THE ROLE OF THE CCR5 Δ32 POLYMORPHISM IN ABDOMINAL AORTIC ANEURYSMS

Thesis submitted for the degree of Doctor of Medicine at the University of Leicester

by

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University of Leicester

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STATEMENT OF ORIGINALITY

I hereby declare that the work contained in this thesis is entirely my own, except where acknowledged, and has not been submitted for a degree to this or any other University.

R.M. Sandford
ABSTRACT

The Role of the CCR5 Δ32 Polymorphism in Abdominal Aortic Aneurysms

By

R.M. Sandford

C-C Chemokine receptor 5 (CCR5) is involved in the regulation of the inflammatory response. Abdominal aortic aneurysms (AAA) may arise as the result of a chronic inflammatory process which is influenced by a genetic predisposition. The CCR5 gene harbours a 32 base pair deletion (the Δ32 polymorphism, rs333). The aim of this study was to investigate the role of the CCR5 Δ32 polymorphism in the development of AAA.

A case control study was conducted including 285 patients with AAA and 273 control subjects. A blood sample was taken from each individual and DNA extracted. CCR5 genotype was determined using the polymerase chain reaction (PCR). Flow cytometry was used to investigate the biological activity of the Δ32 polymorphism.

There was no significant difference between the AAA and the control group in relation to the Δ32 allele frequency (AAA group 10%, control group 12%, P=0.82, chi squared analysis). There was also no significant difference in CCR5 genotype between groups (wild type homozygotes 82% in AAA group vs 77% in control group, heterozygotes 16% in AAA group vs 21% in control group, and Δ32 homozygotes 2% in both groups, P=0.33, chi squared analysis). The polymorphism was shown to be biologically active with the number of Δ32 alleles correlating with cell expression of CCR5 as detected with flow cytometry (P=<0.05).

This study demonstrates that the CCR5 Δ32 is a biologically active genetic polymorphism, however, there is no association between this polymorphism and AAA.
PUBLICATIONS ARISING FROM THIS THESIS


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immuno Deficiency Syndrome</td>
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<tr>
<td>AOD</td>
<td>Aorto-Occlusive Disease</td>
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<tr>
<td>BMT</td>
<td>Best Medical Therapy</td>
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<td>BP</td>
<td>Blood Pressure</td>
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<td>CABG</td>
<td>Coronary Artery Bypass Graft</td>
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<td>CCL</td>
<td>C-C Chemokine Ligand</td>
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<td>CCR</td>
<td>C-C Chemokine Receptor</td>
</tr>
<tr>
<td>COAD</td>
<td>Chronic Obstructive Airways Disease</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>CVA</td>
<td>CerebroVascular Accident</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribo Nucleoside Triphosphate</td>
</tr>
<tr>
<td>DUSS</td>
<td>Duplex UltraSound Scan</td>
</tr>
<tr>
<td>ECD</td>
<td>Extra Cellular Domains</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>EVAR</td>
<td>EndoVascular Aneurysm Repair</td>
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<tr>
<td>FSC</td>
<td>Forward Scatter</td>
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<td>GPCR</td>
<td>G Protein Coupled Receptor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HLA</td>
<td>Human Leucocyte Antigen</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IHD</td>
<td>Ischaemic Heart Disease</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ISH</td>
<td>Isolated Systolic Hypertension</td>
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<tr>
<td>MCP</td>
<td>Monocytes Chemoattractant Protein</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarct</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inhibitory Protein</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
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<tr>
<td>MMP</td>
<td>Matrix MetalloProteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene Tetra Hydro Folate Reductase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PAF</td>
<td>Platelet Activating Factor</td>
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<tr>
<td>PAI</td>
<td>Plasminogen Activator Inhibitor</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PLC</td>
<td>PhosphoLipase C</td>
</tr>
<tr>
<td>PVD</td>
<td>Peripheral Vascular Disease</td>
</tr>
<tr>
<td>QALY</td>
<td>Quality Adjusted Life Years</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activated Normal T cell Expressed and Secreted protein</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitors of Matrix Metalloproteinases</td>
</tr>
<tr>
<td>TMS</td>
<td>Transmembrane Spanning</td>
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<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor Alpha</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue type Plasminogen Activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase Plasminogen Activator</td>
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Chapter One

THE AORTA
1.1 Introduction

In order to give a better understanding of aortic disease, the possible underlying mechanisms for this and potential areas for therapeutic intervention, it is necessary to understand normal aortic structure and function. This chapter focuses on these areas.

1.2 Anatomy: An overview of the macroscopic structure

The aorta is the main artery of the body and carries oxygenated blood from the heart to the rest of the body via the systemic circulation. Its abdominal portion begins at the level of the twelfth thoracic vertebra where it enters the abdominal cavity. It is approximately thirteen centimetres (cm) in length and ends at its bifurcation, becoming left and right common iliac arteries at the level of the fourth lumbar vertebra (see Figure 1.1a). As it travels through the abdomen, it is related to various other major structures: posteriorly lie the twelfth thoracic to the fourth lumbar vertebrae in addition to the left lumbar veins as they pass to the inferior vena cava; anterior to the aorta at the diaphragmatic hiatus is the coeliac plexus of nerves with their associated ganglia, and inferior to this lie the body of the pancreas, the left renal and splenic veins, the second part of the duodenum and the small intestine; to the right and left of the aorta are the respective diaphragmatic crura, and on the right also lies the cysterna chyli, the thoracic duct and the azygos vein (see Figure 1.1b).
Figure 1.1a: Anterior Relations of the Abdominal Aorta (viewed from the anterior aspect)

The aorta can be seen closely related to the inferior vena cava, with the left renal vein crossing its anterior surface. The ureters can be identified by their course over the bifurcation of the common iliac arteries.
Figure 1.1b: Posterior relations of the Abdominal Aorta (viewed from posterior aspect)\(^{(2)}\)
Showing the cisterna chyli immediately posterior to the abdominal aorta as it passes through the aortic hiatus.
The aorta has several branches which are given off in the abdomen. These can be classified as paired or unpaired and visceral or non-visceral (parietal). The three unpaired visceral branches are the coeliac trunk at the level of the twelfth thoracic vertebra, just as the aorta enters the abdomen; the superior mesenteric artery anterior to the first lumbar vertebra; and the inferior mesenteric artery at the level of the third lumbar vertebra. These vessels supply blood to the embryological foregut (lower oesophagus, stomach, and duodenum), midgut (small intestine, caecum, ascending and transverse colon) and hindgut (descending and sigmoid colon and upper rectum) respectively.

There are three sets of paired visceral arteries which arise from the aorta in the abdomen and these are the renal and adrenal arteries at the level of the first lumbar vertebra, and the gonadal arteries at the level of the second. As suggested by their name, these vessels supply the renal and adrenal glands, and the ovaries or testis respectively (see Figure 1.2).

There are then several paired parietal branches which comprise the subcostal arteries, the inferior phrenic arteries and the lumbar arteries, which again all supply their namesake. There is only one unpaired parietal branch which is the median sacral artery that arises at the bifurcation of the aorta at the level of the fourth lumbar vertebra. Any pathology affecting the abdominal aorta may have consequences for any organs supplied by its branches.
Chapter One: The Aorta

Figure 1.2: Branches of the abdominal aorta

- Left adrenal artery
- Left and right renal arteries
- Paired gonadal arteries
- Median sacral artery
- Left and right common iliac arteries
- Coeliac trunk
- Superior mesenteric artery
- Paired lumbar arteries
- Inferior mesenteric artery

(3)
1.3 Histology: An overview of the microscopic structure

All blood vessels follow the same broad structure consisting of three layers: the tunica adventitia (outermost), the tunica media (in the middle) and the tunica intima (innermost) (Figure 1.3).

Different sized arteries tend to have slightly different composition in terms of the structural content of these layers and this can be used for classification. Larger arteries tend to be more
elastic and smaller arteries tend to be more muscular. By definition therefore, the aorta may be referred to as a large, elastic artery.

Elastic arteries have multiple layers of 'elastin', a protein which, as its name suggests, has elastic properties that help it serve as a tube for conduction of blood. Pressure is generated by the muscular left ventricle of the heart which then causes the walls of the aorta to distend. Over distension is prevented by the strength of collagen (a strong structural protein) present in the tunica media and adventitia. During the relaxation phase of the heart beat (diastole), the elastin component in the aortic wall causes recoil of the artery and therefore encourages continuous forward movement of the blood stream.

The tunica adventitia, the outermost covering, consists of a relatively thin layer of connective tissue. This is made up of collagen and elastic fibres in a loose network synthesised by cells called fibroblasts. In addition to blood vessels providing nutrients to the aortic wall, there are other cells present called macrophages which are important for the turnover of the connective tissue mesh or matrix.

The central portion of the aortic wall is termed the tunica media and consists of multiple layers of smooth muscle cells which are separated by sheets of elastin. The elastin is arranged in concentric layers called lamellae. At birth, there are no lamellae present in the aorta, but these form in response to the blood pressure in the aorta and by adulthood there are forty to seventy lamellae present in cross-section. Both the number and thickness of lamellae is increased in people suffering from hypertension (high blood pressure).
Smooth muscle cells are spindle shaped with an elongated nucleus and are also arranged in layers. These layers form a relaxed spiral along the long axis of the vessel. There are no fibroblasts present in the media of the aorta and it is the smooth muscle cells which produce structural proteins such as collagen and elastin. These cells may be stimulated by a number of chemical messengers in the blood. In addition to producing increased amounts of collagen and other proteins, they are also capable of migration in response to stimulation by cytokines, a group of chemical messengers. This ability to migrate towards the intima (innermost layer of the vessel wall) forms part of the normal repair mechanism of the vascular wall, but may also be part of a pathological (disease) process such as atherosclerosis (which is the focal accumulation of lipids and fibrous tissue in the intima of a vessel, associated with smooth muscle proliferation).

The tunica intima is also made up of layers comprising the endothelial cell layer, the subendothelial layer and the internal elastic membrane. Endothelial cells are thin, flattened cells which line the lumen of the blood vessel. They have specialised junctions between cells termed gap junctions and the cells themselves have several specific functions which will be described later in this chapter. The subendothelial layer is another connective tissue layer containing collagen and elastin which are again secreted by smooth muscle cells. These cells provide contractile properties to this layer which is important for regulation of intercellular gap junctions. These protein lined channels connect endothelial cells allowing free communication between cells. In the contracted state, smooth muscle cells open these
junctons, but when relaxed, the gap narrows, closing the communication. The internal elastic membrane is a thin elastic layer which provides flexibility to the intima.

Figure 1.4: Microscopy of arterial wall.\(^{(5)}\)

Endothelial cells in the tunica intima are responsible for the maintenance of a selectively permeable barrier to luminal contents. Movement of molecules between the lumen and the vessel wall is dependent on size and charge. This barrier also prevents exposure of the subendothelial layer to blood which may cause clotting or thrombosis. Endothelial cells produce anticoagulant (thrombomodulin) and antithrombotic substances (prostacyclin and tissue plasminogen activator) in order to prevent intravascular coagulation. Damaged endothelial cells release prothrombotic factors resulting in platelet aggregation and thrombosis in order to prevent blood loss at the site of endothelial damage.
Other substances released from endothelial cells (endothelin, angiotensin converting enzyme, nitric oxide and prostacyclin) aid in the modulation of blood flow and control of vascular resistance. In addition, the endothelial cell layer has immune functions regulated by the interaction of lymphocytes with endothelial cell receptors via adhesion molecules and cytokines. Hormone synthesis also occurs in this layer with the production of haemopoetic colony stimulating factors, fibroblast stimulating factor and platelet derived growth factor.

Endothelial cells therefore have a key role within the aorta, not only due to their physical attributes which form an effective barrier system, but because of their diverse physiological role via the actions of hormones, cytokines and the immune system.

1.4 Physiology

The arterial circulation carries blood from the heart to the rest of the body supplying it with oxygen and other essential nutrients. Blood travels through the vascular tree down a pressure gradient. Arterial compliance is the opposite of elasticity and is determined by the properties of the arterial wall. The abdominal aortic wall has a low compliance due to its high collagen content. During systole (cardiac contraction) the energy of the cardiac impulse is distributed to the aortic wall but because of the low compliance of the aorta, there is minimal distension and therefore the pressure within the vessel increases causing rapid flow of blood.

Blood pressure in the aorta is determined by a number of factors which may be likened to an electrical circuit. Ohms' law states that the power of a system (V) is determined by the current
Chapter One: The Aorta

(I) multiplied by the resistance (R) to flow (V=IR). This same theory can be applied to the vascular system where the blood pressure (power) is determined by the product of cardiac output (current) and total peripheral resistance (resistance). Therefore the blood pressure in the aorta may be affected by changes in the cardiac output (determined by the stroke volume and the heart rate) and the resistance provided by the rest of the vascular system (via vasoconstriction or vasodilatation).

The mean blood pressure, approximated by;

\[ \text{diastolic pressure} + \left(\frac{(\text{systolic pressure} - \text{diastolic pressure})}{3}\right), \]

in the aorta is around 90 mmHg and this decreases steadily throughout the rest of the vascular system. This is because the cross sectional area for passage of blood (approximately 3cm\(^2\) in the aorta) progressively enlarges as the vascular tree divides into a greater number of smaller vessels. As it branches, although the diameter of any individual arteriole is progressively smaller, the total cross sectional area increases to around 800cm\(^2\) at arteriolar level and 3500cm\(^2\) at capillary level. As such, the same volume of blood is distributed through a much larger area and therefore the pressure in each vessel is reduced. Because of the drop in driving pressure, there is a resulting reduction in velocity of blood flow from approximately 30cm/second in the aorta to 0.026cm/second at the capillary bed. This allows for diffusion of substances between the capillary and the surrounding extracellular space.

Some of the pressure resulting from the cardiac impulse is transmitted to the aortic wall itself, termed transmural pressure. This pressure describes the outward force tending to cause the aorta to expand. Stretching of the wall in this way creates an opposite tension which is
determined by the radius of the aorta and the thickness of the vessel wall. This is the tangential wall tension. The transmural pressure in the aorta is higher than in any other vessel and is counterbalanced by the tangential wall tension (determined by structural components of the arterial wall). As will be described in Chapter 2, an aortic aneurysm (abnormal dilatation) causes an increase in the aortic radius in addition to reduced vessel wall thickness and therefore the balance between the transmural pressure and the tangential wall pressure is altered, resulting in the tendency of the vessel wall to expand.

1.5 Cytokine function and tissue repair mechanisms

A cytokine is a soluble protein mediator secreted by one cell to influence another cell, tissue or organ. The cytokine family represents a diverse group of proteins which may be classified according to their function. Chemotaxis is the process of attracting cells towards the area of inflammation, and cytokines which are capable of this may be termed chemokines. A specific group of chemokines have a structural similarity to one another due to four preserved cysteine amino acid residues. These proteins are called the C-C chemokines and act via specific receptors on the cell surface known as C-C Chemokine Receptors (CCRs). Of particular interest in aneurysm development is a central chemokine receptor called CCR5 and this will be discussed in detail in chapter 4. At least twelve C-C chemokines exist, and those acting via the CCR5 receptor include monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP) and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES).
Wound healing involves firstly a temporary repair, often through the coagulation cascade and formation of a clot, and then secondly the laying down of fibrous scar tissue. This consists mainly of collagen which is secreted from fibroblasts migrating into the area via chemotaxis. The initial scar then undergoes a process known as remodelling which involves both degradation of the existing wound matrix and further collagen synthesis and deposition. The balance between these two processes determines the composition of the final healed wound.

Cytokines such as Interleukin-1 (IL-1) and Transforming Growth Factor beta (TGFβ) are profibrotic i.e. they tend to stimulate increased collagen production and fibrosis. In addition to stimulating collagen synthesis, IL-1 is also capable of inhibiting the collagenase enzyme which breaks down collagen, and TGFβ increases the action of protease inhibitors (known as Tissue Inhibitors of Matrix MetalloProteinases, or TIMPs) therefore resulting in a net increase in matrix proteins.

Antifibrotic cytokines such as Tumour Necrosis Factor alpha (TNFα) and Interleukin-6 (IL-6) act to counterbalance these effects by decreasing the accumulation of collagen precursors and down regulating collagen synthesis. They too have effects on TIMPs, but stimulate their production, therefore encouraging breakdown of the protein matrix.

Abdominal aortic aneurysms are known to have weakened walls as a result of matrix changes which reduce the ability of the abdominal aorta to withstand systolic blood pressure. This will be discussed in detail in Chapter 2.
Chapter Two

THE ANEURYSM
Chapter Two: The Aneurysm

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2.1 Introduction

Abdominal aortic aneurysm (AAA) is the abnormal dilatation of the abdominal portion of the aorta and represents a significant health problem. The incidence of aortic aneurysm has been steadily increasing in recent years, despite government led attempts to reduce cardiovascular risk factors such as hypertension and smoking. The most significant risk associated with aortic aneurysm is rupture: as the artery dilates, its wall becomes thin and is at risk of bursting. If this complication occurs, it is life threatening and is associated with a high mortality. Non-ruptured aneurysms can also cause complications such as compression of surrounding structures or embolisation of thrombus arising from turbulent flow within the aneurysm sac. Emboli may cause ischaemic complications in the organs supplied by branches of the aorta, commonly the lower limbs. Surgical repair is the only definitive treatment of AAA and even in the elective setting this carries a significant risk of perioperative morbidity and a mortality of 5-10%.

Although AAA is common, particularly amongst men over 65 years of age, there is little public awareness of its existence. There has been no real change in the mortality from AAA in recent years, and the key to improving survival is early diagnosis and elective repair. Screening programmes have been investigated and if introduced on a national basis, would improve both early detection and public education regarding aneurysms at an acceptable cost (Multicentre Aneurysm Screening Group 2002).

Current research focuses largely on novel strategies of treating aneurysms, which in the past have all required laparotomy and open surgical repair. Since AAA can be detected when small and have a long latent period, there is an opportunity to arrest growth and progression to surgery if a pharmacological treatment could be developed. As part of this, the underlying mechanisms
of AAA formation are being investigated in an attempt to understand the predisposing factors and pathological processes which take place and could potentially be manipulated.

In this chapter, a general overview of aneurysms, their risk factors, pathogenesis and management will be discussed.

2.2 Definition
An aortic aneurysm can be defined as a permanent and irreversible localised dilatation of the aorta and derives from the Greek word aneurusma, meaning 'widening'. A more precise definition has been sought in order to correctly identify patients with small aneurysms who may benefit from surveillance. As such, the International Society for Cardiovascular Surgery set up an ad hoc committee to agree a standard aortic diameter at which an aneurysm could be diagnosed. Prior to this, aortic diameters of anywhere between 2.5 and 3.5 centimetres had been considered aneurysmal in either sex patient (McGregor 1995). As aortic diameter is known to vary according to patient age and sex, the committee agreed a new reporting standard. An aneurysm is therefore now considered to be at least 1.5 times greater than the expected diameter of a normal aorta in a patient of the same age and sex (Johnston 1991). As the normal aortic diameter for a man is approximately 2cm, a diameter of ≥3cm is generally accepted to be aneurysmal.
2.3 Epidemiology

2.3.1 Prevalence

Abdominal aortic aneurysm (AAA) is a common and significant health problem. It affects males more commonly than females and age is also known to be a risk factor (Vardulaki 2000). Prevalence may be difficult to estimate as AAA is usually asymptomatic until the point of rupture. Data from screening studies suggest rates from 1.3 to 12.7% depending on the age group of patients studied and the criteria used to define AAA (Wilmink 1991). Autopsy studies appear to agree with screening studies and male to female ratios of 3.5-6:1 are also reported (Drury 2005). Population based studies looking at trends in aneurysm diagnosis have reported significant increases over the last 20 years and this increase appears to be out of proportion to the ageing population (Naylor 1988) indicating a true rise in diagnoses. There has also been a significant rise in hospital admissions from 52 to 149 per million per year in men from 1979-1999 which represents an 8.2% increase (Filipovic 2005).

Figures taken from the Office of National Statistics show an increase in the number of aortic aneurysm repairs performed between 2002 and 2005 (see Table 2.1).

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of Operations</th>
<th>Percentage Male</th>
<th>Percentage Emergencies</th>
<th>Mean Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000-2001</td>
<td>10,115</td>
<td>76%</td>
<td>26%</td>
<td>68 years</td>
</tr>
<tr>
<td>2001-2002</td>
<td>9,141</td>
<td>76%</td>
<td>27%</td>
<td>68 years</td>
</tr>
<tr>
<td>2002-2003</td>
<td>9,043</td>
<td>76%</td>
<td>26%</td>
<td>68 years</td>
</tr>
<tr>
<td>2003-2004</td>
<td>9,135</td>
<td>78%</td>
<td>26%</td>
<td>66 years</td>
</tr>
<tr>
<td>2004-2005</td>
<td>9,404</td>
<td>77%</td>
<td>26%</td>
<td>66 years</td>
</tr>
</tbody>
</table>

Table 2.1: Number of aortic operations performed between 2000 and 2005.
2.3.2 Incidence

The incidence of symptomatic aneurysm is known to increase with age, occurring in 25 per 100,000 people with a mean age of 50 years, but rising to 78 per 100,000 people in those over 70 years (Drury 2005). The true incidence however is much higher than this as most aneurysms are asymptomatic. In keeping with the increasing prevalence, studies report rises in age-adjusted incidence over recent years (Wilmink 1998), and vary between 3 and 117 per 100,000 people affected per year. The rise in incidence is disproportionately higher in women than in men (12.1% vs 8.2%, Filipovic 2005) however the reasons for this are not fully understood.

2.3.3 Mortality

Abdominal aortic aneurysm has been shown to cause 1.3% of all deaths in men aged 65-85 years old in developed countries (Sakalihasan 2005). Aneurysm related deaths in the Oxford region doubled during the period 1979-1999 (Filipovic 2005). The main cause of aneurysm related death is rupture. In the UK, about 6000 men die from ruptured AAA every year, and this amounts to approximately 2% of men over the age of 65 years (Eamshaw 2005, Henricksson 2005). The mean incidence of ruptured aneurysm is also increasing. Mealy found a rise from 9.2 to 17.5 per 100 000 patient years over an 8 year period (Mealy 1988) and the Goteborg study found a seven-fold increase in the incidence of rupture over a 36 year period (Drott 1992).

Interestingly, the number of deaths due to aneurysm in England and Wales over the past 5 years has fallen slightly (Table 2.2), and although this is unlikely to be significant given the numbers involved, if the trend were to continue, it may reflect the improved anaesthetic and intensive care facilities in addition to the introduction of minimal access surgery for aneurysms.
Table 2.2: Number of deaths due to aortic aneurysm by year.


<table>
<thead>
<tr>
<th>Year</th>
<th>Male Deaths</th>
<th>Female Deaths</th>
<th>Total Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>3582</td>
<td>1619</td>
<td>5201</td>
</tr>
<tr>
<td>2002</td>
<td>3595</td>
<td>1551</td>
<td>5146</td>
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<tr>
<td>2003</td>
<td>3583</td>
<td>1668</td>
<td>5251</td>
</tr>
<tr>
<td>2004</td>
<td>3535</td>
<td>1628</td>
<td>5163</td>
</tr>
</tbody>
</table>

2.4 Risk Factors

2.4.1 Age

Advancing age is now well established as a major risk factor for the development of AAA. Autopsy and screening studies found increasing age to be a significant risk factor with incidence of AAA rising sharply over the age of 55 years and reaching a peak between the ages of 80 and 90 years independent of gender (Krohn 1992, Bengtson 1992). Since that time, observational studies have confirmed these findings of the increasing incidence with age, reporting aneurysmal disease in up to 23% of those over the age of 80 years (Morris 1994).

Aneurysm is likely to be more common in older people as it represents a degenerative disorder characterised by weakening of the aortic wall. This may be the result of a chronic inflammatory process resulting in elastolysis and increased collagen turnover as described later in the chapter (see section 2.5 Pathogenesis). The inflammatory response and subsequent aneurysmal dilatation are likely to arise in response to prolonged exposure to other risk factors such as cigarette
smoking and hypertension. An aneurysm is more likely to develop in those with greater exposure to risk factors and as such age is a very significant risk factor for the presence of an aneurysm.

2.4.2 Gender

Male sex represents a significant risk factor for development of many cardiovascular pathologies including ischaemic heart disease, peripheral vascular disease and abdominal aortic aneurysm. Whilst pre-menopausal women appear to be at a comparatively lower risk, the protective effect of endogenous oestrogens is lost after the menopause.

Screening studies have suggested a six fold increase in the prevalence of AAA in men compared to women (Scott 1995, Pleumeekers 1995), however autopsy studies suggest this may be an overestimate, with the true figure closer to twice as common (McFarlane 1991, Bengtsson 1992).

There may also be differences in the presentation and natural history of aneurysms between men and women. Katz found men to be 1.8 times more likely to undergo surgical repair of an aneurysm and 1.4 times more likely to have a ruptured AAA treated surgically than women (Katz 1997). The reasons for this discrepancy are not clear. One possible mechanism is the later development of AAA in women due to the protective effect of oestrogens in earlier life. In this way, the female patient may be significantly older (and have co-morbidities) than the male equivalent at the point of presentation, thus making surgical repair more risky.

Aneurysms in women may also expand more rapidly than in men. Solberg found that over a five year period, the mean growth rate of aneurysms amongst men was 1.82mm/year in contrast to 2.43mm/year in women (Solberg 2005, Mofidi 2007). This is consistent with the finding of the
UK Small Aneurysm Trial which reported female sex as an independent risk factor for rupture (UK Small Aneurysm Trial Participants 1998).

One proposed mechanism to explain the gender related differences observed suggests that oestrogen may influence cytokine pathways. Ailawadi demonstrated a relative protective effect of administered oestradiol against AAA formation in rats and this was associated with a reduction in MMP-9 production which is thought to be significant in the initiation of AAA (Ailawadi 2004).

2.4.3 Family History

The first report of familial AAA was in 1977 when Clifton observed three brothers who were all affected by aneurysm (Clifton 1977). Since then there has been significant interest in a potential genetic contribution to AAA development and many other authors have found family history to be a significant risk factor.

Increased incidence of AAA is seen particularly in first degree relatives of affected individuals. Baird found that 4.4% of siblings of AAA patients had aneurysms compared with 1.1% of controls. The lifetime cumulative risk of AAA in this group was also greater (60.8% vs 14.9%) (Baird 1995). This is in agreement with Adams who found aortic dilatation in 21% of male first degree relatives over the age of 50 years old, however only 4% of sisters and no daughters were affected (Adams 1993).

Powell found a prevalence of 8% among first degree relatives (7% among siblings) (Powell 1987) and Wanhainen quantified the odds ratio at 4.4 (Wanhainen 2005).
A positive family history may suggest a genetic predisposition to AAA formation, however it must also be considered that family members may have other factors in common, such as exposure to cigarette smoke and socioeconomic status. In addition, other risk factors for AAA such as hypertension, hypercholesterolaemia and ischaemic heart disease may also demonstrate familial clustering and may therefore exaggerate any true genetic predisposition. This topic is covered in greater detail in Chapter 3.

2.4.4 Hypertension

The increase in transmural pressure generated by raised blood pressure is likely to predispose to aneurysm formation and indeed hypertension has been shown to increase the risk of having an aneurysm by 30-40% (Vardulaki 2000). Diastolic pressure in particular is thought to be significant (Lindbald 2005) due to the greater length of diastole (60% of the cardiac cycle) compared with systole. A long term prospective study published in 2003 confirmed that a 1.1 per 20mmHg higher systolic pressure and a 1.12-1.14 per 12mmHg higher diastolic pressure was associated with AAA (Rodin 2003) and a history of treated hypertension conferred a 1.87 increased lifetime risk of aneurysm.

The effects of hypertension can be difficult to separate from those of generalised atherosclerosis. Atheroma leads to a reduction in elastic recoil of the aorta and this may predispose to isolated systolic hypertension and a widened pulse pressure often observed in the elderly. Comparison between patients with isolated systolic hypertension (ISH) and no hypertension has revealed 11.9% prevalence of AAA in the ISH group and 6.5% among normotensive subjects although this difference was not statistically significant (P=0.134) (Naydeck 1999). Multivariate analysis
in this study however did reveal higher pulse pressure to be an independent predictor of AAA which may be indicative of the reduction in medial elastin which characterises the early stages of aneurysm pathogenesis.

Not only is hypertension a significant risk factor for the development of AAA, there is also evidence to suggest that increased expansion rates may be observed amongst hypertensive individuals. Gadowski used an experimental model in rats to demonstrate the effects of systolic BP on AAA diameter. They found that aneurysms in the hypertensive animals were significantly larger on day 7 and day 14 than the normotensive group. Overall, the mean growth rate was nearly twice as high in the hypertensive group (Gadowski 1993). This is in keeping with observational studies which have reported an increased rate of rupture (which in turn is associated with greater diameter and rate of expansion) amongst hypertensive patients (Powell 2001, Brown 1999).

Further confirmation of hypertension as a risk factor comes from the observation that beta-blockade reduces the expansion rate of AAA in experimental studies. This antihypertensive strategy targets beta-adrenergic receptors and therefore causes relaxation of vascular smooth muscle resulting in vasodilatation and reduction of mean blood pressure. In experimental studies beta-blockers have been shown to reduce AAA expansion independently of their effect on BP (Slaiby 1994, Gadowski 1994). Although human studies have produced less convincing results (Propanolol Aneurysm Trial Investigators), good blood pressure control remains important in the management of small aneurysms.
2.4.5 Smoking

Multiple observational population based studies have documented an increased incidence of AAA among smokers (Wilmink 1999, Cornuz 2004, Wanhainen 2005, Lee 1997, Lindbald 2005) with odds ratios varying from 2.4-5.2. Smoking has also been found to be an independent risk factor with the level of exposure being more significant than the duration (Vardulaki 2000). Wilmink reported that current smokers were 7.6 times more likely to have an aneurysm than non-smokers, and ex-smokers were 3 times more likely (Wilmink 1999). However, in contrast to the Vardulaki study, they found that the duration of smoking was significantly associated with an increased risk of aneurysm and there was a clear linear relationship with the duration of smoking and the relative risk of AAA which increased by 4% per year of smoking (Wilmink 1999). Again contradicting the Vardulaki study (Vardulaki 2000), they found duration to be more significant than level of exposure using cotinine (a metabolite of nicotine) levels as an objective measure of exposure.

It has been suggested that nicotine may have a direct effect on neutrophil elastolytic activity leading to aneurysm formation. Murphy examined elastase activity release amongst smokers and non-smokers with aneurysms, peripheral occlusive vascular disease and control groups. They found a direct correlation between elastase activity release and nicotine concentration (Murphy 1998).

Smoking may also affect the rate of aneurysm expansion. MacSweeney found higher growth rates in those who continued to smoke compared with ex- or never smokers (0.16 vs 0.09cm per year (P=0.038)) (MacSweeney 1994). These results were corroborated by Chang and Lindholt
who both found that a history of cigarette smoking was associated with more rapid aneurysm

Experimental evidence also supports this observation. Buckley demonstrated that while all mice
exposed to elastase developed aneurysmal aortas, those which were also exposed to daily
cigarette smoke had aneurysms of 50% greater diameter at 12 weeks (Buckley 2004).

2.4.6 Previous Vascular Disease

The relationship between AAA and vascular disease affecting other major vessels such as the
coronary, carotid or femoral arteries is complex. Although many of the risk factors which
predispose to AAA formation are shared with those predisposing to ischaemic heart disease,
peripheral vascular disease and stroke, it is clear that aneurysm development involves a different
pathological process from generalised atherosclerosis. The risk of AAA remains high among
patients with a history of vascular disease, even when adjustments for common risk factors have
been made (Madaric 2006).

The incidence of AAA among patients admitted for coronary artery bypass grafting has been
found to be as high as 13% compared with 1.4% among a control group (Bergersen 1998).
Although this is helpful to guide focussed screening services, it may simply reflect a higher
prevalence of risk factors such as hypertension and smoking among this group of patients (Rodin
2003).
Chapter Two: The Aneurysm

It seems likely that a previous history of vascular disease is a significant risk factor for AAA development independent of other common risk factors, although age, male sex and smoking continue to be far more significant.

2.5 Pathogenesis

2.5.1 Early theories of aneurysm development

Traditionally, the development of aortic aneurysms was thought to be associated with atherosclerotic degeneration of the aortic wall, causing weakening and susceptibility to aneurysmal dilatation. This theory gained credibility due to the common risk factors shared by both atherosclerotic and aneurysmal disease such as smoking, hypertension and hypercholesterolaemia. However, one major risk factor for occlusive disease, diabetes mellitus, is not associated with aneurysm formation and this has resulted in the atherosclerotic theory of aneurysm formation being challenged. In fact diabetes seems to predispose to aorto-occlusive disease which is likely to be the result of atherosclerotic degeneration, and aneurysm formation is now thought to be the result of an entirely different disease process: inflammation.

2.5.2 Evidence to support an inflammatory process

Inflammatory infiltrates consist of elevated tissue levels of macrophages and lymphocytes and contain certain pro- and anti-inflammatory cytokines. Examination of the cellular composition of both normal and aneurysmal aortic wall has confirmed increased expression of inflammatory cells including T lymphocytes and macrophages (Forester 2005, Ailawadi 2003). Inflammatory cytokines have been shown to be upregulated in aneurysmal aortic walls compared with normal controls (Juvonen 1997). Specifically Interleukin (IL) 1 beta, IL-6 and Tumour Necrosis Factor
(TNF) alpha levels were significantly higher in aneurysm patients than healthy controls. TNF alpha and IL-6 have also been shown to be elevated in aneurysmal disease compared with aorto-occlusive disease (AOD), confirming the different underlying processes involved (Shteinberg 2000). IL-10, an anti-inflammatory cytokine has also been shown to be upregulated in AAA compared with AOD suggesting a level of regulation of the inflammatory process (Davis 2001).

Evidence of an autoimmune trigger for the inflammatory process has been described following work using extracted immunoglobulin G (IgG) from aneurysmal aortic walls. This was found to react to connective tissue components in sections taken from normal aortic tissue (Gregory 1996). In support of this autoimmune theory, Human Leukocyte Antigen (HLA) tissue typing comparing aortic aneurysms with controls found that the HLA-DR B1*0401 allele was more common amongst the aneurysm group, whereas the HLA-DR B1*01 allele tended to be protective against aneurysms (Monux 2003).

Therefore, there is ample evidence of an inflammatory process which underlies aneurysm formation and it is likely that there may be an autoimmune component to this.

2.5.3 The role of cytokines

Cytokines represent a diverse group of proteins which perform a wide range of differing functions. In broad terms, there are three main groups of cytokines known to be involved in aneurysm formation: chemotactic cytokines, predominantly IL-8, profibrotic cytokines such as IL-1 and Transforming Growth Factor beta (TGFβ), and antifibrotic cytokines such as IL-6 and TNFα.
Interleukin-8 is a potent mediator of chemotaxis and has been shown to be upregulated in aneurysms, and indeed associated with aneurysm rupture (Treska 2002). It is likely that IL-8 is secreted early in the inflammatory process in order to recruit appropriate inflammatory cells to the aortic wall. T lymphocytes extracted from AAA tissue and stimulated in vitro demonstrate upregulation of TNFα, interferon (IFN) gamma and IL-6 secretion, suggesting that the cytokine response is mediated by T cell interactions (Forester 2005).

IL-1 and TGFβ have similar effects to one another and together produce a tendency towards excessive collagen deposition, or fibrosis. IL-1 inhibits collagenases and TGF β increases expression of Tissue Inhibitors of Matrix Metalloproteases (TIMPs), which causes the accumulation of collagen within the extracellular matrix. Their role in aneurysm formation follows excessive matrix breakdown and the rapid rate of turnover results in a disorganised matrix which is unable to withstand aneurysm formation. The cytokines IL-6 and TNFα act to counterbalance excessive collagen deposition by the reduction of available precursors for collagen synthesis. The balance between these profibrotic and antifibrotic mechanisms determines the composition of the aortic wall and the propensity for aneurysm development.

2.5.4 Aneurysm formation

Elastolysis appears to be the primary event in aneurysm formation (Wilmink 1998). Matrix metalloproteases (MMPs) are the enzymes responsible for this process. MMPs 2, 9 and 12 are known to have elastolytic functions and are expressed and produced in increased amounts in human aneurysm tissue (Thompson 1996). MMP 12 has been found to be particularly highly expressed along the leading edge of the aneurysm and therefore may be involved in the initial stages of aneurysm formation (Ailawadi 2003). Elastin degradation products may be chemotactic.
for macrophages and the initial loss of elastin produces thinning of the medial wall associated with macrophage infiltration and neovascularisation (Ailawadi 2003). MMP 2 may also be associated with the initial stages of aneurysm formation as it has been found in small aneurysms.

2.5.5 Aneurysm expansion

Although loss of elastin appears to initiate aneurysm formation, expansion seems to be dependent upon collagen destruction by MMPs 1, 8 and 12 (collagenases). The subsequent increase in collagen turnover leaves the aortic wall weakened and less able to withstand high pressure. This produces an increase in wall tension, leading to dilatation and eventually rupture (Ailawadi 2003). It is likely that hypertension is a significant factor in this stage of aneurysm development, and indeed beta blockers have been shown to reduce wall tension and expansion rates in experimental models (Ailawadi 2003). Cytokine levels may also be significant determinants of expansion as higher levels of IL-6, IL-8 and TNFα have been found in the walls of ruptured aneurysms than asymptomatic aneurysms (Treska 2002), and the main risk factor for aneurysm rupture is known to be size. There is significant individual variation in the rate of aneurysm expansion and this may in part be explained by differences in cytokine levels determined by genetic variation. This will be discussed in detail in Chapter 3.

2.6 Clinical Features and investigation

The vast majority of aneurysms are asymptomatic until the point of rupture. Those that do present electively may do so with abdominal pain, back pain or a visible pulsation in the abdomen. This may be associated with rapid aneurysm growth or erosion into surrounding structures such as lumbar vertebrae.
If rupture occurs, the patient is likely to present with abdominal and/or back pain associated with cardiovascular collapse. At this point, it may or may not be possible to palpate the aneurysm itself, making diagnosis difficult. Clues as to the diagnosis may be ascertained from a history of hypertension, smoking, family history, previous vascular disease etc.

If the patient is stable, the investigation of choice is a computerised tomography (CT) scan with intravenous contrast (Figure 2.3) which will confirm the presence of an aneurysm, determine whether rupture has occurred and detail whether the aneurysm may be suitable for endovascular repair.

Figure 2.3: CT scan showing a ruptured abdominal aortic aneurysm.
This will not only confirm the diagnosis but will also demonstrate the anatomy of the aneurysm in terms of its proximity to the renal arteries, the tortuosity and length of the aneurysm neck and the condition of the iliac arteries, all of which are important considerations when planning surgical treatment. If the patient is not stable, it may be that exploratory laparotomy is necessary without prior investigation.

In the elective setting where there is no question of aortic rupture, Duplex Ultrasound Scanning (DUSS) has been found to be around 99% sensitive and specific (Lindholt 1999) and is therefore used routinely in the diagnosis of aneurysm (Figure 2.4). As this is a cheap, non invasive test, it can be used widely and as such has been identified as the investigation of choice for aortic aneurysm screening programmes.
Figure 2.4: DUSS showing abdominal aortic aneurysm. Arrow represents antero-posterior diameter of aneurysm, which in this case appears to be ~7cm.
2.7 Screening

Aneurysms are largely asymptomatic unless complications such as rupture occur. The mortality associated with elective repair of an aneurysm is approximately 5% (Hallett 1993), however the mortality associated with emergency repair of a ruptured aneurysm is closer to 50% (Bown 2002). Because of this, the viability of screening programmes has been explored in order to detect aneurysms at an earlier stage when they can be electively repaired.

The Multicentre Aneurysm Screening Study carried out in 2002 randomised men aged 65-74 years to screening by ultrasound scan or no screening. They demonstrated a 53% relative risk reduction amongst the men who attended screening (Multicentre Aneurysm Screening Group, 2002). This resulted in a reduced rate of emergency operations and lower aneurysm related mortality. Several single centre studies confirm these results on a local level (Collin 1998, Lucarotti 1993, Lindholt 2005).

Cost effectiveness studies have also been performed. These demonstrate acceptable costs in terms of quality adjusted life years (QALYs) gained by screening, both in urban and rural areas (Multicentre aneurysm screening group 2002, Henricksson 2005, Duncan 2005). The additional cost was found to be £63.39 per patient and the projected cost per life year gained after ten years was £8000. This falls within the guidelines for government funding of a screening programme.

It is likely in view of this data and the increasing number of patients affected by aortic aneurysm that pressure will be put on the government to establish a national screening programme. This will enable follow up of aneurysms until they reach 5.5cm when the risks of rupture outweigh those of elective repair (Szilagy 1972) and surgery is recommended (The UK Small Aneurysm
Trial Participants 1998). It may be that new medical therapies can be introduced to prevent rapid aneurysm expansion. This may enable surgery to be deferred in some patients, and others, especially those with small aneurysms, may avoid surgery and its associated risks altogether.

2.8 Natural history and prognosis

The natural progression of small aneurysms is to expand. This is due to the weakened aneurysmal aortic walls which have a increased wall tension compared with normal aorta and therefore cannot resist the transmural pressure (see chapter 1). The rate of aneurysm expansion may be an important factor predisposing to aneurysm rupture and a faster rate of expansion is often seen in larger aneurysms.

The risk associated with AAA is primarily rupture, and this is directly related to aneurysm diameter. In fact, the only reliable predictor of rupture is aneurysm size (Glimaker 1991). The UK Small Aneurysm Trial compared mortality associated with AAA of 4-5.5cm diameter in two groups randomised to early elective surgery or ultrasound surveillance. No survival advantage was shown in either group (Powell 2007). Surgery is therefore reserved for aneurysms reaching 5.5cm diameter.

It is widely accepted that the optimum treatment for AAA is elective repair where possible. The outcome from elective surgery varies between centres, but operative mortality falls within the range of 5-10%. The outcome following rupture is much poorer, with less than 50% of patients reaching hospital alive, and only around 50% of those surviving following emergency surgery (Donaldson 1985, Johansen 1991).
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It is difficult to estimate the mortality associated with conservative management of AAA, but studies of patients considered unfit for surgery do demonstrate a reduced life expectancy (Wilmink 1998). This is both in terms of aneurysm related and all cause mortality (Norman 2004).

In order to address the morbidity and mortality of AAA repair, endovascular techniques have been developed and introduced into clinical practice and are revolutionising the management of aortic aneurysm.

2.9 Management

The first stage in the management of AAA is of course diagnosis, and screening programmes have already been discussed. In a patient who is considered fit for open repair, two options are available for treatment of an aneurysm which has grown to 5.5cm or greater. These are 1) open repair and 2) endovascular repair. If a patient is considered to be unfit for open repair, an endovascular approach may still be considered, although there is doubt that this confers any survival advantage over conservative management alone.

The EndoVascular Aneurysm Repair (EVAR) trial is a large scale, multicentre randomised control trial set up to investigate the role of endovascular repair in the management of AAA. It has two arms: EVAR versus open repair in those who are fit for open repair (EVAR 1); and EVAR versus conservative management in those who are unfit for open repair (EVAR 2). This
trial reported mid-term data in 2005 and although the recruitment stage has been completed, data collection continues.

2.9.1 EVAR vs Open Repair: EVAR 1

The treatment of AAA has traditionally involved placing a prosthetic graft into the aneurysmal section of aorta via open surgery through the abdomen (see figures 2.5 & 2.6).

Figure 2.5: Operative view of an open repair.
Open repair showing prosthetic graft secured in aneurysm sac which is yet to be closed over the graft repair.
Diagram 2.6: Stages of open aneurysm repair.
A. The aneurysm sac is opened. B. Thrombus is evacuated. C. Proximal anastomosis is performed using ‘inlay’ technique. D. Distal anastomosis is performed in same manner. E. Aneurysm sac is closed over graft.

This technique involves cross clamping the aorta which imposes a significant physiological insult, particularly to the cardiovascular system, both in terms of cardiac strain and peripheral ischaemia. A laparotomy in itself may result in respiratory compromise due to inadequate pain
relief and diaphragmatic splinting, and the inevitable haemodynamic changes which occur perioperatively also impact on the kidneys. Consequently, open repair carries an overall operative mortality of approximately 5-10% in addition to significant complications such as post operative myocardial infarction, renal failure or lower limb loss. Post operative pain, chest infections, prolonged ileus, or thromboembolic events are also potential risks which may prolong hospital stay.

Endovascular repair (EVAR) was first introduced by Parodi in 1991 (Parodi & Palmaz, 1991) and avoids many of these potential morbidities through a minimally invasive approach. The femoral arteries are exposed through groin incisions, and the endovascular stent is placed and deployed under radiological guidance via the femoral arteries (see figures 2.7 & 2.8). This technique avoids the need for laparotomy and cross clamping of the aorta, and is therefore thought to cause less surgical stress to the patient. Recovery times and mortality following EVAR are significantly less than after open repair.
Figure 2.7: Operative view of EVAR
The endovascular stent graft device is introduced via the common femoral artery in the groin under x-ray guidance.
Diagram 2.8: Stages of EVAR.
A. The main body of the device is introduced and deployed below the level of the renal arteries. B. A balloon is passed up through the graft and inflated (C) to secure proximal fixation. D. The limb components are placed within the main body and deployed in a similar fashion.
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The EVAR 1 Trial reported 30 day mortality of 1.7% for EVAR compared with 5.7% for open repair. This reduced aneurysm related mortality was sustained throughout the 4 year follow up period. There was no difference however in all cause mortality between the EVAR and open repair groups (EVAR 1 Trial Participants 2005). In addition to this, unique complications have been observed following EVAR which include technical failures of the stent such as displacement, kinking, migration and ‘endoleak’—the persistent flow of blood outside the stent but within the aneurysm sac. Therefore, at four years post operatively, the proportion of patients experiencing complications from their surgery was 41% in the EVAR group and only 9% in the open repair group (EVAR 1 Trial Participants 2005).

Cost effectiveness analyses have suggested that EVAR is significantly more expensive than open repair with an increased cost of £110 000 per QALY compared with open repair (Michales 2005). However, much of this increase in cost is associated with long term follow-up and reintervention, and it is likely that with continued investment and development of stent graft devices in addition to a greater understanding of the natural history of endoleaks, the long term costs will decrease over the next few years.

2.9.2 EVAR vs Conservative Management: EVAR 2

Patients considered unfit for open repair due to significant co-morbidity or age have traditionally been treated by best medical therapy (BMT). This involves management of hypertension and hypercholesterolaemia, cessation of smoking, and low dose aspirin therapy aimed at reducing expansion rates, preventing thromboembolic complications from the aneurysm (Dehlin 2005), and reducing cardiovascular mortality through secondary prevention.
EVAR provides an alternative to BMT which may be of particular benefit to this group of patients. The second arm of the EVAR trial (EVAR 2) randomised patients who were unfit for open repair to receive either best medical therapy or EVAR. Outcomes were assessed on an ‘intention to treat’ basis. No difference was found in all cause or aneurysm related mortality between the two groups and the perioperative mortality of EVAR was 9%, similar to the rupture rate in the best medical therapy group of 9 per 100 person years (EVAR 2 Trial Participants 2005).

Although these data suggest no benefit of EVAR for patients who are unfit for open aneurysm repair, they may be misleading as only 80% of patients adhered to their randomisation and there was a significant amount of crossover between groups. Therefore, up to 20% patients who were randomised to best medical therapy underwent compassionate EVAR when it was thought that their risk of rupture outweighed their risks from surgery, for example if an aneurysm was expanding very rapidly or had reached a very large size. This must be taken into account when interpreting the results of the study.

2.9.3 The role of conservative management

EVAR is likely to enable surgical repair in a much larger group of patients than previously when only open repair was available, however with the proposed introduction of screening programmes to detect small aneurysms, the role of medical therapy is being explored as a means of preventing or slowing aneurysm expansion. This would enable aneurysm surgery to be performed electively in all but a few cases, thus dramatically improving survival. It may also avoid surgery altogether for some patients.
As the process underlying aneurysm formation is now known to be inflammatory, mediators such as cytokines and their receptors are key targets for such a therapy. A drug that could counteract the inflammatory process, perhaps by blocking a key receptor, could potentially be incorporated into the medical management of patients unfit for surgery and those with small AAA.
Chapter Three

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Chapter Three: The Genes

3.1 Introduction

The incidence of abdominal aortic aneurysm (AAA) has been steadily increasing over recent years, with up to 8% of men over the age of 60 now affected (Singh 2001). Aortic aneurysm has been repeatedly linked with smoking, hypertension, hypercholesterolaemia and male sex (Blanchard 2000), and it is believed that these risk factors play a causal role in aneurysm development. However, as medical management of these co-morbidities and anti-smoking campaigns attempt to address these factors, the incidence of aneurysm continues to rise. This is in contrast to other smoking related diseases such as ischaemic heart disease, which have seen a fall in incidence over a similar period (Lampe 2005). This has led to uncertainty as to the significance of these factors in aneurysm pathogenesis.

Clifton in 1977 first reported a possible genetic component of aneurysm disease when he described a family in whom three brothers were all affected by AAA (Clifton 1977). Since then many familial studies have been conducted in an attempt to establish a genetic link. Several authors have also attempted to characterize the mode of inheritance through segregation studies.

The first half of this chapter examines the evidence in support of a genetic predisposition for aneurysm development. As detailed in Chapter 2, there is now a significant body of evidence in support of an inflammatory process underlying aneurysm formation, and the latter part of this chapter reviews the role of biologically plausible candidate genes which may be responsible for the familial clustering of aneurysms observed.
3.2 Familial Studies

One of the earliest population based studies to examine the aetiology of AAA compared coronary heart disease, stroke and aortic aneurysm (Hammond 1969). This was a prospective study of mainly white races from over 1,000 counties. There were approximately 500,000 females and 350,000 males between 40 and 79 years old who were included in the analysis. Subjects completed a questionnaire at enrolment, detailing significant co-morbidity and smoking status, and were then followed up for 6 years. Any deaths occurring during this period were classified by the cause of death given on the death certificate. During the study, 431 men and 88 women died from aneurysm related disease. The death rate from AAA was found to be higher in men who were hypertensive. This study found significant associations between increased weight, low levels of exercise and smoking, and the development of aortic aneurysm. Although these common risk factors with coronary heart disease are still thought to be significant today, Clifton's report of familial aneurysm in 1977 then provoked a series of studies into a possible genetic cause for aneurysms.

In 1986, Johansen compared the family histories of 250 patients with AAA and 250 controls. They found that 19.2% of patients with an aortic aneurysm reported having a first degree relative with a history of AAA. Only 2.4% of controls reported a positive family history of AAA. They therefore calculated an estimated relative risk of developing AAA if a first degree relative has already been affected of 11.6 (Johansen 1986). This represented a significant clustering of AAA in families, and in 1989 Darling conducted a study to determine if two distinct subtypes of aortic aneurysm may exist: familial and non-familial.
This was a nine year prospective study of 542 consecutive patients undergoing abdominal aortic aneurysm repair. Eighty-two of these patients reported a positive family history of AAA in between two and five first degree relatives. Four hundred and sixty patients therefore reported no previous incidence of aortic aneurysm in their family. There were some initial differences observed between the familial and non-familial groups. Firstly, the non-familial group had a higher male preponderance of AAA than the familial group (86% vs 65% male). Secondly, the average age of the non-familial group tended to be higher than that of the familial group (67.8 years vs 62.4 years) and this difference was significant among the male patients. The two groups were however comparable in terms of smoking status, and number of patients affected by either hypertension or diabetes. They also found no difference in aneurysm morphology, either in terms of anatomy or aortic wall composition, suggesting that although some aneurysms may be the result of an unknown genetic factor, the final pathological process is likely to be similar to sporadic aortic aneurysm (Darling 1989).

A similar study conducted by Baird in 1995 utilized ultrasound scanning to screen relatives of both AAA patients and controls, in order to gain a more reliable estimate of family tendency to aneurysm development than relying solely on self reported incidence (which inevitably will miss some affected individuals). They recruited 126 unrelated patients consecutively admitted to hospital with AAA and 100 healthy controls undergoing cataract surgery. All of the controls, and 54 of the 427 siblings of aneurysm patients, underwent ultrasound scan, and this data was added to family history data obtained from interviews. They found that 4.4% of siblings of aneurysm patients had an aneurysm versus 1.1% of controls on the basis of family history alone. Following ultrasound scan, 19% of siblings of aneurysm patients were found to have AAA versus 8% of
controls. The risk of aneurysm was shown to begin earlier and increase more rapidly for siblings of affected individuals compared with controls (Baird 1995).

Also in 1995, LaMorte wrote the first report on racial differences in rates of AAA repair. They observed that atherosclerotic disease was more common in black than white Americans, but aortic aneurysm was more common in a white population. A multivariate analysis comparing aneurysmal disease with a control group (undergoing appendicectomy) confirmed hypertension, age and smoking as significant risk factors for aneurysm disease, and reported a protective effect associated with diabetes. They found black people to be at less risk of aneurysm than white with an odds ratio of 0.29. They therefore concluded that this observation may suggest the influence of genetic factors (La Morte 1995).

Two further studies in 1999 and 2000 confirmed the increased incidence of AAA observed in first degree relatives of affected individuals, and quantified the odds ratios at 4.33 and 4.77 respectively (Salo 1999, Blanchard 2000) (Figure 3.1).

Although these observational studies have identified a clear familial tendency for aortic aneurysm, most do not consider the effects of common familial factors such as socioeconomic status, dietary and lifestyle considerations. Further studies were therefore required to confirm and further investigate the genetic basis of AAA.
1977
Clifton described series of three brothers with AAA

1986
Johansen & Koepsell: Relative risk of AAA in first degree relative 11.6

1989
Darling & Brewster: Familial AAA occurs at younger age and affects more women than sporadic AAA

1995
LaMorte: AAA more common in white than black populations
Baird: 19% of AAA siblings have AAA compared with 8% of control siblings

1999
Salo: Odds ratio for AAA in first degree relative 4.33

2000
Blanchard: Odds ratio for AAA in first degree relative 4.77

Figure 3.1: Summary of familial studies into aortic aneurysm
3.3 Segregation Studies

In view of the overwhelming evidence for a genetic link, several studies have attempted to characterize the pattern of inheritance of AAA. The earliest of these was Tilson in 1984 who studied 50 families with clustering of AAA in 2 or more first degree relatives. Following segregation analysis, they concluded that if only one gene were responsible for aortic aneurysm, it is likely to be autosomal, however they could not exclude multigene mechanisms (Tilson 1984).

Powell in 1987 examined the family histories of 60 consecutive patients undergoing aneurysm repair. Of these, 20 patients had at least one first degree relative with AAA. There were 320 first degree relatives and 192 siblings in total, and they reported 25(8%) and 14(7%) aneurysms respectively. They suggest that the genetic component of aneurysm development is likely to contribute at least 70% of the overall susceptibility (Powell 1987).

Following on from this, Majumder performed a segregation analysis based on a population of patients with ruptured and surgically treated aneurysms. They found strong evidence to reject a sporadic model of aneurysm development and found the multifactorial effect to be minimal. A major gene locus was found to be likely and a recessive model was shown to best fit their data (Majumder 1991).

In contrast, Verloes in 1995 recruited 520 patients treated for AAA at a single centre and studied the 276 patients who had a positive family history of aneurysm. Although they also strongly rejected a sporadic model, and found no better fit with a multifactorial model, a single dominant
allele was felt to give the best explanation of their findings. In addition to this, they described an allele frequency of 1:250 for the morbid allele, with a sex-dependent penetrance which slowly increases with age to 0.3 in women and 0.4 in men over the age of 80 years. This would go some way to explaining the far greater incidence of aneurysms observed in men (Verloes 1995).

In 2003, Kuivaniemi recruited 233 families known to have at least 2 members affected by AAA. They agreed with the Majumder study on the whole, reporting that the majority of their data fitted an autosomal recessive inheritance pattern. However, in approximately 25% of cases, an autosomal dominant pattern better explained their findings in agreement with the work of Verloes. They conclude that the lack of consistency in the mode of inheritance may be indicative of multifactorial disease (Kuivaniemi 2003).

In summary, there is overwhelming evidence of a genetic predisposition to development of AAA shown by clustering of aneurysms in families. Although no clear pattern of inheritance has yet emerged, it is generally agreed that an autosomal gene (or genes) is the most likely, although both dominant and recessive models may be feasible. Although studies have suggested a single gene may be responsible, others have suggested a multifactorial aetiology, and it is likely that the interplay between genetic predisposition and environmental factors such as smoking and hypertension are ultimately responsible for aneurysm formation.
3.4 Candidate Genes

In order to further characterize the potential genetic component of AAA pathogenesis, several investigators have adopted a 'candidate gene' approach. The candidate gene strategy is based on selection of a gene that encodes a protein (i.e. receptor, enzyme, intracellular messenger) that is pathogenetically relevant to the investigated phenotype. In the case of abdominal aortic aneurysms, this had led to the investigation of genes which code for proteins involved in the inflammatory process. Many of these genes have been shown to have polymorphic sites which may in part explain the genetic predisposition of some individuals, not just for aortic aneurysms, but for a whole range of chronic inflammatory conditions.

3.4.1 Elastin and Elastases: MMP-2, -7, -9 & -12

The degradation of medial elastin in the aortic wall appears to be an initiating step in the process of aneurysm formation. Matrix metalloproteinases (MMPs) digest both collagen and elastin, however MMP-2, -7, -9, and -12 are generally considered to be primarily elastases. Elevated levels of these MMPs have been shown to occur in aneurysmal aortic wall compared with normal aorta (van Vlijmen-van Keulen 2002).

MMP-9, also referred to as gelatinase B, is both a type IV collagenase and an elastase. A cytosine to thymidine transition in position 1562 of the promoter region of the MMP-9 gene produces a 1.5 fold increase in promoter activity (Jones 2003). It is therefore plausible that this polymorphism may be responsible for the upregulated MMP-9 activity seen in association with AAA.
Jones compared 414 patients with AAA, 172 patients with peripheral vascular disease, and 203 healthy controls with respect to the C-1562T polymorphism. They found a greater proportion of T alleles amongst the aneurysm population than either of the other groups, and quantified the odds ratio of developing AAA with this allele to be 2.41 in comparison to the control group (Jones 2003). However, other studies investigating the effect of MMP-9 have found no significant association between MMP-9 polymorphisms and AAA (Yoon 1999, Higashikata 2004). It may be that the increased MMP-9 levels observed in the aneurysm wall are the result of increased local stimulus, rather than a genetic predisposition to MMP overproduction.

Experimental studies have examined the effect of MMP-12 on aneurysm development with the use of knockout mice. Longo compared aortic diameter following infusion of calcium chloride to initiate aneurysm formation in wild type and MMP-12 knockout mice. They found that the knockout mice developed less aortic dilatation than the wild type mice. Histological examination showed disruption and fragmentation of medial elastin fibres in wild type mice, whereas knockout mice showed only focal elastic lamellae breakdown. MMP-12 levels were increased in the wild type mice, however, in the knockout mice, no MMP-12 rise was seen following infusion of calcium chloride (Longo 2005). This is suggestive of a role for MMP-12 in aneurysm development in mice.

The MMP-12 gene has a polymorphic site in its promoter region involving a single nucleotide transition from adenine to guanine in position -82. Human studies of this gene polymorphism were initially conducted in reference to coronary artery aneurysms, however the allele frequencies did not differ significantly between coronary aneurysm and control groups (Lamblin
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Eriksson later investigated the genotype-phenotype relationships of MMP-12 in addition to MMP-2, -3, and -9, and found no evidence that any specific MMP polymorphism had a clinically significant effect on aortic aneurysm expansion (Eriksson 2005). Therefore, although MMP-12 is likely to be involved in aneurysm development, it is unlikely that the polymorphic locus is responsible for the upregulation seen.

There is a polymorphic site in the promoter region of the MMP-2 gene with a cytosine to thymidine transition at the -1306 locus. Although this has not been investigated with respect to aortic aneurysm disease, it has been shown to be significant in the development of coronary artery aneurysms, with patients affected by aneurysm tending to have a higher proportion of cytosine alleles than thymidine (Lamblin 2002). This may be a potential area for further investigation.

3.4.2 Collagen and Collagenases: MMP-1, -8 & -13

Types I and III collagen are found in abundance in the aortic wall and provide structural integrity and strength. A point mutation in the collagen I gene can be inherited as an autosomal dominant trait and results in a range of clinical syndromes known as Osteogenesis Imperfecta (brittle bone disease), but this is not known to be associated with vascular defects (van Vlijmen-van Keulen 2002). In contrast with collagen I, alterations in collagen III turnover are likely to be pivotal in the weakening of the aortic wall and aneurysmal dilatation.

Collagen III is secreted as a procollagen, and the aminoterminal propeptide (PIIINP) is then cleaved to produce the mature collagen molecule. The propeptide is detectable in the
extracellular matrix following cleavage, and if collagen turnover increases rapidly, large amounts of PIIINP are found. A study conducted in 1995 by Satta found significantly higher levels of PIIINP in peripheral and aortic blood samples taken from patients with AAA than those with aorto-occlusive disease, indicating increased collagen turnover (Satta 1995). Anderson also described an increased collagen I to collagen III ratio in fibroblast cell cultures taken from skin cells in a patient with multiple large vessel aneurysms. They associated this with a polymorphism of the collagen III gene resulting in an amino acid switch from leucine to phenylalanine (Anderson 1996).

Matrix metalloproteinases are largely responsible for the alterations in matrix turnover seen in AAA. Their activities are regulated at several levels: firstly the transcription and translation of inactive precursors (zymogens); secondly the post-translational activation of zymogens by proteolysis; and lastly by the interaction of mature MMPs with their tissue inhibitors (Tissue Inhibitors of MetalloProteinases, TIMPs). Higashikata used the real-time reverse transcriptase polymerase chain reaction (RT-PCR) to quantify gene expression and found MMP-1 and -3 to be elevated in human aneurysmal compared with normal aorta (Higashikata 2004). They also found the ratio of MMP-1 to TIMP-1 and -3 was significantly elevated, indicating increased tendency to matrix degradation with decreased regulation by the usual control mechanisms. These findings were previously reported by Tamarina in 1997 when they described elevated levels of MMP-1 mRNA and an increased MMP-1 to TIMP-1 ratio in human aneurysmal aorta (Tamarina 1997). MMP-8 is a potent type I collagenase, which has also been shown to be upregulated in the aneurysm wall (Wilson 2005).
To date, there is no clear evidence that the upregulation of MMPs seen in association with AAA results from a genetic predisposition. Although polymorphic sites have been identified in the promoter regions of several MMPs, no particular polymorphism has been found with increased frequency in association with aneurysmal disease. Although experimental work with knockout mice has demonstrated a protective effect from complete lack of MMP-12, it is unlikely that any naturally occurring polymorphism would have such a dramatic effect on the gene function and consequent protection from AAA formation.

3.4.3 Tissue Inhibitors of MetalloProteinases

The balance between MMPs and their tissue inhibitors (TIMPs) determines the composition of the extracellular matrix. There are currently four TIMPs which have been characterized and named TIMP-1 to -4. TIMP-1 is a specific inhibitor of MMP-1 and -9. Eskandari studied the response in TIMP-1 deficient mice when elastase enzymes were infused. They found that TIMP-1 knockout mice grew aneurysms which were significantly larger than the wild type mice, and had an expansion of 208% compared with 153% in the wild type mice (Eskandari 2005).

Tilson in 1993 conducted a study to determine whether the relative decrease in TIMP expression seen in human aneurysmal aorta occurs as a result of decreased tissue expression or a primary genetic predisposition. They found a single nucleotide polymorphism from cytosine to thymidine in the third position of codon 101 (rs2070584), but demonstrated that the amino acid for which it coded was preserved. They also demonstrated normal fibroblast expression of TIMP mRNA in association with this polymorphism, and therefore concluded that there was no evidence to
suggest that the TIMP-1 deficiency seen in aneurysmal aorta results from a primary gene deficiency (Tilson 1993).

More recently however, two studies have demonstrated a significant difference in the allele frequencies expressed in patients with AAA compared with control groups. Ogata found an association between the TIMP-1 polymorphism and AAA in male patients without a family history of aneurysm (Ogata 2005), and Wang found a significant difference between aneurysms and controls in the frequency of the TIMP-2 nt 573 polymorphism, which occurs in the same position as in the TIMP-1 gene, and is also a neutral polymorphism with no effect on translation (Wang 1999).

Although it seems unlikely, given that these single point polymorphisms do not affect the amino acid sequence of the TIMP protein, that a predisposition to aneurysm formation could be transmitted via these polymorphic sites, there is evidence in other areas of significant TIMP polymorphisms affecting phenotype. Several novel single nucleotide polymorphisms of the TIMP-2 gene have been found including the -418 guanine to cytosine locus, the -177 cytosine to thymidine locus and the +34 cytosine to adenine locus. Whilst these have not been investigated in relation to AAA, they have been found to be linked and possibly significant in relation to chronic obstructive pulmonary disease (Hegab 2005). It may be therefore, that although the single polymorphisms studied by Ogata and Wang are not in themselves responsible for the alteration in TIMP activity, they may be markers for another polymorphism which does affect translation (Wang 1999, Ogata 2005).
3.4.4 Plasminogen Activator Inhibitor-1

Regulation of the MMP/TIMP system is partly controlled by the plasmin system. The inactive pro-enzyme of plasmin, plasminogen, is converted to its active form by plasminogen activators, either tissue type plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Plasmin (the active enzyme) in turn activates MMPs. The actions of plasminogen activators are regulated by plasminogen activator inhibitors (PAI-1 and -2).

There is a 4G/5G insertion/deletion polymorphism of the PAI-1 gene in position -675 of the promoter region (rs1799889, SERPINE2). The 4G allele binds only an activator of transcription, whereas the 5G allele binds both an activator and a repressor, and is therefore associated with relatively reduced transcription of PAI-1. The frequency of the 5G allele has been shown to be greatest among patients with familial aneurysms at 53%, in comparison to 39% in a control population and 38% in patients with non-familial aneurysms (Rossaak 2000).

The 5G allele has also been analysed in relation to aneurysm progression and patients homozygous for the 5G insertion were found to have faster aneurysm growth than those with a 4G allele (Jones 2002).

It is likely therefore, that the plasmin system may be involved in progression of AAA and this makes it a potential target for the development of medical therapies to suppress aneurysm growth.
3.4.5 Interleukins

It is now generally accepted that AAA arises as a result of a chronic inflammatory process. Bown therefore studied a range of inflammatory cytokine polymorphisms in relation to their prevalence in AAA. Although polymorphisms of the interleukin (IL)1β, IL-6, IL-10 and tumour necrosis factor alpha (TNFα) genes were studied, only the IL-10 polymorphism (SNP A1082G, rs1800896) appeared in higher frequency among aneurysm patients than healthy controls (Bown 2003). Interleukin-10 is an anti-inflammatory cytokine, and the -1082 polymorphism A allele is associated with decreased IL-10 production, thus limiting the ability of the subject to regulate the inflammatory process which leads to AAA formation.

Several other studies agree with these findings. Marculescu compared 135 patients with aneurysms to 270 patients with coronary artery disease with respect to six known polymorphisms in the IL-1 gene. They found no significant differences in any of the polymorphisms between groups and therefore concluded no role for IL1 polymorphisms in the pathogenesis of AAA (Marculescu 2005).

Similarly, Jones assessed the role of the -174 G to C substitution in polymorphic locus of the IL-6 gene. This is known to be a functionally significant polymorphism as lower IL-6 levels have been reported with the homozygous G genotype than when a C allele is present. The authors found no link between IL-6 genotype and aneurysm progression, however, the G allele did appear to be a predictor of cardiovascular mortality (Jones 2001).
3.4.6 Angiotensin Converting Enzyme

The renin-angiotensin-aldosterone system plays a major role in the conservation of salt and water and as such is a useful therapeutic target for antihypertensive agents. The angiotensin converting enzyme (ACE) which converts angiotensin I into the active angiotensin II has been shown to be highly expressed in human aneurysmal aorta (Daugherty 2000, Nishimoto 2002). This evidence is suggestive that high local levels of angiotensin II may play a significant role in AAA development.

There is a polymorphic site in the ACE gene which consists of the presence or absence of a 287 base pair DNA fragment (corresponding to an alanine repetitive sequence in the ACE protein) in the intron of the ACE gene (rs1799752). The polymorphism has been termed I for insertion of the fragment and D for its deletion. The alleles are co-dominant with an additive effect on plasma levels, so homozygotes for the deletion allele have the highest plasma levels of ACE and homozygotes for the insertion have the lowest. Fatini compared the genotypes of 250 patients with AAA and 250 age and sex matched controls. They found an increased D allele frequency in the AAA group of 0.63 compared with 0.49 in the control group and this difference was found to be highly significant (Fatini 2005).

These findings were confirmed by Pola. They segregated the aneurysm group into those with hypertension and those without, in order to determine whether the ACE polymorphism was acting via an effect on blood pressure (which is already known to predispose to aneurysm formation). Interestingly, they found a high incidence of the DD genotype in normotensive patients with aneurysms (70%), but only 32% of hypertensive patients with co-existent AAA had
this genotype. This does suggest that the polymorphism affects AAA development independently of any association with blood pressure (Pola 2001).

In agreement with this finding, experimental studies have shown that whilst infusion of angiotensin II produces AAA, infusion of incremental doses of aldosterone produces no effect on aortic diameter (Cassis 2005). This is significant as it suggests that the increased ACE levels seen in the aneurysm wall may be stimulated by a different pathway to the physiological renin-angiotensin-aldosterone system.

As angiotensin converting enzyme inhibitors (ACEI) are already in routine clinical use in the treatment of hypertension and are generally well tolerated, studies into the effects of ACEI on aneurysms in patients with differing ACE genotypes would be interesting as this represents a potential therapeutic target for future medical treatment of small aneurysms.

3.4.7 MethyleneTetraHydroFolate Reductase

Homocysteine is a non-protein amino acid which is involved in carbon metabolism and methylation reactions. It is structurally similar to cysteine however possesses an additional methylene group which prevents the formation of stable peptide bonds. High plasma levels are toxic to vascular tissues, and hyperhomocysteinaemia is known to be a risk factor for early onset vascular disease (Brunelli 2000). Excessive homocysteine is re-methylated by methionine synthase and in this reaction, 5-methyltetrahydrofolate serves as a methyl donor. This compound
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is produced by methylenetetrahydrofolate reductase (MTHFR) and therefore a defect in this enzyme may lead to hyperhomocysteinaemia.

There is a polymorphism in the MTHFR gene at the 677 locus which involves substitution of a cytosine nucleotide for a thymidine (rs1801133). Strauss demonstrated an elevated T allele frequency amongst patients with AAA compared to a control group (0.37 vs 0.21) with an odds ratio for AAA of 4.4 if a T allele was present (Strauss 2003). More recently, a large study of 428 patients with aneurysms failed to demonstrate an association between the C677T polymorphism and aneurysm formation, however they did demonstrate significantly larger aneurysms amongst patients who were homozygous for the T allele compared with those who had a C allele (Jones 2005).

3.4.8 Platelet Activating Factor

Platelet activating factor (PAF) has been shown to induce MMP-1, -2 and -9 in epithelial cells and fibroblasts (Shan 1999, Sugano 2000). PAF is inactivated by the enzyme PAF acetylehydrolase (PAF-AH). An autosomal recessive inherited deficiency of PAF-AH has been reported in the Japanese population (Miwa 1991), and this deficiency has now been found to be the result of a single point mutation (a guanine to thymidine substitution at position 994) in exon 9 (Stafforini 1996). With this polymorphism, heterozygotes show reduced enzymatic activity and homozygotes have no PAF-AH activity at all.

Unno conducted a case control study to determine the effect of the PAF-AH polymorphism on AAA. They found a higher proportion of T alleles among the aneurysm group, and the risk of
developing an aneurysm if a T allele was present was 2.48 (Unno 2002). They also quantified enzyme activity and found it to be much greater in homozygous GG patients with an aneurysm than those without, confirming that the enzyme is likely to be involved in AAA development.

3.4.9 Nitric Oxide Synthase
Nitric oxide (NO) is responsible for most vasodilatation induced by the endothelium and a decrease in NO production may cause abnormal vascular function. NO is produced by endothelial NO synthase (eNOS), the gene coding for which is located on chromosome 7. A 27 base pair repeat is present in intron 4 of the eNOS gene and it is possible that this polymorphism may affect function of the enzyme. Kotani studied an aneurysm and a control group, dividing the aneurysm group into surgical and non-surgical patients. They found that 10% of the control group had 4 repeats present, whereas 14% of the aneurysm group had 4 repeats. The remainder in each group had 5 repeats at the polymorphic site. When the aneurysm group was subdivided, 21% of the surgical patients were found to have 4 repeats in contrast to only 4% of the non-surgical group, and the authors conclude that the 4 repeat genotype may be associated with rapid progression of AAA (Kotani 2000).

Two further polymorphisms of the eNOS gene have been described: a T substitution to C at the -786 locus and a G substitution to T at the 894 locus. Although a significant association has been found between the G894T allele and AAA, no such link was demonstrated for the T786C allele (Fatini 2005).

3.4.10 Human Leucocyte Antigens
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The Human Leukocyte Antigen (HLA) system is a recognition system which controls a variety of cell-cell interactions. Although not directly part of the inflammatory response, it determines T cell function, which is in turn central to chronic inflammatory processes. There are two arms to the HLA system: HLA class I which comprises HLA-A and -B molecules and is recognized by receptors on CD8 positive cells (suppressor T cells); and HLA class II (HLA-DR) which are recognized by receptors on CD4 positive T helper cells. Certain HLA subtypes have been associated with a number of chronic inflammatory conditions, and represent the genetic basis of a number of well known diseases such as diabetes and rheumatoid arthritis (Ghodke 2005).

HLA alleles have been studied in relation to aortic aneurysm. Rasmussen studied the HLA-DR B1 locus which has 12 associated alleles. They found B1*02 and B1*04 subtypes to be more common amongst the aneurysm group compared to a control group, and the B1*01, B1*08 and B1*14 alleles to be more frequent among the controls (Rasmussen 2001). These findings were supported by Monux whose results did not reach statistical significance, but suggested HLA-DR B1*01 to be protective and HLA-DR B1*04 to predispose to AAA (Monux 2003). Increased frequency of the HLA-DR2(15) allele has also been described amongst aneurysm patients compared with controls (58.7% vs 28%) in a Japanese population (Hirose 1998).

Class I HLA antigens may also be significant, and a case control study found HLA-A2 to be present in 60.4% of patients with aneurysms but only 42.6% of controls (P=0.036); and HLA-B61 to be present in 30.4% of patients with aneurysms but only 11.4% of controls (P=0.002) (Sugimoto 2003).
Although the precise mechanism by which HLA genotype affects individual susceptibility is not yet fully understood and is likely to be a complex interplay between genetic predisposition and environmental exposure, it may be possible to identify an HLA allele, or combination of alleles, which function as a genetic marker for AAA.

3.4.11 Inflammatory Receptors: CCR2 and CCR5

Many inflammatory cytokines have cell signalling or chemotactic properties and attract inflammatory cells to the site of injury. This family of cytokines are often termed chemokines and share a common structure. This involves 4 cysteine amino acid residues which interact with each other to produce a characteristic three dimensional shape. A subgroup of chemokines, the C-C chemokines which act via the CCR receptors, have 2 adjacent cysteine residues near their amino terminus and induce the migration of monocytes, natural killer cells and other cell types. The CC chemokine subfamily include proteins such as monocyte chemoattractant protein 2 (MCP-2), macrophage inflammatory protein 1 alpha (MIP-1α) and beta (MIP-1β), and regulated upon activation, normal T cell expressed and secreted protein (RANTES).

Chemokine receptors have attracted much scientific interest in recent years as they function as a co-receptor for the entry of Human Immunodeficiency Virus (HIV) into CD4 positive cells, and anti-chemokine receptor therapies are being trialled in this context. As they are central to cell signalling and the inflammatory process, CCR (C-C chemokine receptors) have also begun to be studied with respect to their potential role in chronic inflammatory conditions including AAA.
There are two chemokine receptors in particular, CCR2 and CCR5, which are structurally similar to one another and are known to have non-synonymous gene polymorphisms (Oppermann 2004). Studies of these polymorphisms in relation to HIV susceptibility have demonstrated a relative resistance amongst patients who are homozygous for the polymorphic alleles compared to those who have a wild type gene (Munerato 2003).

CC chemokine receptor 2 (CCR2) is structurally similar to CCR5 and experimental work has recently suggested that it may be directly involved in the pathogenesis of AAA (McTaggart 2007). There is a single nucleotide substitution polymorphism in the CCR2 gene (V64I). This polymorphism has not yet been investigated in humans with respect to AAA formation, but experimental work appears promising.

CCR5 has a 32 base pair deletion (Δ32) polymorphism in the promoter region of the gene. This results in a frameshift and premature termination of the protein (Mueller 2004). The role of this polymorphism in susceptibility to AAA has been investigated, and a higher incidence of the Δ32 allele was observed among patients with aneurysms than patients with other vascular pathology (carotid stenosis and peripheral vascular disease) or healthy controls (Ghilardi 2004).

CCR5 has been extensively studied owing to the interest in HIV therapy and the chapter which follows will review current knowledge of the structure and function of this receptor.
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C-C CHEMOKINE RECEPTOR 5
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Chapter Four: C-C Chemokine Receptor 5

4.1 Introduction

This chapter will review the current literature regarding C-C chemokine receptor five (CCR5) in terms of its structure and function. Research into the role it may play in HIV and other diseases will also be included. The polymorphism known as Δ32 will also be discussed and its possible significance and biological relevance outlined.

4.2 Structure and Function of CCR5

4.2.1 Structure of CCR5

There are six chemokine receptors known to date which all share certain common structural features. They all consist of 340-370 amino acids and contain seven regions containing hydrophobic amino acids. This indicates that they are likely to be members of the seven transmembrane spanning (7TMS) G protein coupled receptor (GPCR) superfamily. The hydrophobic regions correspond with the regions which span the phospholipid bilayer of the cell membrane. Each receptor has an extracellular aminoterminal (N terminal) segment and a cytoplasmic C terminal tail containing structural motifs that are critical for ligand dependent signalling, desensitization and receptor trafficking (Figure 4.1) (Opperman 2004).

CCR5 is a 352 amino acid protein with a calculated molecular mass of 40.6kDa, which is expressed predominantly on resting T-lymphocytes (memory and effector cells), monocytes and macrophages. It has also been found on T cells of a natural killer (NK) subtype which provides further evidence that the receptor is involved in inflammation (Kin 2002). In common with other GPCR it has cysteine residues in all four extracellular domains and a conserved sequence on the second intracellular loop which appears to be important for G protein interaction. All GPCR
have a disulphide bridge between 2 conserved cysteine residues on the second and third extracellular loops, and CCR5 seems to have another disulphide bond between the N terminus and the third extracellular loop which confers stability to the receptor and enables ligand binding.

Figure 4.1: Diagramatic representation CCR5 receptor structure demonstrating 7 transmembrane spanning domains with intracellular and extracellular termini.\(^{(8)}\)
The CCR5 receptor undergoes several modifications after initial translation of the gene. It has several tyrosine residues adjacent to acidic amino acids in the aminoterminus which are post-translationally modified by sulphate and this contributes significantly to the ability of CCR5 to bind with its ligands (Farzan 1999). In addition, CCR5 is also modified by O-linked glycosylation and if this step is prevented, chemokine binding is disrupted (Bannert 2001). Palmitoylation is the covalent attachment of fatty acids to cysteine residues of membrane proteins. CCR5 contains several cysteine residues, 12-25 amino acids away from the plasma membrane, which are suitable for palmitoylation. Studies of the CCR5 receptor have demonstrated that cysteine residues at positions 321, 323 and 324 are modified by palmitoylation and this facilitates the transport of receptors to the plasma membrane. It also aids ligand-stimulated endocytosis, affects the ability of the receptor to couple to signalling pathways and is required for G protein coupled receptor kinase (GPCRK) or protein kinase C (PKC) mediated phosphorylation (Blanpain 2001, Percherancier 2001, Kraft 2001).

Different forms of the CCR5 receptor are known to exist. A 22kDa soluble form of the receptor has been identified and interestingly, a lower proportion of patients infected with the Human Immunodeficiency Virus (HIV) seem to possess this short soluble CCR5 (ssCCR5) than healthy HIV negative individuals. Plasma macrophage inflammatory protein 1 beta (MIP-1β) levels were shown to be twice as high in the ssCCR5 positive individuals than the negative suggesting that this may be a potential source of variation in individuals’ responses to inflammation (Tsimanis 2005).
Suzuki identified two distinct forms of the CCR5 protein; a 62kDa form in addition to the 42kDa. They found that the larger 62kDa form is located on the cell membrane, and the 42kDa molecule is intracellular. This may be the result of post-translational modification or altered mRNA splicing, but appears not to simply be a dimeric receptor (Suzuki 2002).

4.2.2 Ligand binding to CCR5

A two step mechanism for ligand binding to CCR5 has been proposed. According to this theory, there is initial interaction between the chemokine core and the exposed aminoterminal receptor domain. At the same time, there is interaction between the ligand and the second extracellular loop which confers ligand specificity (Samson 1997). The free aminoterminal domain then interacts with the transmembrane helix bundle and triggers receptor activation (Govaerts 2003, Govaerts 2001, Blanpain 2003). This is confirmed by the finding that monoclonal antibodies to the N terminus do not block chemokine binding, but those to the second extracellular loop do, and are effective inhibitors of the CCR5 receptor (Navenot 2001). The second transmembrane domain also appears to be significant for ligand binding. A highly conserved TXP motif exists, and when this is disrupted, the CCR5 response to RANTES binding is greatly reduced (Arias 2003).

Ligand binding to CCR5 may be triggered by different mechanisms. Interleukin-12 (IL-12) is known to upregulate both MIP-1α and -β levels. Experimental studies examining the effect of IL-12 deficiency have shown upregulation of the CCR5 receptor in response to decreased circulating levels of MIP-1α and -β, suggesting a negative feedback type circuit exists (Losana 2002). Oxidative stress is another proposed mechanism of upregulation and CCR5 protein
expression on the cell surface has been shown to be upregulated by hydrogen peroxide (which is produced in response to oxygen deprivation) (LeHoux 2003).

It is thought that chemokine receptors form dimers following chemokine ligand binding (Hernanz-Falcon 2004), although it is not known which amino acids participate in this process. It has been shown that mutation of certain residues in the fourth transmembrane region prevents receptor dimerization and results in a non-functional receptor that does not trigger intracellular signalling.

G proteins have α, β and γ subunits which are mobilised from the main protein to cause activation of intracellular signalling pathways. α, β, γ and other dimers form and can regulate enzymes such as adenylate cyclase or phospholipase C beta (PLCβ) which in turn results in intracellular calcium mobilisation and inositol-1,4,5-triphosphate formation. Activation of PLCβ via CCR5 results in the generation of diacylglycerol and the activation of protein kinase C (PKC).

CCR5 has several known ligands. These are macrophage inflammatory protein-1α (MIP-1α), which is also known as C-C chemokine ligand 3 (CCL3), MIP-1β or CCL4 and RANTES (CCL5) (Onuffer 2002).

Binding of a chemokine (MIP-1α, MIP1β or RANTES) to CCR5 induces actin reorganisation and the formation of cellular lamellipodia enabling cell movement. This is an essential process in the recruitment of macrophages to the sites of inflammation (di Marzio 2005).
4.2.3 Regulation of CCR5

There are a number of mechanisms by which ligand binding to CCR5 is regulated. Firstly, the receptor itself becomes refractory to continued stimulation within seconds to minutes of initial binding, a process known as desensitization. Secondly, following chemokine binding, the CCR5 receptor is internalised within the cell. This process appears to be dependent on the action of β-arrestin (a central regulator of GPCR signalling). If β-arrestin is unable to bind to CCR5 (for example, via a conformational change), then receptor desensitization may be impaired (Perry 2002).

GPCR kinases play a key role in agonist induced receptor phosphorylation and desensitization and are likely to be important in receptor regulation. Following receptor endocytosis, CCR5 accumulates in perinuclear recycling endosomes and is returned back to the plasma membrane in a dephosphorylated form. Recycling and receptor degradation, which result in the reduction of total cellular receptor numbers, may operate over several hours. Recycling does not require dissociation of the chemokine from the resensitized receptor and ligand bound CCR5 can undergo multiple cycles of receptor internalisation and recycling (Signoret 2000).

CCR5 is associated with the T cell receptor CD4 and soluble fragments of each receptor have been shown to interact with one another. Furthermore, the CCR5/CD4 receptor complex has a 3.5-fold lower affinity for MIP-1β, but exhibits enhanced G-protein signalling. This is likely to contribute to the ‘fine tuning’ of the immune response (Staudinger 2003).
4.3 The role of the CCR5 receptor in disease

Because of its central involvement in inflammatory processes, CCR5 has been studied in relation to many chronic conditions. It has been shown to be present in abundance in the synovial tissue taken from patients with arthritis (Haringman 2006); implicated in the severity and prognosis of multiple sclerosis (Elovaara 2006, Martinez-Caceres 2002); and associated with the recruitment of T lymphocytes to the thyroid gland in Graves' disease (Aust 2002) and the lungs in asthma (Kallinich 2005) and Wegener's granulomatosis (Zhou 2003). It has also been linked with gastrointestinal disorders such as ulcerative colitis where upregulated CCR5 appears to correlate with disease activity (Matsuzaki 2003), and Helicobacter pylori infection where the number of CCR5 positive cells detected in children with the bacterium was far higher than in those without (Krauss-Etschmann 2003).

CCR5 has also been identified as a co-receptor with CD4 for the macrophage-tropic (M-tropic) strain of HIV-1 entry into cells (Doranz 1997, Moore 1997, Broder 1997). It forms one of two main modes of entry into the cell, with the CXC chemokine receptor four (CXCR4) being the other which is mainly utilised by T cell tropic (T tropic) strains of the virus. Transformation of the virus from the M tropic to T tropic or syncitium inducing (SI) form occurs in approximately 50% of HIV positive individuals and is often linked with, but not necessary for, disease progression (Westby 2005).

HIV surface glycoprotein 120 (gp120) independently triggers CCR5 activation of mitogen-activated protein kinases which in turn lead to gp120-induced expression of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and MIP-1α and -β (Lee 2003) which may affect
disease manifestation and progression. Wang investigated cytokine regulation of CCR5 and CD4 expression on cells known to serve as reservoirs for viral persistence in patients with AIDS, such as macrophages, microglia and monocytes. They demonstrated that IL-4 and IL-10 enhanced the entry and replication of HIV-1 in microglia through the upregulation of CD4 and CCR5, and that this was associated with increased migration of microglia in response to MIP-1β (Wang 2002).

Quinonez attempted to qualify which regions of the CCR5 protein contribute to its coreceptor function. They used genetic footprinting to establish which extracellular domains (ECD) are important, and found ECD 3 and 4 to be critical for viral entry (Quinonez 2003).

It is thought that CCR5 expression may make cells particularly susceptible to cytopathic effects during HIV-1 infection (Zaunders 2004), and the interaction between CCR5 and the HIV envelope has been shown to trigger apoptosis (or cell death) (Algeciras-Schimnich 2002, Zaunders 2003).

CCR5 clearly plays a central role in a number of chronic inflammatory and infective illnesses. The aetiology of many of these conditions is not fully understood, but a familial trend can often be observed. This has led to investigation of any possible genetic predisposition towards diseases such as diabetes, various cancers, multiple sclerosis, rheumatoid arthritis etc. The CCR5 gene contains polymorphic sites or loci, which may be significant in determining receptor function which in turn may influence susceptibility to disease.
4.4 CCR5 gene polymorphisms

The CCR5 gene was first characterised by Mummidi who described, amongst other things, two previously unidentified promoter regions in the gene, one upstream of exon 1 and one downstream between exon 3 and 4. The latter of these regions demonstrated strong promoter activity in a range of leukocyte cell environments (Mummidi 1997). They went on to describe a polymorphic region in the regulatory region of this gene, and this was further characterised when the gene was sequenced as part of the Human Genome Project in 2001. There are now several non-functional polymorphisms which have been described, however it is the 32-base pair deletion (Δ32, rs333) in the promoter region of the gene which has attracted most interest due to the apparent resistance it confers to infection with the HIV-1 virus (McDermott 1998, Roger 1998, McNicholl 1997). This Δ32 polymorphism causes a frameshift in translation and consequent truncation of the receptor protein (Samson 1996) which results in decreased expression of the receptor at the cell surface.

The CCR5 Δ32 allele is relatively common in Caucasians, but found at lower frequencies in the Middle East and Indian populations. Haplotype analysis indicates that the allele originated quite recently, about 700 years ago, at a single point in North Eastern Europe (Stephens 1998, Libert 1998). A gradual change in allele frequencies is observed in a North to South gradient in Europe, with the highest frequencies seen in Finland and Iceland. This suggests that the allele conferred some kind of selective advantage and therefore passed through populations rapidly. One theory to explain the origin of this allele is that it disseminated from Scandanavian populations during the Viking invasions, which fits both historical and geographical data (Lucotte 2003).
The high frequency of the Δ32 allele does suggest that it confers some form of survival advantage over the wild type gene, and although it has in recent years been associated with resistance to HIV-1 infection, initial appearance of the mutation seems to predate the HIV-1 epidemic. The plague bacillus *yersinia pestis* has been implicated as a potential source of strong selective pressure on European populations during medieval times with the 'Black Death' killing some 25 million people. The bacterium emerged around the estimated time of the CCR5 Δ32 mutation and so a popular theory that the Δ32 allele conferred survival advantage during the plague has emerged. Mecsas conducted experimental studies to test this theory using wild type and CCR5 deficient mice. They found no difference between the wild type and knock out mice in either bacterial load or spread following infection with *yersinia pestis*. Furthermore, they found the CCR5 deficient male mice died more quickly than the wild type mice, thus contradicting the theory that the Δ32 allele was protective (Mecsas 2004).

Further evidence against the Plague Theory of selection came when fragments of CCR5 Δ32 were identified and amplified from the remains of 2900 year old skeletal remains found in burial sites in Central Germany and Southern Italy. This would indicate that the mutation was prevalent already amongst prehistoric Europeans and therefore the plague did not represent a major selective force (Hummel 2005).

The frequency of this allele in modern Caucasian populations is approximately 15-20%, however in certain chronic illnesses, it has been found in altered frequency, suggesting some genetic predisposition through this gene for certain pathologies.
4.5 The role of CCR5 gene polymorphisms in disease

As the CCR5 receptor functions as a co-receptor with CD4 for HIV-1 virus entry into cells, much of the current literature centres around HIV pathogenesis. An early study which described a link between the Δ32 polymorphism and HIV-1 infection susceptibility, genotyped three groups of people. Firstly, healthy donors from North America, Asia and Africa; secondly, HIV positive patients; and thirdly, seronegative individuals with previous high risk exposure. They found the mutation to be common among Caucasians, less common in North American racial groups and not detected in West Africans or Tamil Indians. The frequency of homozygous Δ32 individuals was highest among the highly exposed seronegative individuals (4.5%) and lowest among the HIV-1 positive patients (0%). They concluded that the Δ32 allele may confer protection from HIV-1 infection; a biologically plausible theory as the mutation results in decreased co-receptor expression (Zimmerman 1997). These findings were later confirmed by other authors who found no patients of the Δ32/Δ32 genotype to be infected with HIV-1 (Munerato 2003) and some authors even suggest that the Δ32 molecule itself may scavenge CCR5 and CXCR4 molecules to further prevent viral entry into cells (Aggrawal 2004).

Some HIV seropositive patients have however been found to have the Δ32/Δ32 genotype and it is likely therefore that this only confers partial resistance to the virus. The syncitium inducing form of the virus appears to be more common among this group of patients, which may reflect mutation of the virus in order to utilise the CXCR4 co-receptor for viral entry (Sheppard 2002, Lathley 2001).
The heterozygous state, whilst not protective for infection with HIV-1 (Contopoulos-Ioannidis 2003), does seem to be associated with slower disease progression (Roger 1998). This may in part be due to modulation of host responses to infection via the chemokine system, in addition to possible impairment of viral spread.

The Δ32 polymorphism has been implicated in a number of other chronic conditions. A significant association exists between the Δ32 allele and reduced portal inflammation and fibrosis in patients with hepatitis C infection (Hellier 2003), although it does not appear to alter susceptibility to the illness (Promat 2003). Similarly the allele has been associated with increased risk of progression to nephropathy in association with type I diabetes (Mlynarski 2005), however an early onset of the disease is seen in association with the wild type gene (Kalev 2003), perhaps indicating some protection from the Δ32 allele in this case.

The chronic inflammatory conditions Behçet’s disease and rheumatoid disease have been studied in relation to the Δ32 polymorphism, but neither have shown any association with the allele in terms of susceptibility or progression (Yang 2004, Zuniga 2003).

Therefore, although the CCR5 receptor is known to play a key role in a number of diseases and chronic inflammatory conditions, it seems that a functional polymorphism causing decreased expression of the receptor has little clinical effect, except in HIV-1 susceptibility. This implies that other mechanisms may be upregulated to compensate for the lack of CCR5.
4.6 The role of CCR5 in abdominal aortic aneurysms

Abdominal aortic aneurysms are known to arise as a result of chronic inflammation in the aortic wall, and many cytokine pathways are known to be upregulated as part of this process. It is possible that the CCR5 receptor may play a central role in the regulation of the inflammatory response and that mutation in its gene may therefore alter an individual’s susceptibility to AAA.

Only one study to date has examined the link between the Δ32 allele and aortic aneurysm. It compared 4 groups of patients: 70 patients with AAA; 76 patients with peripheral vascular disease (PVD); 62 patients with carotid stenosis; and 172 healthy controls, who had been referred to the vascular clinic, but had no detectable disease on ultrasound scan. The Δ32 allele frequency among the AAA group was 0.14, compared with 0.06 in the PVD group, 0.05 in the carotid group and 0.06 in the control group. Aneurysm patients with a Δ32 allele were also found to be four times more at risk of aortic rupture than those without a Δ32 allele (Ghilardi 2004). This study does imply a possible link between the Δ32 mutation and both risk and progression of abdominal aortic aneurysm.

4.7 CCR5 inhibitors

The CCR5 receptor has become a potential target for therapeutic modulation in view of the overwhelming evidence particularly for its role in HIV pathogenesis and progression. Because the Δ32 allele is naturally occurring, and homozygotes for the allele do not display any known detrimental effects, attempts have been made to create receptor antagonists that could be used to alter disease progression (Markovic 2006, Ray 2006, Westby 2005, Tamamura 2005).
Chapter Four: C-C Chemokine Receptor 5

current antagonists have reached phase IIb clinical trials. These are Maraviroc (UK-427,857, Pfizer), Aplaviroc (873140, GlaxoSmithKline) and Vicriviroc (SCH-D, Schering-Plough). These had until recently all demonstrated efficacy and tolerability in HIV-infected patients, however the Aploviroc trial was terminated prematurely at the end of 2005 due to severe hepatic toxicity in some patients.

One potential problem with these drugs is that by blocking CCR5, a switch from CCR5- to CXCR4- tropic strains occurs in about 50% of HIV-infected patients, thus bypassing the need for the CCR5 co-receptor and reducing efficacy of the drug.

It is likely that anti-CCR5 therapy will become routine in the foreseeable future in the treatment of patients with HIV. The question remains whether these therapies will provide a novel strategy in the approach to treating other chemokine dependent pathology such as aortic aneurysm.
Chapter Five

HYPOTHESIS AND AIMS OF THESIS
Chapter Five: Hypothesis and Aims of Thesis

5.1 Hypothesis

5.2 Aims of thesis
5.1 Hypothesis

Abdominal aortic aneurysm is an increasingly common and potentially fatal condition. Mortality due to AAA has improved only slightly in recent years despite significant advances in anaesthesia and intensive care. The advent of endovascular aneurysm repair is beginning to impact on the mortality of this condition, however the longevity of this repair is not yet proven and it will not necessarily be the treatment of choice for all patients with aneurysms in the future. Operative treatment is the only option available currently, and this carries a 2-5% risk of death in the elective setting and 50% mortality following rupture.

In order to address the high operative mortality of AAA repair, recent scientific research has concentrated on attempting to understand the underlying mechanisms of aneurysm formation in order to identify pathways which could be manipulated. This has seen new theories emerge and gain credibility, particularly in the area of chronic inflammation and cytokine pathways. There is now indisputable evidence of ongoing upregulation of the inflammatory process in the aneurysm wall when compared with normal aorta. Furthermore, the increased collagen breakdown and alteration in matrix composition which results provides a plausible explanation for the weakened aortic wall and aneurysmal dilatation observed.

It is also important to establish which factors increase individual susceptibility to aneurysm formation, and consequently which factors may in fact be protective, in order to design novel therapies which mimic this protection. Many of the cytokines genes known to be involved in inflammation and aneurysm pathogenesis have polymorphic loci which may confer an inheritable predisposition to aneurysm. However, it is unlikely that any future treatment targeted
at one specific cytokine amongst such complex pathways will succeed in preventing aneurysm formation.

CCR5 has emerged as a central chemokine receptor through which many cytokines act, and this receptor may therefore influence several different pathways. The naturally occurring Δ32 polymorphism causes truncation of the CCR5 receptor and therefore altered receptor function. Possession of this allele has been identified as a protective factor against HIV infection and no detrimental effects have been observed in patients homozygous for the deletion.

Medical treatment of AAA is currently limited to addressing contributing factors such as hypertension and smoking. Although there is some evidence to support this strategy, the majority of patients will eventually need surgery and with this come significant risks. If operative management were not required and the incidence of rupture could be kept negligibly low, the mortality due to AAA would be vastly reduced.

Screening programmes have been introduced in many parts of the UK and there is evidence to suggest that they are cost effective in reducing aneurysm related deaths. If it were possible to treat small, screening detected aneurysms with a medical option which prevented their expansion, it may be possible to avoid surgery altogether. Drugs to block CCR5 are already in development and have reached clinical trials as part of HIV treatment strategies, although it is not known whether these drugs may be successful in other areas, such as AAA.
The hypothesis on which this thesis is based is that:

1. The CCR5 Δ32 polymorphism may confer protection from AAA development and may therefore be observed in higher frequencies among a control population than in patients with aneurysms.

2. The CCR5 Δ32 polymorphism is biologically active and therefore the genotype may be demonstrated to affect receptor expression.

3. There will be a demonstrable difference between patients with aneurysms and a control group with respect to CCR5 expression.

5.2 Aims of Thesis

In order to investigate the role of CCR5 in the development of abdominal aortic aneurysms, a case control study was conducted. This involved the recruitment of 250 patients and 250 healthy control subjects who gave their consent to be included in the study. Clinical information was gathered regarding known risk factors for aneurysm such as age, gender, hypertension, smoking status and family history. A sample of blood was also taken from each patient to be used in laboratory analysis.
Chapter Five: Hypothesis and Aims of Thesis

The primary aim of this thesis was to investigate the CCR5 receptor and its Δ32 gene polymorphism in relation to the pathogenesis of abdominal aortic aneurysm. This was achieved by:

1. Genotyping all patients and control subjects with respect to the CCR5 Δ32 polymorphism and comparing allele frequencies of the polymorphism between groups.
2. Investigating the relationship between genotype and rate of aneurysm expansion.
3. Establishing whether genotype influences cell surface expression of the receptor by flow cytometry.
4. Comparing plasma detection rates of soluble CCR5 molecules between patient and control groups though the use of an enzyme linked assay.
Chapter Six

CLINICAL METHODS
Chapter Six: Clinical Methods

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6.1 Ethical Considerations

The recruitment of patients with aortic aneurysms and healthy control subjects raises ethical issues which had to be addressed and approved prior to commencement of this study. This relates to the mode of recruitment of both patients and control subjects, the nature of the study including samples taken and information gathered, confidential data handling, ongoing storage of samples and the nature of laboratory tests to be carried out. A protocol detailing these factors was submitted to the Leicestershire and Rutland Ethics Committee and ethical approval obtained prior to commencement of the study (see Appendix 1: Ethics Approval Documentation).

As individual participants in the study were not in a position to gain directly from the results, it was important not to cause them any inconvenience. It was therefore decided to approach patients within a routine clinical environment such as an outpatient clinic or ward setting. Control subjects were recruited from aneurysm screening clinics after an ultrasound scan had proven a normal aortic diameter. In this way, no participant was required to attend additional sessions for the purposes of the study. Cases with aneurysms were also recruited from surveillance clinics, but also surgical outpatient clinics and hospital wards.

An interview then took place during which the participant was advised as to the nature of the study, the voluntary nature of their involvement and the procedures which were required for participation. Following this discussion, formal written consent was obtained, ensuring that an opportunity to decline further involvement was offered. At this time, it was clearly explained that the study was investigating the role of genetics in aneurysm development, but that it was unlikely that any meaningful information would be gathered on an individual basis. The participant
therefore understood that they did not stand to gain directly from the study and the results would not be discussed individually.

It was necessary to keep records of the participants’ names in order to prevent replication of recruits, however each was assigned a unique anonymous study identifier code so that the names were not necessary to identify samples or information. A database which de-coded names was stored on a password protected computer and separately from the main database containing participant information.

Following informed written consent, a sample of venous blood was collected from each participant. Where possible this was combined with routine clinical blood tests or collected from the operating theatre whilst the patient was under general anaesthetic (but prior to any blood transfusion) in order to minimise disruption to their routine care.

Samples were stored on ice and transported to the laboratory to be processed. All samples were clearly labelled with the unique study identifier but not with any patient details. Samples were prepared by separating white cell and plasma layers through centrifugation, and then frozen in the Department of Surgery laboratory at the University of Leicester. Boxes were clearly labelled with the investigator's name and the nature of the samples, and were not available for use in any other study or by any other investigator. All samples were stored so as to comply with the regulations of the Human Tissue Act 2004.

DNA was extracted from the white cell layer of each sample and analysed for presence or absence of the CCR5 Δ32 polymorphism (these methods will be described in detail in later
chapters). This information was not reported back to the patient. Further tests were then performed to establish the relationship between the genotype of the patient and the function of the CCR5 receptor in the blood. Again, this information was not reported back individually to the participant involved. DNA was not tested for any other gene or inheritable disease, and the storage of this DNA has only been approved for the investigation of cytokine genes in relation to abdominal aortic aneurysm.

There is significant justification for not revealing individual results. Firstly, it was highly unlikely that a single gene was going to be identified which was single-handedly responsible for the development of aortic aneurysms. Secondly, even if this were the case, the patient involved was already known to have an aneurysm and therefore knowledge of the genotype would not affect their future plans or management. First degree relatives of patients with aneurysms are already advised to attend ultrasound screening, and this protocol would be unchanged by the identification of a specific genetic link. Thirdly, if there was a single gene responsible and a control subject was found to have this gene but not yet have developed an aneurysm, there would be no way of altering this process.

The knowledge gained from this study will be published in the public arena and so will be freely available to all study participants should they wish to read it. All results will remain completely anonymous throughout publication.
6.2 Collection of Data

Each participant in the study was approached in the context of a routine clinical setting through the outpatient department, ward or screening clinic and asked whether they would be prepared to speak to a member of the research team. They were then interviewed privately in a separate room where the details of the study were explained in full, including what their participation would involve. An opportunity was then given to refuse further involvement in the study, however if permission was given to continue, a written consent form was discussed and completed (see Appendix 1: Ethics Approval Documentation).

Questions were then asked regarding the participant’s demographic details (including name, age, gender and ethnicity) in addition to details regarding their medical history. This data was important as factors such as age, male sex, white race, hypertension, smoking status and family history are significant risk factors for the development of abdominal aortic aneurysm (see Chapter 2). In order to separate any true effect that the genotype of each participant may have on their aneurysm status it is important to control for these other risk factors. Data were recorded on a standard proforma which is given in figure 6.1.
### Chapter Six: Clinical Methods

#### Study Ref number

Date seen ..../..../.....

#### Name

U.................

#### Age

Ethnic origin.................

#### Sex

M □  F □

#### Aneurysm size

Date of diagnosis ..../..../.....

#### Expansion

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#### Date of diagnosis

Date seen ..../..../.....

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#### Family History

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#### Meds

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#### Smoking

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Where BP=Hypertension, MI=Myocardial Infarct, CABG=Coronary Artery Bypass Graft, A2=Angioplasty, PVD=Peripheral Vascular Disease, CVA=Cerebrovascular Accident, Carotid=Carotid Endarterectomy, DM=Diabetes Mellitus, COAD=Chronic Obstructive Airways Disease, Ca=Malignancy, Chol=Hypercholesterolaemia

**Figure 6.1: Proforma for data collection**
Definition of the factors recorded relied on participant reporting. In order to reduce the potential for error which this introduced, each medical condition was marked positive only if there was a definite previous episode or if the individual was receiving regular medication for that condition. All patients were interviewed by the same investigator. The co-morbidities included in the proforma represented factors which are known to increase the risk of aneurysm formation in order to control for these factors in the analysis (previous history of MI, hypertension, cerebrovascular or peripheral vascular disease, hyperlipidaemia), but also factors which may cause upregulation of cytokine pathways in the absence of AAA and therefore may confound results (COAD, current or recent malignancy or surgery). Smoking status was recorded as stated by the participant even though self reporting is known to be inaccurate (Coultas 1988, Colletti 1982). As each participant was interviewed by the same investigator and the question phrased in the same way, any bias introduced should apply to the cohort as a whole and therefore not influence the analysis significantly.

All data were anonymised and entered onto a password protected computer database.

6.3 Collection and Storage of Samples
A ten millilitre (ml) sample of venous blood was obtained from each study participant, collected in an EDTA (ethylenediaminetetraacetic acid) containing vial. EDTA was used as an anticoagulant so that cellular and plasma components could later be separated. It is a chelating agent which binds positively charged ions such as calcium and potassium, but does not alter genetic or proteinaceous material in blood. Samples were labelled with the participant’s study identifier (in non-water soluble ink) and stored on ice for transportation to the laboratory.
Each sample was then centrifuged using a Jouan CR422 machine at 2000 revolutions per minute (RPM) (Revolutionary Centrifugal Force of 990g) for 10 minutes. Whole blood consists of cellular and non-cellular components. Exposing whole blood to centrifugation causes the components to separate resulting in three clear layers: the red cell layer at the bottom of the tube; the white cell layer or ‘buffy coat’ forming a thin film on top of the red cells; and the plasma layer which is seen above the buffy coat (see figure 6.2).

Figure 6.2: Separated whole blood forming red cell, buffy coat and plasma layers. The red cell layer can be seen at the base of the test tube with a thin film of ‘buffy coat’ on top which separates it from the clear plasma layer at the top of the tube.

Following centrifugation, the plasma layer and buffy coat were extracted separately for storage and the red cells discarded (as they have no nucleus and therefore contain no genetic material). This was done by carefully pipetting the plasma layer into several 1ml cryovials, ensuring that no white cells were allowed to contaminate the plasma. This is important as there is a theoretical
risk that white cell precipitates may be biologically active ex vivo and therefore may alter the true cytokine levels in the plasma if contamination occurred. In order to ensure no contamination occurred, a very small amount of plasma was left behind with the buffy coat.

Next, the buffy coat was extracted, again using a sterile pipette. As the buffy coat layer is very thin, a small amount of the plasma and red cell layers were also included in order to ensure maximal white cell harvest. This was divided into two 1ml cryovials ready for storage and all samples were clearly labelled with the study identifier code.

Both plasma and buffy coat samples were then ‘snap’ frozen in liquid nitrogen. This involved placing the cryovials into a flask of liquid nitrogen (-195°C) to ensure instantaneous freezing. This is important as a slower freezing process may lead to crystallization of the aqueous components of white cells which may lead to cellular damage owing to the expansion and shrinkage of water crystals during the freezing and thawing processes respectively. Whilst this is unlikely to affect the process of DNA extraction, it may limit the use of buffy coat for other work in the future.

Following freezing, samples were transferred to a storage box and placed in the freezer at -80°C ready for analysis at a later date. It is possible to store blood samples at a range of different temperatures. For the purposes of this study, it was necessary to store buffy coat at -80°C in order to prevent DNA degradation by biological mechanisms prior to extraction. Cytokine mechanisms in plasma may also be active at higher temperatures and so plasma was also stored at -80°C. Once frozen, samples can be stored in this manner for prolonged periods of time without damage (Simon 1977).
Throughout the storage process all samples were handled with gloves and all work areas treated with 70% ethanol prior to sample handling. The work took place in a Gelaire BSB6 Class II flow cabinet. These measures were taken to prevent contamination of samples.
Chapter Seven

PATIENTS AND CONTROL SUBJECTS
Chapter Seven: Patients and Control Subjects

7.1 Introduction

7.2 Patient demographics

7.3 Co-morbidity

7.4 Current medication

7.5 Associations with AAA

7.6 Summary
7.1 Introduction

There were 285 patients with aneurysms and 273 control subjects recruited into the study between 16/11/04 and 28/04/06. Although no formal attempts were made to ‘match’ the patient and control groups, the nature of the recruitment process for control subjects enabled a representative cross section of the community to be studied. The study groups were compared in terms of their demographics and co-morbidities so that any potential differences between the groups could be taken into consideration during later analysis. This chapter provides details of the patients and control subjects included in the study.

7.2 Patient demographics

Following recruitment into the study, each patient was interviewed with respect to their medical history, current medications and smoking status. This data was recorded anonymously as described in Chapter 6. Details of the demographic data for each study group are given in Table 7.1. The two groups were compared using Chi squared analysis (except where stated otherwise) to demonstrate any significant differences between the populations recruited.

Table 7.1: Demographic data

<table>
<thead>
<tr>
<th>Category</th>
<th>Aneurysm Group n=285</th>
<th>Control Group n=273</th>
<th>P Value (Fishers Exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median (range)</td>
<td>72 (50-89)</td>
<td>66 (65-79)</td>
<td>&lt;0.0001 (Mann Whitney U Test)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>263 (92)</td>
<td>273 (100)</td>
<td>0.007</td>
</tr>
<tr>
<td>Ethnicity (% white)</td>
<td>272 (95)</td>
<td>267 (98)</td>
<td>0.44</td>
</tr>
<tr>
<td>Family history of AAA (%)</td>
<td>37 (13)</td>
<td>16 (6)</td>
<td>0.17</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>77 (27)</td>
<td>37 (14)</td>
<td>0.05</td>
</tr>
<tr>
<td>Ex-smoker (%)</td>
<td>172 (60)</td>
<td>156 (50)</td>
<td>0.46</td>
</tr>
</tbody>
</table>

P=≤0.05 taken to represent a significant difference between the two groups.
Patient demographics were largely similar, however differences were observed between the groups in terms of age, gender and current smoking status. This reflects known risk factors for the development of AAA (see Chapter 2), and the effect of any potential bias will be discussed later in this chapter and also in detail in Chapter Nine.

7.3 Co-morbidity

Any significant co-morbidities were recorded firstly to allow known risk factors for aneurysm development to be controlled for in any later analysis, but also to examine alternative explanations for differences observed.

The co-morbidities for aneurysm and control subject groups are given in Table 7.2. This demonstrates significant differences between the groups in terms of the incidence of previous MI, coronary angioplasty or coronary artery bypass graft, the presence of hypertension and hypercholesterolaemia, all of which were found to be more prevalent amongst the aneurysm group than the control group. This is in keeping with known risk factors for the development of abdominal aortic aneurysm as discussed in Chapter 2. Previous history of peripheral vascular disease did appear to be more common amongst the aneurysm group but this trend failed to reach statistical significance. Interestingly, there was no difference in prevalence of chronic obstructive airways disease, which could be expected to reflect smoking habits, between the two groups. No differences were observed between the groups in terms of diabetes, malignancy or cerebrovascular disease.
Chapter Seven: Patients and Control Subjects

<table>
<thead>
<tr>
<th>Category</th>
<th>Aneurysm Group</th>
<th>Control Group</th>
<th>P Value (Fishers Exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischaemic Heart Disease (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angina</td>
<td>34 (12)</td>
<td>19 (7)</td>
<td>0.33</td>
</tr>
<tr>
<td>MI</td>
<td>79 (28)</td>
<td>18 (7)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Coronary Artery Bypass Graft</td>
<td>31 (11)</td>
<td>8 (3)</td>
<td>0.05</td>
</tr>
<tr>
<td>Angioplasty</td>
<td>13 (5)</td>
<td>4 (1)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>140 (49)</td>
<td>87 (32)</td>
<td>0.02*</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin dependent</td>
<td>9 (3)</td>
<td>6 (2)</td>
<td>1</td>
</tr>
<tr>
<td>Non-insulin dependent</td>
<td>15 (5)</td>
<td>18 (7)</td>
<td>0.77</td>
</tr>
<tr>
<td>Diet controlled</td>
<td>10 (4)</td>
<td>4 (1)</td>
<td>0.37</td>
</tr>
<tr>
<td>Hypercholesterolaemia (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>93 (33)</td>
<td>47 (17)</td>
<td>0.01*</td>
</tr>
<tr>
<td>Malignancy (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>23 (8)</td>
<td>5 (2)</td>
<td>0.11</td>
</tr>
<tr>
<td>During previous 12 months</td>
<td>4 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral Vascular Disease (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (7)</td>
<td>3 (1)</td>
<td>0.07</td>
</tr>
<tr>
<td>Cerebrovascular Disease (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21 (7)</td>
<td>7 (3)</td>
<td>0.33</td>
</tr>
<tr>
<td>Chronic Obstructive Airways Disease (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 (7)</td>
<td>10 (4)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

P < 0.05 taken to represent a significant difference between the two groups (*).

Table 7.2: Patient and control subject co-morbidity.

This table demonstrates increased prevalence of ischaemic heart disease, hypercholesterolaemia and hypertension amongst the aneurysm group compared with the control group.

7.4 Current medications

In keeping with the number of co-morbidities observed, many patients were taking medications on a regular basis. These were recorded in order to ensure any effect they may have exerted on aneurysm development or expansion (eg. β-blockade which has been thought to play a role in limiting aneurysm expansion (Gadowski 1994)) could be considered. Medications such as aspirin or other anti-inflammatory preparations may also interfere with the inflammatory pathways in
which the CCR5 receptor is involved and so it was important to collect this data to aid analysis of laboratory data regarding the CCR5 protein. The long term medications of the study groups are given in Table 7.3.

Table 7.3: Patient and control group medication

<table>
<thead>
<tr>
<th>Class of Drug</th>
<th>Aneurysm Group</th>
<th>Control Group</th>
<th>P Value (Fishers Exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
<td>Number</td>
</tr>
<tr>
<td>Aspirin</td>
<td>131</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>β-blocker</td>
<td>99</td>
<td>35</td>
<td>48</td>
</tr>
<tr>
<td>Statin</td>
<td>132</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>Nitrates</td>
<td>30</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>25</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Digoxin</td>
<td>10</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Warfarin</td>
<td>26</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Diuretic</td>
<td>94</td>
<td>33</td>
<td>34</td>
</tr>
<tr>
<td>ACE Inhibitor</td>
<td>94</td>
<td>33</td>
<td>47</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>25</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>

*P =<0.05 taken to represent a significant difference between the two groups (*).
Where ACE = Angiotensin Converting Enzyme

This table demonstrates significant differences between the groups in terms of current drug therapy with aspirin, beta blockers, statins, clopidogrel, diuretics and ACE inhibitors. This is likely to reflect the increased incidence of cardiovascular co-morbidity in terms of ischaemic heart disease, hypertension and hypercholesterolaemia seen in the aneurysm group.
7.5 Associations with AAA

Differences in age, gender, co-morbidity, medications, smoking status and family history between the case and control groups were examined using univariate analysis with Chi squared tests. The results are given in Table 7.4. Several factors were found to be significantly associated with AAA, namely history of hypertension, myocardial infarction, angina, stroke and hypercholesterolaemia. Family history of AAA, current smoking, and drug treatment with antiplatelet therapy, statin therapy, beta blockers, nitrates, warfarin, diuretics or ACE inhibitors were also found to be significant. Age was analysed separately using a Mann Whitney U test and also found to be highly significant (P=<0.0001).

<table>
<thead>
<tr>
<th></th>
<th>Degree of Freedom</th>
<th>Chi squared value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension</td>
<td>1</td>
<td>17.51</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MI</td>
<td>1</td>
<td>46.07</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Angina</td>
<td>1</td>
<td>4.52</td>
<td>0.033*</td>
</tr>
<tr>
<td>CVA</td>
<td>1</td>
<td>7.27</td>
<td>0.007*</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1</td>
<td>0.85</td>
<td>0.357</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1</td>
<td>19.85</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Family history</td>
<td>1</td>
<td>8.96</td>
<td>0.003*</td>
</tr>
<tr>
<td>Smoker</td>
<td>1</td>
<td>61.7</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>1</td>
<td>61.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>1</td>
<td>23.68</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Statin</td>
<td>1</td>
<td>48.66</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1</td>
<td>10.63</td>
<td>0.001*</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>1</td>
<td>11.21</td>
<td>0.004*</td>
</tr>
<tr>
<td>Digoxin</td>
<td>1</td>
<td>3.81</td>
<td>0.051</td>
</tr>
<tr>
<td>Warfarin</td>
<td>1</td>
<td>7.49</td>
<td>0.006*</td>
</tr>
<tr>
<td>Diuretic</td>
<td>1</td>
<td>36.19</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Ace inhibitor</td>
<td>1</td>
<td>20.42</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Where MI=Myocardial infarction, CVA=cerebrovascular accident, cholesterol=hypercholesterolaemia and ACE=angiotensin converting enzyme.
P=<0.05 taken to represent a significant difference between the two groups (*).

Table 7.4: Univariate analysis of demographic data and co-morbidities
Gender was not found included in this analysis as there were no female subjects amongst the control population. This would have inevitably led to a selection bias and statistical analysis would have been meaningless.

Factors which were found to be significant on univariate analysis were subjected to multivariate analysis using binary logistic regression and the results are given in Table 7.5.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>Degree of Freedom</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td>6.57</td>
<td>2.93-14.72</td>
<td>1</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>3.51</td>
<td>1.78-6.96</td>
<td>1</td>
<td>0.0003*</td>
</tr>
<tr>
<td>MI</td>
<td>3.73</td>
<td>1.63-8.56</td>
<td>1</td>
<td>0.0009*</td>
</tr>
<tr>
<td>Statin</td>
<td>2.33</td>
<td>1.24-4.39</td>
<td>1</td>
<td>0.0086*</td>
</tr>
<tr>
<td>Diuretic</td>
<td>2.39</td>
<td>1.24-4.64</td>
<td>1</td>
<td>0.0093*</td>
</tr>
<tr>
<td>Family history</td>
<td>2.88</td>
<td>1.09-7.54</td>
<td>1</td>
<td>0.0314*</td>
</tr>
<tr>
<td>CVA</td>
<td>3.88</td>
<td>1.02-14.78</td>
<td>1</td>
<td>0.0472*</td>
</tr>
</tbody>
</table>

B = 27.74, SE 3.35, 1 degree of freedom, p=<0.001

Where MI=myocardial infarction and CVA=cerebrovascular accident.
P=≤0.05 taken to represent a significant difference between the two groups (*).

Table 7.5: Factors associated with aortic aneurysm
Multivariate analysis confirms the significant associations of smoking, aspirin therapy and previous MI with the presence of AAA.

This suggests a true association between aneurysms and smoking, previous history of myocardial infarction (MI) and stroke (CVA), family history of aneurysm and drug therapy with aspirin, statins and diuretics. The p value gives a measure of how significant each factor is,
demonstrating smoking to be the most significant risk factor for AAA in the study group (P=<0.0001). Interestingly, hypertension does not remain a significant factor on multivariate testing of the study population, in contrast to previously published work (Chapter 2). The reason for this is not clear, but may be related to under-reporting of hypertension in patients on drug therapy for other cardiovascular co-morbidity. Diuretic therapy does appear to be significantly associated with AAA and this may reflect a subgroup of patients on treatment for hypertension. Although certain drug therapies have been found to be significantly associated with AAA, it is unlikely that this is via a direct effect on aneurysm formation. Therefore, amongst this study group, smoking (P=<0.0001), family history of AAA (P=0.0314), and previous history of MI (P=0.0009) or CVA (P=0.0472) are significantly associated with the development of abdominal aortic aneurysm.

7.6 Summary
This chapter details the demographic information for the group of aneurysm patients and control subjects in the study. There were significant differences observed between the groups in terms of age, gender and smoking status, history of hypertension, ischaemic heart disease and hypercholesterolaemia, and medication with aspirin, beta-blockers, statins, clopidogrel, diuretics and ACE inhibitors. This is in keeping with recognised risk factors for AAA.

Univariate analysis confirmed significant associations between AAA and age, history of hypertension, hypercholesterolaemia, myocardial infarction and stroke, family history of AAA, smoking, and drug therapy with antiplatelet agents (aspirin and clopidogrel), antihypertensives (beta blockers, diuretics and ACE inhibitors), nitrates, statins and warfarin.
When subjected to multivariate testing, smoking, previous history of MI and stroke, family history of AAA, and drug therapy with aspirin, statins and diuretics remained significant. The most significant risk factor was smoking with an odds ratio of 6.57, indicating that smoking increases the chance of having an aneurysm by more than six times.

Age in particular has been a difficult factor to control for as the control group were recruited from screening appointments at the age of 65 years whereas the aneurysm group were often much older than this. Although this difference is significant, it can be justified by the evidence to support a single screening ultrasound scan at the age of 65 as adequate to exclude aneurysm for the remainder of that individual’s life (Crow 2001).

In summary, this chapter highlights some clear differences between the control group and the aneurysm group in terms of known risk factors for the development of AAA. This confirms that this study population are representative of the larger aneurysm population in general and highlights the association between AAA and increasing age, hypertension, previous MI and CVA, family history and smoking.
Chapter Eight

LABORATORY TECHNIQUES: DNA extraction and quantification
Chapter Eight: DNA Extraction

8.1 Introduction

8.2 Methods

8.3 Results

8.4 Discussion
8.1 Introduction

Genetic analysis using whole blood requires extraction of the genetic material, i.e. DNA (deoxyribose nucleic acid) from the nuclei of white blood cells. This technique was first described in 1956 by Palmade (Palmade 1956) and since then several different methods have been developed, each with their own advantages. Early techniques involved the isolation of DNA by digestion of cells with Proteinase K, a protein degradation enzyme (Gross-Bellard 1973), and then the addition of phenol which yielded DNA fragments of 100-150 kilobases (Blin 1976). Although this successfully extracted genetic material, protein contamination was problematic and a modification of this technique using dialysis to dissociate the DNA-protein complexes was developed. This unfortunately produced a lower DNA yield, and Bowtell in 1987 used guanidine hydrochloric acid to disrupt cells, which resulted in the rapid extraction of good quality DNA of around 80 kilobases (Bowtell 1987).

Modern methods of DNA extraction from blood avoid the use of enzymatic cell degradation, relying on hypertonic solutions for cell lysis and salt precipitation of DNA. This method was used for the extraction of DNA from blood samples in this project and the method is described in detail below.
8.2 Methods

8.2.1 Protocol

A commercial kit (Gentra Systems Puregene DNA Extraction Kit, Gentra Systems Inc) was used for the extraction of DNA from buffy coat according to the protocol given below.

**Protocol** (See also section 8.2.2: Reagents and Solutions, and Figure 8.1)

1. 1ml of frozen buffy coat was placed in a water bath at 37°C and incubated for 15 minutes until thawed.

2. This was added to 3 parts (3ml) of Red Cell Lysis Solution containing ammonium chloride, EDTA and sodium bicarbonate, and incubated for 10 minutes at room temperature.

3. Following incubation the solution was centrifuged at 2000rpm for 10 minutes, after which the supernatant was discarded leaving the white cell pellet visible in the base of the tube along with a small amount of residual fluid.

4. The tube was then vortexed for several minutes to re-suspend the white cells in the remaining fluid. This step greatly aided white cell lysis.

5. After re-suspension, 3ml of Cell Lysis Solution containing Tris (hydroxymethyl) aminomethane, EDTA and sodium dodecyl sulphate were added to the solution and pipetted up and down in order to lyse cells.

6. The tube was then placed in a water bath and incubated for 1 hour at 37°C and overnight at room temperature as this was found to aid cell lysis and therefore improve DNA yield.

7. Following cell lysis, 1ml of protein precipitation solution containing ammonium acetate was added to the solution and vortexed for 20 seconds in order to mix the solutions. This
was then centrifuged at 2000rpm for 10 minutes allowing the protein precipitate to gravitate to the base of the tube, leaving a clear solution containing DNA.

8. This supernatant was then transferred to a clean tube containing 3ml of 100% isopropanol and the solutions were mixed by gently inverting the tube to allow DNA precipitation.

9. Following a further centrifugation at 2000rpm for 3 minutes, the supernatant was discarded leaving a DNA pellet at the base of the tube.

10. The DNA pellet was then washed with 3 ml of 70% Ethanol, centrifuged for 1 minute at 2000rpm, and inverted on clean dry paper to air dry for a few minutes.

11. The DNA pellet was then re-suspended in 250µl of DNA Hydration Solution containing tris (hydroxymethyl) aminomethane and EDTA, incubated for 2 hours at 65°C in a water bath, and then left to incubate overnight at room temperature before transfer to a suitable cryovial ready for storage.
Add 1ml buffy coat to 3ml red cell lysis solution and incubate for 10 mins

Centrifuge for 10mins at 2000G

Vortex to re-suspend cells and add cell lysis solution

Pipette and incubate for 1 hour at 37°C

Add 1ml protein precipitation solution and vortex to mix

Centrifuge for 10 mins at 2000rpm

Transfer supernatant to clean tube containing 3ml isopropanol and invert to mix

Discard supernatant leaving DNA pellet at base

Wash with 70% ethanol, air dry tube and add DNA rehydration solution

Incubate at 65°C for 2 hours and overnight at room temperature

Quantify and transfer for storage

Figure 8.1: DNA extraction protocol
8.2.2 Reagents and Solutions

**Red Cell Lysis Solution** (containing ammonium chloride, EDTA and sodium bicarbonate)

The main active ingredient in the red cell lysis solution is ammonium chloride. Red cell membranes are permeable to ammonium chloride (NH\textsubscript{4}Cl) allowing entry of this reagent into the cell. This results in cell lysis due to intracellular hypertonicity and cell rupture (Phillips 1983).

EDTA is a polyprotic acid containing 4 carboxyl groups and 2 amine groups with one lone pair of electrons. It is able to chelate metal ions in 1:1 metal to EDTA complexes. Binding of EDTA to magnesium and chloride in this way destabilizes the cell membrane and aids cell lysis. It may also contribute to a buffering effect through the binding of free magnesium and chloride ions. Sodium bicarbonate functions primarily as a buffer for the cell lysis reaction but may also aid cell membrane destabilisation through competition with magnesium and calcium ions.

**Cell Lysis Solution** (containing Tris (hydroxymethyl) aminomethane, EDTA and sodium dodecyl sulphate (SDS))

Sodium dodecyl sulphate (SDS) is a sodium salt which is capable of disrupting non-covalent protein bonds. This causes denaturation of the protein and disruption of the cell membrane. SDS anions bind to the peptide at a ratio of 1 SDS anion per 2 amino acid residues resulting in an increased negative charge on the protein which is proportional to its molecular mass. This new negative charge is significantly greater than the original charge of that protein and electrostatic repulsion forces cause the protein to unfold, thus further denaturing the cell membrane.

The EDTA in this solution functions as before to bind membrane cations and destabilize the membrane (see ‘Red Cell Lysis Solution’ above).
Chapter Eight: DNA Extraction

The cell lysis solution includes Tris (hydroxymethyl) aminomethane (THAM) as a buffer in place of sodium bicarbonate. This is because THAM is able to diffuse into the intracellular space, therefore buffering reactions within as well as outside the cell, and also contributing to cell lysis.

**Protein Precipitation Solution (containing ammonium acetate)**

Ammonium acetate (CH₃COONH₄) is produced by the reaction of ammonia with acetic acid and provides a good buffer for protein and nucleic acid purifications. The stability of protein in solution is dependent on electrostatic interactions (between amino acid side chains and salts), hydrogen bridges (between amino acid side chains and water) and hydrophobic interactions of the protein itself. Alteration of any one of a number of factors such as temperature, pH and salt concentration, or addition of agents such as lyophilic substances (eg alcohol), cross linking agents (eg protamine) or water extracting agents (eg polyethylene glycol), may cause the protein to precipitate out of solution. The addition of the ammonium acetate salt therefore disrupts the stability of the soluble protein and results in precipitation.

**Isopropanol**

Isopropanol (CH₃CHOHCH₃) is a secondary alcohol where the alcohol carbon is attached to 2 other carbon atoms. It is an isomer of propanol. When isopropanol is added to water it forms an azeotrope (a mixture of 2 or more molecules). It is used to react with water in this way to aid DNA precipitation. DNA molecules become condensed in the presence of alcohols (in addition to other substances such as magnesium, polyamines and basic polypeptides) and therefore form a visible precipitate.
Chapter Eight: DNA Extraction

Ethanol

The final ethanol wash in this method is used to remove any contaminating protein salts. A 70% ethanol solution is used as this concentration retains the nucleic acid salt as a precipitate whilst readily dissolving contaminating salts.

8.2.3 Optimisation Experiments

There were initial problems with this protocol resulting in a low yield of DNA. In order to address this, several experiments were conducted to assess the different stages involved and what effect they had on DNA yield.

Experiment 1: Variation of buffy coat concentration

Firstly, the amount of buffy coat added to the reaction was varied. Early experiments used only 250μl of buffy coat added to 750μl of red cell lysis solution in accordance with the manufacturers’ recommendations. Although the DNA produced from this extraction was sufficient to use in later experiments, the yield was very low with values of between 10-20 nanograms per microlitre (ng/μl) of DNA. This limited both the supply of DNA and its possible uses. A series of four experiments were performed to assess the effect of adding a larger amount of buffy coat and the results are presented in Table 8.2. Using 1ml of buffy coat was found to produce a significant improvement in the DNA yield and so this protocol was adopted and the early samples re-extracted (Figure 8.3). The amount of DNA extracted was out of proportion to the increased amount of buffy coat and this may reflect the technical difficulties in dealing with a very small DNA pellet at lower buffy coat volumes.
Table 8.2: DNA Yield

This table demonstrates increasing DNA yield with larger volumes of buffy coat used.

<table>
<thead>
<tr>
<th>Amount of buffy coat used (μL)</th>
<th>DNA concentration (ng/μL)</th>
<th>Mean DNA concentration (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>250</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>250</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>95</td>
<td>84</td>
</tr>
<tr>
<td>500</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>455</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>110</td>
<td>205</td>
</tr>
<tr>
<td>750</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>330</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>335</td>
<td>266</td>
</tr>
<tr>
<td>1000</td>
<td>315</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8.3: DNA Yield.
This boxplot demonstrates increasing DNA yield with increasing buffy coat volume. The error bars represent the range of DNA yield in each experiment, and the boxes represent the mean DNA concentration.

DNA Yield (ng/microlitre)

Experiment 2: Variation of cell lysis step
Secondly, again to address the issue of DNA yield, the cell lysis step was examined. The manufacturers' instructions recommended a brief incubation period of 15 minutes in cell lysis solution, only if cell clumps were visible after pipetting. In order to assess the effect of cell lysis on DNA yield, three experiments were performed.

1. All samples were incubated at 37°C for 15 minutes in cell lysis solution.
2. Samples were incubated at 37°C for one hour in cell lysis solution.
3. Samples were incubated at 37°C for one hour in cell lysis solution as in experiment 2, and then incubated at room temperature overnight.

All experiments were performed using 1ml of buffy coat. The results from this series of experiments are given in Table 8.4. As shown in Table 8.4 and Figure 8.5, a longer cell lysis
period did improve the DNA yield, and a period of 1 hour at 37°C and then overnight at room temperature was therefore adopted.

Table 8.4: Cell Lysis Incubations
This table demonstrates increasing DNA yield with longer cell lysis incubation time.

<table>
<thead>
<tr>
<th>Experiment and incubation period</th>
<th>DNA concentration (ng/μL)</th>
<th>Mean DNA Yield (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Incubation period 15 minutes at 37°C</td>
<td>590 450 265 640 600</td>
<td>509</td>
</tr>
<tr>
<td>2. Incubation period 1 hour at 37°C</td>
<td>220 525 470 780 635</td>
<td>520</td>
</tr>
<tr>
<td>3. Incubation period 1 hour at 37°C followed by overnight at room temperature.</td>
<td>530 585 615 775 765</td>
<td>654</td>
</tr>
</tbody>
</table>
Where Experiment 1 = incubation for 15 minutes at 37°C; experiment 2 = incubation for 1 hour at 37°C; and experiment 3 = incubation for 1 hour at 37°C and then overnight at room temperature.

**Figure 8.5: Cell Lysis Incubations.**

This boxplot demonstrates greater DNA yield with longer cell lysis incubation. The boxes represent the mean DNA yield and the error bars represent the range of values in each experiment.
Variation of DNA re-suspension

Once the issue of low DNA yield had been addressed, a further problem was revealed as the larger DNA pellets were difficult to re-suspend in the rehydration solution. This resulted in an excessively viscous suspension with incompletely dissolved DNA. This viscosity was difficult to quantify, however a number of measures were taken to overcome this problem. Initially, it was thought that the solution may have become rapidly saturated with DNA preventing further dissolution. However, when this theory was tested by the addition of increased volumes of DNA Rehydration Solution, it was found to have no effect and it was therefore concluded that the difficulty with re-suspension was caused by another factor.

The length of time that samples were left to air dry following washing with ethanol was then varied. A long drying time was found to be detrimental to re-suspension and the initial recommendation of 15 minutes was therefore reduced to 1-2 minutes, just long enough for the alcohol to evaporate before adding the rehydration solution.

Finally, the length of incubation at 65°C was varied and found to aid re-suspension. The protocol initially included a 1 hour incubation period, however, where samples remained incompletely suspended following this, a further hour’s incubation was used. This resulted in much more uniform re-suspension of DNA with no detrimental effects on quantity or quality and so the incubation period was increased to 2 hours for all samples.

The final protocol detailed above has been found to produce a good DNA yield of 100-400ng/μl which is sufficient for all genotyping techniques. It has also been found to be of good quality with minimal protein contamination.
8.2.4 DNA Quantification

Following DNA extraction, it was important to quantify the amount of DNA produced in order to ensure a fixed concentration of DNA could be used in genotyping experiments. Different methods of DNA quantification exist. It is possible to run the DNA on an agarose gel with a DNA ladder of known sizes for comparison. This allows estimation of the total amount of DNA produced and has the advantage of demonstrating the quality of DNA by the nature of the bands and degree of smearing (good quality DNA produces discrete bands and minimal smearing).

Another common method uses a spectrophotometer to measure the absorbance of DNA at different light wavelengths. This perhaps gives a more accurate reading and was the chosen method of quantification for this study. It can also be used to give an indication of the purity of DNA, but not the quality in terms of degree of fragmentation.

It is known that DNA with a concentration of 50 ng/μl has an optical density (the absorbance of an optical element for a given wavelength (λ) per unit distance) of 1 at a wavelength of 260 nanometres (nm). DNA can therefore be quantified by measuring the optical density at 260nm and comparing this with the known standard of 50ng/μl (DNA co-efficient). A measurement taken at 280nm also allows for calculation of the level of protein contamination.

The DNA concentration was calculated using the following equation:

\[(A_{260}) \times (\text{DNA coefficient}) \times \text{dilution factor} = [\text{DNA}] \text{ in ng/μl}\]

where \(A_{260} = \) absorbance at 260; DNA coefficient at 260 = 50; and the dilution factor = the dilution of the DNA sample in water prior to quantification, which in this case was 100.
Chapter Eight: DNA Extraction

The purity of DNA was then calculated by dividing the absorbance at 260nm by the absorbance at 280nm (A260/A280). Pure DNA gives a ratio of 1.7-2, however readings lower than this indicate protein contamination and higher readings suggest RNA contamination.

After quantification, samples were diluted to a final concentration of 100ng/μL for use in genotyping experiments.

8.3 Results

The final concentrations of DNA extracted prior to dilution are given in Appendix 3 and summary data are given in Table 8.6 and Figures 8.7a&b below. This demonstrates extraction of good quality DNA in sufficient quantity to perform repeated analysis using the polymerase chain reaction and other techniques. There was greater variation observed in the control group in terms of both the yield and purity of DNA obtained. This may be related to the longer transit and storage time involved in the collection of control samples compared to aneurysm samples.
Table 8.6: Analysis of DNA quantification.
This table summarises the DNA yield which was of adequate quantity and good quality among both the groups.

<table>
<thead>
<tr>
<th>Statistics using Mann Whitney U Tests</th>
<th>Aneurysms (n = 284)</th>
<th>Controls (n = 263)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/µl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>329</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>258</td>
<td>213</td>
<td></td>
</tr>
<tr>
<td>Standard Error</td>
<td>15</td>
<td>13</td>
<td>P=&lt;0.0001</td>
</tr>
<tr>
<td>Median (range)</td>
<td>272.5 (0 - 1190)</td>
<td>190 (-50 - 1015)</td>
<td></td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>299-359</td>
<td>212-263</td>
<td></td>
</tr>
<tr>
<td>A260/280 Ratio (Purity)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.75</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.38</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.02</td>
<td>0.07</td>
<td>P=0.0009</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1.78 (0-4)</td>
<td>1.71 (-0.33-18)</td>
<td></td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>1.71-1.8</td>
<td>1.56-1.84</td>
<td></td>
</tr>
</tbody>
</table>

NB. Mann Whitney test used for this data as normality testing confirmed it to be of non-parametric distribution.
Chapter Eight: DNA Extraction

Figure 8.7a: Mean DNA Concentration

This figure demonstrates the higher mean DNA concentration amongst the aneurysm group than the control group, which is statistically significant ($P < 0.0001$). The boxes represent the mean DNA concentration and the error bars represent the standard error of the mean.
Figure 8.7b: Mean DNA purity (A260/280)

This figure demonstrates the comparable mean DNA purity amongst both groups however highlights the greater range of values in the control group. The boxes represent the mean DNA purity and the error bars represent the standard error of the mean.
In each group there were some samples which failed to yield any DNA or only produced a very low concentration. In some cases, this was due to an error in the extraction process. Samples BC1-10 suffered damage during the thawing process causing coagulation of the buffy coat sample which impaired the DNA extraction process. In other samples however, there was no obvious cause for the low DNA yield observed and this will be discussed in more detail in the next section (8.4 Discussion).

Although there were a few spuriously high or low A260/280 values, the DNA extracted from aneurysm samples was good quality overall with a mean value of 1.81 which indicates a good level of purity (see Figure 8.8a&b). The ratio was somewhat lower for the control samples and it may be that the storage and transportation involved in processing the control samples reduced the quality of DNA extracted.

8.4 Discussion

The salt precipitation method of DNA extraction is a well established technique and has the advantages of producing good concentrations of DNA with little contamination by either protein or RNA. A commercial kit was used for extracting DNA for the purposes of this project as it was felt to be convenient and cost effective. The method recommended by the manufacturer was however adapted and optimised for use in our centre and a new protocol established which produced a greater DNA yield than using the standard manufacturers’ instructions.

Using this refined protocol produced an acceptable DNA yield (mean 283ng/μl) which allowed samples to be diluted to a standard concentration of 100ng/μl. This was important as it allowed
accurate and consistent amounts of DNA to be added to the later genotyping experiments (see Chapter 9). There is a general trend towards greater amounts of better quality DNA extracted from the later samples which reflects both the alterations to protocol and more experience with the technique.

DNA extracted in this way was also found to be pure with the majority (58%) of A260/280 values falling between 1.7 and 2 (see Figure 8.8.a&b). This indicated good quality DNA which may also be suitable for use in other forms of genetic analysis in addition to the polymerase chain reaction which was used in this project.

There was a tendency towards lower concentrations and poorer quality of DNA extracted from the control subjects. Control subjects were often recruited in large numbers from peripheral clinics and therefore whole blood was stored on ice for transportation to the laboratory. Aneurysm samples tended to be collected in smaller numbers from hospital areas close to the laboratory thus avoiding prolonged storage times for whole blood. This may go some way to explaining the differences in DNA yield between the two groups.
Chapter Eight: DNA Extraction

Figure 8.8a: Purity of DNA extracted from aneurysm samples
This scatter plot demonstrates largely consistent levels of DNA purity with a few cases falling outside of the expected range.
In spite of the differences observed, the control group also yielded sufficient quantities of good quality DNA to allow PCR experiments to be performed and therefore the slightly poorer results seen are unlikely to adversely affect the genotyping experiments performed.

At the end of the experiments, the DNA extraction process had been optimised and therefore the genotyping experiments could begin.
Chapter Nine

LABORATORY TECHNIQUES: CCR5 Genotyping
Chapter Nine: CCR5 Genotyping

9.1 Introduction

9.2 Methods

9.3 Results

9.4 Discussion
Chapter Nine: CCR5 Genotyping

9.1 Introduction

The polymerase chain reaction (PCR) may be used to amplify a segment of DNA in order to create multiple copies of a discrete region of DNA and can be modified to enable genetic analysis or the end products used in later experiments for genetic analysis. The segment to be amplified lies between regions of known base pair sequences. Oligonucleotide primers possessing complimentary sequences to the host DNA are used to flank the segment of DNA to be amplified. The template DNA is denatured by heating in the presence of the two oligonucleotide primers and nucleotide triphosphate bases (dNTPs). The reaction mixture is then cooled to allow the primers to anneal to their target sequences. An added DNA polymerase enzyme then extends the primers, and this cycle of denaturation, annealing and DNA synthesis is repeated. The major product produced from this process is a segment of double stranded DNA which lies between the two primers and has been multiplied many times over thirty cycles.

A PCR therefore requires a reaction mix consisting of the template DNA, the oligonucleotide primers, dNTPs, a DNA polymerase enzyme and a reaction buffer to maintain the pH of the reaction as the temperature varies. These components may be largely prepared in advance in the form of a ‘mastermix’ containing the primers, buffer and dNTPs, to which the template DNA and DNA polymerase may be added individually for each reaction.

A PCR reaction was developed which reliably amplified the CCR5 gene. Initially, reaction conditions and mastermix details were taken from published studies, however this was unsuccessful and so a novel method was established. This chapter describes the initial results with published methods, the development and optimisation of a new method and the genotyping results produced.
9.2 Methods

9.2.1 Published methods

There were two separate published methods for amplifying the CCR5 gene which were trialled in these experiments. Firstly, the method of Munerato (Munerato 2003) was followed. A 100μl reaction mix was made up containing:

- 50mM Magnesium Chloride; 10mM dNTP, 20 picomols (pmol) of each primer, 0.5 units of Taq polymerase and made up to the final volume of 100μl with reaction buffer.

Primer sequences were as follows:

- Forward primer: CAA AAA GAA GGT CTT CAT TAC ACC
- Reverse primer: CCT GTG CCT CTT CTT CTC ATT TCG

These primers anneal to chromosome 3 flanking the region from 46389905 to 46390093 (189 base pairs). Each amplification consisted of 40 cycles. Initially, five cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes; followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds were performed.

The second method was published by Ghilardi in 2004 (Ghilardi 2004). This method used a 25μl final reaction mix containing:

- 5μl of extracted genomic DNA; 100μmol/L of dNTPs; 1.5 mmol/L of magnesium chloride, 1 unit Taq polymerase; and two primers each at a concentration of 80nmol/L.
Primer sequences were as follows:

Forward primer: CTG TGT TTG CGT CTC TCC CA
Reverse primer: CCT CTT CTT CTC ATT TCG ACA CCG

The PCR reaction was started with 10 minutes incubation at 94°C to activate the enzyme, followed by 35 cycles of 20 seconds at 94°C, 20 seconds at 55°C and 30 seconds at 72°C.

Primer sets were ordered for both the published methods described and both methods were carried out according to the authors' methods. Results are given below.

9.2.2 Initial results

Using the Munerato method, a mastermix was used containing 40μl of 10mM dTNP mix, 10μl reaction buffer, 5μl forward and 5μl reverse primer, 29.8μl sterile water and 0.2μl (0.5 units) Taq DNA polymerase. This resulted in a 90μl mastermix, which was sufficient for 4 PCR reactions, each using 2.5μl template DNA, with a final reaction volume of 25μl per reaction.

Following amplification the PCR products were run on a 3% agarose gel containing ethidium bromide (see later notes) and viewed under ultraviolet light. Unfortunately, no products were seen, suggesting that the amplification had not been successful (see Figure 9.1). Non-specific binding of primers frequently occurs and can result in a failed reaction. It can be due to repeat sequences in the DNA template, non-specific binding between the primer and the template and incomplete primer binding leaving the 5' end of the primer unattached to the template. Manipulation of the annealing temperature and magnesium concentration are known to stabilise DNA interactions and can increase specificity of binding. The reaction conditions were therefore altered, firstly to vary the amount of magnesium chloride present in the buffer solution in 0.5μl increments between 0 and 2.5μl.
Secondly the annealing temperature was varied based on the primer melting point (Tm) calculated by

\[ Tm = 4(G+C) + 2(A+T). \]

Where \( G \) = number of guanine residues, \( C \) = number of cytosine residues, \( A \) = number of adenine residues and \( T \) = number of thymine residues in each primer.

The optimal annealing temperature should be approximately 5°C below primer melting point and the reaction conditions were therefore varied to 10°C, 5°C and 2°C below Tm respectively.

Once again, blank gels were observed and the reaction had failed.

---

**Figure 9.1: Blank gel using Munerato method.**

In this figure, the wells can be seen faintly at the top of the gel and the darker lines are the gel loading buffer which has migrated to the middle of the gel. There are no bright DNA bands indicating that the PCR was unsuccessful.
It was then decided to use the Ghilardi primers and so a reaction mix containing 10μl 10mM dNTP mix, 1.25μl forward and 1.25μl reverse primers, 2.5μl reaction buffer, 1μl magnesium chloride, 2.5μl template DNA, 0.05μl Taq polymerase and sterile water to total 25μl was used. Again this produced no clear PCR products and variation of magnesium concentrations and annealing temperature in the same manner as before had no effect.

In order to identify why the reaction was unsuccessful, a further PCR was performed using a method developed in our own lab and using primers for a different gene which have been shown to produce products (interleukin-10 gene (IL-10)). It was hoped that this experiment would demonstrate a successful amplification of the IL-10 gene and so verify that the reaction mix, template DNA and reaction conditions were capable of producing results. The reaction was performed using a set of IL-10 primers and also the Munerato CCR5 primers for comparison. The reaction conditions used were as follows: 5 mins at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C and a final step of 5 mins at 72°C.

This amplification produced 2 distinct products as demonstrated by gel electrophoresis (Figure 9.2), one produced by the IL-10 primers (left lanes) and another produced by the CCR5 primers (centre lanes). Different concentrations of the CCR5 primer were used in order to establish the maximal dilution required for a successful reaction.
Figure 9.2: Successful amplification of IL-10 and CCR5 genes.

This gel shows 2 distinct groups of bands: the two left hand bands have migrated a little further and represent successful amplification of the IL-10 gene. The three fainter bands to the right indicate successful amplification of the CCR5 gene, with higher concentrations of substrate appearing more effective than dilute.
9.2.3 Development of a new mastermix

Following on from the success of this ‘home-grown’ reaction, a new mastermix was developed, based on the IL-10 mix. Each reaction tube contained:

- 0.18 µl magnesium chloride
- 2.5 µl 10 µM dNTP mix
- 2.5 µl reaction buffer
- 2.5 µl forward and 2.5 µl reverse primer
- 0.25 µl Taq polymerase
- 2.5 µl template DNA
- 12.07 µl sterile water to give a final reaction volume of 25 µl.

The reaction conditions were as follows: an initial denaturation step of 5 minutes at 95°C, followed by 30 cycles of 95°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute. A final extension of 5 minutes at 72°C was also included. This protocol appeared to give consistent bands which were between 20 and 40 base pairs apart on agarose gel (Figure 9.3). This was indicative of a successful amplification of the CCR5 gene and its Δ32 base pair deletion.
Figure 9.3: Successful amplification with CCR5

This gel demonstrates 2 different bands. The bands towards the top of the gel represent the larger wild type CCR5 allele and the 3 extra bands which have migrated represent the smaller CCR5 Δ32 allele. This indicates that of the subjects tested in this experiment, there were 7 homozygotes for the wild type gene and 3 heterozygotes.
9.2.4 Optimisation of new mastermix

Once the amplification reaction was working consistently, it was important to optimise the reaction mix and conditions. The concentration of magnesium chloride in the reaction buffer is important as it affects the ability of the buffer to control the pH of the reaction mix as the temperature varies. The acidity of the reaction in turn affects the ability of the enzyme to catalyse the DNA synthesis reaction. The reaction buffer provided with the Taq DNA polymerase (REDTaq™ DNA Polymerase, Sigma-Aldrich. Inc) contained 100mM Tris-HCL pH 8.3, 500mM potassium chloride (KCl), 11mM magnesium chloride (MgCl₂) and 0.1% gelatin. A series of optimisation reactions were performed, varying the amount of 50mM MgCl₂ added to the reaction mix in 0.1μl increments (Figure 9.4). Smaller concentrations of MgCl₂ appeared to give brighter bands indicating a more successful amplification, and 0.14μl 50mM MgCl₂ produced the brightest band (seen in the third lane from the left on this gel). This was therefore used for all future amplifications.
Figure 9.4: Magnesium concentration optimisation with CCR5 amplification.

In this experiment, increasing volumes of magnesium chloride have been added to the mastermix and are seen in the gel from left to right. Although all of the reactions worked successfully, the brightest band is seen in the second lane from the left, indicating that this is the optimum amount of magnesium to add.
Primer concentrations were also varied as recommended dilution factors seemed to vary. The Munerato primers were used and these were supplied by Sigma-Genosys Ltd (Appendix 6) in a desalted form. They were re-suspended according to the manufacturers' instructions with 500μl sterile water added to the forward and 463μl sterile water added to the reverse primer, resulting in a final stock solution of 100μM. An amplification reaction was performed using varied primer concentrations at first by a factor of 10-100, and then by a factor of 1-10 to 'fine tune' the optimal primer concentration (Figure 9.5). A 1:10 dilution of stock primer solution to sterile water was found to be optimal and this was used for all further experiments.

Therefore, the final PCR reaction mix used in all genotyping experiments contained:

- 0.14μl 50mM MgCl₂; 2.5μl 10mM dNTP mix; 2.5μl 10x reaction buffer (as above); 2.5μl 1:10 forward and 2.5μl 1:10 reverse primer; 2.5μl template DNA;
- 0.25μl Taq DNA polymerase; and 12.11μl sterile water giving a final reaction volume of 25μl.
In this experiment, increasing dilutions of the primers were used to establish the optimal primer concentration. The smaller concentrations of primer seen on the right hand side of the gel failed to produce a successful PCR reaction and therefore the neat solution seen in the first lane with a bright band was used.
Once the reaction mix had been finalised, the reaction conditions also had to be optimised. The annealing stage of the PCR reaction is crucial to successful amplification. Optimal annealing temperature should be approximately $5^\circ C$ lower than the melting point for the primer. Primer melting point (Tm) can be calculated by:

\[ Tm = 4(G + C) + 2(A + T) \]

Therefore for the forward primer;

\[ Tm = 4(3+6) + 2(10+5) = 36 + 30 = 66^\circ C \]

and for the reverse primer;

\[ Tm = 3(3+9) + 2(1+11) = 48 + 24 = 72^\circ C. \]

The initial annealing temperature of $57^\circ C$ was therefore increased initially to $61^\circ C$ which is $5^\circ C$ lower than the forward primer melting temperature of $66^\circ C$ (Figure 9.6a), and then to $65^\circ C$ which is closer to the theoretical optimal annealing temperature for the reverse primer of $67^\circ C$, but not above the forward primer melting point (Figure 9.6b).
Chapter Nine: CCR5 Genotyping

Control samples run at annealing temperature of 61°C

Control samples run at annealing temperature of 65°C

Figure 9.6a: Annealing temperature optimisation for forward primer

Figure 9.6b: Annealing temperature optimisation for reverse primer

Figures 9.6 a and b showing faded (9.6a) or absent (9.6b) bands at a higher annealing temperature which indicates a less successful amplification reaction than at lower temperature.
Chapter Nine: CCR5 Genotyping

As shown in figures 9.6a & b, the bands seen were much fainter with the increased annealing temperature and disappeared with the higher 65°C experiment demonstrating that the reaction had failed. It was therefore decided to use the initial annealing temperature of 57°C for all genotyping experiments.

Therefore, the final reaction conditions for all genotyping experiments were as follows:

An initial step of 5 minutes at 95°C; followed by 30 cycles of 95°C for 1 minute,
57°C for 1 minute and 72°C for 1 minute; and a final extension of 5 minutes at 72°C.

9.2.5 Contamination of PCR and its treatment

Using the above reaction mix and conditions, 147 aneurysm patients were genotyped for the CCR5 gene, and subsequently 143 control subjects were also genotyped. The control population appeared to have a much higher proportion of heterozygotes than the aneurysm group. In addition to this, the allele frequency for the Δ32 polymorphism amongst the control group was significantly higher than either the aneurysm group or the expected population frequency (Table 9.7).
Chapter Nine: CCR5 Genotyping

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Aneurysm Group N=147 Number (%)</th>
<th>Control Group N=143 Number (%)</th>
<th>P Value</th>
<th>Chi Squared</th>
<th>Degrees of Freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT</td>
<td>121 (82.3)</td>
<td>71 (49.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT/Δ32</td>
<td>24 (16.3)</td>
<td>71 (49.5)</td>
<td>&lt;0.0001</td>
<td>26.16</td>
<td>2</td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>2 (1.4)</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Frequency of Δ32 allele

| Frequency | 28 (10) | 72 (25) | P=0.0092 | 6.79 | 1 |

Table 9.7: Initial genotyping results.

This table indicates a significantly higher expression of the CCR5 Δ32 allele amongst the control group than the aneurysm group which would suggest that the Δ32 allele may exert a protective effect against aneurysm formation.

The frequencies were out of Hardy-Weinberg equilibrium (p=0.000254 (Pearson)), suggesting a major selective force acting on the CCR5 Δ32 allele. As the control group were felt to be representative of the local population, it seemed unlikely that they would have been subjected to different selective forces from the aneurysm group and therefore it was suspected that an error had occurred in the genotyping process. As the control subjects were genotyped after the aneurysms, and the higher Δ32 allele frequency was observed in this group, it was considered that the PCR reaction may have become contaminated. If this were the case, then a higher proportion of heterozygotes would be observed as fragments of DNA from previous reactions may have been repeatedly re-amplified. Even if there were only a small number of heterozygous samples, a Δ32 allele which has contaminated the equipment or solutions would produce enough DNA when amplified to produce the heterozygous pattern on all subsequently analysed gels.
In order to test this theory, a series of negative control reactions were performed, using identical reaction conditions to the genotyping experiments, but without the addition of template DNA. These experiments produced a clear heterozygous pattern when the products were run on agarose gel (Figure 9.8). Although this provided a satisfactory explanation for the increased allele frequency observed, it was necessary to identify the source of contamination, in order to eliminate it from future experiments.

Figure 9.8: Negative controls demonstrating contamination.

This gel demonstrates a heterozygous pattern in all lanes however no template DNA was used in this experiment and so the gel should appear blank. This indicates contamination of equipment and/or solutions with successfully amplified PCR products from previous experiments.
A further series of negative control experiments were performed, testing each pipette in turn. All pre-mixed solutions were discarded and replaced in order to eliminate these as a source of contamination. All the pipettes were found to be affected.

Once the source of contamination had been identified, it was necessary to treat the pipettes in order to prevent the problem from recurring. All pipettes used for PCR were therefore immersed in a 1mg/ml DNase 1 solution and incubated for 1 hour at 37°C. They were then re-tested with a series of negative control PCR reactions. This time the gels were blank, indicating that the pipettes and solutions had been effectively decontaminated.

In order to prevent recurrence of this problem, separate work areas were created for preparatory work prior to the PCR reaction and for gel electrophoresis of PCR products. In this way, the risk of contamination of the pre-PCR solutions with PCR products was reduced. In addition, pipettes were clearly labelled as PRE-PCR or POST-PCR and all were handled with gloves at all times. Standard pipette tips were also replaced by filter tips in order to prevent aerosol contamination of pipettes.

All genotyping experiments were then repeated with the above precautions in place. In addition to this, both a positive (a known homozygote, genotyped during the initial experiments) and a negative (no template DNA) control were included with every PCR reaction performed in order to confirm that no further contamination had occurred.
9.2.6 Gel electrophoresis of PCR products

The standard method for separating DNA fragments of differing sizes is electrophoresis through either agarose or polyacrylamide gel. Fragments are loaded into wells at the proximal end of the gel and an electric current is passed through the gel. As DNA is negatively charged, molecules will migrate towards the positively charged electrode. Larger fragments will tend to migrate more slowly than smaller due to frictional properties of the gel. Molecules therefore migrate at a rate which is inversely proportional to log_{10} of the number of base pairs (Helling 1974). In order to visualise the DNA fragments, an intercalating dye such as ethidium bromide is added to the gel. This intercalates with DNA fragments in the gel and fluoresces under ultraviolet light so that molecules as small as 1 ng of DNA can be detected.

Agarose gel electrophoresis was used for this project as it is cheaper than polyacrylamide and is able to adequately separate the fragment sizes required. Agarose is a linear polymer which is extracted from seaweed. Gels were prepared by adding 3 g of agarose powder to 100 ml buffer solution of TBE (Appendix 1: Reagents and solutions). This solution was then heated until boiling for several minutes. The solution was then allowed to cool slightly and 15 μl of ethidium bromide were added. After re-boiling briefly, the gel was poured into a pre-prepared mould ensuring that the comb was not in contact with the base (Figures 9.9a&b). The gel was then allowed to cool until set (approximately 45 minutes) and transferred to a buffer tank for electrophoresis.
Figure 9.9a: Gel preparation by heating

Figure 9.9b: Gel setting in mould
The PCR products were prepared for electrophoresis by adding 15µl of PCR product to 5µl of gel loading buffer (Appendix 1: Reagents and solutions). This buffer is visible with the naked eye as a blue mark in the gel and ensures that the system is working correctly. The buffer migrates slightly more rapidly than the PCR products and so can be used to guide the length of time needed for electrophoresis.

Once the gel was set, the prepared products were carefully loaded into the well, taking care to avoid damage to the wells. A clean pipette tip was used for each sample to avoid contamination. Positive and negative electrodes were attached and a voltage applied across the gel (Figure 9.10). Optimum DNA migration occurs at voltages of 5 volts (V) per centimetre (cm) of gel, and therefore 60V were used for these 12cm gels.

Figure 9.10: Fragment separation by electrophoresis.
The gel is placed in an electrophoresis tank and a current passed through a liquid buffer. DNA fragments have a negative charge and are therefore drawn towards the positive electrode, seen here in red.
Once the gel loading buffer had migrated approximately half the length of the gel, electrophoresis was stopped and the gel read under UV light (Figure 9.11). This was found to be adequate for separation of the wild type fragment from the Δ32 fragment, and longer electrophoresis times resulted in difficulty in interpretation of gels due to feint DNA bars.

Photographs were taken of each agarose gel and both electronic and paper copies were kept. All results were recorded and analysed using the anonymised study sample reference number and contained no patient data.
9.2.8 Sequencing of PCR products

DNA sequencing was first introduced in 1977 by Sanger (Sanger 1977), who later shared the Nobel Prize for Chemistry in 1980. DNA polymerases are used to synthesize fragments of DNA. The synthesis reaction is halted when a particular base appears. Modern adaptations of this technique now use fluorescent dye labels which are incorporated into the DNA extension products. Automated DNA sequencers then detect fluorescence from four different dyes that are used to identify the A, C, G and T extension reactions. Each dye emits light at a different wavelength when exposed to an argon ion laser. In this way, all four bases can be detected and distinguished.

In order to sequence the PCR product and confirm that it was the CCR5 gene which had been amplified, it was necessary to process the product through a 'clean up' kit to remove any excess dNTPs, Taq, mineral oil etc. This was done using a commercially available kit purchased from Sigma (GenElute PCR Clean Up Kit, product number NA1020), and the method is described below.

1. A GenElute miniprep binding column was inserted into a collection tube and 0.5ml of column preparation solution was added. The tube was centrifuged at 12,000g for 30 seconds and the eluate discarded.
2. 500μl of binding solution were added to 100μl of PCR reaction and the solutions mixed.
3. The solution was then transferred to a binding column and centrifuged at 12,000g for 1 minute and the eluate discarded.
4. The binding column was then placed into the collection tube and 0.5ml of diluted wash solution was added to the column which was then centrifuged at 12,000g for 1 minute. The eluate was discarded but the collection tube retained.
5. The column was placed into the collection tube and centrifuged for 2 minutes at 12,000g. The eluate was then discarded along with the collection tube.

6. The column was then transferred to a fresh 2ml collection tube and 50μl of elution solution added. This was then incubated for 1 minute at room temperature and centrifuged at 12,000g for 1 minute.

7. The sample was then transferred on ice to the University of Leicester Protein and Nucleic Acid Sequencing Laboratory for analysis on an automated DNA sequencing machine.

Figure 9.12 gives results of the sequencing reaction and the sequence of products analysed, thus confirming that CCR5 had been successfully amplified.
Sequence: ... NNN TAA NNA NNN TCA TCT TGG GGC TGG TCC TGC CGC TGC TTG TCA TGG GGA ATC CTA AAA ACT CTG CTT CGG TGT CGA AAT GAG AAG AAG AGG CAC AGG AAN NNN...

Where A= adenine, C=cytosine, G=guanidine, T=thymidine and N=indeterminate

Figure 9.12a: DNA Sequencing Results for sample BC78 (Δ32/Δ32 Genotype).

The DNA sequence is determined by emissions of light at different wavelengths for each nucleotide in order. This is displayed as a graph as shown and the sequence determined according to the occurrence of different coloured peaks. Where there is no clear dominant peak, and ‘N’ represents the unidentified nucleotide.
Sequence: ... NNN TTT TAN NNN NCC GAG TAG CAG ATG ACC ATG ACA AGC AGC GGC AGG ACC AGC CCC CAA GAT GAC TAT CTT TAA TGT CTG GAA ATT CTT CCA GAA TTG ATA CTG ACT GTA TGG AAA ATG ANA GCT GCA GGT GTA ATG AAG ACC TTC TTT TTG GAN NNA NCT GTT TCT GAN NAG CCT TCA NGA TTA CAN GAT TCA AAG TGG GCT GCG GGG GCC GGN NCG ANN CTC CTT CTT CNC CAA AAG CAC TCT GAT TGA CAN ATA CAN TTT ATC TAA TTT ANA NGA AAT TGC TTC TGG NGG CGC TCC CCT CTC TAA NGA AGT CGG GGA NCG GTT GCC AAG ANG TTC CAT CTG CCA NNT ATC AGG CAN GGA TAT GGG CTC ACT NNN ACT ACA TCA CCT ATT CTG ATT ACA CCC GAN GGG GAT GAT AAA CCG GGC GCG GTC GGT AAN GTT GNN NNN ...

Where A = adenine, C = cytosine, G = guanidine, T = thymidine and N = indeterminate

Figure 9.12b: DNA Sequencing Results for sample BA150 (WT/WT Genotype)
Chapter Nine: CCR5 Genotyping

These sequencing results were referenced against the NCBI ‘BLAST’ database and the results sequence confirmed to be human CCR5, located on chromosome 3 with 100% homology.

9.3 Results

9.3.1 Genotype and allele frequencies

There were 285 patients with aneurysms and 273 control subjects recruited into the study. Of these, 284 aneurysm patients and 273 control subjects had extracted DNA available for analysis. Each subject was genotyped for the CCR5 gene as described in order to establish whether a relationship exists between the Δ32 polymorphism and abdominal aortic aneurysm. Genotyping data are given in Appendix 4: Genotyping Results.

There were some PCR reactions that did not work and therefore patients for whom the genotype is unknown. In cases where genotyping failed, the experiment was repeated twice using the same DNA and reaction conditions. In a number of cases this remained unsuccessful and the experiment was therefore repeated using newly extracted DNA. This yielded a result in the majority of cases however there remained a minority of samples (54 samples) for which genotyping failed. The reasons for this are unclear and possible explanations are reviewed in the discussion at the end of this chapter.

A summary of the genotyping results is given in Table 9.13. It can be seen from this that there is no significant difference observed in the CCR5 genotype or Δ32 allele frequency between the aneurysm group and the control group (using Chi Squared). There was no evidence of deviation from the Hardy-Weinberg equilibrium in either group (Aneurysms: P=0.27; Controls: P=0.76). The armitage trend test gave odds ratios of 0.79 for the Δ32 allele
and 1.26 for the wild type allele thus confirming the suspicion that the Δ32 allele would tend to be protective against the development of AAA (although this is not significant).

Table 9.13: Summary of genotyping results.

This table confirms a similar allele frequency amongst the aneurysm and control groups for the Δ32 allele and demonstrates no significant difference between groups in terms of allele frequency or genotype.

Subgroup analysis of large (>5cm) versus small (<5cm) aneurysms also revealed no significant effect of the CCR5 genotype or Δ32 allele frequency on size of aneurysm when seen (see Table 9.14).

Table 9.14 Genotype and aneurysm diameter.

This table confirms no significant association between the CCR5 genotype or Δ32 allele frequency and aneurysm diameter.
9.3.2 Genotype and expansion

In order to examine the effect of the CCR5 Δ32 allele and genotype on aneurysm progression, cases were compared in terms of average (geometric mean) growth rate. This assumes a linear model of aneurysm expansion. Some debate exists regarding the growth pattern of aneurysms with some authors suggesting an exponential trend (Limet 1991), however to establish a model of growth for this data was beyond the scope of this thesis. For the purposes of comparison between different genotypic groups therefore, a linear model was assumed.

Serial measurements for each aneurysm were recorded at relevant time points and the average growth rate at each time point calculated by dividing the increase in aneurysm size in centimetres by the number of months since diagnosis for each patient. This figure was then converted and reported as centimetre per year. Data were analysed and found to be non-parametric in distribution (Figure 9.15) and therefore the medians of the average growth rates were compared between groups using a Kruskal Wallis test (see Table 9.16). There were 146 patients with 2 or more serial measurements for analysis from this series. There were a mean of 6.2 observations per patient at a mean time period of 31 months follow up.
Figure 9.15: Expansion data plotted against a normal distribution curve. This graph confirms that the expansion data retrieved is not normally distributed and must undergo nonparametric statistical testing.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median expansion (cm/yr)</th>
<th>Range (cm/yr)</th>
<th>Mean Rank</th>
<th>Kruskal Wallis</th>
<th>Degrees of freedom</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT</td>
<td>0.24</td>
<td>-8.8 - 45.6</td>
<td>422.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT/Δ32</td>
<td>0.16</td>
<td>-0.7 - 26.4</td>
<td>410.5</td>
<td>5.11</td>
<td>2</td>
<td>0.775</td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>0.18</td>
<td>0 - 2.5</td>
<td>443.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9.16: Comparison of median expansion rates by genotype. This table demonstrates that there is no significant association between CCR5 genotype or allele frequency and rate of aneurysm expansion.
In order to address the possibility of an exponential pattern of growth, the natural log of each expansion rate was taken and the statistical testing repeated. Results are given in Table 9.17.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Median expansion (cm/yr)</th>
<th>Range (cm/yr)</th>
<th>Mean rank</th>
<th>Kruskal Wallis</th>
<th>Degrees freedom of P = value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT</td>
<td>-11.3</td>
<td>-19.9 - 6.6</td>
<td>236.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT/Δ32</td>
<td>-10.6</td>
<td>-19.4 - 4.2</td>
<td>246.7</td>
<td>0.639</td>
<td>2</td>
</tr>
<tr>
<td>Δ32/Δ32</td>
<td>-11.3</td>
<td>-18.4 - 6</td>
<td>218.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9.17: Comparison of Log median expansion rates by genotype.
This data confirms no association between CCR5 and log median expansion.

As shown in Tables 9.13 and 9.14, whether an exponential or a linear growth pattern is assumed, the CCR5 genotype does not seem to significantly influence aneurysm expansion.

### 9.4 Discussion

The polymerase chain reaction is used to amplify a gene or region of interest in order that it might be viewed on gel electrophoresis for analysis. It relies on specific primers which anneal to regions of template DNA flanking the gene of interest. Although it is a reliable and reproducible method, it is fairly sensitive to a number of factors such as temperature and pH which need to be optimised in order to produce quality results.

Two published methods of amplification of the CCR5 gene were trialled in this project. Neither produced results and this highlights the sensitivity of the technique. Differences in brand of reagents used, storage facilities and PCR machines (affecting temperature and cycling times) may all have contributed to the lack of results. It is likely that the novel method used was more successful because it had previously been developed in this laboratory, thus eliminating many of the variables which may have impaired the success of
the published methods. Although this reaction worked immediately, it was still necessary to optimise both the reaction mix and conditions in order to produce clear results for interpretation.

There were some PCR reactions which failed to produce results in spite of good quality template DNA and identical reaction conditions to other samples. The reason for this is unclear, however as PCR relies on a standard primer annealing site, it is possible that a group of patients had polymorphisms within this region preventing primer annealing. This would explain the consistent lack of results with repeated reactions using re-extracted DNA.

Unfortunately, problems were encountered with contamination during the early genotyping experiments for this project. PCR is very sensitive to contamination and as little as one molecule of DNA can be amplified to produce a false result. Initially, few measures were in place to prevent contamination, which often occurs as a result of aerosol deposits of DNA within the barrel of pipettes. Once this problem had been identified and rectified through treatment with DNA degredation enzymes, several measures were put in place to prevent a further problem occurring. These included the use of separate areas of the laboratory for PCR preparation and post-PCR electrophoresis (ideally these areas would be in separate rooms), clear labelling of pipettes, routine inclusion of positive and negative controls with each PCR reaction and importantly the use of filter tips with all PCR work. Using these precautions, no further problems with DNA contamination have been observed.

Analysis of the genotyping results has demonstrated no significant difference between genotype or allele frequencies observed amongst the aneurysm group and the control group. In addition to this, univariate analysis has not demonstrated any significant association between CCR5 genotype and presence or absence of AAA. Although CCR5 appeared to be a
promising gene to investigate in view of the central role of the CCR5 receptor in cytokine inflammatory pathways and the promising results seen with its blockade in HIV work, it does not seem to play a significant role in the development of abdominal aortic aneurysm. This is likely to be due to the highly complex nature of inflammatory interactions which have the ability to upregulate other pathways in the absence of functional CCR5.

In keeping with the lack of influence of the CCR5 Δ32 polymorphism on presence of aortic aneurysm, the expansion rate amongst patients of all genotypes appeared very similar and certainly presence of the CCR5 Δ32 allele did not seem to significantly influence aneurysm growth.

In conclusion, the Δ32 polymorphism of the CCR5 gene does not seem to be significantly associated with the presence or expansion of abdominal aortic aneurysms. This is likely to relate to the highly complex nature of cytokine interactions which may effect their actions via similar functional receptors such as CCR2 in the absence of functional CCR5.
Chapter Ten

LABORATORY TECHNIQUES: Detection of soluble CCR5 by ELISA
10.1 Introduction

10.2 Methods and Results

10.3 Discussion
10.1 Introduction

Enzyme Linked Immunosorbant Assay (ELISA) is a method for the identification and quantification of protein. It relies on an antibody-antigen interaction followed by an enzymatic reaction to produce a detectable product which is proportional to the number of antibody-antigen complexes. A plate, usually containing 96 wells, is coated with the primary antibody which binds the protein of interest. The wells are then washed and exposed to a secondary antibody which also interacts with the protein, creating a ‘sandwich’ of antibody-protein-antibody complexes. The wells are washed again and then exposed to an enzyme which reacts with the secondary antibody with the formation of a product in those wells expressing the protein of interest. The optical density of each well is then analysed using an ELISA plate reader and values can be compared against known standards to give quantitative data.

Although CCR5 is usually a membrane bound receptor, a soluble form has previously been identified using a sandwich ELISA technique (Tsimanis 2005). This was found to be a 22kDa protein in contrast to the 40kDa membrane-bound form and seemed to affect an individual’s susceptibility to infection with the HIV1 virus (see Chapter 4). Population frequency of this protein varied between 5% and 20%, and so at least 5 positive samples could be expected from 100 analysed.

It was proposed that such a protein may be detectable amongst the study groups of patients and that the presence of this soluble CCR5 receptor may be correlated with presence of an abdominal aortic aneurysm. In order to investigate this theory, an ELISA was developed based on the published method used by Tsimanis and plasma samples
assayed for soluble CCR5.

10.2 Methods

10.2.1 ELISA protocol

Initially, the method of Tsimanis was followed and all antibodies and reagents purchased in keeping with the technique described. This method is given below.

1. ELISA plates (Nunc-Immuno™, Maxisorp, Denmark) were coated with purified mouse anti-human CCR5 monoclonal antibody by the addition of 200μl of antibody to each well and incubation overnight at 4°C.

In each plate, a negative control was used, leaving 2 wells untreated to assess background level of fluorescence.

2. The wells were washed with 0.05% Tween in phosphate buffered saline (PBS) (see section 11.2.2 Antibodies and Reagents) and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature.

3. The wells were washed with PBS and 100μl of each test sample was added to two wells and incubated for 16 hours at 4°C.

4. The wells were again washed with PBS and the secondary Biotinylated mouse anti-human CCR5 antibody added (200μl per well) and incubated for 1 hour at room temperature (Figure 10.1).
Chapter Ten: Detection of Soluble CCR5 by ELISA

5. The wells were washed and 200μl of alkaline phosphatase-conjugated streptavidin was added for 1 hour at room temperature. Streptavidin binds to biotin (and therefore the secondary antibody) and an enzymatic reaction between the alkaline phosphatase and phosphate is then used to demonstrate fluorescence.

6. The wells were then washed 3 times with a buffer containing Tris-HCl and NaCl and then again with Tris-HCl, NaCl and MgCl₂ (see section 11.2.2 Antibodies and Reagents).
Chapter Ten: Detection of Soluble CCR5 by ELISA

7. 100μl p-nitrophenyl-phosphate (PNP) in diethanolamine buffer was then added to each well, providing alkaline phosphatase substrate.

8. 50μl of 0.6M NaOH was added to each well to stop this reaction (Figure 10.2).

9. The plates were read at 405nm using a microtitre plate spectrophotometer.

Figure 10.2: Completed ELISA plate to be read.
Once the ELISA reaction is completed, the wells become varying shades of yellow which are read and quantified on an optical plate reader.

10. In keeping with the method of Tsimanis, an optical density of greater than three times the background reading was taken to be positive.
10.2.2 Antibodies and reagents

All antibodies and reagents were purchased and made up according to the method of Tsimanis (Tsimanis 2005).

**Primary Antibody**

The primary antibody was purchased from BD Pharmingen and was purified mouse anti-human CCR5 monoclonal antibody 2D7, diluted to 1µg/ml in PBS. It recognises a conformation-dependent epitope on the second extracellular loop (ECL2) of CCR5.

**Secondary Antibody**

The secondary antibody, also purchased from BD Pharmingen, was an anti-CCR5 biotinylated mouse monoclonal antibody which recognised ECL2 and was also dependent on regions in extracellular loop 1 (Lee 1999). This was also diluted to 1µl/ml in accordance with the manufacturer’s instructions.

**Streptavidin-Alkaline Phosphatase**

Streptavidin bound alkaline phosphatase was purchased from Sigma and diluted in Tris-HCl at pH7.5 to a concentration of 2µg/ml according to the manufacturer’s instructions. Streptavidin is a tetrameric protein purified from *Streptomyces avidinii* that binds very tightly to biotin, a vitamin of the B (B7) family which is important for fatty acid synthesis and gluconeogenesis. The alkaline-phosphatase is used to react with the phosphate substrate (PNP). In this way, the amount of reactivity between the alkaline phosphatase and the PNP is dependent on the amount of streptavidin bound to the secondary, biotinylated antibody. The amount of product produced by this reaction is measured by its optical density.
Alkaline Phosphatase Substrate

$P$-nitrophenyl-phosphate (Sigma) was used as a substrate for the streptavidin alkaline phosphatase and this was diluted in 1M diethanolamine buffer and 0.24 mmol $\text{MgCl}_2$ at pH 9.8.

Wash Solutions

Several wash buffers were used which were based around phosphate buffered saline solutions. The basic PBS was purchased from Sigma, as was the Tween in PBS, pH 7.4.

The bovine serum albumin was purchased from Sigma and diluted to a 1% solution with PBS.

Two Tris-HCl buffers were used, the first containing 0.1M tris-HCl and 0.15M sodium chloride ($\text{NaCl}$) at pH 7.5 and the second containing 0.1M Tris-HCl, 0.1M $\text{NaCl}$ and 50mmol magnesium chloride ($\text{MgCl}_2$) at pH 9.5. These solutions were made up from laboratory stock solutions.
10.2.3 Initial Results

An ELISA plate was set up and performed using duplicate plasma samples from aneurysm subjects BA1-BA46 (n = 46 in duplicate). The method of Tsimanis was followed as above. Results are given in Table 10.3. As seen, one sample, BA5, had an optical density reading of greater than three times the background level and was therefore taken to be positive. Although more samples could be expected to be positive if indeed the protein was expressed in 5% or more of the subjects, this was an encouraging start.

Table 10.3: Initial ELISA results for samples BA1-BA46

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median OD</th>
<th>Range OD</th>
<th>Median OD: Background OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>0.049</td>
<td>0.048-0.05</td>
<td>-</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.104</td>
<td>0.092-0.116</td>
<td>2.12</td>
</tr>
<tr>
<td>BA1-4 &amp; BA6-46</td>
<td>0.074</td>
<td>0.059-0.131</td>
<td>1.51</td>
</tr>
<tr>
<td>BA5</td>
<td>0.345</td>
<td>0.329-0.362</td>
<td>7.04</td>
</tr>
</tbody>
</table>

Where OD = optical density

Following on from this, an experiment was conducted to determine the variation in optical density seen in both the positive control (BA5) and negative controls, and a background variation was also included. This was performed in order to establish the likelihood of false positives or false negatives occurring with the current threshold for a positive value set at three times the background level. Results are given in Table 10.4.
Although one sample continued to give a higher reading than the others, the high coefficient of variation suggests that this is a relatively insensitive test and therefore false positives are likely.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Standard Deviation</th>
<th>Mean</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background (n=64)</td>
<td>0.033</td>
<td>0.074</td>
<td>45.3</td>
</tr>
<tr>
<td>Negative control (n=40)</td>
<td>0.061</td>
<td>0.143</td>
<td>42.46</td>
</tr>
<tr>
<td>BA1 (n=32)</td>
<td>0.017</td>
<td>0.098</td>
<td>18.29</td>
</tr>
<tr>
<td>BA5 (n=22)</td>
<td>0.158</td>
<td>0.376</td>
<td>42.06</td>
</tr>
</tbody>
</table>

Where OD = optical density

As demonstrated, BA5 continued to give a reading greater than three times the background optical density. The coefficient of variation was then calculated according to the equation given below.

\[
\text{Coefficient of variation} = \frac{\text{standard deviation} \times 100}{\text{Mean}}
\]

This allows direct comparison of the variation between the different samples. As seen the coefficient for all samples is fairly high indicating a significant variation in the range of values observed and therefore a relatively insensitive test.

Having confirmed the presence of a consistently positive sample that could be used as a control, the next batch of samples were analysed, BA47-BA90 (n=43 in duplicate).
Unfortunately, although the 'positive control' BA5 sample did yield consistently higher optical densities than the background and negative readings, it no longer reached the threshold value of >3 times the OD. In addition, no further samples produced positive readings. In view of this, the experiment was repeated however, the same negative results were confirmed (see Table 10.5).

Table 10.5: Samples BA47-BA90.
A further ELISA experiment failed to confirm the positivity of the previously positive sample and also failed to identify any other positive samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median OD</th>
<th>Range OD</th>
<th>Median OD:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Background</td>
<td>Background OD</td>
</tr>
<tr>
<td>Background</td>
<td>0.142</td>
<td>0.138-0.146</td>
<td>-</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.078</td>
<td>0.075-0.08</td>
<td>0.55</td>
</tr>
<tr>
<td>BA47-90</td>
<td>0.079</td>
<td>0.071-0.111</td>
<td>0.56</td>
</tr>
<tr>
<td>BA5</td>
<td>0.219</td>
<td>0.197-0.24</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Where OD = optical density

A further attempt to analyse more samples in the hope of determining further positive samples which could be used as controls was performed however no further positives were identified using BA90-BA136 (n=46 in duplicate).

In order to clarify whether the lack of soluble CCR5 detected was due to the presence of an aneurysm rather than a technical problem with the ELISA method, a plate of control subjects was tested, but again no positive samples were identified (Table 10.6).
A further ELISA experiment was performed using control samples but this also failed to identify any positive samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Median OD</th>
<th>Range OD</th>
<th>Median OD: Background OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>0.077</td>
<td>0.072-0.082</td>
<td>-</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.136</td>
<td>0.136-0.136</td>
<td>1.77</td>
</tr>
<tr>
<td>BC1-45</td>
<td>0.142</td>
<td>0.105-0.184</td>
<td>1.84</td>
</tr>
<tr>
<td>BA5</td>
<td>0.185</td>
<td>0.171-0.199</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Where OD = optical density

As there had been no consistently positive samples identified, a series of experiments were conducted to alter the protocol by firstly increasing the capture antibody concentration, then the streptavidin concentration and finally the incubation time for the final reaction to develop. Results are given in Tables 10.7-10.9. In each case, duplicate plasma samples were used however it was not possible to use the same sample for each experiment due to limited sample volumes, therefore 46 different samples (duplicated) were used per plate and the median reading calculated.
Chapter Ten: Detection of Soluble CCR5 by ELISA

Table 10.7: Increasing capture antibody concentration
This table demonstrated no significant increase in optical density achieved with increasing capture antibody concentration and again demonstrates no positive samples.

<table>
<thead>
<tr>
<th>Capture antibody concentration</th>
<th>Median OD</th>
<th>Range OD</th>
<th>Median OD: Background OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>0.106</td>
<td>0.052-0.159</td>
<td>-</td>
</tr>
<tr>
<td>1μl/ml</td>
<td>0.105</td>
<td>0.094-0.211</td>
<td>0.99</td>
</tr>
<tr>
<td>2 μl/ml</td>
<td>0.103</td>
<td>0.095-0.108</td>
<td>0.97</td>
</tr>
<tr>
<td>3 μl/ml</td>
<td>0.104</td>
<td>0.097-0.117</td>
<td>0.98</td>
</tr>
<tr>
<td>4 μl/ml</td>
<td>0.105</td>
<td>0.093-0.127</td>
<td>0.99</td>
</tr>
<tr>
<td>5 μl/ml</td>
<td>0.108</td>
<td>0.097-0.136</td>
<td>1.02</td>
</tr>
<tr>
<td>6 μl/ml</td>
<td>0.111</td>
<td>0.098-0.133</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Where OD = optical density

Table 10.8: Increasing streptavidin concentration.
This table demonstrates that although increasing the streptavidin concentration does increase the optical densities achieved, the experiment has still failed to identify a positive control.

<table>
<thead>
<tr>
<th>Streptavidin Concentration</th>
<th>Median OD</th>
<th>Range OD</th>
<th>Median OD: Background OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>0.056</td>
<td>0.055-0.057</td>
<td>-</td>
</tr>
<tr>
<td>1μl/ml</td>
<td>0.102</td>
<td>0.096-0.234</td>
<td>1.82</td>
</tr>
<tr>
<td>2 μl/ml</td>
<td>0.106</td>
<td>0.1-0.129</td>
<td>1.89</td>
</tr>
<tr>
<td>3 μl/ml</td>
<td>0.112</td>
<td>0.091-0.155</td>
<td>2</td>
</tr>
<tr>
<td>4 μl/ml</td>
<td>0.127</td>
<td>0.104-0.166</td>
<td>2.27</td>
</tr>
<tr>
<td>5 μl/ml</td>
<td>0.142</td>
<td>0.12-0.158</td>
<td>2.54</td>
</tr>
<tr>
<td>6 μl/ml</td>
<td>0.147</td>
<td>0.116-0.166</td>
<td>2.63</td>
</tr>
</tbody>
</table>

Where OD = optical density

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Increasing capture antibody concentration produced a trend towards a higher median optical density, as did increasing streptavidin concentration, however the range of readings also widened so this trend was not significant. Although BA5 did produce a reading of more than three times the background level after 3 hours incubation at 37°C, it remained the only sample to do so. It is of course possible that BA5 was the only subject possessing the soluble form of the CCR5 receptor, however in view of the large number of samples studied and the much higher expression of this protein previously reported, it seems that this is unlikely and that variations to the ELISA protocol failed to convincingly demonstrate the presence of this receptor.
Chapter Ten: Detection of Soluble CCR5 by ELISA

10.2.4 Development of a positive control

In order to continue with this work, it was felt necessary to find a confirmed and reliable positive control. No commercially available soluble CCR5 exists and therefore it was necessary to find a substitute. As the antibodies used in the ELISA were developed against membrane bound CCR5, it was decided to use white cells as a source of positive control. Buffy coat was taken from 3 samples which were selected as they had a large amount of buffy coat available for experiments.

Red cell lysis solution was added to buffy coat as described in Chapter 8. The samples were centrifuged and the supernatant discarded. The white cell pellet was then resuspended and a cell lysis buffer added to lyse the white cells. This solution was then diluted to various concentrations using PBS and a further ELISA plate prepared. Results are given in Figure 10.10. Although a pattern of higher OD associated with both extremes of dilute and concentrated white cell solutions was demonstrated, it was not clear whether this was due to antibody binding with CCR5 or more non-specific binding to plasma proteins. It was therefore decided to perform a western blot in an attempt to confirm the presence of the CCR5 protein before work with the ELISA continued.
Figure 10.10a&b: Variation of optical density with decreasing concentration of lysed white cells.
Chapter Ten: Detection of Soluble CCR5 by ELISA

Figure 10.10a, b&c: Variation of optical density with increasing concentration of lysed white cells.
The graphs show that optical density of wells after the ELISA experiment varied with increasing concentration of lysed white cells used. Both very dilute and very concentrated solutions appear to give high OD readings in graphs a & b, however in graph c, only dilute white cells appear to produce a high OD.

Western blotting is an immunostaining technique in which plasma proteins are separated by gel electrophoresis and then transferred to a nitrocellulose membrane for detection using fluorescent labelled antibodies. This technique relies on the separation of plasma proteins according to size and then antibody specific binding to detect the desired protein.

A western blot was performed using re-suspended buffy coat as described above. A protein ladder was used to indicate when proteins of fixed molecular weights had migrated to the bottom of the gel. The various concentrations of buffy coated were loaded into a 10% SDS polyacrylamide gel and underwent electrophoresis for 60 minutes at 150v until the 50kDa
marker had reached the base of the gel. The CCR5 protein has a molecular weight of 55kDa and should therefore be retained in the gel at this time.

Proteins were then transferred to a nitrocellulose membrane by electrophoresis at 200mA overnight. The membrane was then blocked with 5% milk for 1 hour. Following this, it was incubated with the primary antibody (anti-CCR5/anti-Factor IX) for one hour at room temperature. It was then incubated with the secondary antibody (1:2000 anti-mouse IgG) for a further one hour. The antibodies were then detected using a locally developed version of the commercially available ‘ECL’ (enhanced chemiluminescence) detection system from Amersham Biosciences (see Appendix 2: Reagents and Solutions).

Results are given in Figure 10.11. As shown, although clear bands were seen using anti-factor IX antibody, when the anti CCR5 antibody was added, it tended to cross react with many proteins present and therefore failed to give a clear band which could be identified as CCR5. This non-specific binding may explain the non-specific trend observed on the ELISA curves performed with buffy coat, and is likely to be due to a generic binding site for the antibody which is common to many plasma proteins.
Figure 10.11a&b: Western blot using anti-CCR5, anti-Factor IX as control and re-suspended buffy coat.

The western blot a. on the left hand side shows clear bands as marked which represent specific protein binding of factor IX with the antibody. When the re-suspended buffy coat is added however, the bands are lost and only smears are seen (right hand picture, b.) demonstrating non-specific binding.
10.3 Discussion

In the absence of a confirmed positive control and with no samples consistently demonstrating positive results, it is impossible to draw any meaningful conclusions from the ELISA work. There has been no evidence in support of the presence of a soluble CCR5 protein, however without a positive control it is impossible to confirm that the technique is working properly. It may be that the cell lysis buffer used to lyse white cells in these experiments could have disrupted the membrane protein itself. The red cell buffer containing ammonium chloride works simply by osmosis to create a hypertonic intracellular environment which causes influx of water at cell lysis. The white cell buffer on the other hand contains sodium dodecyl sulphate (SDS) which is capable of disrupting non-covalent protein bonds. This may have therefore altered the conformation of the CCR5 protein so that it was not detected by anti-CCR5 antibodies. Further work to develop a positive control is beyond the scope of this thesis, however if one were to be identified, it would be interesting to repeat this series of experiments comparing the frequency of a soluble CCR5 protein amongst and aneurysm and a control population.
Chapter Eleven

LABORATORY TECHNIQUES: Detection of CCR5 receptor by flow cytometry
11.1 Introduction

11.2 Methods

11.3 Results

11.2 Discussion
Chapter Eleven: Detection of CCR5 receptor by Flow Cytometry

11.1 Introduction

11.1.1 Principles of Flow Cytometry

Flow Cytometry is used to determine optical and fluorescence characteristics of individual cells. Fluorescent-labelled antibodies may be used to investigate a particular cell line or cell surface antigen, and the cell population of interest identified by the pattern of 'scatter' generated.

A flow cytometer works by drawing cells in suspension into a single file stream past a beam of monochromatic light (usually a laser). Light emitted from the laser is reflected by the cells, giving a forward scatter reading (FSC) and a side scatter reading (SSC). The forward scatter reflects the size of the cell and the side scatter indicates the degree of granularity of the cell. The information detected is displayed as a dot-plot of FSC and SSC. This allows selection of a specific cell group to be included in further analysis (Figures 11.1&11.2).
Figure 11.1: Schematic Representation of Flow Cytometer
Chapter Eleven: Detection of CCR5 receptor by Flow Cytometry

Figure 11.2: Leucocyte pattern on FSC/SSC Plot.
This diagram indicates the typical position of different white cell groups when seen on a flow cytometry scatter plot.

Once the cell line of interest has been identified by its characteristic pattern of forward and side scatter, it can be further studied with the use of fluorescent-labelled antibodies. The antibody is incubated with the cell sample and binds to cells possessing the relevant antigen. The cytometer measures emission and absorption of light and calculates the fluorescence using Stokes Shift which states:

\[ \text{Emission} - \text{Absorption} = \text{Fluorescence} \]

Cells which stain positively with the antibodies of interest can be selected using ‘gating’ which isolates a cell group to be analysed. The percentage of positively stained cells is then determined using quadrant statistics. The details of gating and quad statistics will be discussed in more detail in the protocol section of this chapter.
11.1.2 Flow cytometry and CCR5

This thesis aims to examine the relationship between the CCR5 Δ32 allele and the presence of abdominal aortic aneurysm. In order to produce meaningful results, it was necessary to establish whether the Δ32 allele is biologically active, i.e. does possession of one or more Δ32 alleles influence the functional amount of CCR5 receptor present on a cell? Flow cytometry was used to investigate this.

CCR5 is predominantly a CD3 positive T-lymphocyte cell surface receptor and therefore lends itself to being detected by flow cytometry. Whole blood samples were incubated with an antibody to CD3 which distinguishes T-lymphocytes from B-lymphocytes. Incubation with an anti-CCR5 antibody was used to demonstrate the presence of the CCR5 protein.

In this way, the amount of functional CCR5 protein available on T-lymphocytes to interact with the anti-CCR5 antibody was quantified and expressed as a percentage. This chapter describes a series of qualitative experiments performed on patients of known genotype examining the relationship between CCR5 genotype and receptor expression on T-lymphocytes.

11.2 Methods

11.2.1 Protocol for flow cytometry

The protocol used for this set of experiments was adapted from a clinical protocol used in assessing effectiveness of anti-lymphocyte therapy for patients with acute rejection of transplant kidneys. Firstly, whole blood was incubated with the antibodies of interest. Lysis solution was then added to disrupt red cells. This supernatant was then discarded leaving a white cell pellet, which was re-suspended for analysis.
**Antibody Incubation Protocol**

1. 50μl of whole blood was added to the bottom of a test tube.

2. 10μl of PE (phycoerythrin labelled)-CD3 monoclonal antibody and 10μl of FITC (fluorescein isothiocyanate labelled)-CCR5 monoclonal antibody were added to the tube.

3. The tubes were incubated in a room temperature incubator in the dark at 22°C for 30 minutes.

4. 1ml of FACSLyse reagent was added to each tube in the fume cupboard (see section 10.2.2 Antibodies and Reagents).

5. The tubes were capped and vortexed briefly to mix.

6. The tubes were incubated for a further 9 minutes in the room temperature incubator.

7. 3ml of PBS/0.1% azide were added to each tube, and the tubes were centrifuged at 1100rpm (200G) for 10 minutes at room temperature.

8. In the fume cupboard, the tubes were uncapped and most of the supernatant discarded.

9. The tubes were re-capped and vortexed to re-suspend the white cell pellet.

10. The cells were washed with 4ml PBS/0.1% azide at 4°C and re-centrifuged at 1100rpm for 10 minutes at room temperature.

11. The supernatant was again discarded in the fume cupboard and the tubes vortexed to re-suspend the cells.

12. 250μl of FACSFlow solution was added to each tube and the samples analysed immediately.

**Flow Cytometer Analysis Protocol**

A Becton-Dickinson FACSCalibur Flow Cytometer was used for this work in conjunction with an Apple Macintosh computer.

1. The flow cytometer was set up according to the laboratory protocol ensuring adequate levels of sheath fluid and an empty waste container.

2. ‘Acquisition mode’ was selected from the appropriate computer menu.
3. 20,000 events were acquired ungated using a threshold level of 150 which was sufficient to include all cells but exclude the majority of debris from the analysis.

4. The acquired events were displayed on a FSC:SSC dot plot.

5. A broad 'gate' was defined by circling the lymphocytes (identified by their position on the dot plot) and excluding other cells.

6. This gate, termed R1 was then applied to the FSC:SSC dot plot and a further gate (R2) was applied to isolate the lymphocytes more precisely.

7. This second R2 gate was then applied to the FL1:FL2 dot plot which displays antibody fluorescence.

8. This gives at least 2 distinct populations of cells, those in the lower left quadrant representing non-fluorescent cells ie. B-lymphocytes, and those in the upper left quadrant representing CD3 positive cells (ie T-lymphocytes). Cells stained positively with the FITC-CCR5 antibody were seen in the upper right quadrant of the dot plot and were clearly visible as a distinct population from the CD3 positive cells in the upper left quadrant.

9. Quadrant statistics were then applied to calculate the percentage of cells seen in the upper right quadrant therefore expressing both CD3 and CCR5.

11.2.2 Antibodies and reagents

There were 2 antibodies used in this set of experiments. Firstly an anti-CD3 antibody which was used to select T-lymphocytes from B-lymphocytes, and secondly an anti-CCR5 antibody which was used to quantify the CCR5 receptor protein present. Both antibodies are commercially available and were purchased from Becton Dickinson Biosciences.
The CD3 antibody used was labelled with phyco-erythrin, a chemiluminescent dye which emits pink light (wavelength 575nm) and can be detected by the flow cytometer. Following identification of the total lymphocyte population by ‘gating’ around a cell population with typical FSC and SSC characteristics, a more specific gate was applied therefore isolating a lymphocyte population for analysis.

The anti CCR5 antibody used was also a chemiluminescent labelled mouse anti-human antibody. The dye used in this case was fluorescein isothiocyanate (FITC) which emits green light of wavelength 521nm. It was important to use different chromophores in order to distinguish between the total T-lymphocyte population and the subpopulation possessing the CCR5 receptor.

Several commercially available solutions were used during the antibody incubation stage. These were also purchased from Becton Dickinson and were designed for use with the FACSCalibur flow cytometer.

1. FACSLyse Reagent. This solution contains 30% diethylene glycol and 10% formaldehyde and is used for red cell lysis.

2. Phosphate buffered saline (PBS) with 0.1% azide. The PBS is simply used as a wash and the 0.1% azide functions as a preservative for this solution.

3. FACSFlow solution. This contained 10mM PBS and 150mM sodium chloride at pH 7.4 and was used to re-suspend the cells for analysis. It was also used as the sheath fluid of the cytometer itself.
11.2.3 Optimisation experiments

Before this protocol was used, a series of optimisation experiments were carried out to ensure that the incubation temperatures and volume of antibody used in the standard protocol were appropriate for the detection of CCR5.

Firstly, the temperature of incubations was varied, using 4°C and 22°C. Each experiment was set up using different volumes of antibody in order to establish which combination produced the clearest results. The volume of PE-CD3 added was kept constant as 10μl is known to produce reliable results in this laboratory.

Therefore, 6 experiments were included, all using the same sample:

1. 10μl FITC-CCR5, 10μl PE-CD3 incubated at 4°C
2. 15μl FITC-CCR5, 10μl PE-CD3 incubated at 4°C
3. 20μl FITC-CCR5, 10μl PE-CD3 incubated at 4°C
4. 10μl FITC-CCR5, 10μl PE-CD3 incubated at 22°C
5. 15μl FITC-CCR5, 10μl PE-CD3 incubated at 22°C
6. 20μl FITC-CCR5, 10μl PE-CD3 incubated at 22°C

The results are given in Appendix 5 (example in Figure 11.3). As seen by the distribution in these dot plots, the addition of extra antibody did not increase the sensitivity of the technique and therefore a final antibody volume of 10μl per sample was used. Table 11.4 demonstrates that incubating at 4°C actually impaired the FITC-CCR5 antibody interaction with membrane bound CCR5 receptor and the percentage of positive cells detected was significantly less than those
incubated at 22°C. Therefore all incubations were carried out in the room temperature incubator at 22°C as stated in the original protocol.

Figure 11.3 Example of dot plot from optimisation experiments.

This dot plot shows on the left hand side, a subgroup of leucocytes (recognisable by their characteristic position in relation to the forward and side scatter scales), which are then isolated and displayed on the right hand plot. The T-lymphocytes are seen in the top half of the grid (identified by their characteristic fluorescence with PE-CD3) and those in the top right hand corner represent the CCR5 expressing cells (recognised by their fluorescence with FITC-CCR5).

<table>
<thead>
<tr>
<th>Incubation (°C)</th>
<th>Temperature</th>
<th>Volume of FITC-CCR5 (µl)</th>
<th>Percentage of CCR5 positive cells detected</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>10</td>
<td>3.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.34</td>
<td></td>
<td>P=0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Using unpaired t test, t=8.04 with 4 degrees of freedom</td>
</tr>
<tr>
<td>22°C</td>
<td>10</td>
<td>9.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>9.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>9.77</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11.4: Optimisation experiments.

This table summarises the optimisation experiments indicating a better yield of CCR5 positive cells with room temperature incubations and no additional benefit to using larger volumes of antibody.
11.3 Results

There were 17 patients included in this set of experiments. It was hoped to recruit 6 patients of each genotype into this arm of the study, however as there were only very few subjects homozygous for the Δ32 allele, this was not possible. In this group of 17, there were 6 homozygotes for the wild type gene, 6 heterzygotes and 5 homozygotes for the deletion.

Quadrant statistics describe the number of events (cells) which have been detected by fluorescence from both of the antibodies. In this case, cells expressing both CD3 and CCR5 were identified in this way in the right upper quadrant of the dot plot. The quadrant statistic represents the number of cells which fell within the right upper quadrant as a proportion of the total number of cells detected. The results for each patient are given in Table 11.5 demonstrating the percentage of cells which stained positive with FITC-CCR5. The dot plots are given in detail in Appendix 5.
Table 11.5: Quadrant statistics demonstrating percentage of cells expressing CCR5.

<table>
<thead>
<tr>
<th>Patient Code</th>
<th>Genotype</th>
<th>% CCR5 positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA16</td>
<td>Δ32/Δ32</td>
<td>0.27</td>
</tr>
<tr>
<td>BA22</td>
<td>WT/Δ32</td>
<td>5.25</td>
</tr>
<tr>
<td>BA48</td>
<td>WT/Δ32</td>
<td>6.86</td>
</tr>
<tr>
<td>BA69</td>
<td>WT/Δ32</td>
<td>8.84</td>
</tr>
<tr>
<td>BA78</td>
<td>WT/Δ32</td>
<td>23.84</td>
</tr>
<tr>
<td>BA101</td>
<td>WT/Δ32</td>
<td>12.13</td>
</tr>
<tr>
<td>BA111</td>
<td>Δ32/Δ32</td>
<td>0.07</td>
</tr>
<tr>
<td>BA128</td>
<td>WT/WT</td>
<td>8.53</td>
</tr>
<tr>
<td>BA145</td>
<td>WT/WT</td>
<td>18.87</td>
</tr>
<tr>
<td>BA155</td>
<td>WT/WT</td>
<td>17.03</td>
</tr>
<tr>
<td>BA164</td>
<td>WT/Δ32</td>
<td>19.30</td>
</tr>
<tr>
<td>BA170</td>
<td>WT/WT</td>
<td>45.82</td>
</tr>
<tr>
<td>BA171</td>
<td>WT/WT</td>
<td>10.51</td>
</tr>
<tr>
<td>BA174</td>
<td>WT/WT</td>
<td>10.92</td>
</tr>
<tr>
<td>BA228</td>
<td>Δ32/Δ32</td>
<td>0.36</td>
</tr>
<tr>
<td>BC74</td>
<td>Δ32/Δ32</td>
<td>1.01</td>
</tr>
<tr>
<td>BC78</td>
<td>Δ32/Δ32</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Although this data appears to fit a normal distribution, it has been subjected to non-parametric statistical testing as the standard deviations differ greatly between groups. Median values by CCR5 genotype were 14% in the group homozygous for the wild type gene, 10.5% in heterozygotes and 0.36% amongst subjects homozygous for the Δ32 allele. In view of this, and accounting for the small numbers in each group, a Kruskal Wallis test has been used with a post-hoc Dunn's multiple comparison test. The Kruskal Wallis statistic was found to be 10.21 with a significant P value of 0.006. When this was subjected to the multiple comparison test, 2 of the three comparisons remained significant (see Table 11.6).
Table 11.6: Multiple comparison testing of CCR5 expression by genotype. This table demonstrates a significant difference in cell surface expression of CCR5 by genotype in all but the homozygous wild type vs heterozygous comparison.

Although only two of the subgroup analyses remain significant, there is a clear trend seen towards greater CCR5 expression with increasing numbers of the wild type allele (see Figure 11.7) and in view of the small numbers involved in this arm of the project, this is strong evidence to support the theory that CCR5 Δ32 is a biologically active polymorphism.
Figure 11.7a: CCR5 expression by genotype
Where the boxes represent the mean values and the error bars represent the mean +/- 1 standard deviation.
Chapter Eleven: Detection of CCR5 receptor by Flow Cytometry

Figure 11.7b: CCR5 expression by genotype
Where lines represent the median, boxes represent the 25-75th percentile and whiskers represent the 95th percentile. The asterisks represent extreme outlying values.

These graphs demonstrate a clear trend towards lower CCR5 expression with fewer CCR5 wild type alleles, indicating that the CCR5 Δ32 allele impairs cell surface expression of CCR5.
11.4 Discussion

This chapter describes a small qualitative study to determine whether the CCR5 Δ32 polymorphism is biologically active. Success with demonstrating CCR5 has been described previously (Wang 2004, Segerer 1999) but no reports characterise the biological activity of the Δ32 polymorphism.

The technique used was adapted from a clinical protocol and optimised for use with the FITC-CCR5 mouse antihuman monoclonal antibody. Optimisation confirmed the incubation temperatures and concentration of antibody to be used.

Flow cytometry is a reliable and reproducible way of demonstrating cell surface proteins. There is an element of subjectivity introduced however, when applying both the lymphocyte gates, but more importantly the quadrant which allows statistical analysis. The quadrant is placed manually aiming to leave no acquired events in the lower right quadrant of the dot plot. Although with some populations of cells, there is a distinct and separate group in the area of interest (ie. the upper right quadrant in this case), the CCR5 positive population appeared to produce more of a spectrum, with clearly distinct populations observed but a ‘grey’ area lying between. In order to minimise the user error and bias introduced by this, samples were anonymised for analysis so that the operator did not know the sample genotype, and the right hand limit of the B lymphocyte population in the lower left quadrant was used as a guide to the positioning of the quadrant.

This work has produced strong evidence to suggest that the Δ32 allele is biologically active and that possession of one or more Δ32 alleles leads to decreased expression of functional CCR5 receptor protein.
Chapter Twelve

DISCUSSION AND CONCLUSIONS
Abdominal aortic aneurysm is a significant health problem affecting up to 12% of men over 65 years and is responsible for over 1% of male deaths in the 65-85 year age group in the western world. Mortality from aneurysm rupture continues to be up to 50% in spite of recent improvements in perioperative and intensive care. The most significant risk factor for aneurysm related death is aneurysm rupture and this is directly related to aneurysm size.

Currently, there is limited understanding of the factors responsible for aneurysm development, expansion and rupture and the main focus of research is aimed towards identifying underlying mechanisms for aneurysm development and from this, identifying potential therapeutic targets for their prevention or medical treatment. Following on from this, work into a potential genetic component to aortic aneurysm began in the 1960s and continues to suggest an inheritable element to this condition.

Population based studies have proposed differing theories and models of inheritance and some have suggested a single gene polymorphism may be responsible. Many candidate genes have been studied and some do appear to demonstrate a significantly different pattern amongst subjects with aneurysms to those without, however, no single gene polymorphism to date has been identified which fully explains the complex inheritance patterns seen.

Other risk factors such as smoking and hypertension are well established to be associated with an increased prevalence of abdominal aortic aneurysm and it is likely that the interplay between a genetic predisposition and environmental triggers is ultimately responsible for individual susceptibility to aneurysm formation.
Experimental work exploring the genetic component has to date focused largely on a candidate gene approach centred around inflammatory cytokine genes as cytokine pathways are now well established to be part of the inflammatory upregulation associated with aortic aneurysm. Cytokine pathways represent a highly complex interplay of systems and it therefore seems unlikely that one single step will, in isolation, be responsible for the whole process of aneurysm pathogenesis right from initiation through expansion and eventually to rupture.

CCR5 is a central chemokine receptor which modulates a variety of different cytokine pathways and functions. A deletion polymorphism, the Δ32 polymorphism, exists in the promoter region of the gene which is responsible for a frameshift and truncation of the mature protein. The role of this polymorphism in the development of AAA was investigated.

Firstly, a large number of cases and controls were recruited into the study and blood taken and stored for later study. They were selected randomly rather than ‘matched’ although because of the large numbers involved, the populations were broadly matched except for known aneurysm risk factors such as smoking which were found with greater frequency among the aneurysm group. DNA was extracted from the stored blood and a series of genotyping experiments performed. Initially there were problems with DNA contamination of the PCR reaction and this was dealt with using DNA degradation enzymes. The work was repeated ensuring several measures were in place to prevent recurrence of this problem, and the CCR5 genotype compared between groups in terms of presence or absence of aneurysm.
Chapter Twelve: Discussion and Conclusions

No difference was identified in allele frequency between the aneurysm and the control group and therefore a set of experiments to examine the biological activity of the Δ32 polymorphism were conducted. Flow cytometry was used to examine the percentage of lymphocytes expressing CCR5 and comparisons were made across genotypes. This revealed greater expression of CCR5 with increasing number of wild type CCR5 alleles, suggesting that presence of the Δ32 polymorphism does indeed reduce the amount of functional CCR5 protein produced. It is therefore likely that the CCR5 protein and Δ32 polymorphism do not play a significant role in the modulation of cytokine activity resulting in AAA.

Reports of a soluble 22kDa CCR5 protein were therefore investigated using a combination of ELISA and western blot techniques, however it was not possible to reproduce the published results and it has therefore not been possible to confirm the presence of such a protein. In fact, the antibody binding was found to be very non-specific in the series of experiments performed and it was therefore not reliable to assume that any increase in fluorescence was related to CCR5 binding rather than cross reactivity with other plasma proteins.

There are two major conclusions which can be drawn from this thesis. Firstly that the CCR5 Δ32 polymorphism is biologically active and exerts a significant effect on the amount of functional membrane bound CCR5 protein expressed on lymphocytes in human peripheral blood; and secondly that there is no significant difference in expression of this polymorphism between subjects with aneurysms and healthy controls, suggesting that the CCR5 receptor itself does not play a crucial role in the presence of or indeed prevention of abdominal aortic aneurysms.
These conclusions do not come as any surprise when the complexity of the inflammatory process is considered. It seems highly probable that a combination of genetic factors may predispose to aneurysm formation and these may at least in part be in the form of single nucleotide polymorphisms, but it is highly unlikely that one single cytokine gene polymorphism will be solely responsible for an inherited propensity to AAA. It is far more logical to assume that in the absence of one fully functional gene, another similar gene is upregulated to compensate for the defective gene, and this would be in keeping with the apparently normal immune function observed in patients who develop aneurysms.

There are significant limitations in adopting this candidate gene approach to research complex genetic diseases. It relies on the existing knowledge of a subject being sufficient to identify genes which are likely to be involved. This may lead to a lengthy and inefficient search of individual genes and may not ultimately provide any new insight into the condition of interest. In many areas of genetic research, there has been a trend towards whole genome analysis using candidate gene investigation only with the prior evidence of linkage. In this way, fewer assumptions are relied upon during the early stages of research. Future work into AAA is likely to follow this pattern and new cellular pathways may be found to be of interest. In the immediate future, aneurysm mortality will continue to be addressed through screening programmes to identify subjects with aneurysms which can be monitored and electively repaired, thus reducing the risk of rupture with its associated significant morbidity and mortality.
APPENDIX 1: Ethical Approval Documentation
Dear Mr Sayers

ID: 08246 The role of cytokines and cytokine gene polymorphisms in the development of abdominal aortic aneurysms.

LREC Ref: 6819 MREC Ref:

We have now been notified by the Ethical Committee that the proposed amendment to this project listed below has been given ethical approval (please see the attached letter from the Ethical Committee).

Amendment form (unsigned and dated) ethics approved copy dated 27th June 2006
Protocol version 4 dated June 2006
Information Sheet (controls) version 5 dated June 2006
Consent form (controls) version 5 dated June 2006
Information Sheet (non-operative) version 4 dated June 2006
Consent form (non-operative) version 4 dated June 2006
Information Sheet (operative) version 3 dated June 2006
Consent form (operative) version 3 dated June 2006

I can therefore now re-confirm the full approval of this project on behalf of the University Hospitals of Leicester NHS Trust.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your original notification form (and subsequent amendments).

The project continues to be covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

Please make sure if you or other researchers have an honorary contract with the Trust that this stays within date whilst working on the research study.

In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.
Below is a list of the Researchers Approved to work on this Application within UHL.
Mr Gersint Lloyd
Prof Kilian Mellon
Mr Gaiaask Beeharry Panray
Miss R Sandford
Mr Robert Sayers

Yours sincerely,
Nicola Tomer
R&D Office Manager
11th July 2006

Mr R D Sayers
Consultant Vascular Surgeon
University Hospitals of Leicester
Clinical Sciences Building
Leicester Royal Infirmary
Leicester, LE2 7LX

Dear Mr Sayers

Study title: The role of cytokines and cytokine gene polymorphisms in the development of abdominal aortic aneurysms

REC reference: 6819

Amendment number: 3
Amendment date: June 2006

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on 7th July 2006.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

- Notice of Substantial Amendment Form
  Dated: 27th June 2006

- Covering Letter
  Dated: 27th June 2006

- Protocol
  Version 4
  Dated: June 2006

- Patient Information Sheet
  Version 3
  Dated: June 2006

- Patient Consent Form
  Version 3
  Dated: June 2006

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority
Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Research governance approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects research governance approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

I 6819: Please quote this number on all correspondence

Yours sincerely

Ms Linda Ellis
Committee Co-ordinator

E-mail: linda.ellis@rushcliffe-pct.nhs.uk

Copy to:

R&D Department for NHS care organisation at lead site - UHL

Enclosures
List of names and professions of members who were present at the meeting and those who submitted written comments

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority
An investigation into candidate genes for abdominal aortic aneurysms

RD Sayers
Reader in Surgery, University of Leicester, Leicester, UK


Introduction

Abdominal aortic aneurysm (AAA) is predominately a disease of elderly males, occurring in 6.7% of males over 65 years of age (Loh 1989). They are the 7th commonest cause of death in England and Wales and cause 14,123 deaths per annum (National Statistics 2003). The number of deaths caused by AAA is increasing and despite advances in surgery and intensive care medicine there has been little reduction in the mortality of ruptured AAA over the last four decades (Bown 2002). In addition, this increase has been largely among those aged over 80 years who are a high risk for major surgery, currently the only treatment for AAA. A better understanding of the mechanisms of pathogenesis may translate into better and possible non-surgical treatments such as pharmacotherapy (drug treatment) for small AAAs to prevent growth and rupture.

There is evidence that genetics contribute to the pathogenesis of AAA. Patients with first degree relatives with AAA are at increased risk of AAA themselves (Johansen 1986; Darling 1989; Baird 1995). The majority of laboratory studies aiming to identify genes
associated with AAA to date have adopted a candidate gene approach. A deletion/interstitial in the angiotensin converting enzyme (ACE) gene that is associated with elevated plasma ACE levels has been shown to be more common in patients with AAA than in control groups (Fatini 2005; Pola 2001). Other genetic polymorphisms that have been shown to be associated with AAA are an oestrogen receptor beta (ER) single nucleotide polymorphism (SNP) (Massart 2004), a haem oxygenase 1 (HOI) promoter dinucleotide repeat (Schillinger 2002), an interleukin-10 promoter SNP (Bown 2002), a methylene tetrahydrofolate reductase (MTHFR) SNP (Strauss 2003), a platelet activating factor acetylhydrolase (PAFAH) SNP (Unno 2002), a common chemokine receptor-5 (CCR-5) insertion/deletion (Ghilardi 2004), a matrix metallo-proteinase-9 (MMP-9) SNP (Jones 2003) and two tissue inhibitor of metalloproteinase-1 (TIMP-1) SNPs (Ogata 2005). In addition some genetic polymorphisms have been shown to be associated with growth of small AAA but not necessarily the presence of AAA per se (Yeung 2002 – ACE).

In the majority of the studies above the findings are biologically plausible. An ACE promoter deletion associated with high plasma ACE levels is commoner in patients with AAA and since high ACE leads to high Angiotensin II and this is over-expressed in the wall of AAA the authors suggest that this may be a causal link (Fatini 2005; Pola 2001). Patients with AAA have been shown to have a higher frequency of long HOI promoter dinucleotide repeats which impairs the ability of cells to upregulate the production of HOI in response to stimuli. This reduction in HOI which is an anti-oxidant and anti-inflammatory was postulated by the authors to be related to the inflammatory degradation
genetic information get shorter and shorter. In order to protect the genetic information on
the chromosomes from becoming compromised by this successive shortening the ends of
each chromosome are capped with areas called telomeres that do not carry genetic
information that can safely be sacrificed as the cells age. However once these telomeres
become too short the cell is unable to divide any longer and becomes non-functional
(senescent) and may actually die. The length of a persons telomeres can be measured and
is thought to reflect true biological age.

Current evidence suggests that telomere shortening is associated with age-dependent
dysfunction of blood vessels (Aviv 2002) and may play a role in the development of
hardening of the arteries (atherosclerosis) which shares common risk factors with
AAA (Brouilette 2003). Patients who develop AAA may do so because the abdominal
aorta becomes unable to repair itself as the cells within it become senescent with age and
cumulative damage results in the formation of and aneurysm. It may be that by measuring
telomere length the presence of or potential to develop an AAA could be determined.

Our hypothesis is that the mean telomere length is decreased in patients with abdominal
aortic aneurysm. Although there may be slight difference between different types of
tissues, white blood cells can be used as a marker for the extent of telomere length
shortening. Therefore, our null hypothesis is that there is no difference in telomere length
between patients with abdominal aortic aneurysm and without aneurysm.
This project will investigate telomere lengths and the following candidate genes for abdominal aortic aneurysms:

<table>
<thead>
<tr>
<th>Candidate Gene</th>
<th>Reference</th>
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<tr>
<td>(HUGO nomenclature)</td>
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<tr>
<td>PAFAH</td>
<td>Unno 2002</td>
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<tr>
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<td>Massart 2004</td>
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<td>TIMP-1</td>
<td>Ogata 2005</td>
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<td>ACE</td>
<td>Fatini 2005</td>
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<td>Jones 2003</td>
</tr>
<tr>
<td>HO-1</td>
<td>Schillinger 2002</td>
</tr>
<tr>
<td>IL-10</td>
<td>Bown 2002</td>
</tr>
</tbody>
</table>

Aims of the Project

1. To determine whether the genetic polymorphisms listed above and white blood cell telomere lengths are associated with abdominal aortic aneurysms.

2. To investigate a possible relationship between the genetic polymorphisms above and their protein products.

3. To determine whether any of the gene products produced by the genes of interest are expressed in the aneurysm wall, and if so by which part of the wall and
whether aortic wall telomere lengths are related to white blood cell telomere lengths.

4. To investigate whether any of the gene products produced by the genes of interest plasma levels differ significantly between patients with abdominal aortic aneurysms and those who have a normal aorta

5. To determine whether any of the gene products produced by the genes of interest are produced by the arteries of patients with aneurysms at sites remote from the aneurysm.

Methods

1. Patients who are known to have aneurysms will be selected as cases from the aneurysm screening program, out-patient clinics and in-patients prior to aneurysm repair. Age and sex matched controls who are known not to have aneurysms following abdominal ultrasound or CT scans (as part of their medical care) will be selected. Venous blood samples will be taken from each participant and separated into plasma and white cells and stored for subsequent analysis. Polymerase Chain Reaction (PCR) based techniques will be used to examine the genetic polymorphisms of interest and telomere lengths in the 2 groups.

2. In a sub-group of patients undergoing open aneurysm repair a 1x1cm specimen of aneurysm wall will be removed and analysed for protein levels using
immunohistochemistry and in addition the mean telomere lengths will be assayed. Specimens of aneurysm wall are currently being removed and examined as part of another study in Leicester. We also seek approval to use reverse transcriptase polymerase chain reaction (RT-PCR) to study mRNA expression in the aorta. mRNA is produced by cells as a template for protein synthesis and gives information on which genes are switched on in the tissue at a specific time. This would complement the work on protein expression shown by ELISAs. Furthermore we would like to culture these tissue samples to measure cytokines, inflammatory markers and MMPs in an explant model system. These additional techniques only affect the tissue after removal from the patient and do not affect the participant’s health in any way.

3. The aneurysm wall will be examined for the production and expression of MMPs using immunohistochemistry, in-situ hybridisation and in-situ zymography. The techniques employed have been previously used in the University of Leicester Department of Surgery laboratory.

4. In patients undergoing aneurysm repair, a 1cm length of inferior mesenteric artery (IMA) will be removed (the IMA is disconnected during most aneurysm repairs). A similar number of patients without aneurysms undergoing left sided colonic resections in which the IMA is also removed will form the control group. The IMA specimens will be examined for IL-10 production again using immunohistochemistry.
5. Twenty patients who have previously given their permission and participated in the study will be re-approached and a further 10ml sample of venous blood taken. This will then be used for flow cytometry (which requires fresh rather than frozen blood) to quantify the level of the cytokine receptor CCR5 on the surface of T lymphocytes. It is anticipated that these patients will be seen initially at their pre-operative assessment visit, genotyped, and then re-approached when admitted to hospital for their surgery.
References


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Agarose
Supplied by Sigma, product code A9539

Alkaline Phosphatase-Conjugated Streptavidin
Containing:
  Streptavidin alkaline phosphatase from Streptomyces Avidinii, supplied as lyophilized powder and reconstituted according to manufacturers instructions.
Supplied by Sigma, product code S2890

'Blue Juice' Gel Loading Buffer
Supplied by Invitrogen, product code 10816-015

Bovine Serum Albumin
Supplied as lyophilized powder by Sigma, product code A7906 and reconstituted according to manufacturers instructions in 1L distilled water.

Cell Lysis Solution
Containing:
  Tris (hydroxymethyl) aminomethane
  Ethylenediaminetetraacetic acid (EDTA)
  Sodium Dodecyl Sulphate
Supplied by Gentra, product code D50K2-1L

Deoxyribonuclease I
Supplied by Invitrogen, product code 18047-019

Developing Solution 'ECL'
9ml water
1ml 1M Tris-HCl, pH 8.5
22µl 90mM p-coumaric acid in DMSO (dimethyl sulfoxide)
50µl 250mM luminal in DMSO (dimethyl sulfoxide)

Diethanolamine
Supplied by Sigma, product code D8885

DNA Rehydration Solution
Containing:
  Tris (hydroxymethyl) aminomethane
Ethylendiaminetetraacetic acid (EDTA)
Supplied by Gentra, product code D0K4-500ml

dNTPs
Supplied by Invitrogen, product code 10297-018

Ethidium Bromide
Supplied by Invitrogen, product code 15585-011

Ethylendiamine tetraacetic acid (EDTA)
Containing:
186g EDTA
800ml distilled water
pH adjusted by adding Sodium Hydroxide until pH=8 (when EDTA pellets dissolve readily).

Isopropanol 99+%
Supplied by Sigma, product code IS 497-0

Mineral Oil
Supplied by Sigma, product code M5904

Monoclonal Antibodies
Mouse antihuman CCR5 MAb 2D7
Supplied as 0.1mg dry antibody by BD Biosciences, product code 555991 and reconstituted according to manufacturers instructions.

Biotinylated mouse anti-human CCR5 MAb FAB181B
Supplied as 500µg reconstituted antibody by R&D Systems, product code MAB181

P-nitrophenyl Phosphate
Containing:
SIGMAFAST p-nitrophenyl phosphate tablets and reconstituted according to manufacturers instructions.
Supplied by Sigma, product code N2770

PBS/Tween
Supplied in tablet form by Sigma, product code O8057 and reconstituted according to manufacturers instructions in 500ml distilled water.

**PCR 20 base pair low loop ladder**  
Supplied by Sigma, product code P1598

**Phosphate Buffered Saline (PBS)**  
Supplied in tablet form by Sigma, product code P3813 and reconstituted according to manufacturers instructions in 1L distilled water.

**Primers**  
Containing:  
CCR5 primer sequences as detailed below, supplied as desalted form and reconstituted according to manufacturers instructions.  
Forward primer: CAA AAA GAA GGT CTT CAT TAC ACC  
Reverse primer: CCT GTG CCT CTT CTT CTC ATT TCG  
Supplied by Sigma, made to specifications.

**Protein Precipitation Solution**  
Containing:  
Ammonium Acetate  
Supplied by Gentra, product code D0K3-500ml

**Red Cell Lysis Solution**  
Containing:  
Ammonium Chloride  
Ethylenediaminetetraacetic acid (EDTA)  
Sodium Bicarbonate  
Supplied by Gentra, product code D50K1-1L

**Red Taq DNA Polymerase**  
Supplied by Sigma, product code D4309

**TBE Buffer**  
Containing:  
54g Tris Base  
27.5g Boric Acid  
20ml 0.5M EDTA  
Dissolved in 1 litre distilled water to give a 5 times concentration.
Tris (hydroxymethyl) aminomethane
Supplied by Sigma in powder form, product code 252859
APPENDIX 3: DNA Quantifications
Table 8.3a DNA yield from aneurysm samples

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<th>Sample ID</th>
<th>A260</th>
<th>A280</th>
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<th>Conc (ng/μl)</th>
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BA44

Genotype
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Sample
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Genotype
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Sample
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Genotype
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WTAVT

W here WT = w ild type a llele and A 32= A32 polym orphic allele

244

Genotype
WTAVT
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Where WT = wild type allele and Δ32 = Δ32 polymorphic allele

245
APPENDIX 5: Flow Cytometry Dot Plots
Optimisation experiments

1. 10µl anti-CCR5 incubated at room temperature

2. 15µl anti-CCR5 incubated at room temperature
3. 20μl antiCCR5 incubated at room temperature

4. 10μl antiCCR5 incubated at 4°C
5. 15μl antiCCR5 incubated at 4°C

6. 20μl antiCCR5 incubated at 4°C
Dot plots for patients 1-17

Patient 1

Patient 2
Patient 3

Patient 4
Patient 5

Patient 6
Patient 7

![Graphs for Patient 7]

Patient 8

![Graphs for Patient 8]
Patient 13

Patient 14
Patient 17
APPENDIX 6: Manufacturers’ Details
Becton Dickinson
Becton Dickinson Biosciences
United Kingdom & Ireland
21 Between Towns Road
Cowley
OX4 3LY Oxford, UK

Gentra
Gentra Systems, Inc
13355 10th Avenue N
Suite 120
Minneapolis
MN 55441
USA

Invitrogen
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park,
Paisley
PA4 9RF
UK

Sigma-Aldrich
Sigma-Aldrich Company Ltd,
The Old Brickyard,
New Road,
Gillingham
Dorset
SP8 4XT
UK
BIBLIOGRAPHY


cells, and infection levels are regulated by compound CCR5 polymorphisms. Proc Nat Acad Sciences Unit Stat Amer. 100:8401-8406, 2003.


Mummidi S, Ahuja SS, McDaniel BL, Ahuja SK. The human CC chemokine receptor 5 (CCR5) gene. Multiple transcripts with 5'-end heterogeneity, dual promoter usage, and


292


295


Wang J, Crawford K, Yuan M, Wang H, Gorry PR, Gabuzda D. Regulation of CC chemokine receptor 5 and CD4 expression and Human Immunodeficiency Virus type I


'Nobody said it was easy, no-one ever said it would be this hard...
I'm going back to the start.'

Chris Martin
From The Scientist, Coldplay