recruitment of IL-1 receptor accessory protein (IL-1RAcP) which triggers intracellular signalling resulting in the activation of nuclear factor κB (NF-κB) transcription factor and mitogen-activated protein kinases (MAPKs). These then act to cause downstream up-regulation of pro-inflammatory cytokines such as IL-6, TNFα, chemokines and adhesion molecules.(138,139) Upon release, IL-1α is regulated via two receptors: a naturally occurring IL-1 receptor antagonist (IL-1Ra) which binds to IL-1R1 to inhibit IL-1α, and IL-1R2, which functions as a decoy receptor by failing to up-regulate downstream IL-1α signalling pathways as it lacks a signal-transducing cytosolic domain.(140) The IL-1α signalling receptor complex consists of IL-1R1 and IL-1

Receptor Accessory Protein (IL-1RAcP), and once bound to this, activated IL-1α phosphorylates IL-1 receptor associated kinase (IRAK) via links formed through myeloid differentiation factor 88 (MyD88). Phosphorylated IRAK then dissociates from the receptor complex and associates instead with TNF receptor associated factor 6 (TRAF6), activation of which induces two further kinase pathways in a manner that is not yet fully understood. These two kinase pathways, the IkappaB kinase (IKK) complex, and the p38 MAPK act by inducing phosphorylation of IκB protein, causing its ubiquination and degradation (in the case of the IKK pathway) which then liberates NF-κB, enabling its translocation into cell nuclei where it induces target gene expression. The MAPK pathway, once activated, leads to activator protein-1 (AP-1) transcription factor activation through c-Jun kinase (Jnk). Activation of IL-1RI triggers multiple and sequential phosphorylations that result in nuclear translocation of transcription factor. Post receptor amplification is responsible for the potent effects of IL-1 signalling despite the relatively low expression of IL-1RI in cells.(141)

**IL1α in atherosclerosis and cardiovascular disease**

Atherosclerosis is a chronic, insidious disease process affecting the arterial wall, which becomes thickened over time, thus losing its normal functional abilities. Although any artery of the body may be affected, lesions typically manifest at vessel bifurcations or sites where there is turbulence in normal, laminar blood flow. Risk factors for the disease can be sub-grouped into constitutional factors: increasing age, male sex and a positive family history, or acquired: smoking, hypertension, diabetes and hypercholesterolaemia. Pathogenesis is the result of interplay between several mechanisms, including inflammatory, biochemical, metabolic and thrombotic events, although historically it was thought to be solely the result of lipid accumulation.(25)

Although no single inflammatory cascade is thought to directly result in atherosclerotic lesions, there is an emerging role for IL-1α in the development and propagation of the disease.

There has been interest in IL-1 in the context of inflammation and cardiovascular disease. IL-1 is thought to play a part in atherosclerosis through vessel wall inflammation, leukocyte chemotaxis and adhesion and increased levels of IL-1 have been found within atheromatous plaques.(103,142–147) IL-1 may increase expression of MMPs leading to collagenous breakdown within plaques and instability. Studies in mice have shown an absence of IL-1β decreases the severity of atherosclerosis.(142,148) In the context of AAA, studies have shown that patients with aortic disease have elevated IL-1β titres.(89,94) One study has looked at the inflammatory activity of IL-1α and found that patients had an elevated titre which was reversed after EVAR.(137)

As atherosclerotic lesions form, localised ischaemia results in necrosis and the release of intra-cellular IL-1α. This then binds to its receptor on adjacent macrophages and epithelial cells to induce a response such as localised infiltration of neutrophils and monocytes.(133) Additionally, IL-1α stimulates the production and release of endothelial adhesion molecules and proteases as well as inducing the release of messenger cytokines such as IL-6, which in parallel, then stimulate hepatic release of acute-phase reactants such as CRP.(26) Both IL-6 and CRP have been found to be elevated in patients with advanced atherosclerotic plaques.(149,150) Direct monocyte activation by IL-1α also increases endothelial cell (EC), monocyte, and MMP-9 production and subsequent leukocyte infiltration.(151) MMP-9 is an enzyme involved in elastin degradation, and has been shown to correlate positively

with aortic stiffness, causing hypertension, as well as having been shown to directly correlate with AAA progression.(152)

It has previously been demonstrated that patients with asymptomatic AAA exhibit a hypercoagulable and hypofibrinolytic state characterised by increased thrombin generation, activity and fibrin turnover.(83,153,154) It has further been hypothesised that this prothrombotic diathesis results from the release of biologically active moieties from the AAA wall and intra-luminal thrombus, and this is supported by the observation that exclusion of the AAA following OR and EVAR leads to a normalisation of coagulation in most patients.(155,156) In further work, serum IL-1α, IL-1β, Il-4, IL-6, IL-8, IL-10, interferon (IFN)-γ, MCP-1 and tumour necrosis factor (TNF)-α and TNF-β were measured before and 6-months after EVAR repair.(157) IL-1β, IL-6, IL-10, IFN-γ, TNF-α and -β were detectable pre-operatively and did not significantly change after EVAR, but by contrast, serum IL-1α and IL-8 levels fell significantly by 6 months after EVAR although only IL-1α, but not IL-8, positively correlated with AAA size.(137)

The methods through which IL-1α is measured however remains a bone of contention, as will be shown in greater detail as part of our own work in Chapter 7. A review of the literature identified that a variety of research groups, studying different pathologies, have shown a range of IL-1α titre values, raising the question whether is variability between manufacturers' kits and also between batches of kits produced by the same company:

- One study evaluated serum IL-1α in patients with ovarian epithelial cancer against healthy controls. Using an ELISA immunoassay (R&D Systems, Minneapolis, USA), they reported that of the 35 patients with disease, 30 had serum titres <50pg/ml and 5 between 100-500pg/ml, whilst in the control

group of 36 patients, 33 had titres <20pg/ml, and the remaining 3 titres between 50-200pg/ml.(158)

- Another study, using the same immunoassay, studied serum IL-1α in 66 patients with systemic sclerosis (SS) against 19 age- and sex-matched controls. It reported IL-1α titres in the SS group of mean 4.95±0.28pg/ml, and in the control group 4.52±0.29pg/ml, and differences were statistically significant.(159)
- A further study using the same immunoassay compared serum IL-1α in patients with acute viral hepatitis to controls. It reported mean titres of 15 ±8.8pg/ml in those with the disease against 1.1±2.7 in the control group.(160)
- Another study, using a different immunoassay (LINCOplex® Multiplex immunoassay, Linco Research Inc., MO, USA), evaluated serum IL-1α in patients with rheumatoid arthritis (RA) against age-matched controls. It reported mean IL-1α titres in 169 patients with the disease of 144pg/ml (range 48-380), and in the 92 controls, mean titres of 97.8pg/ml (range 33-398.4).(161)

These show that there is a dynamic range of results in those with pathology between kits produced by one or different companies. The difficulty is however interpreting whether this is due to a 'true' difference in results, because of the disease process' being studied, or if it due to variations between kits. The variability of results between the studies in their respective control groups is therefore of particular interest, as one would intuitively expect similar titre ranges, particularly in kits from the same manufacturer in 'healthy' control subjects. One possible reason for the differences across kits in this group may be that the age-range of the patients studied

was not consistent, however there is no strong evidence that IL-1α titres should vary with age.

# Chapter 6: Results from an analysis of the natural history of AAA in our own patient population at HEFT

## Introduction

The UK Small Aneurysm Trial (UKSAT) and the Veterans Affairs Aneurysm Detection and Management (ADAM) study have shown that the risk of rupture in asymptomatic AAA <55mm MAPD is approximately 1%/year and that routine surgical intervention in such patients confers no benefit.(36–38) For this reason, asymptomatic AAA <55mm in MAPD are usually entered into a US-based surveillance programme and subsequently offered intervention when the risks of rupture, based upon absolute AAA size and growth rate, are believed to exceed those of OR or EVAR. As would be expected, the interval gap between surveillance scans decreases the larger the AAA MAPD. UKSAT indicates that patients with AAA of <40, 41 to 45, and >45mm MAPD can safely be offered US at 24, 12 and 3-6 monthly intervals.(36–38) However, more recent evidence suggests that these intervals can be safely increased to 3, 2 and 1-yearly for AAA of MAPD 30 to 39, 40 to 44, and 45-54mm respectively thus significantly improving patient acceptability and the cost-effectiveness of AAA surveillance, whilst maintaining a <1% risk of AAA size exceeding 55mm between screening visits.(162–165) In fact, the most recent Society of Vascular Surgery guidelines, published in 2009 and the American College of Cardiology/American Heart Association (ACC/AHA) 2005 guidelines on surveillance scans recommend US or CT-based surveillance every 6 to 12 months for aneurysms 40 to 54mm in diameter, and for AAA 30 to 40mm, suggest that imaging every two-to-three years is sufficient.(166,167) If such recommendations are internationally adopted, then this will have significant implications for screening

programmes, such as the UK National AAA Screening Programme (UK-NAAASP), where the surveillance intervals (annually for AAA measuring 30 to 44mm and 3-monthly for those with MAPD between 45 to 54mm) are still based on the UK Multicentre Aneurysm Screening Study (MASS).(36–38) Given the conflicting data and recommendations in the literature, it is perhaps unsurprising that surveillance intervals vary considerable at international, national and regional levels.(163) At present our local HEFT surveillance protocol requires annual scans for AAA with MAPD ≤39mm, 6-monthly scans for AAA MAPD between 40 and 49mm, and 3-monthly scans for AAA with MAPD between 50 and 55mm. Once the AAA exceeds 50mm the patient is usually referred on to a vascular surgeon for further investigations pending a decision for intervention. The increased frequency of scans as the AAA enlarges is deemed reasonable on the grounds that US-imaging carries a relatively low-cost and reduces the potential risk of losing patients to follow-up from extended intervals between scans, as gaps in surveillance scans have been shown to be the factor most predictive of rupture in patients with known AAA.(168)

Although forthcoming UK NICE national guidelines on AAA (including surveillance) will reduce non-evidence-based variability in practice and improve cost-effectiveness, it is important that clinical decision-making regarding AAA intervention takes into account potentially important individual patient factors such gender, smoking status, diabetes, blood pressure, hypercholesterolaemia, other co-morbidity, and patient preferences.(169–171)

**Aims**

The aim of this study, therefore, was to examine factors affecting AAA growth in patients entered into the surveillance programme at HEFT.

**Methods**

Retrospective interrogation of the prospectively gathered and maintained computerised HEFT AAA surveillance database was undertaken for the period 1st February 1993 to 31st August 2013. All patients with AAA of MAPD ≥30mm and at least one follow-up scan were included. Those patients with AAA ≥55mm at first scan and those with only one measurement were excluded from analysis of growth rates. For the former it was due to the inherent selection bias of maintaining such patients in surveillance despite having reached threshold intervention MAPD, and for the latter because growth rates could not be calculated from isolated MAPD measurements. Those with MAPD <30mm at time of first scan were excluded from the analysis until such a time, if it occurred, as their aortic measurements were greater than 30mm, as prior to that they were not defined as having an AAA. Overall, 5363 AAA measurements were analysed (4906 US, 442 CT, and 15 MRI). The average duration of follow-up was 5 years 7 months with a mean (median; range) of 10 (9; 2 to 29) measurements per person. Patient demographics including co-morbidity, smoking status, use of BMT, death and cause of death were collected through review of HEFT electronic patient records (EPR) and other hospital databases. For each patient a MAPD growth rate was derived by dividing the change in MAPD between the first and last scans by the time elapsed between them. Growth rates were censored at MAPD of 55mm, as most patients went on to have OR or EVAR at this stage. Large negative (>0.5mm/month) and positive (>1.0mm/month) growth rates were excluded as they were believed to be spurious. The effect of patient factors on growth rates was analysed using independent sample t-tests and one-way ANOVA for comparisons of two, and more than two, groups respectively. A survival analysis was performed using the compound outcome of all-cause

mortality or OR/EVAR. Univariate analysis was performed using Kaplan-Meier survival curves, followed by a multivariable Cox regression model with a backwards stepwise entry method. All analyses were performed using IBM SPSS Statistics 22 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance. Bonferroni or Šidák corrections were not made to adjust the p-values when performing multiple comparisons, so it is possible that some of the results obtained occurred simply through chance alone.

**Results**

Twenty patients with initial AAA MAPD >55mm, nine with only one scan, six with negative growth rates >0.5mm/month and two with positive growth rates >1mm/month were excluded leaving 692 patients. Of these, 575 (83.1%) were men of mean (range, [SD]) age 72 (45-89, [±6.7]) years at time of entry into the AAA surveillance programme for analysis. Mean (range, [SD]) MAPD at first and last scans was 37 (30-44 [SD±6]) and 40 (30-55, [±10]) mm respectively. There was no significant difference between men and women in starting AAA MAPD. Mean (median/range) AAA MAPD growth rate was 2.3 (1.9/0-13.1) mm/year overall; 2.2mm/year for men and 2.7mm/year for women ($p = 0.05$, t-test)

A further 73 patients were entered into surveillance despite having MAPD <30 (range 15 to 29mm) and underwent 109 (106 US, 3 CT) scans. Four remained in surveillance for up to 10 years, undergoing a total of 18 US scans, but never reached MAPD ≥30mm before being discharged. Of the 69 patients whose AAA MAP progressed to >30mm, 10 went on to OR or EVAR.

Co-morbidities, smoking status and BMT are shown in **Table 1**. Most patients (n = 608, 87.9%) were ≥65 years at the time of entry into surveillance and had associated

cardiovascular risk factors such as hypertension (71.4%), IHD (44.7%) and a positive (ex-, or current) smoking history (66.9%). Additionally, most (83.8%) were maintained on some form of BMT.

| Co-morbidity | N | Data missing |
|---|---|---|
| BP | 492 (71%) | 24 (3.5%) |
| IHD | 309 (44.7%) | 23 (3.3%) |
| AF | 100 (14.5%) | 1 (0.1%) |
| COPD | 163 (23.6%) | 25 (3.6%) |
| DM | 107 (15.5%) | 25 (3.6%) |
| Hypercholesterolaemia | 170 (24.6%) | 32 (4.6%) |
| ESRF | 89 (12.9%) | |
| CCF | 77 (11.1%) | |
| Active history of cancer | 138 (19.9%) | |
| Dementia | 36 (5.2%) | |
| **Smoking status** | | **50 (7.2%)** |
| Never | 165 (23.8%) | |
| Ex-smoker | 290 (41.9%) | |
| Current | 187 (27.0%) | |
| **BMT** | | **35 (5.1%)** |
| No BMT | 77 (11.1%) | |
| Aspirin/Statin | 445 (64.3%) | |
| Aspirin only | 40 (5.8%) | |
| Statin only | 56 (8.1%) | |
| Warfarin/Statin | 22 (3.2%) | |
| Other combination | 17 (2.5%) | |

**Table 1. Characteristics of the 692 patients in the historical database of HEFT AAA surveillance**

BP: hypertension; IHD: ischaemic heart disease; AF: atrial fibrillation; COPD: chronic obstructive pulmonary disease; DM: diabetes mellitus; ESRF: end-stage renal failure; CCF: congestive cardiac failure; BMT: best medical therapy

Elective AAA repair (57 OR, 82 EVAR) was undertaken in 139 (20.1%) patients at a mean (range, [SD]) age of 75 (54-87, [±6]) years and mean MAPD of 58mm. There were 124 men of mean age of 75 years and 15 women of mean age of 77 years. There was no significant difference between men and women in terms of MAPD at time of surgery ($p = 0.914$). Those who eventually underwent surgery grew on average at 4mm/year while those that did not grew at 1.7mm/year ($p<0.001$, t-test).

*Death in surveillance*

Of the 692 patients, there were 213 (30.8%) deaths in 164 men of mean (range, [SD]) age 80 (61-94, [±6]) and 50 women of mean 81 (66-92, [±6]) years at time of death.. Of these, only 25 (11.7%) were AAA-related (**Table 2)**. Of those who died, 171 (80.3%) patients, 131 men of mean (range, [SD]) age 80 (61-94, [±7]) and 41 women of mean (range, [SD]) age 82 at time of death (66-92, [±6]) years died in surveillance having never been offered AAA surgery. A total of 133 (62.1%) deaths occurred in patients who never reached a MAPD >55mm. Of these, 102 were men of mean [SD] age 80 [±6] years and mean growth rate of 1.9mm/year.

A further 38 patients died without having surgical AAA repair despite having MAPD ≥55mm at time of death. Surgery in this group was precluded on the grounds of advanced age, significant cardiovascular co-morbidity and metastatic malignancy or patient refusal (n=1). Mean age at time of death in this group was 82 [±7] years. Of these, 34.2% (n=13) died of AAA rupture.

A total of 42 patients died within the follow-up period having undergone AAA repair, 8 of whom were recorded as post-operative 'in-hospital' deaths (3 emergency OR, 2 elective OR, and 3 elective EVAR). Excluding the 8 post-operative deaths, 26/34 of these patients were men, with mean age at time of surgery being 77 years.

Mean time to death was 49 [±28] months post-operatively, and mean age at death was 77 years [±5].

| | All deaths (n = 214) | Deaths (MAPD ⩽55mm at last scan) (n = 133) | Deaths (MAPD > 55mm at last scan and no AAA surgery) (n = 39) | Deaths (after AAA repair) (n = 42) |
|---|---|---|---|---|
| Unknown* | 30 | 20 | 6 | 4 |
| **Non-AAA-related** | | | | |
| Cardiac arrest | 19 | - | - | - |
| IHD/MI | 8 | 20 | 4 | 4 |
| Malignancy | 53 | 36 | 7 | 9 |
| Pneumonia | 32 | 21 | 2 | 10 |
| CVA | 7 | 5 | 1 | 1 |
| Sepsis | 11 | 9 | 1 | 1 |
| PE | 3 | 2 | - | - |
| ESRF | 3 | 2 | 1 | - |
| COPD | 9 | 6 | 1 | 1 |
| CCF | 8 | 6 | 1 | 1 |
| Other | 5 | 2 | 1 | 2 |
| **AAA-related** | | | | |
| AAA repair (elective) | 5 | - | - | 8 |
| Rupture (operated) | 3 | - | - | - |
| Ruptured (not operated) | 17 | 4 | 13 | - |

**Table 2. Cause of death in the 214 patients who died during HEFT AAA surveillance as recorded on death certification**

*Where cause of death is recorded as unknown the death certificate was unavailable and interrogation of individual patient records also yielded no obvious cause.

HT: hypertension; IHD: ischaemic heart disease; MI: myocardial infarction; CVA: stroke; PE: pulmonary embolus; ESRF: end-stage renal failure; COPD: chronic obstructive pulmonary disease; CCF: congestive cardiac failure

*AAA rupture*

Overall, only 27/692 (3.9%) patients went on to rupture. Of these, 11/27 (40.7%) ruptured with a last recorded MAPD <55mm. Of these, seven were men with mean AAA size at time of last scan 51mm (47-54, [±2]) and mean growth rate 2.7mm/year. Seven of these proceeded to emergency surgery, with two deaths in the immediate post-operative period. The mean time between last known surveillance scan and rupture date was 531 days. One patient had been lost to AAA surveillance follow-up prior to re-presenting with rupture 4 years later. At the time of her last surveillance scan, the AAA measured 47mm and she underwent successful emergency OR. Another patient had been discharged from AAA surveillance follow-up due to age (91 years) and co-morbidities and presented 3years later with a rupture but was not taken forward for emergency surgery.

Of those 16 patients who ruptured with AAA >55mm, 11 were men with mean AAA size of 66mm at last scan (55-120, [±15]) and mean AAA growth rate of 6.1mm/year. This difference in growth rates between those who ruptured <55mm and those who ruptured at >55mm was statistically significant ($p = 0.004$ t-test).

A total of ten patients underwent emergency surgery (6 OR, 4 EVAR) for ruptured AAA, of whom 3 died in hospital. Of the remaining 17, four did not survive to the operating theatre, and the remaining 13 were deemed unsuitable for emergency surgery on the grounds of advanced age, significant co-morbidity or because they refused surgery (n=1). Mean age in these 13 patients was 82 (67-94, [±8]) years and mean AAA size at time of last scan was 68mm (55-120, [±16]).

Comparing mean growth rates in those who ruptured (0.38 mm/year) and those who did not (0.18 mm/year), we found no significant difference in mean AAA growth rate ($p = 0.838$, t-test).

*Baseline AAA MAPD on entry into surveillance*

There was no statistically significant difference in baseline MAPD between men and women ($p=0.687$) (**Table 3**). Younger patients had significantly smaller MAPD upon entry to the surveillance programme ($p<0.001$) Patients with a baseline MAPD >40mm were significantly more likely to be offered surgery ($p<0.001$). Specifically 14.3% (n = 62) patients with AAA <40mm at point of entry into surveillance proceeded to undergo surgical repair whilst 34.4% (n = 87) of those with AAA between 40mm and 50mm at first surveillance scan went on to require surgical intervention. Those who went on to be offered elective AAA repair had 4mm larger MAPD at baseline than those who did not (40mm vs. 36mm) ($p<0.001$). Additionally, those who went on to rupture had 4mm larger MAPD at baseline compared to those who did not (41mm vs. 37mm) (0=0.002).

| | N | First AAA (mm) | p-Value |
|---|---|---|---|
| **Age at First Scan** | | | **<0.001*** |
| < 65 | 95 | 35 (6) | |
| 65 - 69 | 178 | 36 (6) | |
| 70 - 74 | 203 | 37 (6) | |
| 75+ | 216 | 39 (6) | |
| **Sex** | | | 0.687 |
| Male | 575 | 37 (6) | |
| Female | 117 | 37 (7) | |
| **Surgery** | | | **<0.001*** |
| No | 543 | 36 (6) | |
| Yes | 149 | 40 (7) | |
| **Rupture** | | | **0.002*** |
| No | 667 | 37 (6) | |
| Yes | 25 | 41 (6) | |

**Table 3. Baseline AAA measurements of patients in HEFT AAA surveillance**

Data reported as: "mean (SD)", with p-values from t-tests, for comparisons of two groups, or one-way ANOVA for more than two groups. *Significant at $p<0.05$ (bold)

*AAA growth rates (mm/year)*

Mean growth rate for all 692 patients was 2.3mm/year (**Table 4)**. Females were associated with significantly higher AAA growth rates compared to men (2.7 vs. 2.2mm/year) (p=0.025). Analysis of co-morbidity revealed no significant differences except that those with diabetes had significantly lower growth rates (1.7 vs. 2.5mm/year) (p=0.002). Additionally, although the result did not quite reach significance, active smokers also had higher AAA growth rates compared to those who were ex-smokers or those who had never smoked.

Those who were subsequently offered intervention had significantly higher growth rates than those who were not (4.0 vs. 1.8mm/year) ($p<0.001$) and those with more rapid growth rates were more likely to rupture (4.6mm vs. 2.2mm/year) ($p<0.001$).

| | N | Growth rate (mm/year) | p-Value |
|---|---|---|---|
| **Age at First Scan** | | | 0.350 |
| < 65 | 95 | 2.2 (2.1) | |
| 65 - 69 | 178 | 2.1 (1.7) | |
| 70 - 74 | 203 | 2.2 (1.8) | |
| 75+ | 216 | 2.5 (2.6) | |
| **Sex** | | | **0.025*** |
| Male | 575 | 2.2 (2.0) | |
| Female | 117 | 2.7 (2.7) | |
| **Death in surveillance** | | | **<0.001*** |
| No | 478 | 2.1 (2.0) | |
| Yes | 214 | 2.8 (2.4) | |
| **Surgery** | | | **<0.001*** |
| No | 543 | 1.8 (1.8) | |
| Yes | 149 | 4.0 (2.3) | |
| **Rupture** | | | **<0.001*** |
| No | 665 | 2.2 (2.0) | |
| Yes | 27 | 4.6 (3.3) | |
| **IHD** | | | 0.231 |
| No | 360 | 2.4 (2.2) | |
| Yes | 309 | 2.3 (2.1) | |
| **COPD** | | | 0.606 |
| No | 504 | 2.3 (2.1) | |
| Yes | 163 | 2.4 (2.3) | |
| **DM** | | | **0.002*** |
| No | 560 | 2.5 (2.3) | |
| Yes | 107 | 1.7 (1.9) | |
| **BP** | | | 0.220 |
| No | 176 | 2.5 (2.3) | |
| Yes | 492 | 2.3 (2.1) | |
| **Smoking status** | | | 0.086 |
| Never | 165 | 2.1 (2.1) | |
| Ex | 290 | 2.2 (1.9) | |
| Current | 187 | 2.7 (2.3) | |
| **Cholesterol** | | | 0.379 |
| No | 490 | 2.4 (2.3) | |
| Yes | 170 | 2.1 (1.7) | |
| **BMT** | | | 0.237 |
| None | 77 | 2.4 (2.2) | |
| Aspirin & statin | 445 | 2.4 (2.1) | |

| | | | |
|---|---|---|---|
| Aspirin only | 40 | 2.0 (2.2) | |
| Statin only | 56 | 2.0 (1.9) | |
| Warfarin & Statin | 22 | 2.4 (2.3) | |
| Other combination | 17 | 2.1 (1.8) | |
| **ESRF** | | | 0.340 |
| No | 603 | 2.3 (2.2) | |
| Yes | 89 | 2.4 (2.1) | |
| **CCF** | | | 0.962 |
| No | 615 | 2.3 (2.1) | |
| Yes | 77 | 2.3 (2.2) | |
| **AF** | | | 0.402 |
| No | 591 | 2.3 (2.2) | |
| Yes | 100 | 2.1 (2.1) | |
| **Active/History of CA** | | | 0.881 |
| No | 554 | 2.3 (2.2) | |
| Yes | 138 | 2.3 (2.1) | |

**Table 4. Factors affecting AAA growth rates in the HEFT AAA surveillance database patient cohort**

Data reported as: "mean (SD)", with p-values from t-tests, for comparisons of two groups, or one-way ANOVA for more than two groups. *Significant at $p<0.05$ (bold)

***Kaplan-Meier estimated surgery-free survival***

Those patients who entered surveillance at a younger age with smaller, more slowly growing AAA were significantly more likely to be alive and free of AAA intervention at 5 and 10-years (**Table 5**). Of those aged > 75 years upon entry to surveillance, only 67.8% and 31.3% were alive without AAA-intervention at 5 and 10-years. Of those who entered with a MAPD 44 to 49mm, 51.1% and 19.7% were alive without AAA-intervention at 5 and 10years. Significantly fewer women than men were alive without AAA-intervention at 5- (70.1 vs. 78.1% respectively) and 10-years (28.2% vs. 44.1%).

There was no relationship between a diagnosis of ischaemic heart disease (IHD), chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM), hypertension, hypercholesterolaemia, congestive cardiac failure (CCF) or dementia and 5 or 10-year AAA intervention-free survival. Those with atrial fibrillation (AF) and end-stage renal failure (ESRF) were significantly more likely to die, or need intervention, than those who did not. Those who were active smokers also had significantly lower 5- and 10-year surgery-free survival rates than those who were ex-smokers, who in turn had lower rates than those who had never smoked. There was no significant difference found across the range of BMT regimes.

| | N | 5 Year Surgery-Free Survival | 10 Year Surgery-Free Survival | p-Value |
|---|---|---|---|---|
| **Age at First Scan** | | | | **<0.001*** |
| **< 65** | 95 | 0.816 | 0.495 | |
| **65 - 69** | 178 | 0.824 | 0.530 | |
| **70 - 74** | 203 | 0.791 | 0.376 | |
| **75+** | 216 | 0.678 | 0.313 | |
| **Initial AAA Size (mm)** | | | | **<0.001*** |
| **< 30** | 72 | 0.942 | 0.697 | |
| **30 - 44** | 518 | 0.801 | 0.419 | |
| **44 - 49** | 80 | 0.511 | 0.197 | |
| **50+** | 22 | 0.120 | . | |
| **AAA Gradient (mm/month)** | | | | **<0.001*** |
| **< 0.1** | 252 | 0.882 | 0.614 | |
| **0.1 - 0.2** | 223 | 0.846 | 0.482 | |
| **>0.2** | 217 | 0.558 | 0.116 | |
| **Sex** | | | | **0.023*** |
| **Male** | 575 | 0.781 | 0.441 | |
| **Female** | 117 | 0.701 | 0.282 | |
| **Rupture** | | | | **<0.001*** |
| **No** | 665 | 0.773 | 0.438 | |
| **Yes** | 27 | 0.640 | 0.080 | |
| **IHD** | | | | 0.518 |
| **No** | 360 | 0.738 | 0.444 | |
| **Yes** | 309 | 0.782 | 0.351 | |
| **COPD** | | | | 0.553 |
| **No** | 504 | 0.769 | 0.383 | |
| **Yes** | 163 | 0.726 | 0.423 | |
| **DM** | | | | 0.354 |
| **No** | 560 | 0.737 | 0.413 | |
| **Yes** | 107 | 0.868 | 0.302 | |
| **BP** | | | | 0.165 |
| **No** | 176 | 0.700 | 0.411 | |
| **Yes** | 492 | 0.780 | 0.389 | |
| **Smoking status** | | | | **0.002*** |
| **Never** | 165 | 0.884 | 0.484 | |
| **Ex** | 290 | 0.755 | 0.378 | |
| **Current** | 187 | 0.671 | 0.340 | |
| **Cholesterol** | | | | 0.951 |
| **No** | 490 | 0.744 | 0.415 | |
| **Yes** | 170 | 0.816 | 0.357 | |
| **BMT** | | | | 0.806 |
| **None** | 77 | 0.769 | 0.483 | |
| **Aspirin & Statin** | 445 | 0.761 | 0.398 | |
| **Aspirin only** | 40 | 0.711 | 0.309 | |
| **Statin only** | 56 | 0.789 | 0.417 | |
| **Warfarin & Statin** | 22 | 0.727 | 0.302 | |
| **Other** | 17 | 0.857 | . | |
| **ESRF** | | | | **0.008*** |
| **No** | 603 | 0.772 | 0.442 | |
| **Yes** | 89 | 0.741 | 0.285 | |
| **CCF** | | | | 0.177 |
| **No** | 615 | 0.769 | 0.425 | |
| **Yes** | 77 | 0.757 | 0.376 | |

| | | | | |
|---|---|---|---|---|
| **AF** | | | | **0.030*** |
| **No** | 591 | 0.771 | 0.439 | |
| **Yes** | 100 | 0.749 | 0.315 | |
| **Active/History of CA** | | | | **0.006*** |
| **No** | 554 | 0.773 | 0.450 | |
| **Yes** | 138 | 0.745 | 0.296 | |
| **Dementia** | | | | 0.954 |
| **No** | 656 | 0.772 | 0.417 | |
| **Yes** | 36 | 0.69 | 0.434 | |

**Table 5. Kaplan-Meier estimated surgery-free survival**

Data reported as Kaplan-Meier estimated survival, with p-values from log-rank tests.

*Significant at $p<0.05$ (bold)

***Cox-regression multivariable analysis***

A further multivariable survival analysis looking at survival to death or surgery was then performed using all the covariates identified in the Kaplan-Meier analysis to look at patient survival related to AAA surgery, or death (**Table 6**). Analysis was performed to determine whether any factors could be used to predict a future need for AAA surgical intervention or patient death. As expected, patients with larger AAA at point of entry into surveillance were significantly more likely to undergo intervention ($p < 0.001$). The same applied to those who were older ($p = 0.005$). Interestingly, AAA growth rate (mm/month) was also a significant predictor for surgical intervention, with a hazard ratio of 1.57 for patients with an AAA growth rate of 0.1 – 0.2 mm/month ($p = 0.007$) and 4.21 if the growth rate was >0.2 mm/month ($p < 0.001$), relative to those with a growth rate of <0.1 mm/month. Additionally, those who continued to smoke were also more likely to undergo intervention when compared to those who had never smoked (HR=1.97, $p<0.001$). Interestingly, even in those who claimed to have stopped smoking, the risk of intervention remained significantly increased ($p<0.001$), with a hazard ratio of 1.78 relative to those who had never smoked. Those with a history of AF or a known diagnosis of malignancy were also more likely to undergo intervention, or die in surveillance. The effect of COPD did not reach statistical significance after accounting for the other factors in the model (HR 0.79, $p=0.086$).

| | Hazard Ratio (95% CI) | p-Value |
|---|---|---|
| **Age at First Scan** | | **0.005*** |
| *< 65* | - | - |
| *65 - 69* | 0.75 (0.49 - 1.14) | 0.182 |
| *70 - 74* | 1.09 (0.72 - 1.64) | 0.682 |
| *75+* | 1.35 (0.90 - 2.03) | 0.146 |
| **Initial AAA Size (mm)** | | **<0.001*** |
| *< 30* | - | - |
| *30 - 44* | 2.46 (1.52 - 3.97) | <0.001* |
| *44 - 49* | 6.92 (3.89 - 12.32) | <0.001* |
| *50+* | 23.22 (11.60 - 46.49) | <0.001* |
| **AAA Gradient (mm/month)** | | **<0.001*** |
| *< 0.1* | - | - |
| *0.1 - 0.2* | 1.57 (1.13 - 2.17) | **0.007*** |
| *>0.2* | 4.21 (3.10 - 5.71) | <0.001* |
| **COPD** | | 0.086 |
| *No* | - | - |
| *Yes* | 0.79 (0.60 - 1.03) | 0.086 |
| **Smoking status** | | **<0.001*** |
| *Never* | - | - |
| *Ex* | 1.78 (1.31 - 2.43) | <0.001* |
| *Current* | 1.97 (1.41 - 2.75) | <0.001* |
| **AF** | | **0.004*** |
| *No* | - | - |
| *Yes* | 1.53 (1.14 - 2.05) | 0.004* |
| **Active/History of CA** | | **0.007*** |
| *No* | - | - |
| *Yes* | 1.46 (1.11 - 1.93) | 0.007* |

**Table 6. Cox-regression multivariable analysis**

Results from a backwards Cox regression model, with all of the variables in the Kaplan-Meier estimated survival analysis considered for inclusion. COPD = chronic obstructive pulmonary disease; AF = atrial fibrillation; CA = cancer; CI: confidence interval; *Significant at $p<0.05$ (bold).

**Limitations of study**

The presented analysis is a comprehensive description of the natural history of AAA in our local HEFT AAA patient population between February 1993 and August 2013. However, the dataset failed to capture those patients who had moved away from our patient catchment area during surveillance, or who were lost to follow-up for whatever reason. Additionally, as data was sourced from EPR, it was not possible to account for when, during the course of their surveillance, the variables used in the analysis (e.g.: co-morbidity and smoking status) could be defined. For example, patients may not have been diagnosed as diabetic at the time of entering the surveillance programme, but may have gone on to develop diabetes at a later date. The data entry did not account for this; they were simply recorded as being diabetic, as if they had been so for the full duration of surveillance. Likewise, they may have been commenced on BMT at any time during surveillance, but our analysis did not differentiate between a difference in e.g. AAA growth rates before or after BMT initiation; it simply recorded them as being on said medication as if they had been on it for the duration of their time in surveillance.

**Discussion**

This study has provided much useful information regarding the natural history of AAA in our local HEFT patient population, which can hopefully be used to progress the future delivery of surveillance services in our Trust. The analysis covers a twenty year period, and excluding those who were lost to follow-up or who left our patient catchment area during the course of this period, provides exhaustive data on all patients with AAA. It is our belief that the AAA surveillance programme at HEFT is probably not greatly different to those found at other NHS Trusts managing AAA patients, and so our results are generalisable. Historically surveillance scans at HEFT

were performed by local radiological departments, but with the advent of trained (vascular) clinical nurse specialists (CNSs) and improvements in US/employment of dedicated vascular-trained US-technicians, there has been a shift towards CNS-led surveillance clinics. This has greatly eased the burden on stretched radiology services and vascular surgeon out-patient consultant clinics, as patients can now have their AAA monitored in a separate setting.

The main finding of this study is that, over a 20-year period, a huge number of scans are performed for AAA surveillance, a significant proportion of which are on patients who are kept in the surveillance programme inappropriately. Our results report 73 patients being entered into surveillance and undergoing 109 scans despite not actually having AAA. This represents an inappropriate use of increasingly scarce healthcare resources. Although it is impossible to retrospectively calculate the individual financial burden this will have placed on HEFT in terms of staffing and costs, a simple calculation at today's current national tariff of £43 per US (for an US which takes less than 20 minutes, as most surveillance scans do) including reporting fees, (taken from the 2016/17 National Tariff Payment system report from produced by Monitor and NHS England) yields a figure of £4558 wasted performing these 109 US scans alone. Additional to the financial cost to HEFT is the unquantifiable financial, social and emotional cost to the patients involved, in particular for the four patients who remained under US-surveillance for up to a decade without ever reaching aortic diameters definable as aneurysmal.

That only 20% of patients entered into the surveillance programme underwent elective surgery also raises interesting questions about the frequency of screening intervals and what such rigorous surveillance achieves. Less than 4% of patients experienced rupture, of whom 41% had AAA which ruptured at MAPD less than

55mm at time of last scan and of all the ruptured cases, less than half (37%) proceeded to emergency repair.

Interestingly, of the many patient-related factors investigated, none were significantly correlated with rupture risk. This highlights the need to look elsewhere; specifically at AAA-specific factors such as biological markers within patients which may relate to aneurysm 'activity' or growth and rupture rates.

Our results identified that those with growth rates >4.6mm/year were significantly more likely to rupture. However identifying such patients in a large, busy unit such as ours requires close, individual monitoring of individual growth patterns, which is unlikely to be sustainable or feasible. Additionally, it raises the question that even if such patients were identified, what to do with them? There is no existing Level 1 evidence to compel us to offer early intervention in patients with rapidly expanding AAA, and until such a point where there is, simply identifying patients at increased risk does not necessarily confer patient-benefit. One could argue that at least in identifying such patients, they could be offered more regular imaging to monitor growth, but perhaps the bigger issue here is to say that size alone is not a sufficiently accurate marker of AAA-associated mortality risk. Arguably the decision to perform elective OR or EVAR should not be based on AAA MAPD as the absolute factor, but should also take into account individual patient-related factors such as expansion rates. Our results show that patient-related factors have little role in identifying 'at-risk' sub-groups who are more likely to succumb form AAA rupture.

Our results corroborate previously published work in that they suggest that female gender and smoking are associated with significantly faster AAA expansion and that DM appears to have a 'protective' role, in that it slows AAA expansion. Results from

other studies have shown that women experience a higher rate of AAA rupture when compared to men with the same AAA MAPD.(172)

Few published papers comment on the non-AAA related causes of death in patients with AAA, whilst ours clearly show that most patients who die in surveillance do so of non-AAA-related causes, most commonly malignancy.

**Conclusions**

Analysis of the HEFT AAA surveillance database has highlighted areas for service improvement. Specifically, it is important not commence surveillance in patients who are very unlikely to ever grow to a point where AAA surgery would be contemplated on grounds or age and/or co-morbidity. Similarly, patients need to be discharged from surveillance when this likelihood becomes apparent.

# Chapter 7: Results of a failed run of serum IL-1α analysis and the subsequent methodology study from which later studies were derived

In a pilot study we previously demonstrated that some patients with AAA have elevated serum IL-1α levels and that EC activation *in vitro* in response to serum from these patients, can be blocked by anti-IL-1α antibodies.(137) For these reasons, we postulated that IL-1α may represent a clinically useful biomarker of AAA progression and response to treatment. In order to further investigate this hypothesis, we anticipated undertaking a large prospective cohort study examining the relationship between serum IL-1α and various AAA- and patient-related variables.

A number of commercially available assays are available to measure serum IL-1α levels in humans. The majority work on standard sandwich enzyme-linked immunosorbent (ELISA) principles, involving a micro-titre plate pre-coated with a monoclonal anti-IL-1α specific antibody. Following addition of antigen-containing serum, a biotinylated polyclonal antibody specific to IL-1α is then added to complete the 'sandwich'. An avidin-conjugated complex, which has a high binding affinity to biotinylated products, allows signal amplification prior to addition of an organic enzyme substrate that reacts with the conjugated complex to induce a colour change. This can then be quantified using chromogenic techniques.

An alternative to the micro-titre plate based immunoassays is the bead-based multiplex system, which works on the same principles described above, but utilises magnetic microspheres ('beads') pre-coated with the capture antibody instead of the solid surface of the plate and streptavidin-phycoerythrin (SA-PE) as the final conjugate prior to quantification using flow cytometry.

**Methods**

The previous, published pilot study undertaken by other members of our research group was performed on serum samples collected and immunoanalysis performed by the Clinical Immunology Service (CIS) at the University of Birmingham using their standard IL-1α measuring platform, a Bio-Rad Bio-Plex Pro commercially available immunoassay. With that in mind, we therefore embarked on collecting serum samples from patients with AAA as they attended our surveillance clinics at HEFT. In order to maximise the volume of patients recruited, we collaborated with vascular surgical colleagues at City Hospital, part of Sandwell and West Birmingham Hospitals NHS Trust (SWBH) with a plan to run the first cohort batch analysis once n = 100 patients had been recruited. As this was a large cohort of patients, in the interests of serum processing standardisation and to reduce the risk of technical error in a larger study, conducted over a long period of time, we utilised the services of a local bio-bank, the Human Biomaterials Resource Centre (HBRC), based at the University of Birmingham to process the whole blood samples.

Once 96 patients had been recruited, and samples processed, an initial batch run was performed using a Bio-Rad immunoanalysis kit ('Bio-Rad kit #1'). Surprisingly, results from this immunoanalysis yielded a completely negative set of results, with all 96 sample titres reported as below the detectable range of the ELISA assay. The standard curve readings, prepared by performing serial dilutions of one known standard concentrate of IL-1α as provided by the manufacturer prior to generating a standard curve using linear regression models as directed, demonstrated a 'good fit' meaning the kits worked appropriately (**Figure 3**). In light of this finding, we went back to the original paper to check for differences within the methodology. We found that in the original pilot study, the immunoanalysis kit used was manufactured by

Millipore and assays were measured on their Milliplex MAP immunoassay (Millipore, Billerica, Mass.), which is a bead-based multiplex system. Additionally, as the pilot study had used only a small number (n = 17) patients, serum processing of whole blood had been performed 'in-house' within the facilities of HEFT's vascular research laboratories by the chief researcher, and slight differences in serum processing existed. In the 2011 study, samples were collected and immediately centrifuged at RT at 12K RPM for 10 minutes in serum separating tubes (SST) prior to aliquoting and storage at -80C. In using HBRC however, samples were collected at the end of clinics and transferred on ice, with a possible delay of no greater than six hours, before centrifugation and storage. Standard HBRC operating procedures were then followed for obtaining serum from whole blood (centrifugation at room temperature for 10 minutes at 4600RPM). In light of the literature suggesting that IL-1α in serum is relatively stable when collected in commercial tubes and stored at 4C, it was not thought prior to the first run of assays that samples collected using the HBRC protocol would adversely affect results.(173)

Thinking that the differences in titre values seen between the original pilot study and Bio-Rad kit #1 were attributable, for the most part, to the different immunoassay manufacturers' kits, a subsequent re-run was performed using another Bio-Rad immunoassay ('Bio-Rad kit #2') and a Millipore immunoassay at CIS.

This was performed on 41 of the samples tested on Bio-Rad kit #1 and 43 samples from the original 2011 study ('Original 2011 Study') from our research group which had detected positive IL-1α titres in serum samples of patients with AAA.(137) These samples had been stored since collection at -80C, but had been through an undetermined number of freeze/thaw cycles. Results from this were disappointing, with mostly negative results from this re-run (**Table 7**), showing inconsistent results

between the same samples run between the two kits, and that samples that were positive in both yielded markedly different values. Again, the standard curve readings from the kits suggested that they worked appropriately (**Figures 4 and 5**). The standard curve from the original 2011 study was not available, but one can only assume that the researchers at the time had checked its suitability. We also found that IL-1α titre values from the original study were much reduced when compared to their original 2011 values, although this may in part have been due to degradation of IL-1α as a result of prolonged storage at -80C. Discussion with the manufacturer Bio-Rad subsequently confirmed that there had been a change in the production of the kits, and that a different antibody was being used, however we were unable to identify the exact nature of the change. A methodology study to validate the collection, storage and processing of samples and to evaluate potential variability between different nine commercially available IL-1α immunoassays was then performed on a small (n = 10) cohort of patients.

| Standards expected concentration (pg/ml) | Observed concentration (pg/ml) |
|---|---|
| 23534 | >OOR |
| 5883.5 | >OOR |
| 1470.88 | 1400.91 |
| 367.72 | 370.29 |
| 91.93 | 91.07 |
| 22.98 | 23.52 |
| 5.75 | 5.59 |
| 1.44 | 1.47 |

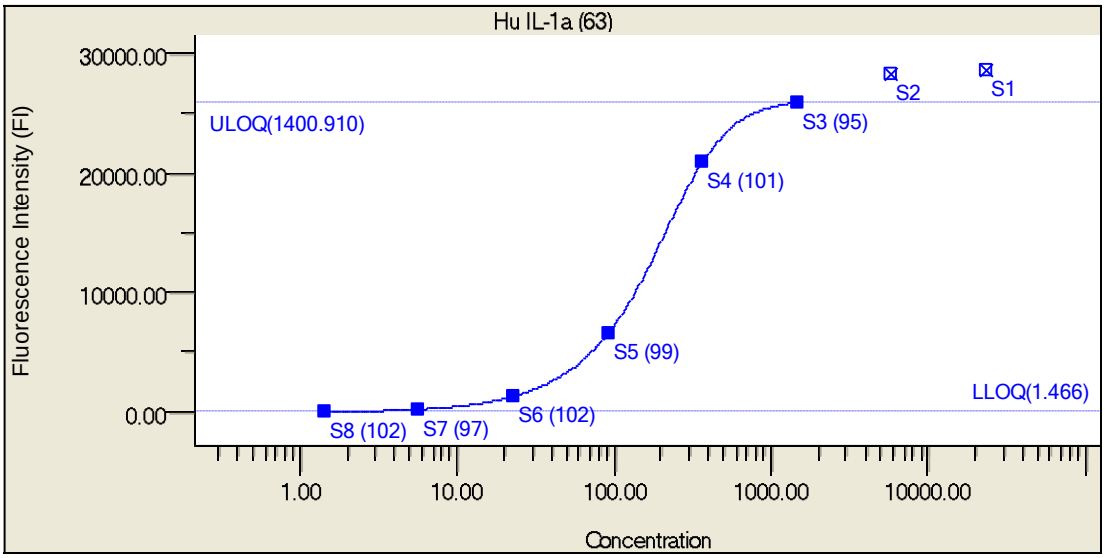


**Figure 3. Standard curve readings and graph from the first immunoassay run using Bio-Rad Kit#1**

All serum samples (n=96) reported as out of range

| Sample | Bio-Rad Kit #2 HBRC samples (pg/ml) | Bio-Rad kit #2 Original 2011 Study samples (pg/ml) | Millipore kit HBRC samples (pg/ml) | Millipore kit Original 2011 Study samples (pg/ml) | Previous recorded titres from Original 2011 Study titres (pg/ml) |
|---|---|---|---|---|---|
| 1 | UND | UND | **0.6** | **11.22** | 40.84872 |
| 2 | UND | UND | **0.91** | UND | 77.999 |
| 3 | UND | UND | **0.91** | **46.04** | 60.44681 |
| 4 | UND | UND | **2.19** | **37.11** | 109.5758 |
| 5 | UND | UND | **3.49** | **59.36** | 164.874 |
| 6 | UND | UND | **6.48** | **7.15** | 77.999 |
| 7 | UND | UND | **8.16** | **7.15** | 60.44681 |
| 8 | UND | UND | 11.56 | **1.87** | 77.999 |
| 9 | UND | UND | UND | **1.23** | 138.2029 |
| 10 | UND | UND | UND | UND | 40.84872 |
| 11 | UND | **8.29** | UND | **3.49** | 109.5758 |
| 12 | UND | **127.75** | UND | **16.03** | 60.44681 |
| 13 | UND | UND | UND | UND | 60.44681 |
| 14 | UND | UND | UND | UND | 40.84872 |
| 15 | UND | UND | UND | **6.48** | 40.84872 |
| 16 | UND | UND | UND | **4.48** | 40.84872 |
| 17 | UND | UND | UND | UND | 124.1792 |
| 18 | UND | UND | UND | **85.2** | 77.999 |
| 19 | UND | UND | UND | **6.14** | 60.44681 |
| 20 | UND | UND | UND | **1.23** | 40.84872 |
| 21 | UND | UND | UND | UND | 77.999 |
| 22 | UND | UND | UND | UND | 60.44681 |
| 23 | UND | UND | UND | **52.15** | 77.999 |
| 24 | UND | UND | UND | UND | 40.84872 |
| 25 | UND | UND | UND | **15.69** | 77.999 |
| 26 | UND | UND | UND | UND | 77.999 |
| 27 | UND | UND | UND | UND | 60.44681 |
| 28 | UND | UND | UND | **0.3** | 40.84872 |
| 29 | UND | UND | UND | **1.87** | 94.25367 |
| 30 | UND | UND | UND | **6.81** | 77.999 |
| 31 | UND | UND | UND | **5.48** | 40.84872 |
| 32 | UND | UND | UND | **7.82** | 151.7441 |
| 33 | UND | UND | UND | UND | 40.84872 |
| 34 | UND | UND | UND | **6.81** | 77.999 |
| 35 | UND | UND | UND | **4.15** | 109.5758 |
| 36 | UND | UND | UND | **7.15** | 77.999 |
| 37 | UND | UND | UND | **0.91** | 124.1792 |
| 38 | UND | UND | UND | **12.59** | 40.84872 |
| 39 | UND | UND | UND | **74.61** | 111.9797 |
| 40 | UND | UND | UND | **55.75** | 98.2595 |
| 41 | UND | UND | UND | **66.61** | 56.30869 |
| 42 | | UND | | **10.88** | 139.1327 |
| 43 | | UND | | **28.61** | 56.30869 |

**Table 7. IL-1α titres for immune-analysis using two kits, with HBRC and original 2011 study samples.** <UND = levels too low to be quantifiable

Values in red indicate positive results from re-run

| Standards expected concentration (pg/ml) | Observed concentration (pg/ml) |
|---|---|
| 23534 | 15581.42 |
| 5883.5 | 9039.34 |
| 1470.88 | 1470.63 |
| 367.72 | 339.07 |
| 91.93 | 94.56 |
| 22.98 | 24.49 |
| 5.75 | 5.02 |
| 1.44 | 1.76 |

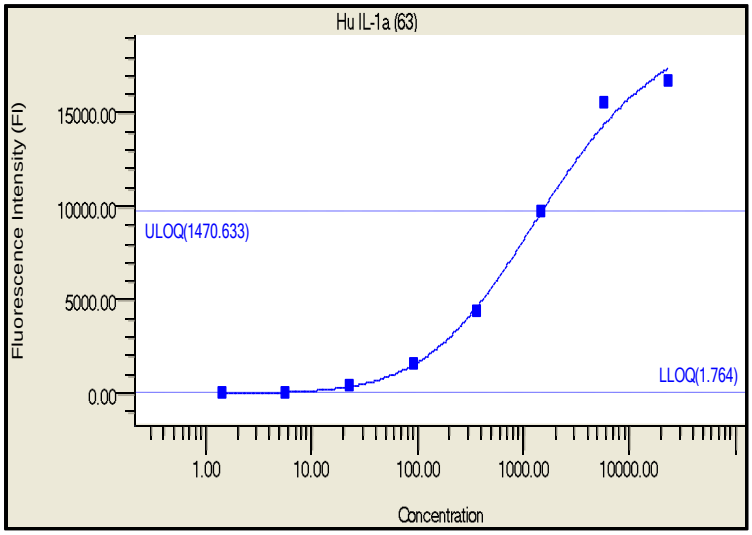


**Figure 4. Standard curve for Bio-Rad kit #2**

| Standards expected concentration (pg/ml) | Observed concentration (pg/ml) |
|---|---|
| 2000 | 2827.7 |
| 400 | 273.74 |
| 80 | 167.52 |
| 16 | 27.2 |
| 3.2 | 3.49 |
| 0.64 | <OOR |
| 0 | - |
| | |

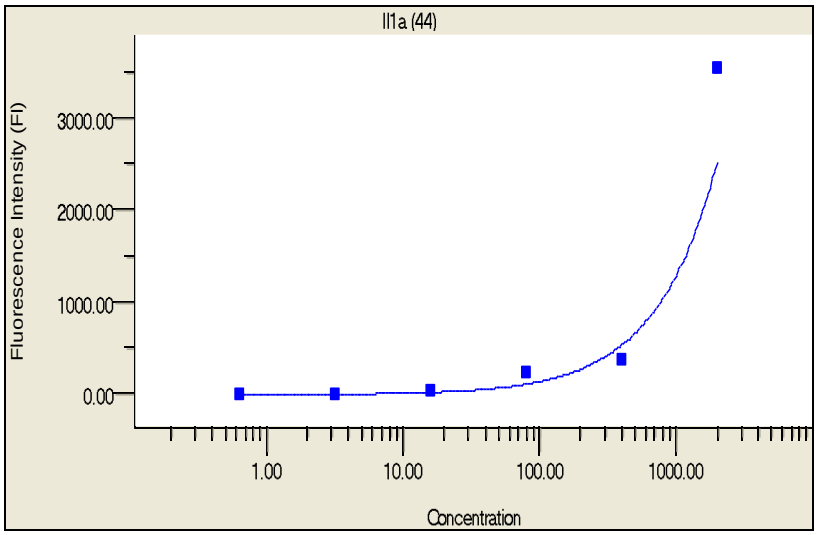


**Figure 5. Standard curve for Millipore Kit HBRC samples**

Before embarking on our larger study, we wished to establish that we could measure IL-1α in the serum of patients with AAA in a practical, reliable and reproducible manner. The aim of this next study, therefore, was to investigate the effect of two different serum processing methodologies and nine different (7 ELISA and 2 bead-based) commercial immunoassays on serum IL-1α levels in patients with AAA. We simultaneously compared recombinant IL-1α (both 33kDa (Cal +ve) and 17kDa (Cal -ve) and to the best of our knowledge, are the first group to do so.

**Patient recruitment and sample collection and preparation**

Ten patients with asymptomatic AAA were recruited from the vascular out-patient clinics at HEFT. All were male, with mean age 70 years (range 64-83). Patient demographics are shown in **Table 8**.

| Patient | AAA MAPD (mm) | AAA type | Smoking status | BMT (anti-platelet/ statin) | Ischaemic heart disease (IHD) | Hypertension | Chronic obstructive pulmonary disease (COPD) | Diabetes Mellitus | Hyper-cholesterolaemia | Congestive heart failure (CCF) | Other co-morbidity of note |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | **44** | Infra-renal AAA | No | No | No | No | No | No | No | No | No |
| **2** | **36** | Infra-renal AAA | Ex | Warfarin and statin | Yes | Yes | No | No | No | No | CABG 2008 |
| **3** | **33** | Infra-renal AAA | Ex | Warfarin and statin | Yes | Yes | No | No | No | No | AF |
| **4** | **32** | Infra-renal AAA | Ex | Yes | No | Yes | No | No | No | No | Hypothyroidism |
| **5** | **32** | Infra-renal AAA | Ex | No | No | No | No | No | No | No | No |
| **6** | **48** | Infra-renal AAA | Yes | Yes | No | No | No | No | No | No | Hypothyroidism |
| **7** | **50** | Infra-renal AAA | Ex | Yes | No | No | No | No | No | No | No |
| **8** | **59** | TAA | Ex | Yes | Yes | Yes | Yes | No | No | No | 5/12 post OR with bilateral renal bypass |
| **9** | **100** | TAA | Ex | Yes | Yes | Yes | No | No | No | No | TB OR 1999 |
| **10** | **68** | Infra-renal AAA | Ex | Yes | Yes | Yes | No | No | No | No | No |

**Table 8. Patient demographics of the ten patient recruits for the IL-1α immunoassay methodology study**

Following skin preparation with an alcohol-based antiseptic, whole blood was drawn from an antecubital fossa vein using a tourniquet, Vacuette® Multi-Sample Blood Collection Needles (21G-green) and Vacuette® Standard Tube Holders into multiple Vacuette® Z Serum Clot Activator tubes (Greiner Bio-One, Kremsmünster, Austria).

In accordance with manufacturer's guidelines, collection tubes were inverted 8 times to allow mixing and the whole blood permitted to clot at RT over 30 minutes.

Two sample processing methodologies were then undertaken, one involving immediate, and the other delayed centrifugation.

- **Method A** comprised immediate centrifugation of clotted whole blood (10 min, 4600 RPM, RT) followed by aliquoting of serum into cryovials which were then transported on ice to the Human Biomaterials Resource Centre (HBRC) at the University of Birmingham where they were stored at -80C (all completed within 6 hours of specimen collection).
- **Method B** comprised transfer of clotted whole blood on ice to the HBRC followed by centrifugation (10 min, 4600 RPM, RT), and then aliquoting of serum into cryovials for storage at -80C (all completed within 6 hours).

Sufficient volumes of serum were placed in at least 9 cryovials to ensure that no samples were subjected to freeze/thaw cycles during immunoanalysis.

Upon receipt, the nine immunoassays (**Table 9**) were stored in accordance with the manufacturers' instructions.

Immunoanalysis was performed on all of the kits, on consecutive days, within 6-months of sample collection to minimise the risk of IL-1α degradation during long-term storage.

| Manufacturer | Name | Cat no. | Batch/Lot no. |
|---|---|---|---|
| **Cusabio** | Human Interleukin 1α (IL-1α) ELISA Kit | CSB-E04620h | I14153067 |
| **BioLegend** | Legend Max™ Human IL-1α | 434907 | B154870 |
| **Biosensis** | Human Interleukin 1 alpha Kit | BEK-2033-1P | BA01-30-2033 TK141212 |
| **BioVendor** | Human Interleukin-1 alpha ELISA | RBMS243/2R | X12-170 |
| **Thermo Scientific** | Human IL-1α ELISA Kit | EH21LA | NJ175968 |
| **Boster** | Human IL-1α ELISA Kit | EK0389 | 111871104 |
| **RayBiotech** | Human IL-1α ELISA Kit | ELH-IL1alpha-001 | 1228120199 |
| **Millipore** | MILLIPLEX® MAP Kit | HCYTOMAG-60K-01 | 2115942 |
| **Bio-Rad** | Bio-Plex Pro™ Assay for IL-1α | 171-304070 | 310011908 |

**Table 9. Commercially available IL-1α kits tested in methodology study**

All assays were run as per the manufacturers' protocols provided, and optical density (OD) was measured using plate spectrophotometry at 450nm. Wavelength correction was performed when specified by individual manufacturers.

Microplates were loaded with manufacturers' standards as provided, undiluted serum samples (in triplicate) and known positive rIL-1α controls, cellular 33kDa and 17kDa subunits [calpeptin positive (Cal +ve) and calpeptin negative (Cal -ve) respectively].[**1]

ELISA endpoint absorbance was measured on a BioTek Synergy® multi-detection microplate reader utilising KC4® v3.4 software. Loading of the ELISA plates is shown in **Figure 6**.

[**1] rIL-1α, Cal+ve and Cal-ve controls were generously provided by Dr Murray Clarke, Division of Cardiovascular Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK

| | A | B | C | D | E | F | G | H | I | J | K | L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | |
| 5 | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | |

**Figure 6. ELISA 96-well plate layout**

| **Key** | |
|---|---|
| | Standards |
| | Method A |
| | Method B |
| | Controls |

Bead-based assays were analysed using the Luminex(r) 100 system (Luminex Corp, USA) with Bio-Plex Manager 6.0 software (Bio-Rad, UK).

IL-1α titres were calculated against standard curves generated from each assay using reference concentrations supplied by manufacturers. OD readings were converted to units (pg/ml) with GraphPad PRISM v5.0.

In cases where bioassay results for the r-IL1α, Cal + ve or Cal - ve controls were out of an assay's detectable range (UND), dilution studies were not subsequently performed to obtain quantifiable titre values as the purpose of running the controls was simply to verify that the immunoassays could detect IL-1α and both the 33kDa and 17kDa subunits.

**Results**

Ten male patients of mean (range, [standard deviation (SD)] age 70 (64-83, [±6.8]) years with asymptomatic, infra-renal AAA or Crawford extent 2 thoracoabdominal aortic aneurysms (TAA) (n=2) were studied. Mean AAA size as recorded on the day of serum sampling by US was 65mm (range 32 to 100mm [±22]). Patient demographics are shown in **Table 10**.

| Patient | AAA MAPD (mm) | AAA type | Smoking status | BMT | IHD | HT | COPD | DM | Hyper-cholesterolaemia | CCF | Other co-morbidity of note |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 44 | Infra-renal AAA | No | No | No | No | No | No | No | No | No |
| 2 | 36 | Infra-renal AAA | Ex | Warfarin and statin | Yes | Yes | No | No | No | No | CABG 2008 |
| 3 | 33 | Infra-renal AAA | Ex | Warfarin and statin | Yes | Yes | No | No | No | No | AF |
| 4 | 32 | Infra-renal AAA | Ex | Yes | No | Yes | No | No | No | No | Hypothyroidism |
| 5 | 32 | Infra-renal AAA | Ex | No | No | No | No | No | No | No | No |
| 6 | 48 | Infra-renal AAA | Yes | Yes | No | No | No | No | No | No | Hypothyroidism |
| 7 | 50 | Infra-renal AAA | Ex | Yes | No | No | No | No | No | No | No |
| 8 | 59 | TAA | Ex | Yes | Yes | Yes | Yes | No | No | No | 5/12 post OR with bilateral renal bypass |
| 9 | 100 | TAA | Ex | Yes | Yes | Yes | No | No | No | No | TB OR 1999 |
| 10 | 68 | Infra-renal AAA | Ex | Yes | Yes | Yes | No | No | No | No | No |

**Table 10**. **Demographics of the ten patients recruited for the immunoassay methodology study**

HT: hypertension; IHD: ischaemic heart disease; CABG: coronary artery bypass graft; CCF: congestive cardiac failure; AF: atrial fibrillation; DM: diabetes mellitus; COPD: chronic obstructive pulmonary disease; OR: open aneurysm repair; TB: tuberculosis; BMT: best medical therapy; TAA: thoracoabdominal aneurysm (Crawford extent 2)

Serum IL-1α titres (pg/ml) using Method A and B on each immunoassay are shown in **Table 11**. Results of rIL-1α, Cal + ve and Cal - ve controls, including dilutions, are shown in **Table 12**. IL-1α titre results comparing Method A to Method B are shown in **Figures 7** and **8**.

| Patient | Cusabio | BioLegend | Biosensis | BioVendor | ThermoSci | Boster | RayBio | Millipore (Bead-assay) | Bio-Rad (Bead-assay) |
|---|---|---|---|---|---|---|---|---|---|
| 1A | UND | UND | 9.7 | UND | UND | UND | UND | UND | UND |
| 1B | UND | UND | 2.1 | UND | UND | 1.0 | 0.65 | UND | UND |
| 2A | UND | UND | 18.2 | UND | UND | 67.8 | UND | UND | UND |
| 2B | 64.9 | UND | 24.0 | UND | UND | 79.3 | 0.41 | UND | UND |
| 3A | UND | UND | 17.1 | UND | UND | 17.5 | 0 | UND | UND |
| 3B | 28.4 | UND | 20.4 | UND | UND | 30.7 | UND | UND | UND |
| 4A | UND | UND | UND | UND | UND | 105.0 | UND | UND | UND |
| 4B | 114.4 | UND | 0.7 | UND | UND | 116.4 | UND | UND | UND |
| 5A | UND | UND | UND | UND | UND | UND | UND | UND | UND |
| 5B | 33.6 | UND | UND | UND | UND | 4.9 | UND | UND | UND |
| 6A | UND | UND | 5.9 | UND | 40.1 | 0.9 | 0 | UND | UND |
| 6B | 31.4 | UND | 8.0 | UND | UND | 1.4 | UND | UND | UND |
| 7A | UND | 23.0 | 27.0 | UND | UND | 59.5 | 0 | UND | UND |
| 7B | 68.2 | 2.0 | 27.4 | UND | UND | 33.2 | UND | UND | UND |
| 8A | UND | 3.8 | UND | UND | UND | UND | UND | UND | UND |
| 8B | 27.4 | UND | UND | 0.9 | UND | 1.3 | UND | UND | UND |
| 9A | UND | 7.8 | 7.6 | UND | 5.4 | 11.5 | UND | UND | UND |
| 9B | UND | UND | 8.3 | UND | UND | 20.0 | UND | UND | UND |
| 10A | UND | 12.8 | 27.7 | 2.3 | UND | UND | 0 | UND | UND |
| 10B | UND | 6.7 | 23.7 | 2.5 | UND | 1.0 | 0 | UND | UND |

**Table 11. IL-1α titres (pg/ml) for each of the 10 patient serum samples processed using Method A (immediate centrifugation and processing) or Method B (delayed centrifugation and processing using a regional biobank) on each of the immunoassays tested**

UND=levels below manufacturer's detectable range.

| Controls | Cusabio | BioLegend | Biosensis | BioVendor | ThermoSci | Boster | RayBio | Millipore (multiplex) | Bio-Rad (multiplex) |
|---|---|---|---|---|---|---|---|---|---|
| **Cal +ve** | 0 | OOR | OOR | OOR | OOR | 1163.2pg/ml | 2.3pg/ml | 1443.1pg/ml | 1091.7pg/ml |
| **Cal –ve** | 0 | OOR | OOR | OOR | OOR | 412.7pg/ml | 3.2pg/ml | 4039.8pg/ml | 2673.6pg/ml |
| **rIL-1α (concentration)** | | | | | | | | | |
| **500ng/ml** | - | - | OOR | OOR | OOR | - | 3.9pg/ml | - | - |
| **250ng/ml** | - | - | - | - | - | - | 3.8pg/ml | - | - |
| **200ng/ml** | - | - | OOR | OOR | OOR | - | - | - | - |
| **100ng/ml** | 0 | - | OOR | 146.5(pg/ml) | OOR | OOR | - | 1499.6pg/ml | 1214.7pg/ml |
| **50ng/ml** | 0 | - | 179.7pg/ml | 100.7pg/ml | 458.6pg/ml | 1315.8pg/ml | - | 745.9pg/ml | 596.6pg/ml |
| **25ng/ml** | 0 | - | - | - | - | - | - | - | - |
| **5ng/ml** | - | OOR | - | - | - | - | - | - | - |
| **3ng/ml** | - | OOR | - | - | - | - | - | - | - |
| **2ng/ml** | 0 | OOR | - | - | - | 6.8pg/ml | - | - | - |
| **1ng/ml** | 0 | OOR | - | - | - | 5.1pg/ml | - | - | - |

**Table 12. IL-1α titres (pg/ml) of positive rIL-1α (rIL-1α) controls and calpeptin-positive (Cal + ve) 33kDa IL-1α and calpeptin-negative (Cal - ve) 17kDa IL-1α subunits**

OOR= levels above manufacturer's detectable range,

'-' = concentration of rIL-1α not tested at this concentration

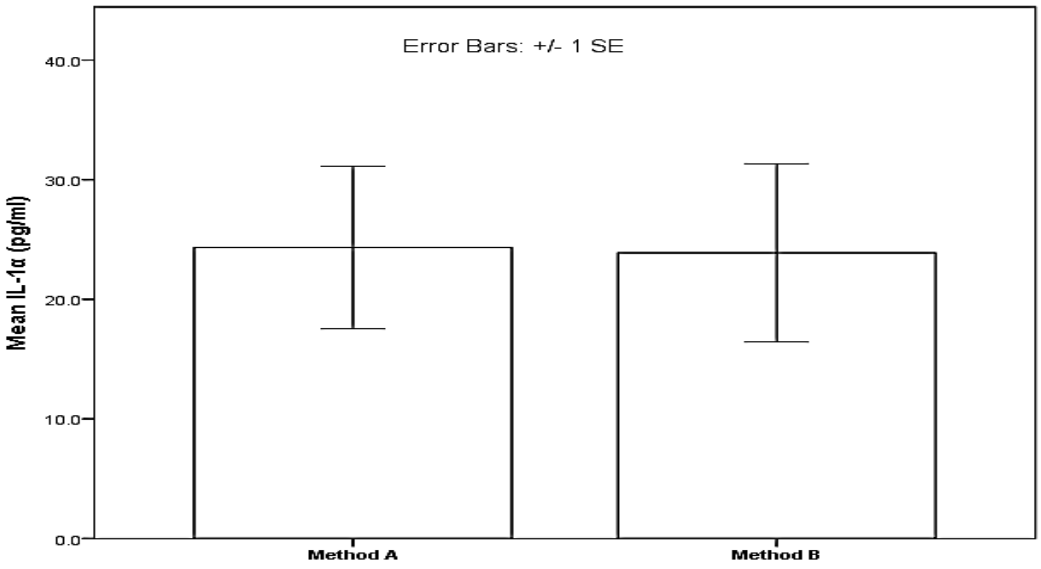


**Figure 7. Box plot comparing mean IL-1α titre concentrations obtained across all immunoassays using Method A (immediate processing) and Method B (delayed processing via a regional biobank)**

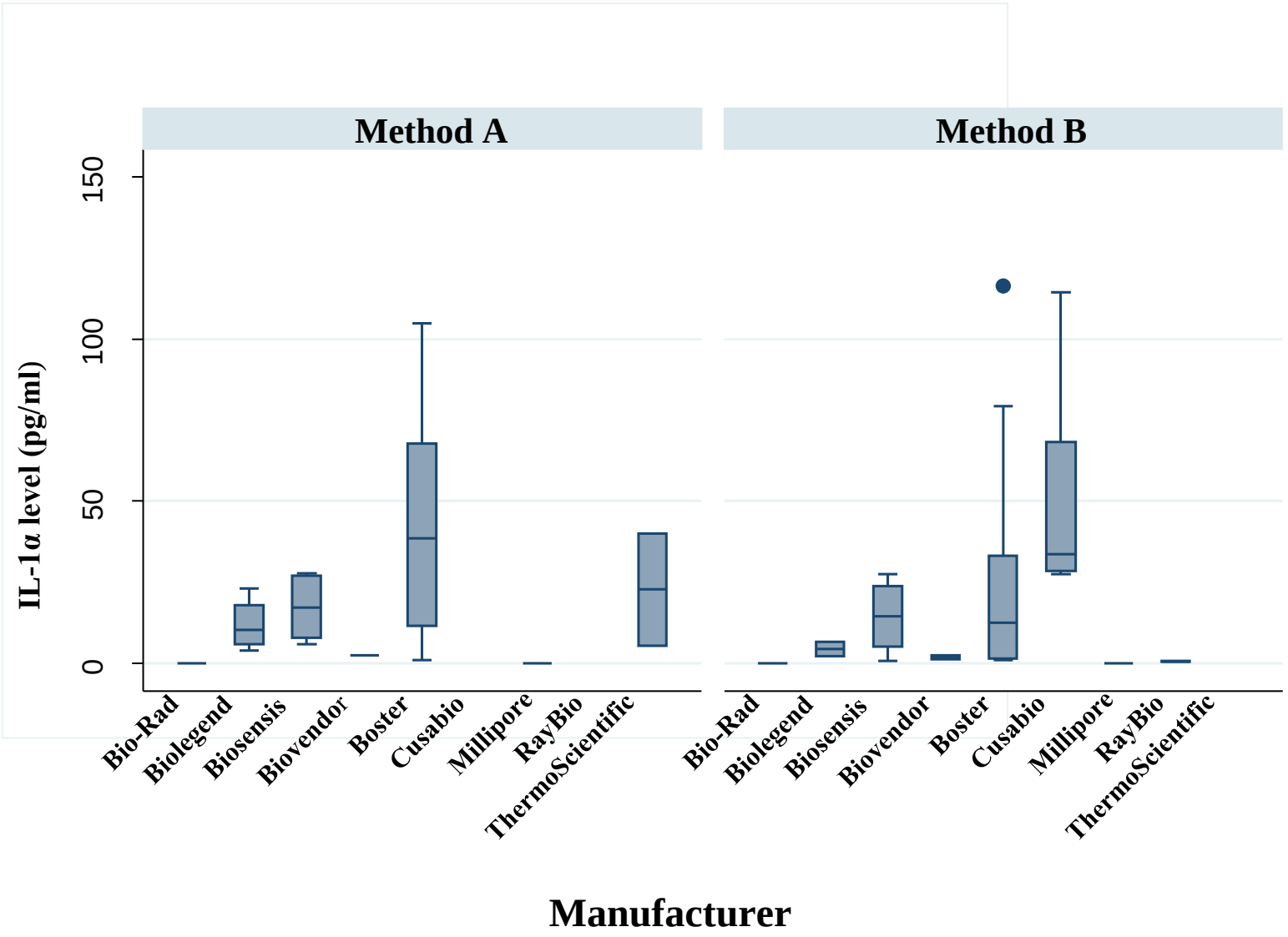


**Figure 8. Box plot of IL-1α titre against manufacturer via methods**

## Discussion

Our study has compared two methods of whole blood processing using nine commercially available immunoassays for the detection and quantification of IL-1α in the serum of patients with AAA. Our aim was to identify a reliable and reproducible means by which to measure IL-1α ahead of a large cohort study investigating IL-1α as a biomarker of AAA progression and response to treatment.

Our results show that although differences in serum processing techniques can affect results, the impact is not uniform and appears to be dependent on the immunoassay used. For example, Cusabio's immunoassay was unable to quantifiably detect IL-1α in serum processed using Method A, but reported high titre results in seven samples processed using Method B. Possible explanations for why immediate processing (Method A) may result in low titres compared to delayed centrifugation (Method B) include sustained cellular release, or production, of IL-1α after blood has been drawn due to progressive, or continued, cell lysis.(174,175)

In complete contrast however, ThermoSci's immunoassay was able to quantify IL-1α in four samples processed using Method A, but none processed using Method B. Explanations for delayed serum processing methods resulting in low titres include the possibility of IL-1α degradation in the time taken for processing and storage. There is some evidence to suggest that IL-1α remains relatively stable, when stored at 4°C, for up to 24 hours, however environmental interactions or artefact effects within whole blood during processing may affect titre levels, or render detectable components with a low affinity for individual immunoassay epitope binding agents.(174,175)

However, on aggregate, we found that there appears to be no real difference in mean IL-1α titre concentrations obtained across all immunoassays using either processing Method A or Method B. From this we have concluded that it is safe and feasible for researchers analysing IL-1α to utilise regional biobanks for sample processing and storage.

One of the most striking findings in our results was the variability of results seen between samples from individual patients across the different immunoassays. Explanations for these findings include a lack of routine disclosure of the exact components of immunoassays by manufacturers, specifically the morphology of standards supplied, antibodies used and their corresponding epitope binding sites. Differences in these may affect an individual immunoassay's ability to detect IL-1α, with falsely elevated, or low concentrations, due to non-specific binding.(176–179)

Additionally, individual manufacturers may use different recombinant antibodies to produce each of their kits, which may cause incongruous results between assays or even inter-batch variation across assays from one manufacturer. This lack of concordance can pose problems to researchers, who may find that different kits produce markedly varied results.(176)

Although there is a paucity of studies in the literature specifically looking at IL-1α, studies have shown that cross-technology comparisons are difficult. Significant differences in results have been found when measuring IL-1β using ELISA immunoassays and explanations for this include physical differences in binding sites for each isoform and limitations in ELISA recognition, or binding-site, profiles.(178,180,181)

Candidate markers may well be present in samples, but differences in detection or capture antibodies may result in failure due to incompatible binding sites or undetermined competitive antigen binding from other components in patient serum.(182) Additionally, endogenous proteases may fragment precursors in ways which render them undetectable.(178–181,183,184)

The presence of auto-antibodies within serum samples causing interference within assays is a recognised flaw in immune-analysis and can lead to misleading results. It is as yet unknown whether such potential competitor auto-antibodies exist in the context of IL-1α assay binding sites, and there are no published studies investigating whether this is the case in the literature.

In the case of IL-1α, which has multiple isoforms capable of exerting biological effects, the ability of an immunoassay to detect both pro- and mature-IL-1α is important as it is not yet known whether one, or both, have a role in AAA.(131,185)

Our results show that, with the exception of the Cusabio immunoassay, all of the kits were able to detect IL-1α as well as both Cal +ve and Cal -ve isoforms. Possible explanations for the anomalous Cusabio results include differences in the structure or pharmacokinetics of these non-endogenous proteins which render them undetectable by the immunoassay, or which prevent epitope binding.(186,187) Technical malfunction is of course another possibility, either as a result of operator error or due to components within the individual assays failing to work correctly, perhaps as a result of product degradation during shipment and delivery.

Our results identify and highlight a potentially serious problem for basic scientists embarking on studies such as ours. It is unlikely that the variability in results represents a failure on our, or the CIS's part, to perform immunoanalysis techniques

correctly, yet we have shown that a variety of manufacturers sell (expensive) kits which may, or may not, be able to accurately measure the moiety they claim to be able to quantify. The wider consequences of this appear to be profound; it is probable that there may be research groups investigating a variety of molecules in various pathologies who are largely unaware that the assays they are buying (at great expense) can produce such varied results. The implications of this are that this leads to (false) 'positive' data which can subsequently mislead researchers, or generates (false) 'negative' data which effectively stops further research in a field where had other immunoanalysis kits been used, could have resulted in positive biological results.

**Conclusions**

Our results demonstrate marked variability in IL-1α titre concentrations when using different serum processing techniques and between IL-1α immunoassays produced by different manufacturers. They suggest that researchers should be cautious when interpreting published data as absolute concentrations of IL-1α from different vendors are not interchangeable and differences in methodologies can impact results considerably. This may result in misleading conclusions being drawn about fundamental biological processes underpinning a wide range of inflammatory diseases.

Our study suggests that conclusions of IL-1α's role in pathological processes should not be drawn from the absolute titre values, but rather from general trends seen.

# Chapter 8: Study 1 looking at serum IL-1α titres in patients with asymptomatic, infra-renal AAA size, morphology and growth rates

Infra-renal abdominal aortic aneurysms (AAA) are usually asymptomatic until complications arise, most commonly rupture. AAA size, and in particular MAPD, together with growth rates, are widely believed to be the best current predictors of rupture and so are the variables most often used in clinical practice to select patients for open surgical (OR) or endovascular aneurysm repair (EVAR).(36,37,42,43)

AAA is widely recognised to be a chronic inflammatory condition that is separate from, but which often co-exists with, atherosclerosis due to shared risk factors.(188) Previous work from our and other groups has shown patients with AAA to have abnormalities in serum markers of inflammation and coagulation associated with a pro-thrombotic, pro-inflammatory, hypercoagulable diathesis.(79–82) However, it remains unclear whether AAA development and progression is primarily a result of biomechanical (blood pressure, AAA size and shape, shear and stress forces) or biological (disintegration of the aortic wall due to inflammation and disordered tissue re-modelling) factors, or both.

An AAA intervention strategy based on size alone may leave patients with small (generally regarded as <55mm in MAPD) but nevertheless 'biologically active' AAA unprotected from rupture, whereas patients with large but biologically 'quiescent' AAA may be offered and possibly even harmed by unnecessary surgery. There is, therefore, an urgent need to identify better predictors of rupture so that the available health care resources can be used in the safest and most clinically and cost-effective manner. One particular field of current interest is identification of reliable clinical biomarkers in AAA.

Interleukin-1 alpha (IL-1α) is a pro-inflammatory cytokine that is not normally detectable in the circulation in health.(127) IL-1α has been linked with cardiovascular disease in general and it is known that some patients with aortic disease have detectable IL-1α titres.(142,134,103,89,94) In a pilot study, we demonstrated that patients with AAA had detectable serum IL-1α levels which appeared to be related to AAA diameter. Additionally, we found that EC activation *in vitro* in response to serum from these AAA patients was blocked by anti-IL-1α antibodies.(137)

**Aims**

In a pilot study, we reported a relationship between abdominal aortic aneurysm (AAA) diameter and serum interleukin (IL)-1α levels and that EC activation *in vitro* in response to serum from AAA patients was blocked by anti-IL-1α antibodies. We now aim to further investigate the relationship between serum IL-1α and asymptomatic infra-renal AAA size, morphology and growth rates.

**Methods**

**Patient recruitment and serum sample collection**

Research ethical committee (REC) approval was obtained and all patients provided fully-informed, written consent. Patients with asymptomatic, infra-renal AAA were recruited from vascular clinics at HEFT and SWBH. Following skin preparation with an alcohol-based antiseptic, whole blood was drawn from an antecubital fossa vein using a tourniquet, Vacuette® Multi-Sample Blood Collection Needles (21G-green) and Vacuette® Standard Tube Holders into multiple Vacuette® Z Serum Clot Activator tubes (Greiner Bio-One, Kremsmünster, Austria).

Tubes were inverted 8 times to allow mixing and the whole blood permitted to clot at RT over 30 minutes prior to transfer, on ice, to the HBRC. Standard HRBC operating procedures were used to process and store samples. Specifically, within 6 hours of specimen collection, samples were centrifuged at room temperature (RT) for 10 minutes at 4600RPM and then stored at -80C for later batch analysis.

**Measurement of IL-1α**

From the work in Chapter 7, we decided to utilise the Boster immunoassay for the purpose of our main analysis as it had provided the most dynamic range of results which were comparable across both serum methodology studies. Serum IL-1a was thus measured using a single batch (no.111945418712) of Boster immunoassay kits (EK0389, Boster Biological Technology Co. Ltd., Fermont, CA) according to the manufacturer's instructions. Microplates were loaded with manufacturer's standards and undiluted serum samples in triplicate. Optical density (OD) was measured using plate spectrophotometry at 450nm. Endpoint absorbance was measured on a BioTek Synergy® multi-detection microplate reader utilising KC4® v3.4 software. IL-1α titres were calculated against standard curves generated from each assay using reference concentrations supplied by manufacturers. OD readings were converted to units (pg/ml) with GraphPad PRISM v5.0. Sample results which fell below the detection limit of the assay were considered to have undetectable levels and assigned the value of 0pg/ml.

**Measurement of AAA size and morphology**

AAA MAPD was recorded by means of US on the date of serum sampling. Patient records were reviewed and in cases where CT-imaging was performed within ±3-months of serum sampling, or where AAA CT size was ±5mm of US size, volume measurements and morphological characteristics were analysed using Aquarius

iNtuition software (TeraRecon, San Mateo, Calif, USA). In patients where suitable CT imaging was available, morphological characteristics were calculated by first using the workstation software to draw a centre line through the AAA, then individual parameters were measured by manually determining cut-off points within the scans such as ‘lowest renal artery’, ‘aortic bifurcation’ and so forth. An example case of AAA morphology measurements is shown in **Figures 9a, 9b and 9c**.

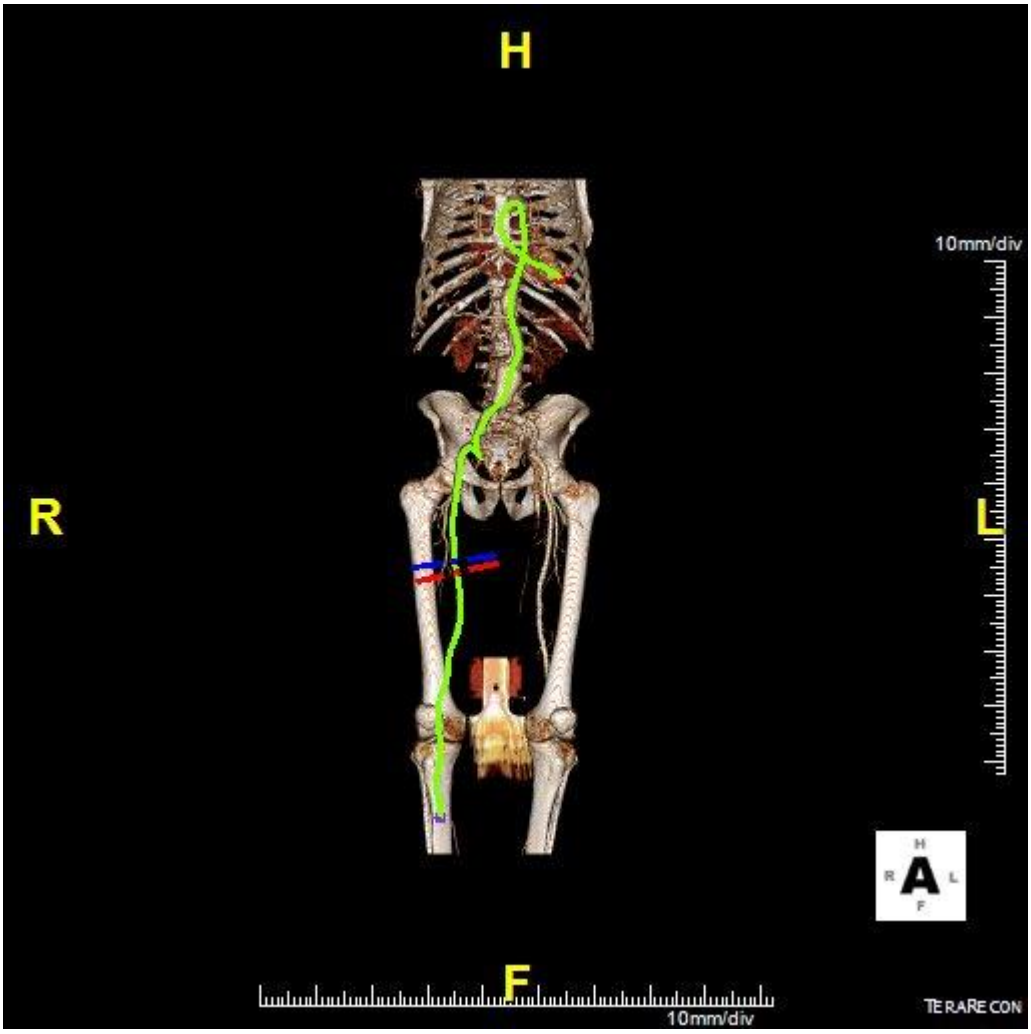


**Figure 9a. Example case of TeraRecon software used to calculate morphological aneurysm features in patients with available CT angiogram (CTA) imaging.**

Here the centre lumen line through a patient's AAA, using the dedicated software is shown.

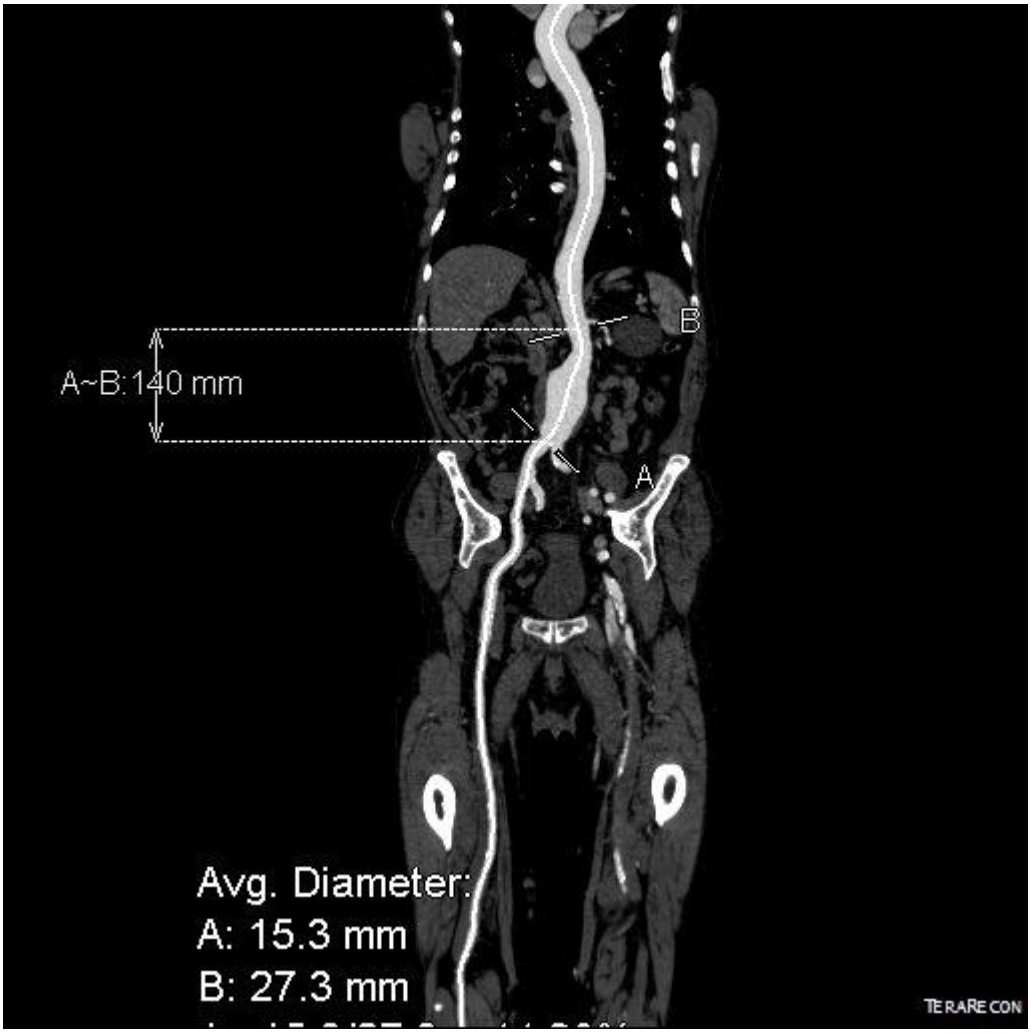


**Figure 9b. Reconstructed stretch view of an example aorta used to calculate AAA morphological features.**

This allows length measurements from the lowest renal artery (A) to the aortic bifurcation (B) to be made as well as optimal diameter measurements.

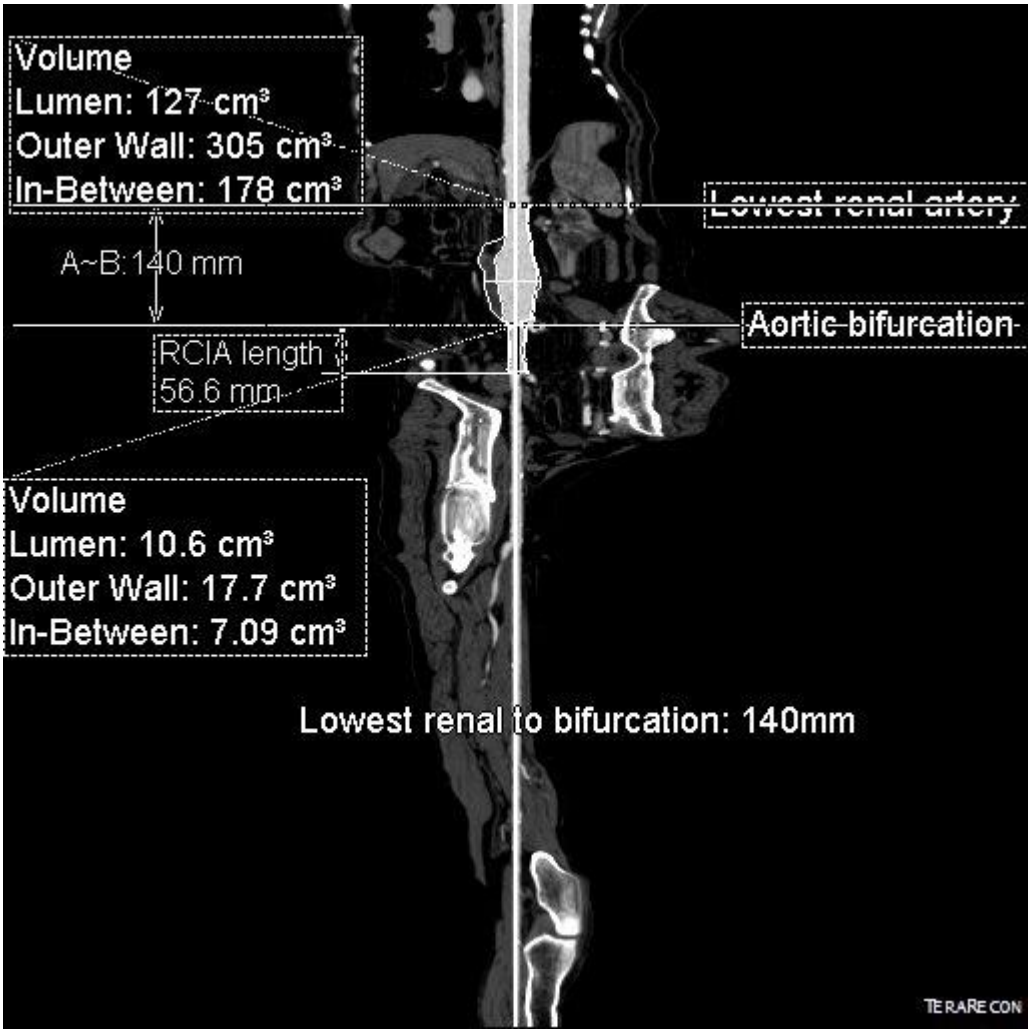


**Figure 9c. Demonstration of example patient AAA morphological measurements using the aortic stretch view.**

The use of dedicated software (in this case provided by TerraRecon) allows detailed assessment of aortic lengths as well as volumetric measurements (in $cm^3$) of the vessel using automatic 3-dimensional reconstructions

A~B = measurement from lowest renal artery to aortic bifurcation, RCIA = right common iliac artery

**Measurement of AAA growth rates**

Retrospective interrogation of a prospectively gathered AAA surveillance database was performed to obtain growth rates in those patients who had at least two scans. In total, 890 AAA measurements were analysed (783 US, 107 CT). The mean (range) duration of follow-up was 61 months (0 to 185 months), with a mean (range) 9.2 scans per person (2 - 34). Data from the first and last scans were used to calculate absolute (mm) change in MAPD and absolute (mm/year) change in MAPD over time. Negative growth rates between serial scans were not automatically excluded. Data were censored at 31 December 2014.

**Statistical analysis**

Since IL-1α titres were highly skewed, they were $\log_{10}$-transformed, after adding one to all values to remove zeros. The relationship between serum IL-1α levels and AAA size and growth rates was investigated using Spearman's rank correlation coefficients. In addition, regression models were produced, with $\log_{10}$IL-1α set as the independent variable.

The subgroup of patients who had received CT scans were then analysed in further detail. Spearman's correlation coefficients were produced to test the relationship between IL-1α titres and a range of morphological AAA-related factors. Due to the high number of patients with undetectable IL-1α, the analysis was also performed with these patients excluded. Bonferroni or Šidák corrections were not made to adjust the p-values when performing multiple comparisons, so it is possible that some of the results obtained occurred simply through chance alone.

In addition, the patients with undetectable levels were compared to those with detectable titres using Fisher's exact tests for rates, t-tests for normally distributed

factors, and Mann-Whitney tests for non-parametric data. The demographics of patients in the study were then compared to those in the previously published pilot study using the same approach.

All analyses were performed using IBM SPSS Statistics 22 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance.

**Results**

Serum IL-1α was measured in 101 patients (90 men) of mean (SD) age 74 (±7.0) years with asymptomatic infra-renal AAA. There was no statistically significant difference in mean (SD) age (years) between men (74±7.1) and women (77±6.6) ($p = 0.180$, t-test). Median ($Q_1$-$Q_3$) IL-1α was 0.57 (0.00-4.91) pg/ml.

IL-1α was detectable in 63 (62.4%) patients (58 men) of mean (SD) age 74 (±7.2) years with mean (range, [SD]) MAPD of 46 (32-70, [±9]) mm. There was a significant age difference between men and women in this group, with women being older than men (mean, [±SD] 81 [±4.7] years *vs*. 73 [±7.1] respectively, $p = 0.020$). Mean (SD) AAA MAPD in this group was 45 (±9) mm for men and 51 (±11) mm for women. This difference was not statistically significant ($p = 0.168$, t-test). Median ($Q_1$-$Q_3$) IL-1α titre was 3.26 (0.80-19.1) pg/ml.

IL-1α was undetectable (0pg/ml) in 38 (37.6%) (32 men) patients of mean (SD) age 74 (±6.9) years with AAA of mean (range, [SD]) MAPD of 46 (30-71, [10]) mm. There was no significant difference in age ($p = 0.905$), gender ($p = 0.260$) or MAPD ($p = 0.879$) between patients with detectable and undetectable IL-1α (t-test), and the gender distribution was also similar in the two groups (Fisher's exact test $p = 0.323$).

**Relationship between IL-1α and MAPD**

Of the 101 patients, 48 had AAA <45mm, 41 had AAA 45 to 55mm, and 12 had AAA>55mm. Mean (range, [SD]) MAPD as measured by US on the day of serum sampling was 46 (30-71, [±9]) mm. There was no statistically significant difference in MAPD between men and women in this group (p = 0.816, t-test).

Overall, there was no statistically significant relationship between IL-1α and MAPD as measured by US on the day of IL-1α serum sampling (R-squared = 0.002, p = 0.649) (**Figure 10**).

Considering just those patients with detectable IL-1α, there was no statistically significant relationship between IL-1α and MAPD on US (p = 0.816, Spearman correlation).

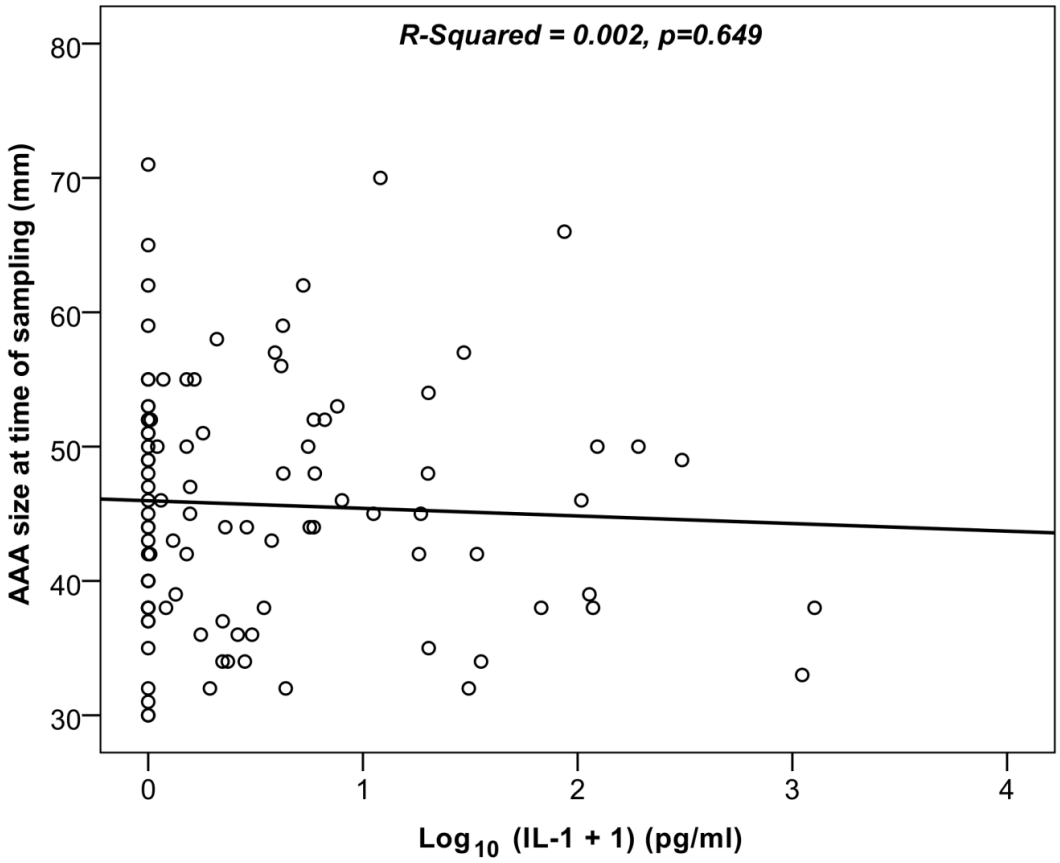


**Figure 10. Relationship between MAPD (mm) as measured on US and $log_{10}$ serum IL-1α levels (pg/ml) in 101 patients with asymptomatic, infra-renal AAA**

The line on the graph is based on a regression model using the resulting variable, which found that a one log (i.e. ten-fold) increase in IL-1α was associated with a non-significant (p=0.649) change of -0.06mm (95% CI: -0.30, 0.19) in AAA size.

**Relationship between IL-1α and AAA morphology on CT**

CT scans were available in 28 patients (24 men) of mean (SD) age of 74 (±7.1) years (74 (±7.2) for men and 72 (±7.2) for women). All CT scans were performed for non-AAA related reasons. Internal morphological AAA features recorded from CT imaging and median IL-1α titres are shown for those with, and without, detectable IL-1α in **Table 13**. Spearman's rank correlations were performed to establish relationships between IL-1α and morphological features. The only significant difference was detected for RCIA length ($p = 0.045$), which was greater in patients with undetectable IL-1α (median: 65 vs. 51mm).

| | All patients (n=28) | Detectable IL-1α (n=13) | Undetectable IL-1α (n=15) | p-Value |
|---|---|---|---|---|
| **Men (%)** | 24 (86%) | 13 (100%) | 11 (73%) | 0.102[#] |
| **Mean age in years (±SD)** | 74 (±7.2) | 72 (±7.5) | 75 (±6.7) | 0.385* |
| **Median ($Q_1$-$Q_3$) IL-1α (pg/ml)** | 0 (2.64) | 3.16 (33.4) | - | - |
| **Mean AAA maximum AP diameter on US [±SD] (mm)** | 50 [±10] | 51 [±11] | 49 [±10] | 0.542* |
| **Mean AAA maximum AP diameter on CT [±SD] (mm)** | 53 [±11] | 51 [±10] | 54 [±12] | 0.457* |
| **Aortic length (mm)** | 126 (93-172) | 126 (103-155) | 122 (93-172) | 0.394 |
| **LCIA length (mm)** | 65 (34-100) | 65 (34-100) | 63 (45-88) | 0.940 |
| **RCIA length (mm)** | 63 (43-83) | 51 (43-83) | 65 (48-74) | **0.045** |
| **Aortic sac volume ($cm^3$)** | 180 (48.7-539.0) | 196 (71.0-394.0) | 177 (48.7-539.0) | 0.381 |
| **Aortic luminal volume ($cm^3$)** | 90.0 (26.2-157.0) | 76.6 (46.0-157.0) | 90.8 (26.2-98.2) | 0.717 |
| **LCIA sac volume ($cm^3$)** | 17.4 (5.8-53.7) | 16.1 (5.8-53.7) | 19.9 (7.5-36.2) | 0.138 |
| **LCIA luminal volume ($cm^3$)** | 7.3 (2.2-35.9) | 7.1 (2.2-35.9) | 8.0 (2.5-20.1) | 0.202 |
| **RCIA sac volume ($cm^3$)** | 18.0 (5.7-71.0) | 18.5 (8.8-40.1) | 18 (5.7-71.0) | 0.899 |
| **RCIA luminal volume ($cm^3$)** | 9.5 (2.4-26.6) | 8.4 (3.2-36.6) | 10.2 (2.4-16.8) | 0.486 |
| **Aortic thrombus volume ($cm^3$)** | 83.1 (22.4-441.0) | 88.3 (25.4-322.0) | 82.4 (22.4-441.0) | 0.650 |
| **LCIA thrombus volume ($cm^3$)** | 9.4 (3.6-17.8) | 9.2 (3.6-17.8) | 9.4 (4.3-14.1) | 0.467 |
| **RCIA thrombus volume ($cm^3$)** | 8.2 (3.3-54.2) | 7.8 (4.6-25.2) | 8.7 (3.3-54.2) | 0.569 |

**Table 13. Internal architectural AAA features and median IL-1α titres recorded from 28 patients with available imaging. P-values result from comparison between detectable IL-1α and undetectable IL-1α groups**

Results are described as median (range), unless stated otherwise. SD=standard deviation, $Q_1$ = 1st quartile, $Q_3$ = 3rd quartile

All p-values calculated by Mann-Whitney U unless otherwise indicated, $p<0.05$ (bold) taken to be significant

[#]Fisher's exact test

[*]t-test

**Aortic and common iliac morphological features**

Aortic length was defined as length (cm) from the lowest renal artery to the aortic bifurcation and common iliac artery (CIA) length was defined from the aortic bifurcation to the CIA bifurcation. The relationship between IL-1α and a range of aortic and CIA morphological features was investigated, including length, sac and luminal volumes and aortic thrombus volume. No significant correlation between IL-1α serum titres and any of these factors was found (**Table 14**), although the correlation between RCIA length and IL-1α was negative and close to significance, in concordance with the previous analysis (rho: -0.364, $p = 0.067$).

| | All patients (N=28) | | Detectable IL-1a (N=13) | |
|---|---|---|---|---|
| | **Rho** | **p-Value** | **Rho** | **p-Value** |
| **Aortic length (mm)** | 0.205 | 0.296 | 0.342 | 0.253 |
| **Aortic sac volume ($cm^3$)** | 0.192 | 0.329 | 0.140 | 0.648 |
| **Aortic luminal volume ($cm^3$)** | 0.046 | 0.818 | 0.517 | 0.070 |
| **Aortic thrombus volume ($cm^3$)** | 0.027 | 0.891 | -0.182 | 0.553 |
| **Left CIA length (mm)** | -0.011 | 0.958 | 0.014 | 0.966 |
| **Left CIA luminal volume ($cm^3$)** | -0.193 | 0.356 | 0.278 | 0.408 |
| **Left CIA sac volume ($cm^3$)** | -0.244 | 0.241 | 0.116 | 0.733 |
| **Left CIA thrombus volume ($cm^3$)** | -0.130 | 0.534 | 0.077 | 0.821 |
| **Right CIA length (mm)** | -0.364 | 0.067 | -0.161 | 0.617 |
| **Right CIA luminal volume ($cm^3$)** | -0.053 | 0.798 | 0.405 | 0.191 |
| **Right CIA sac volume ($cm^3$)** | 0.057 | 0.781 | 0.413 | 0.182 |
| **Right CIA thrombus volume ($cm^3$)** | -0.073 | 0.724 | 0.088 | 0.787 |

**Table 14. Relationship between serum IL-1α and morphological AAA-related features as measured on CT-imaging for all patients with available CT-imaging (n=28) and those with detectable serum IL-1α titres (n=13)**

P-values calculated using Spearman's rank correlation, Rho = correlation co-efficient

**Aortic thrombus volume**

There was no evidence of a significant relationship between IL-1α and absolute (p = 0.891) or relative (thrombus volume / sac volume) aortic thrombus volume (p = 0.967).

**Relationship between IL-1α and changes in AAA diameter**

Growth rate data were available in 97 patients (87 men) of mean (SD) age 74 (±7) years at the time of first scan. Mean MAPD (range) at first and last scans were 39 (24 - 69) mm and 51 (31 - 78) mm respectively. Mean (range) absolute increase in MAPD was 11 (0 - 41) mm. Median (range) absolute AAA growth rate was 1.9mm/year (0 - 69). No statistically significant relationship was detected between IL-1α and absolute AAA increase in MAPD (rho: -0.127, p = 0.214) or absolute growth rate (mm/year) (rho: -0.123, p = 0.230).

**Comparison of study results against published pilot study**

Comparison of our results between the previously published pilot study (137) demonstrated no statistical difference in patient age (mean 80 [±5] years, t-test p = 0.168). However, in the pilot study the AAA were significantly larger (mean 68mm [±14], t-test p = 0.039), and four were juxta-renal. Comparison of the 28 patients in this study with the pilot study data demonstrated no statistically significant difference in aortic ILT-volume (Mann-Whitney, p = 0.160). Performing a sub-group analysis of our results on the basis of the patient demographics described by Yates *et al* still returned a non-significant result (p=0.343), although the effect was in the correct direction (**Figure 11**). Although the pilot study did not specifically analyse titres according to gender, or perform a sub-group analysis of concentrations in infra- vs.

juxta-renal AAA, most likely due to small patient numbers, we have not been able to demonstrate any significant association between IL-1α and these factors.

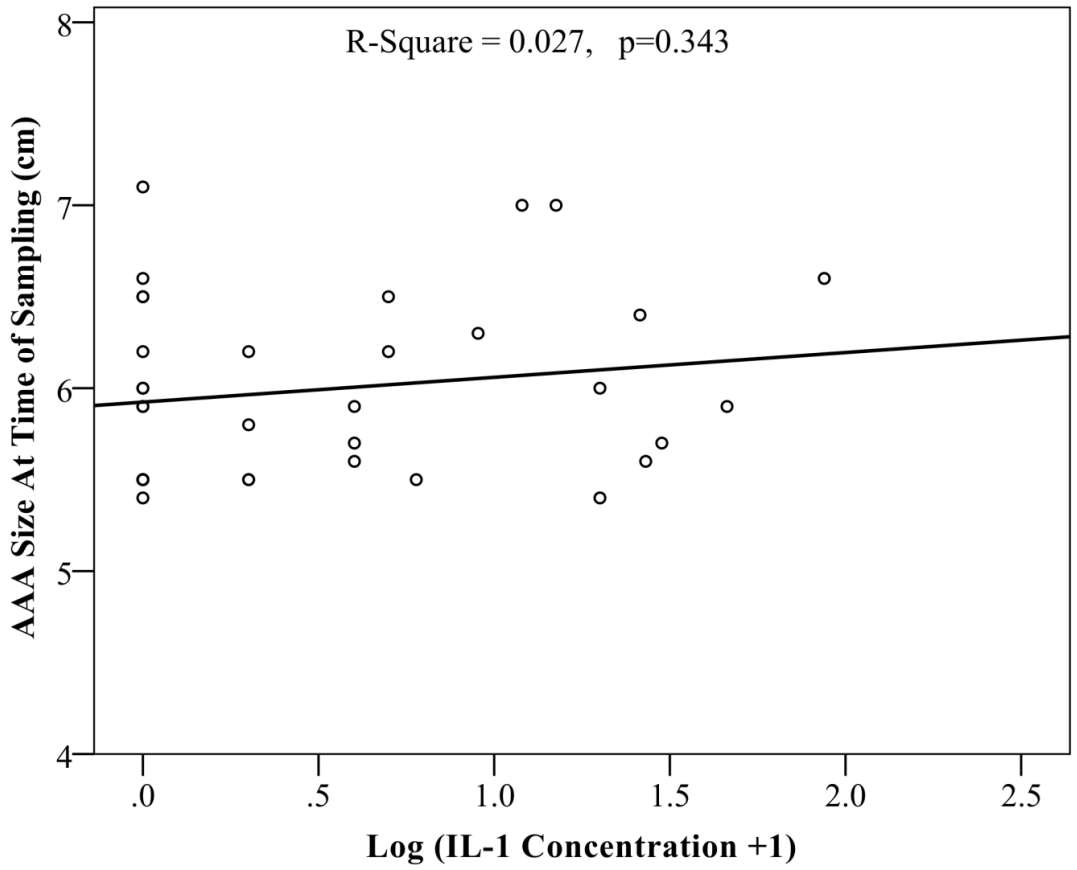


**Figure 11. Patients limited to pilot study paper. i.e.: those pre-op with infra-renal or juxta-renal AAA sized between 54mm and 100mm (inclusive) (n = 34pts).**

The line on the graph is based on a regression model with the Log10 of IL-1α as a predictor. This found that a one log (i.e. ten-fold) increase in IL-1α was associated with a non-significant (p=0.343) increase of 20mm (95% CI: -0.23, 0.63) in aneurysm size.

**Discussion**

The natural history of AAA, and in particular the factors influencing progression, growth and risk of rupture, remain a focus of intense research interest. Identification of circulatory biomarkers which could be used as an adjunct, or alternative, to current predominantly size-based surveillance and intervention strategies are of particular interest. Although there is a relatively large published body of work on AAA biomarkers and the role of IL-1α in cardiovascular disease, there is very little information on IL-1α in patients with AAA. In this study we sought to examine the relationship between IL-1α and AAA MAPD and morphological features in a large cohort of patients with asymptomatic, infra-renal AAA and established that serum IL-1α concentrations are detectable in most (62.4%), patients.

IL-1α is a pro-inflammatory cytokine that is largely undetectable in health but is present within many cell-lines and released in response to cell-damage.(189) Additionally, it has been shown that macrophages within the intima of atherosclerotic arterial lesions produce IL-1α.(190) AAA is a chronic inflammatory condition, characterised by atherosclerosis, with increased expression of both inflammatory cells within the aneurysmal wall, and elevated serum pro-inflammatory cytokines.(191,192) Cytokines such as MMPs and Insulin-like Growth Factor 1 (IGF-1) have been shown to correlate with AAA size and growth rate, and as such have been postulated to be potential biomarkers of AAA development.(82,193–195)

In light of the above, it is reasonable to hypothesize that IL-1α may have a role in the development and progression of AAA. Indeed, we have previously reported a significant relationship between AAA diameter and serum IL-1α levels in a small cohort of AAA patients and that EC activation *in vitro* in response to serum from AAA patients is blocked by anti-IL-1α antibodies.

However, in this larger study we have been unable to demonstrate a significant relationship between IL-1α and AAA across a range of pre-operative MAPD as measured by US or to reproduce the data from the pilot study of 17 patients with infra- and juxta-renal AAA which showed a significant association between IL-1α and MAPD on US.(137)

Comparison of current data against pilot study data demonstrated no significant difference in patient age or ILT volume. However, the patients recruited at that time were older (mean 80 years, range 69-88years) with larger AAA (mean 6.6cm, range 5.4-10cm) AAAs, and the sample size (n=17) much smaller. Additionally, our pilot study reported much higher levels of serum IL-1α (5-100pg/ml) which we have been unable to replicate. Nevertheless, even performing a sub-group analysis of our results on the basis of the patient demographics described by Yates *et al* still returned a non-significant result ($p = 0.343$) when looking at AAA MAPD and serum IL-1α (**Figure 8**). One possible explanation of this is the use of a different immunoassay to measure IL-1α. Inconsistencies in ELISA immunoassays may, in part, account for the differences in results found between this study and the pilot work. However, we consolidated our methodology ahead of this work and believe the Boster immunoassay to be robust in its abilities to detect and measure IL-1α.(196) Nevertheless, variability between manufacturers' ELISA assays is known to exist.(184) The large number of patients where IL-1α was undetectable (0pg/ml) may have masked an underlying relationship between AAA size, morphology, growth and IL-1α. However, no relationship was found even with these "IL-1α negative" patients excluded from the analysis.

The lack of statistically significant findings may also be as a result of the low statistical power of the analyses, on account of the small sample size. A post-hoc

power calculation found that the minimal detectable correlation coefficient was 0.27 for the analyses of the whole cohort (n = 101), and 0.48 for the analysis of the subset of patients with CT scans (n = 28) at 80% power and 5% alpha. Hence, weak to moderate correlations between factors could have been missed (false negative errors). Despite this, the majority of correlation coefficients detected were small (<0.2), and so unlikely to represent meaningful relationships.

ILT activity and extra-cellular aortic wall degradation and adventitial abnormalities are recognised features of AAA disease. However, although there is experimental evidence to suggest that ILT influences the inflammatory process in AAA, this has not been confirmed by clinical studies.(197–200,67,62) Our measurements of internal AAA morphological features (aortic and common iliac artery lengths) are in-keeping with published results (201), but we have been unable to demonstrate a significant relationship between aortic or iliac aneurysmal size and IL-1α. This suggests that the luminal and sac volumes do not affect the pro-inflammatory AAA state, at least through an IL-1α-mediated pathway.

Although the relationship between IL-1α and MAPD observed in our pilot study may represent chance findings, it is harder to discount the observation that EC activation *in vitro* in response to serum from the AAA patients was blocked by anti-IL-1α antibodies while antibodies against the other inflammatory markers studied had no effect.(137) One could argue that the small sample size in the pilot study (n = 17) and the positive results found in that group were fortuitous. However, the small sample size would not necessarily make it any more likely of making chance positive findings in fact, it would be potentially less likely given the reduction in statistical power. As far as we know, there was no biased selection of patients in the pilot study,

and to that end we are unable to explain the pilot study's results given our own negative results.

We have shown that the majority of patients with AAA have detectable serum IL-1α., but the exact nature of the IL-1α profile abnormalities seen remain to be determined.

As IL-1α is an important component of the systemic inflammatory response, our results may, therefore, simply be reflecting an association between IL-1α and a generalised systemic pro-inflammatory, pro-thrombotic state which may be related to the presence of AAA in some patients and to other disease processes in others. At present, the reasons for this are unclear and the subject of on-going studies, including determining the relationship between IL-1α, co-morbidities and other markers of inflammation to establish what the biological and clinical significance of these high serum IL-1α levels may be in terms of cardiovascular outcomes in general and, more specifically, AAA progression.

# Chapter 9: Study 2, looking at serum IL-1α as a marker of chronic inflammation in patients with asymptomatic AAA

**Aims:** To investigate the relationship between serum IL-1α, co-morbidity, smoking status, and BMT in patients with asymptomatic, infra-renal AAA.

## Introduction

AAA usually remain asymptomatic until complications develop, most commonly rupture. MAPD is currently believed to be the best predictor of rupture and so is the variable most often used to select patients for OR or EVAR.(36,37,42,43)

Aortic aneurysmal disease is widely considered to be a chronic inflammatory condition that is separate from, but which often co-exists with, atherosclerosis due to shared risk factors, in particular smoking and high blood pressure.(188)

IL-1α is a pro-inflammatory cytokine that is not normally detectable in the circulation (127) and which has been previously linked with cardiovascular and aortic diseases.(142,134,103,89,94)

Several groups, including our own, have previously demonstrated that patients with AAA have elevated serum markers of inflammation and coagulation in association with a systemic pro-thrombotic, pro-inflammatory diathesis.(79–82) It has been hypothesised that these moieties may originate from within the AAA wall and/or ILT.

Alternatively, or additionally, these factors may enter the systemic circulation as a result of other inflammatory vascular and non-vascular diseases that frequently co-exist in AAA patients. Other factors such as smoking and drug treatment may also influence these processes and affect AAA progression.

It remains unclear whether AAA progression is primarily due to localised biomechanical (blood pressure, aneurysm morphology) or biological factors within the aneurysm wall/thrombus and to what extent other chronic inflammatory processes occurring outside the aorta contribute to AAA growth and rupture.

An intervention strategy based on biomechanical factors (size) alone may leave patients with small, but nevertheless 'biologically active', AAA unprotected from rupture whereas patients with large but biological quiescent AAA may be offered unnecessary surgery.

There is therefore an urgent need to identify better predictors of rupture so that available health care resources can be used in the most clinically and cost-effective manner.

We have previously demonstrated that some patients with AAA have raised serum IL-1α levels and that endothelial cell (EC) activation *in vitro*, in response to serum from these AAA patients, is blocked by anti-IL-1α antibodies.(137)

The aim of this study was to investigate the relationship between serum IL-1α, co-morbidity, smoking status, and BMT in patients with asymptomatic, infra-renal AAA.

## Methods

### Patient recruitment and serum sample collection

Research ethical committee (REC) approval was obtained (reference 12/WM/0204) and all patients provided fully-informed, written consent.

A cohort of 101 patients with asymptomatic infra-renal AAA was recruited from vascular clinics at HEFT and SWBH.

Following skin preparation with an alcohol-based antiseptic, whole blood was drawn from an antecubital fossa vein using a tourniquet, Vacuette® Multi-Sample Blood Collection Needles (21G-green) and Vacuette® Standard Tube Holders into multiple Vacuette® Z Serum Clot Activator tubes (Greiner Bio-One, Kremsmünster, Austria).

Tubes were inverted 8 times to allow mixing and the whole blood permitted to clot at room temperature (RT) over 30 minutes. They were then transferred, on ice, to a regional biobank facility: the HBRC.

Standard HBRC operating procedures were used to process and store samples. Specifically, within 6 hours of specimen collection, samples were centrifuged at RT for 10 minutes at 4600RPM and then stored at -80C for later batch analysis.

**Measurement of IL-1α**

Serum IL-1α was measured using a single batch (no: 111945418712) of Boster ELISA immunoassay (Cat no: EK0389, Boster Biological Technology Co. Ltd., Fermont, CA) according to the manufacturer's instructions.

Microplates were loaded with manufacturers' standards and undiluted serum samples in triplicate. Optical density (OD) was measured using plate spectrophotometry at 450nm. Endpoint absorbance was measured on a BioTek Synergy® multi-detection microplate reader utilising KC4® v3.4 software.

IL-1α titres were calculated against standard curves generated from each kit using reference concentrations supplied by the manufacturer. OD readings were converted to units (pg/ml) with GraphPad PRISM v5.0. Sample results which fell below the detection limit of the assay were considered to have undetectable levels and assigned the value of 0 pg/ml.

**Coding of co-morbidity**

Patient records were reviewed for the presence, or absence of co-morbidities. A diagnosis of hypertension (HT) was recorded if the patient was receiving anti-hypertensive medication; ischaemic heart disease (IHD) if there was record of a past myocardial infarction, or angina; cerebrovascular accident (CVA) if they had a history of transient ischaemic attack, or stroke; diabetes mellitus (DM) and chronic obstructive pulmonary disease (COPD) if they were recorded as having and/or on medication for these diagnoses; hypercholesterolaemia if they had a record of elevated serum cholesterol and/or were on a cholesterol-lowering agent; peripheral arterial disease (PAD) if the diagnosis had been made by a vascular specialist based on symptoms, clinical examination or imaging (e.g. duplex or angiography); and atrial fibrillation (AF), hypothyroidism and gout if they had been recorded as having and/or were on appropriate medication for these diagnoses. Smoking status, and history of coronary artery bypass grafting (CABG), deep venous thrombosis (DVT) and malignancy was confirmed at time of sampling.

BMT was defined as the use of an anti-platelet and statin therapy. Confirmation of BMT regime was recorded at the time of serum sampling. Anticoagulation therapy (e.g. with warfarin) was also noted.

**Analysis of other serum markers of inflammation**

Patient records were interrogated for additional markers of inflammation (neutrophil, lymphocyte eosinophil and platelet counts) taken within 3-months of serum sampling for IL-1α. C-reactive protein (CRP) was only available in 5 patients, so excluded from analysis. All additional serum samples had been taken for non-AAA related reasons.

**Statistical analysis**

Since IL-1α titres were highly skewed, they were $\log_{10}$-transformed after adding one to all values to remove zeros, with the resulting variable set as the covariate in the model. Patient age and AAA size were grouped to allow cross-comparison. Age groups were set as: ≤69 (n = 26), 70 ≥ n ≤ 74 (n = 26), 75 to 79 (n = 28) and ≥ 80 (n = 21) years. AAA sizes were grouped as ≤ 44 (n = 48), 45 to 55 (n = 41) and ≥ 56 (n = 12) mm. The relationships between serum IL-1α levels and co-morbidities, smoking status and BMT for cardiovascular disease and other markers of inflammation were investigated. Analyses were performed using the Mann-Whitney and Kruskal-Wallis tests and IBM SPSS Statistics 22 (IBM Corp. Armonk, NY).

**Results**

Serum IL-1α was measured in 101 patients (90 men) of mean (standard deviation, SD) age 73 (±7.0) years with non-operated, asymptomatic, infra-renal AAA. Patient demographics and co-morbidities are shown in **Table 15**. Overall, median (inter-quartile range, IQR) serum IL-1α was 1.00pg/ml (4.91pg/ml). There was no statistically significant relationship between serum IL-1α levels and patient gender (p = 0.598) or age (p = 0.620). IL-1α was detectable in 63 (62.4%) patients (58 men) of mean (SD) age 74 (±7.1) years with AAA of mean (range, [SD]) AP diameter of 46 (32–70, [±7]) mm. In this group, median (IQR) serum IL-1α was 3.26 (18.27) pg/ml. IL-1α was undetectable (0pg/ml) in 38 (37.6%) of patients.

| Group size [n] | 101 (%) |
|---|---|
| **Co-morbidity [n] (%)** | |
| **HT** | 67 (66.3) |
| **IHD** | 36 (35.6) |
| **CABG** | 10 (9.9) |
| **CVA** | 8 (7.9) |
| **CCF** | 3 (3.0) |
| **AF** | 9 (8.9) |
| **DM** | 17 (16.8) |
| **COPD** | 14 (13.9) |
| **Hypercholesterolaemia** | 23 (22.8) |
| **DVT** | 4 (4.0) |
| **PVD** | 5 (5.0) |
| **History of active/past malignancy** | 10 (9.9) |
| **Hypothyroidism** | 3 (3.0) |
| **Gout** | 5 (5.0) |
| **Smoking status** | |
| **Never** | 13 (12.9) |
| **Ex-smoker** | 63 (62.4) |
| **Active smoker** | 24 (23.8) |
| **Data missing** | 1 (1.0) |
| **Best medical therapy ('BMT')** | |
| **No BMT** | 8 (7.9) |
| **Aspirin/Statin** | 70 (69.3) |
| **Aspirin only** | 2 (2.0) |
| **Statin only** | 10 (9.9) |
| **Warfarin/Statin** | 7 (6.9) |
| **Other combination** | 2 (2.0) |
| **Data missing** | 2 (2.0) |

**Table 15. Patient co-morbidities and aneurysm-related risk factors as recorded at time of IL-1α serum sampling**

Values in (parentheses) are % of group

HT: hypertension; IHD: ischaemic heart disease; CABG: coronary artery bypass graft; CVA: stroke; CCF: congestive cardiac failure; AF: atrial fibrillation; DM: diabetes mellitus; COPD: chronic obstructive pulmonary disease; DVT: deep venous thrombosis; PVD: peripheral vascular disease; BMT: best medical therapy

**Relationship between IL-1α and co-morbidities**

There was no significant relationship between IL-1α and any of the co-morbidities analysed, smoking status or BMT regime, results are shown in **Table 16**.

**Relationship between IL-1α and other serum markers of inflammation**

Additional serum markers for active or chronic inflammation were available in 69 (68.3%) patients (60 men) of mean age (range, [SD]) 74 (56-87, [±7]) years and mean (range, [SD]) AAA MAPD (range, [SD]) 46 (30-71, [9]) mm. IL-1α was detectable in 45/69 (65.2%) patients (40 men) of mean (SD) age 74 (±7.1) years and mean (range, [SD]) AAA MAPD 45 (32-70 [±9]) mm. In this group, median (IQR) serum IL-1α was 2.05 (13.82) pg/ml. IL-1α was undetectable in 24 (34.8%).

There was no statistically significant relationship between serum IL-1α titres and patient neutrophil ($p = 0.726$), lymphocyte ($p = 0.545$), eosinophil ($p = 0.747$) or platelet ($p = 0.585$) counts in the cohort overall (**Figures 10 – 15)**. There was no statistically significant relationship between serum IL-1α titres and patient neutrophil ($p = 0.841$), lymphocyte ($p = 0.935$), eosinophil ($p = 0.935$) or platelet ($p = 0.488$) counts in those with detectable IL-1α (Pearson correlation).

| | Median IL-1α concentration (pg/ml) | IL-1α Range (pg/ml) | P-value |
|---|---|---|---|
| **Co-morbidity** | | | |
| **HT** | 1.0 | 0-1267.99 | 0.810 |
| **IHD** | 0.33 | 0 - 1110.51 | 0.235 |
| **CABG** | 3.31 | 0 - 1110.51 | 0.222 |
| **CVA** | 0.16 | 0 - 1110.51 | 0.397 |
| **CCF** | 0 | 0-19.24 | 0.618 |
| **AF** | 2.46 | 0-305.29 | 0.123 |
| **DM** | 0.64 | 0-305.29 | 0.845 |
| **COPD** | 2.86 | 0-305.29 | 0.201 |
| **Hypercholesterolaemia** | 0.03 | 0-1267.99 | 0.322 |
| **DVT** | 2.54 | 1.09-85.77 | 0.190 |
| **PVD** | 0 | 0-7.0 | 0.504 |
| **History of active/past malignancy** | 0.05 | 0-122.51 | 0.480 |
| **Hypothyroidism** | 0 | 0-0.18 | 0.167 |
| **Gout** | 1.82 | 0-4.56 | 0.635 |
| **Smoking status** | | | |
| **Never (n=13)** | 0.03 | 0-28.5 | |
| **Ex (n=63)** | 1.29 | 0.02-1268.0 | 0.134* |
| **Current (n=24)** | 0.26 | 0-66.7 | |
| **Data missing (n=1)** | | | |
| **Best medical therapy** | | | |
| **None** | 0.43 | 0-4.9 | |
| **Aspirin+Statin** | 0.54 | 0-1268.0 | |
| **Aspirin only** | 0.61 | 0-1.22 | 0.096* |
| **Statin only** | 0.17 | 0-103.1 | |
| **Warfarin+Statin** | 3.26 | 1.8-305.3 | |
| **All else** | 0 | 0 | |
| **Data missing (n=2)** | | | |
| **Statin** | | | |
| **Yes (n=89)** | 0.43 | 0-4.91 | |
| **No (n=10)** | 0.57 | 0-1267.0 | 0.578 |

**Table 16. Relationship between patient co-morbidity and IL-1α**

Analyses performed using Mann-Whitney U test unless otherwise specified.

* Kruskal-Wallis test.

HT: hypertension; IHD: ischaemic heart disease; CABG: coronary artery bypass graft; CVA: stroke; CCF: congestive cardiac failure; AF: atrial fibrillation; DM: diabetes mellitus; COPD: chronic obstructive pulmonary disease; DVT: deep venous thrombosis; PVD: peripheral vascular disease; BMT: best medical therapy

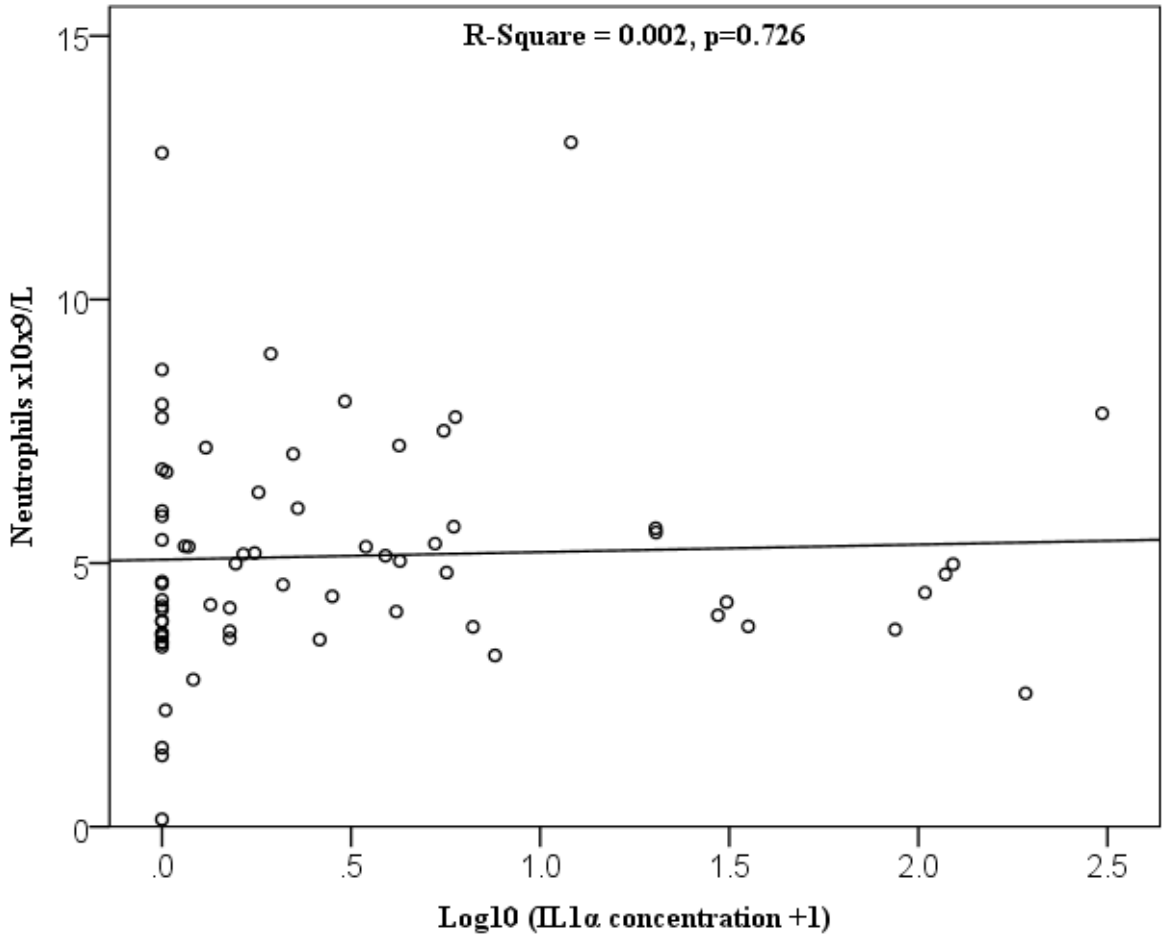


**Figure 10. Relationship between patient neutrophil count and IL-1α**

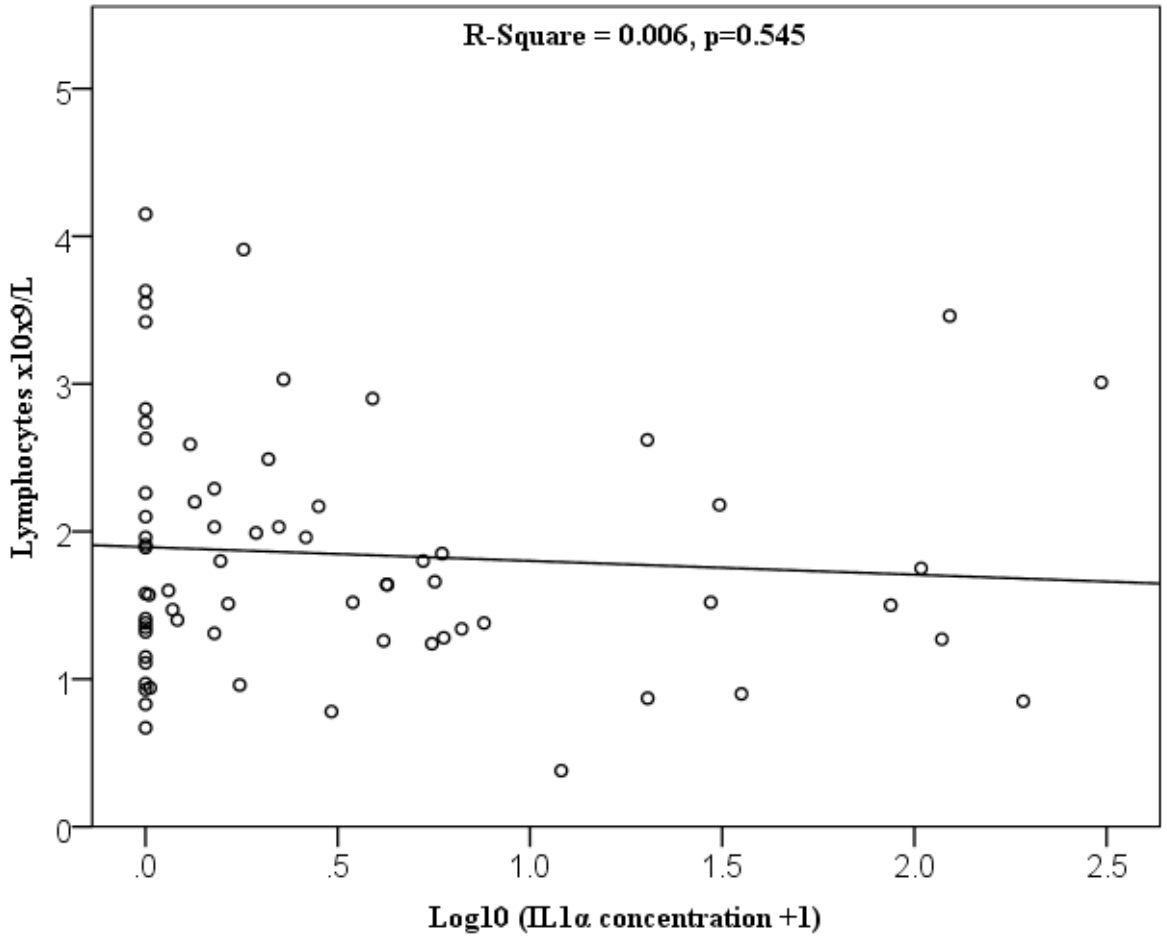


**Figure 13. Relationship between patient lymphocyte count and IL-1α**

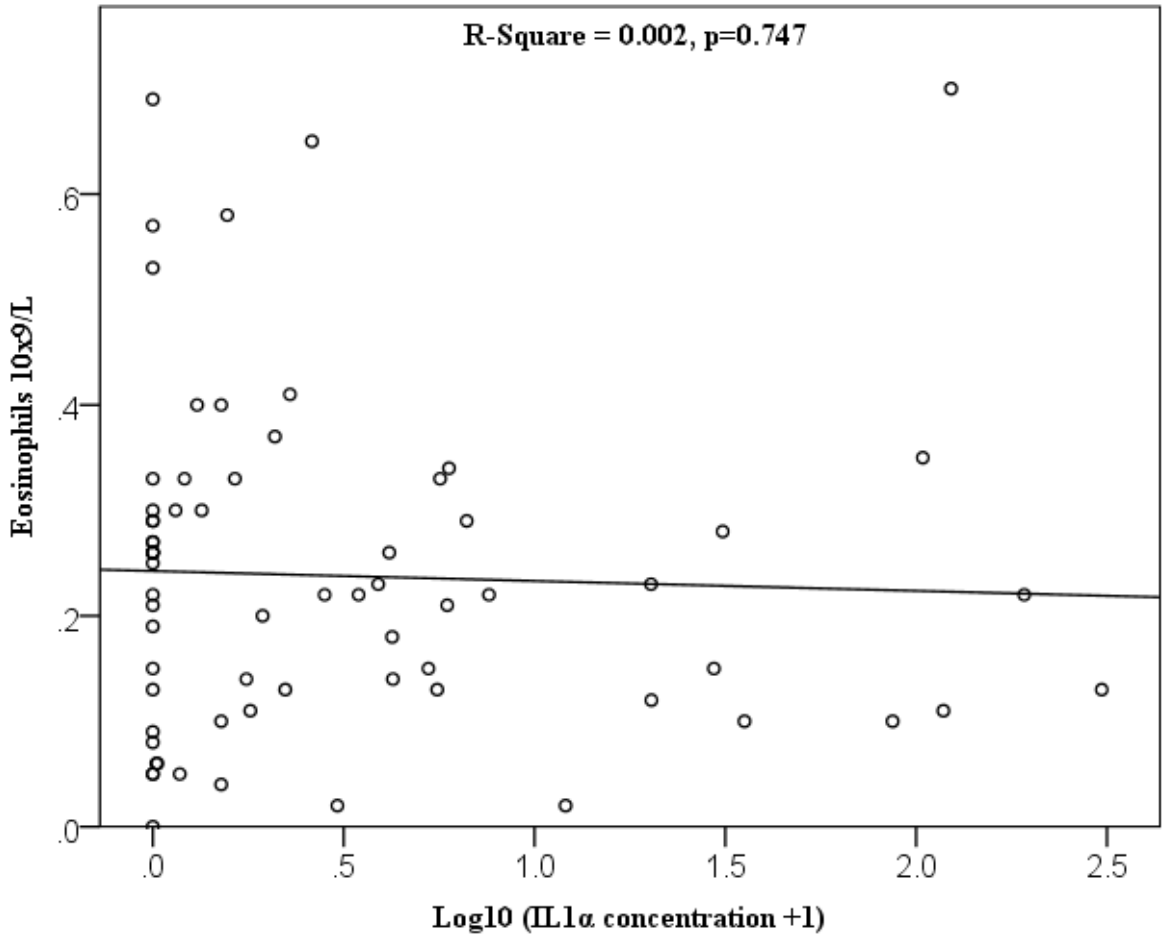


**Figure 14. Relationship between patient eosinophil count and IL-1α**

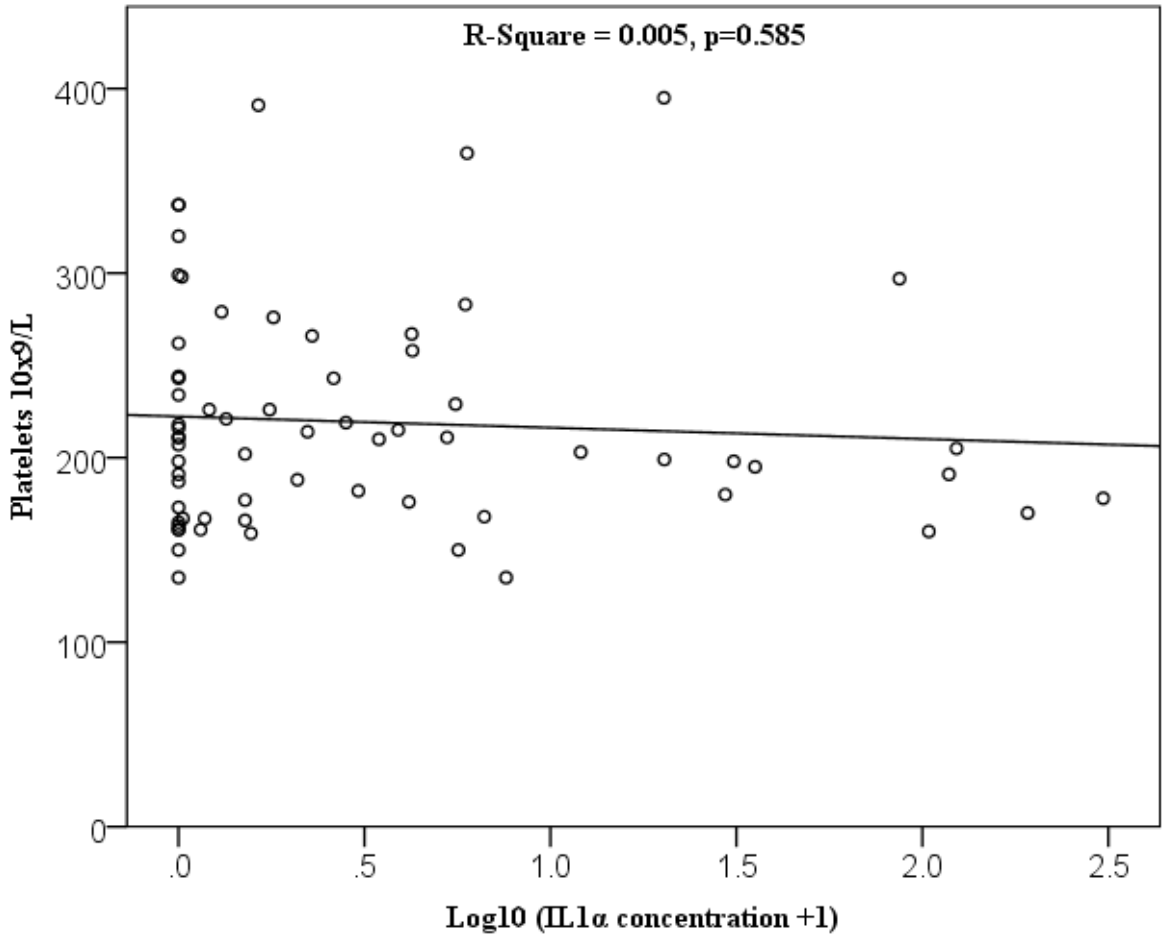


**Figure 15. Relationship between patient platelet count and IL-1α**

## Discussion

The natural history of AAA, and in particular the factors influencing progression and risk of rupture, remain a focus of intense research interest. One line of study focuses on the identification of circulatory biomarkers which could be used as an adjunct, or alternative, to current, predominantly size-based, surveillance and intervention strategies. Such biomarkers may also provide a biological signal that may lead to novel drug therapies that will prevent AAA development, growth and rupture.

The current study arises from previous work which demonstrated that some patients with AAA have raised serum IL-1α levels (a cytokine not normally detectable in health) and that EC activation *in vitro*, in response to serum from these AAA patients is blocked by anti-IL-1α antibodies.(137) The question is whether the IL-1α detected in AAA patients emanates from the AAA (aortic wall and/or ILT) or whether it reflects other inflammatory processes that frequently co-exist with the AAA in this patient group, or both.(197–200,67,62)

In this study, we have again shown that serum IL-1α levels are raised in some, but not all, patients with AAA. However, we have not been able to demonstrate a significant relationship between IL-1α and co-morbidity, smoking status and BMT or other simple markers of inflammation.

Interestingly, however, there was a suggestion that those patients who were receiving warfarin for AF or DVT had higher IL-1α titres than those who were not. Whether there is a link between a pro-thrombotic tendency, requiring warfarin therapy, and raised IL-1α, is the subject of further on-going study. Also of interest was the lack of a relationship between IL-1α and smoking status, with levels tending to be higher in those who were ex-smokers compared to those who had never smoked, or were

current smokers when one might have supposed that active smokers would have higher levels due to the known low-grade systemic inflammatory response, which can persist up to twenty years after cessation.(202)

In conclusion, the current study suggests that the markedly elevated levels of IL-1α observed in about two-thirds of patients with AAA appear not to be related to extra-aortic patient characteristics or co-morbidities. In other words, it appears likely that the IL-1α observed in this group is being generated within the AAA, either from the aortic wall and/or the ILT. The relationship between IL-1α and the characteristics of the AAA in this patient cohort is the subject of on-going study.

## Chapter 10: General discussion

The central hypotheses of this Thesis are that IL-1α can be utilised as a surrogate marker for disease progression and risk of rupture in those with asymptomatic, infra-renal AAA. Specifically, on the basis of previously published pilot work, we hypothesised that IL-1α titres are elevated in those with AAA, and that these titres correlate with AAA size, morphology and patient-dependent factors such as co-morbidity and to that end, IL-1α can be used as a surrogate marker to identify those most likely to need surgical intervention.

These hypotheses have been tested robustly, having first undertaken a thorough methodology study assessing the current IL-1α immunoanalysis assays commercially available on the market to measure IL-1α. I can conclude that, at present, although IL-1α clearly participates in the AAA pathway as most patients with AAA have elevated titres, there is insufficient evidence to say that it is a clinically effective or useful surrogate marker of biological activity.

It took the best part of 18 months to conduct the methodology study, and identify an immunoassay sufficiently reliable to conduct the subsequent analyses. There were two main reasons for this. Firstly, we had strong faith in the methodology used for the published results from the pilot study, and so did anticipate a problem when it came to the initial immunoanalysis. The disappointing results from our initial run were a real obstacle to us progressing with the proposed project work. Our acceptance of the results in the pilot study meant that we were caught unawares by our negative results, and were then obligated to spend time analysing the possible discrepancies between the pilot work and our own patient analysis to try to establish why the results were so different. A review of the literature beforehand had not

identified any obvious reason why processing our samples via HBRC should have resulted in negative titres, as IL-1α has been found to be a relatively stable molecule. Additionally, in our minds, we did not feel there would be great variation amongst different immunoassays, all of which are marketed as being reliable in detecting serum IL-1α. We were thus satisfied in using the assay most commonly used by the CIS, the Bio-Rad Bio-Plex Pro assay in our initial run. The reason why, in our run, CIS used the Bio-Rad immunoassay, and not the Millipore Milliplex MAP kit was that my predecessor had found the positive IL1-α signal during a fortuitous 'fishing screen' of various cytokines in his AAA patient cohort. When analysing for IL-1α alone, the CIS recommended the Bio-Rad Bio-Plex Pro kit as it was the measuring platform they used most commonly for IL-1α. Again, because we had no idea at that point of the differences between kits, we were satisfied that it should not pose any real problems. Our negative results were disappointing, and the purchasing of new Millipore and Bio-Rad kits, identifying my predecessor's stored samples and running both together was a frustrating process which added significant delays to our work progressing. The (again) disappointing results from these re-runs also highlighted an interesting result; although we could not confirm how many freeze/thaw cycles my predecessors samples had been through, logically we could see no reason why they would have been subjected to more than three or four at most. As the literature suggested that IL-1α is relatively stable up to six freeze/thaw cycles, we were unable to explain the differences in titre values found, other than to attribute them to possible degradation of IL-1α as a result of prolonged storage at -80C.(173)

Communication with Merck Millipore enquiring as to whether they had changed their immunoassay between the pilot study run in 2009 and our yielded no useful information other than "the recently ordered kit was not available in 2009, and may

have contained different antibodies, so it is not always possible to compare data" and further enquiries to clarify the nature of antibody differences were unsuccessful. Hence we can but assume that there was a difference, and that this in part must have been a cause of the discrepancies seen between our results and the pilot study.

The main empirical findings of this work are chapter specific, and were summarised within the respective chapters:Chapter 6, Chapter 8 and Chapter 9. Using the findings from these two studies to answer the Thesis hypotheses, we can say that there is a need to identify a better, more cost-effective means of monitring AAA progression and need for surgical intervention in patients as only 20.1% of a cohort of 692 patients in surveillance at HEFT proceeded to elective AAA repair, despite 5363 scans being performed as part of the surveillance protocol. This represents a huge potential waste of resources.

Our results show that current intervention strategies based on MAPD alone also leave a significant proportion of patients with AAA smaller than the current intervention threshold of MAPD ≥55mm unprotected from timely surgery, with 40.7% of patients with ruptured AAA had last recorded MAPDs less than 55mm. Although, in general those with AAA requiring intervention had faster AAA growth rates than those who did not this did not hold true for those who ruptured their AAA at sub-threshold MAPD size. Hence, these small, but clinically important sub-group of patients are not appropriately protected by a surveillance strategy based on MAPD alone. Additionally, analysis of various patient-related factors did not identify any that could be reliably used to predict rupture risk. This confirmed the need to identify other, biological, markers of AAA progression.

Our work on IL-1α however demonstrated that although it is present in most (62.4%) of patients with AAA, it does not correlate with MAPD, or other AAA-related

morphological features, including ILT. Additionally, we did not find any meaningful correlation between it and patient-related factors, including other serum markers of inflammation.

On the basis of this, we can therefore conclude that although the presence of IL-1α in patients in AAA remains undetermined, it cannot be said at present to be a suitable surrogate marker for AAA disease.

**Future studies**

Despite the fact that we initially struggled to find a reliable immunoassay to measure IL-1α in our serum samples, we believe that the Boster kit used provided us with sufficiently reliable and reproducible results to perform the work. There is some merit in continued investigation of the role IL-1α has in AAA disease, in particular the most logical step would be measuring it in those with a recorded positive pre-operative titre in the post-operative period, to see whether surgical repair impacts IL-1α values. Additionally, the source of IL-1α in those with positive titres merits further investigation with histological analysis of aortic wall and intra-luminal thrombus as potential sources in those undergoing OR.

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