THE DIFFERENTIAL EXPRESSION OF MICRORNA IN ABDOMINAL AORTIC ANEURYSMS

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Abstract

Abdominal aortic aneurysms (AAA) account for 5,251 deaths per year in the UK (2010). Their current incidence in the NHS AAA screening programme is 1.8%, however the vast majority are small. These patients therefore undergo surveillance prior to the need for surgical intervention.

Biomarkers for AAA have been extensively studied, with a meta-analysis, conducted herein, identifying a significant association between several biomarkers and AAA (upregulation of matrix metalloproteinase-2, matrix metalloproteinase-9, tissue inhibitor of matrix metalloproteinase-1, interleukin-6, interleukin-10, tumour necrosis factor-α, osteoprotegerin, osteopontin, interferon-γ, intercellular adhesion molecule-1, vascular cell adhesion protein-1, D-dimer, C-reactive protein, alpha-1 antitrypsin, fibrinogen, triglycerides, and lipoprotein(a), and downregulation of apolipoprotein-A and high density lipoprotein). However, the sensitivity and specificity of these biomarkers is poor, therefore this thesis aimed to look at the expression of microRNAs (miRNAs) in AAA, hitherto previously unstudied.

MiRNAs are short, non-coding RNA sequences which are transcribed from DNA, however they are not translated into protein. They exert their effect by attaching to messenger RNA (mRNA) and causing repression of translation into protein, and deadenylation, thus causing mRNA degradation. MiRNAs are capable of interacting with over 60% of known genes.

The studies within this thesis have undertaken a case control discovery and validation study into miRNAs in AAA, identifying a significant upregulation of 29 miRNAs within the discovery study, 4 of which were validated in blood (let-7e, miR-15a, miR-196b, miR-411), and miR-196b being further validated in plasma. There was however no miRNA dysregulation found in aortic tissue. In addition, these 4 miRNAs were found to have significant interactions with previously studied AAA biomarkers identified through earlier systematic review and meta-analysis.

The 4 miRNAs identified within this thesis were similarly dysregulated in patients with peripheral arterial disease, therefore they may be dysregulated due to generalised atherosclerosis rather than AAA, and must be interpreted with caution.

Statement of Authenticity

I certify that the work within this thesis is of my own creation and represents my own efforts and ideas, with the following exceptions. The literature search for aneurysm biomarkers (Chapter 2) was undertaken independently by myself and Mr David Sidloff, with the statistical analysis undertaken in combination, and I am indebted to Dr Nicolas Sylvius for his assistance regarding RNA processing and PCR. I understand that use of another individuals work without acknowledgement is plagiarism, and is grounds for disqualification. I certify that the writing of this thesis was solely by me, and is my original work.

Philip Stather

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3 - Sidloff DA, Stather PW, Choke E, Bown MJ, Sayers RD. A systematic review and meta-analysis of the association between markers of hemostasis and abdominal aortic aneurysm presence and size. Journal of Vascular Surgery. 2014;59(2):528-535

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2 - Stather PW, Sylvius N, Sidloff D, Wild JB, Butt HZ, Choke E, Sayers RD, Bown MJ. MicroRNAs let-7e, -15a, -196b and -411 Circulating regulators of atherosclerotic abdominal aortic aneurysms. Circulation Supplement (To be published)

3 - Stather PW, Sylvius N, Wild JB, Choke E, Sayers RD, Bown MJ. MicroRNAs implicated in peripheral vascular disease. British Journal of Surgery. 2013;100(S2):15-16.

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1 – Post-transcriptional dysregulation of extracellular matrix modifying genes in patients with abdominal aortic aneurysms. Stather PW, Sylvius N, Sidloff D, Wild JB, Butt HZ, Choke E, Sayers RD, Bown MJ. Society of Academic and Research Surgery, 08/01/2014.

2 - Post-transcriptional dysregulation of extracellular matrix modifying genes in patients with abdominal aortic aneurysms. Stather PW, Sylvius N, Sidloff D, Wild JB, Butt HZ, Choke E, Sayers RD, Bown MJ. The Vascular Society of Great Britain and Ireland, 27-29/11/2013.

3 - MicroRNAs let-7e, -15a, -196b and -411 circulating regulators of atherosclerotic abdominal aortic aneurysms. Stather PW, Sylvius N, Sidloff D, Wild JB, Butt HZ, Choke E, Sayers RD, Bown MJ. American Heart Association 17-20/11/2013.

4 - MicroRNAs implicated in peripheral vascular disease. Stather PW, Sylvius N, Wild JB, Choke E, Sayers RD, Bown MJ. European Society of Vascular Surgery 20-22/9/2012

5 - Markers of haemostasis, a potential prognostic indicator for patients with abdominal aortic aneurysm – A systematic review and meta-analysis. Sidloff DA, Stather PW, Choke E, Bown MJ, Sayers RD. British Society of Endovascular Therapy, 27-28/6/2013.

6 - MicroRNAs implicated in peripheral vascular disease. Stather PW, Sylvius N, Wild JB, Choke E, Sayers RD, Bown MJ. Vascular Society 28-30/11/2012.

Abbreviations

AAA – Abdominal Aortic Aneurysm
ACE – Angiotensin Converting Enzyme
AngII – Angiotensin II
antagomiR – miRNA antagonist
AOD – Aorto-Occlusive Disease
Apo – Apolipoprotein
CAD – Coronary Artery Disease
CAD – Cadaveric Donor
Cat – Cathepsin
cDNA – Complementary DNA
CI – Confidence Interval
COL - Collagen
COPD – Chronic Obstructive Pulmonary Disease
CRP – C-Reactive Protein
CT – Computerised Tomography
CTGF – Connective Tissue Growth Factor
DAB2IP – Disabled Homologue 2 interacting protein
DNA – Deoxyribonucleic Acid
DNase – Enzyme degrading DNA
ECM – Extracellular Matrix
EDTA – Ethylenediaminetetraacetic Acid
ELISA – Enzyme Linked Immunosorbant Assay
ELN - Elastin
EVAR – Endovascular Aneurysm Repair
GTP – Guanosine Triphosphate
GWAS – Genome-Wide Association Study
hAFB – Human Aortic Adventitial Fibroblast
hASMC – Human Aortic Smooth Muscle Cell
HCV – Hepatitis C Virus
HDL – High Density Lipoprotein
hsa – Abbreviated suffix for human miRNAs
ICAM-1 – Intercellular Cell Adhesion Molecule 1
IFN-γ – Interferon γ
IFU – Instructions For Use
IGF – Insulin-like Growth Factor
IGFBP - Insulin-like Growth Factor Binding Protein
IL – Interleukin
LATS – Large Tumour Suppressor
LDL – Low Density Lipoprotein
LDLR – Low Density Lipoprotein Receptor

LRP1 – Low Density Lipoprotein Receptor Related Protein
IQR – Interquartile Range
kD – kiloDalton
LA – Large Aneurysm (>55mm)
MAPK – Mitogen-Activated Protein Kinase
MASS – Multicentre Aneurysm Screening Group
MMP – Matrix metalloproteinase
mmu – Abbreviated suffix for mouse miRNAs
miR – Abbreviated suffix denoting a miRNA
miRNA – microRNA
miRNA seed – base pairs through which miRNAs interact with mRNAs
mRNA – messenger RNA
miRNome – miRNA transcriptome
MTHFR – Methylenetetrahydrofolate Reductase
NIHR – National Institute For Health Research
OPG – Osteoprotegerin
OPN – Osteopontin
OSR – Open Surgical Repair
PAD – Peripheral Arterial Disease
PAI – Plasminogen Activator Inhibitor
PCR – Polymerase Chain Reaction
PO – Post-operative
PPE – Porcine Pancreatic Elastase
Pre-miRNA – Precursor miRNA
Pri-miRNA – Primary microRNA
RCT – Randomised Controlled Trial
RISC – RNA-Induced Silencing Complex
RNA – Ribonucleic Acid
RNase – Enzyme degrading RNA
ROC – Receiver Operator Characteristic
RT – Reverse Transcription
RT-qPCR – Real-Time Quantitative PCR
SA – Small Aneurysm (30-54mm)
SNP – Single Nucleotide Polymorphism
SD – Standard Deviation
SEM – Standard Error of the Mean
siRNA – Short Interfering RNA
SI unit – International System of Units
SMAD – Mothers Against Decapentaplegic homologue
SMD – Standardised Mean Difference
SORT1 – Sortilin 1

TGF – Transforming Growth Factor
TIMP – Tissue Inhibitor of Matrix Metalloproteinase
TLDA – TaqMan Low Density Array
TNF – Tumour Necrosis Factor
tPA – Tissue Plasminogen Activator
USA – United States of America
USS – Ultrasound Scan
UTR – Untranslated Region
VCAM-1 – Vascular Cell Adhesion Molecule 1
VSMC – Vascular Smooth Muscle Cell

Contents

Chapter 1

Introduction to Abdominal Aortic Aneurysms

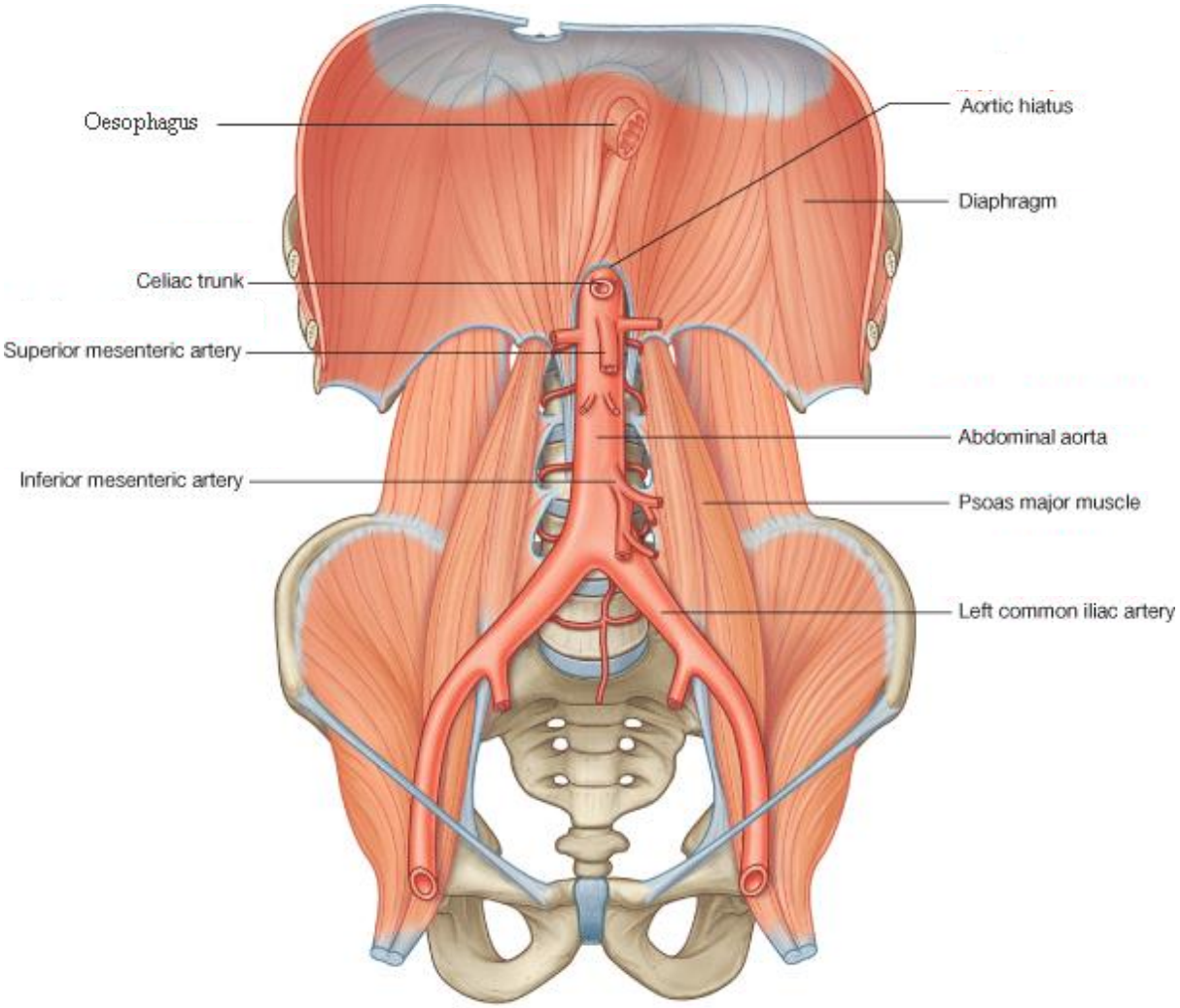
1.1 Abdominal Aortic Aneurysm

1.1.1 Normal Anatomy of the Abdominal Aorta

The aorta is the largest artery in the human body, beginning directly after the aortic valve, and terminating at its bifurcation into the common iliac arteries. The abdominal aorta commences at the level of the 12th thoracic vertebra where the aorta passes through the diaphragmatic hiatus. It then passes into the retroperitoneal space anterior to the vertebral bodies, just to the left of the midline. The abdominal aorta is approximately 13cm in length, and gives rise to several smaller vessels as it descends (Figure 1-1). The first branches are the paired inferior phrenic arteries which supply blood to the inferior surface of the diaphragm and the adrenal glands. The coeliac trunk then arises anteriorly to supply the stomach, liver, oesophagus, spleen, and superior halves of the pancreas and duodenum. The superior mesenteric artery also arises anteriorly, supplying blood to the foregut. The paired renal arteries arise at the level of the 1st lumbar vertebra supplying blood to the kidneys. The paired gonadal arteries arise at the level of the 2nd lumbar vertebra supplying blood to the ovaries in females and the testicles in males. Four or five pairs of lumbar arteries arise at the level of the 1st to 4th lumbar vertebrae supplying the spinal cord, vertebrae, and muscles of the back and posterior abdominal wall. The inferior mesenteric artery commences anteriorly at the level of the 3rd lumbar vertebra supplying blood to the hindgut. The median sacral artery stems from the posterior aspect of the abdominal aorta just superior to the level of its bifurcation supplying blood to the posterior surface of the rectum. Finally at the level of the 4th lumbar vertebra the aorta divides

into the left and right common iliac arteries supplying blood to the lower limbs and pelvis.

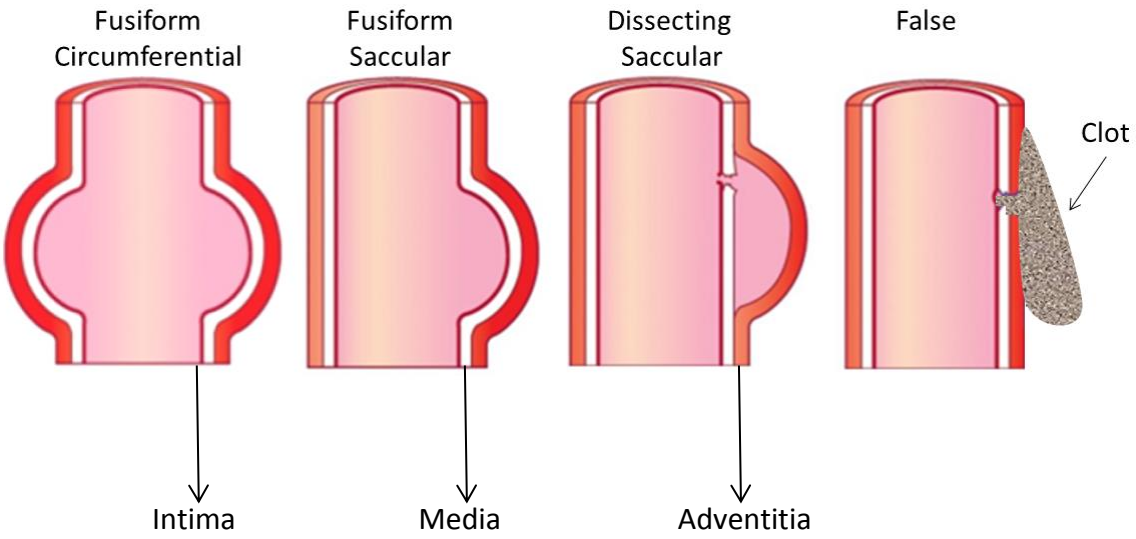
Figure 1-1 – Normal anatomy of the abdominal aorta (Reprinted with permission, © Elsevier. Drake et al: Gray's Anatomy for Students[1])



1.1.2 Aneurysm Classification

An aneurysm is an abnormal localised dilatation of an artery. Aneurysms may be true or false. A true aneurysm involves the intima, media, and adventitia of the arterial wall, and may be congenital, as a result of trauma, or due to infection. A false or pseudo aneurysm is a collection of blood around a vessel confined by the surrounding tissue. This typically occurs in the femoral artery due to its use for arterial catheterisation[2]. Aneurysms may also be classified by their type (Figure 1-2). Saccular aneurysms are roughly spherical in shape, and often filled by thrombus, whereas fusiform aneurysms are variable in their diameter and length[3]. Aneurysms may occur in any artery; however they are most common in the cerebral arteries, coronary arteries, thoracic and abdominal aorta, iliac vessels, femoral artery or popliteal artery[3].

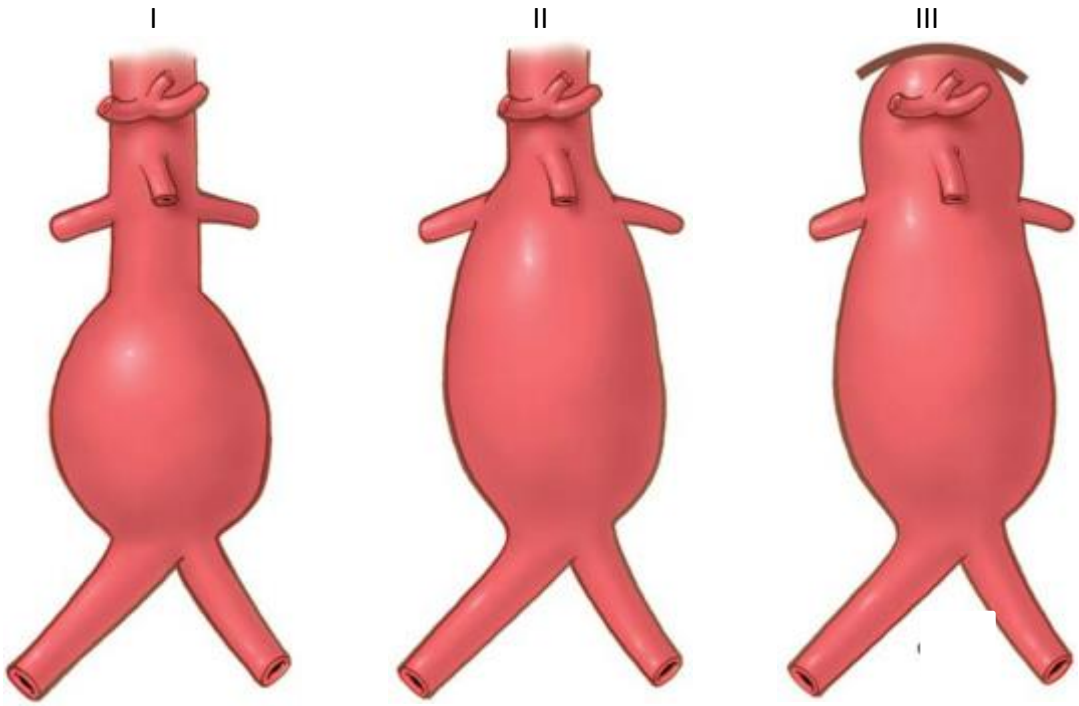
Figure 1-2 – Aneurysm classification



1.1.3 AAA Definition

An abdominal aortic aneurysm (AAA) is a localised dilatation of the abdominal aorta of greater than 50% of its normal diameter[4]. The normal diameter of the abdominal aorta varies between individuals, according to the patients' age, sex, and body surface area[5], although it is typically less than 25mm. The American Association for Vascular Surgery / Society of Vascular Surgery guidelines define an AAA as a diameter of 30mm or greater[6] as this is well above the average diameter for both sexes. AAAs may occur at any point in the abdominal aorta, however the most common location is just below the origin of the renal arteries, causing an infra-renal aneurysm (90%) (Figure 1-3). Supra-renal or para-renal aneurysms may also occur, and although the dilatation typically terminates prior to the aortic bifurcation, it may continue into the iliac arteries leading to an aorto-iliac aneurysm.

Figure 1-3 – Abdominal aortic aneurysm classification



I – infrarenal, II – pararenal, III – suprarenal (By permission of Mayo Foundation for Medical Education and Research. All rights reserved)[7]

1.1.4 History of AAA

The first written evidence of the existence of aneurysms was recorded in 1550BC in the 'Book of Hearts' from the Eber Scolls of ancient Egypt. In the 2nd century AD, the first description of the cause and treatment of an aneurysm was recorded by Antyllus[8], a Greek surgeon living in Rome. Antyllus is also credited with performing the first attempt at a AAA repair having described ligation of the aorta above and below an aneurysm, followed by incision into and emptying of the sac. In 1555, Vesalius was the first to diagnose an abdominal aortic aneurysm, with Lancisi publishing a text on the pathology of aneurysms in 1782[9]. It was not until the 18th Century that significant advances in both the physiology and surgical repair of blood vessels were made by

William and John Hunter, and the first surgeon to successfully ligate the abdominal aorta was one of John Hunter's students, Astley Cooper, doing so for a ruptured iliac aneurysm in 1817[10]. Several further techniques were also tried, including external compression, wiring and electrocoagulation, and cellophane wrapping, used most famously by Rudolf Nissen for the attempted treatment of Albert Einstein in 1949[11]. In 1888 the concept of endoaneurysmorrhaphy was developed by Rudolph Matas, obliterating the aneurysmal sac and oversewing collaterals, whilst preserving a lumen for blood flow[12]. Alexis Carrel pioneered anastomotic techniques, receiving a Nobel Prize in 1912 for demonstrating the feasibility of suturing arteries. With these techniques established, aneurysm repair could be conducted by suturing a graft to the aorta proximal and distal to the aneurysm, which was first performed in 1952 by Dubost[13]. Open AAA repair has remained largely unchanged over the past 60 years. The most significant recent major advance in AAA surgery was when Nikolay Volodos attempted an endovascular AAA repair (EVAR), however this was complicated by a graft occlusion requiring immediate conversion to open repair. Juan Parodi reported the first successful EVAR in 1991[14], thus beginning the modern era of minimally invasive vascular surgery.

1.1.5 AAA Epidemiology

Throughout the 20th century, both the incidence of AAA and its related mortality steadily increased in England and Wales[15], with screening programmes identifying a prevalence of 5% in men between the ages of 65-74[16]. More recent publications however have reported a much lower prevalence of AAA, being 1.9% in England[17] and 2.2% in Sweden[18] at the age of 65. There has been a decline in reported AAA mortality,

ruptured AAA admissions, and emergency AAA repair in recent years[19,20], and it is thought that risk factor modification has increased the age at which AAAs become clinically relevant by 5-10 years[21]. The decrease in smoking and improved control of both hypertension and hypercholesterolaemia have all been implicated as reasons for the decreasing prevalence of AAA[22,23].

1.1.6 Risk Factors for AAA

There are several risk factors for AAA formation, including gender, age, smoking, family history, ethnicity, cholesterol, hypertension and diabetes.

The prevalence of AAA in males is significantly increased compared to females, however, the risk of aneurysm rupture is almost 4 times higher in women than men[24]. The Rotterdam Study[25] offered 5419 participants an abdominal ultrasound scan, diagnosing an AAA if the aortic diameter exceeded 35mm, or more than 50% of normal aortic diameter (normal aortic diameter measured at the level of the superior mesenteric artery). They found a prevalence of 4.1% men, and 0.7% of women had an AAA. Another study assessed 5394 participants finding a prevalence of 7.6% in men and 1.3% in women[26]. The male to female ratio of AAA has been reported between 3.1:1[27] to 8:1[28], however the association of male sex is confounded by its correlation with several additional risk factors for AAA including peripheral arterial disease, hypertension, hypercholesterolaemia, coronary artery disease, immune diseases, smoking, sex hormones, alcohol, obesity, and physical activity[29].

A study looking at the influence of screening on AAA rupture[26] identified age as a significant risk factor for AAA, with men aged 65-70 years having a prevalence of 5.9%, increasing to 9.2% for those aged 76-80 years. Similar results were also seen in

women, reporting an incidence of 1% in those aged 65-70 years, increasing to 1.6% for those 76-80 years old. Recent studies have identified an increasing rate of non-ruptured AAA admission in elderly populations, with an increasing rate of non-emergency AAA repairs in this age group[19]. It is therefore likely that AAA prevalence is shifting to a more elderly population, potentially due to risk factor modification.

Smoking is one of the main risk factors implicated in cardiovascular disease[30,31], although the exact mechanism and constituent of cigarette smoke causing atherosclerotic disease remains unknown[32]. It has been shown to increase the growth rate of AAAs by 0.35mm per year[24], and double the rupture rate[24]. The association between smoking and AAA has also been shown to increase significantly with the number of years of smoking, and decrease significantly with the number of years after smoking cessation[33]. Although the mechanism through which smoking increases cardiovascular risk remains unknown, recent work has identified nicotine as the constituent of cigarette smoke which causes AAA development[34], therefore it is not only smoking cessation but avoiding the use of nicotine patches which is required to decrease this risk. It may therefore be nicotine increasing the risk of AAA, and other constituents of cigarette smoke causing atherosclerotic disease.

The effect of family history on AAA was first hypothesised by Clifton[35] with further studies identifying the frequency of AAA in first degree relatives as 15-20%[36,37], with familial AAAs more likely to occur at a younger age, and lead to rupture[38]. Evidence from the Swedish Twin Registry[39], containing 265 twins with AAA, identified the probandwise concordance rates (the probability that a twin is affected given that his/her co-twin is affected) for monozygotic and dizygotic twins to be 24% and 4.8%

respectively, providing robust evidence that heritability contributes to aneurysm formation. It is however clear that the underlying genetics causing susceptibility to AAA are not all that is required for the aneurysm to develop, and the pattern of inheritance of AAA remains poorly understood. A study of 50 families including 3 pairs of monozygotic twins suggested a single autosomal dominant gene was responsible for AAA inheritance[40]. A further study of 60 patients, 25 with a positive family history, assumed a multifactorial model with 70% heritability (i.e. 70% of the variation between individuals can be accounted for by their genes, with 30% due to environmental factors or random chance). Additional studies have assumed an autosomal recessive inheritance[41], and an autosomal dominant inheritance with low penetrance[42]. Therefore, clear evidence exists as to a genetic predisposition to AAA, however there remains no clear pattern of inheritance, and it is likely that it is a combination of genetic and environmental factors that predispose to aneurysmal dilatation.

AAA are typically a disease of the western world, with studies revealing that Caucasian men are affected significantly more than Asian immigrants[43] with reported prevalence decreasing from 4.69% in Caucasian men to 0.45% in Asians[44]. There is also a lower prevalence in African-Americans[33], Japanese[45] and Chinese[46], which are likely to be due to genetic variation rather than environmental factors as these populations do not increase their risk of aneurysm formation through migration. Maori males also have a lower prevalence of AAA, whereas the prevalence in Maori females is significantly higher than European females. In addition both male and female Maori's tend to

develop AAA at a younger age (8.3 years younger), and have higher mortality rates and rupture rates[47,48].

Hypercholesterolaemia has been reported to be a risk factor for AAA, with a meta-analysis of 8 studies revealing a significantly lower serum HDL cholesterol level and significantly higher serum LDL cholesterol in the AAA group compared to controls[49].

The correlation between hypertension and AAA remains uncertain, with inconsistency seen between trials. A meta-analysis of 9 studies in 2004 concluded that the presence of hypertension increased the risk of AAA although the association was weak (Odds Ratio 1.33, 95% Confidence Interval 1.14 to 1.55)[50]. A more recent meta-analysis concluded that mean arterial blood pressure had no impact on AAA growth rates, however increased pulse pressure was associated with slower growth rates[24]. Hypertension is however associated with an increase in rupture rates[24]. This study also determined that the use of medications to treat hypertension did not significantly reduce aneurysm growth rates.

Diabetes mellitus is consistently found to be a negative risk factor for AAA, since early studies from the 1990's[25,51,52] were validated by the Aneurysm Detection and Management (ADAM) study[33]. When examining aneurysms with a diameter greater than 40mm this association becomes highly significant (Odds Ratio 0.50, 95% Confidence Interval 0.39 to 0.65)[53], and there is also evidence that diabetics are less likely to have an AAA that is ruptured at the time of repair[54]. Studies looking at the growth rate of AAAs have revealed that diabetes decreases aneurysm growth rates by 0.51mm per year[24]. The reasons for this remain unclear, although studies have hypothesised that diabetes leads to cross-linking of aortic lattices in the aortic media

which resists proteolysis and inhibits matrix metalloproteinase (MMP) secretion, which is thought to mediate AAA development[55]. Diabetes also suppresses plasmin generation (an activator of MMPs) through effects on plasminogen activator inhibitor-1[56]. This results in a decrease in the rate of aortic wall degradation, resulting in the thicker aortic wall observed in patients with diabetes[57].

The prevalence of AAA in patients with peripheral arterial disease (PAD) is reported to be as high as 9%[58], with a meta-analysis of 8 studies identifying an increased risk of AAA in patients with PAD (Odds Ratio 2.50, 95% Confidence Interval 2.21 to 2.95)[50]. The strength of this association has led to the introduction of screening for PAD alongside AAA screening in 2 trials currently on-going in Denmark (ClinicalTrials.gov NCT00662480) and Norway (NCT01248533).

1.1.7 AAA Pathogenesis

The aortic wall consists of the innermost tunica intima, the tunica media, and the outermost tunica adventitia. The tunica intima consists of a single layer of endothelial cells and a basal membrane. The tunica media is the thickest layer, consisting of smooth muscle cells, collagen, and elastic fibres. The tunica adventitia consists of connective tissue and the vasa vasorum, supplying nutrients to the arterial wall. AAA is a degenerative condition of the aneurysm wall[59], which was originally thought to be due to atherosclerosis. However, as aneurysmal disease is distinct from other atherosclerotic processes, most notably in the fact that diabetes is a negative risk factor, and many patients develop occlusive disease of the infra-renal aorta without aneurysmal disease, a more specific degenerative process is now believed.

In contrast to atherosclerosis which affects the intima of the vessel, the outer 2 layers of the aortic wall are most affected by aneurysm progression, with these regions undergoing extensive remodelling and weakening due to a combination of local chronic inflammation, apoptosis leading to a decrease in the number of smooth muscle cells, fragmentation of the extracellular matrix through proteolysis, and oxidative stress[60-63]. Macrophages, neutrophils, mast cells, natural killer cells, and T-cells have all been shown to contribute to AAA development[64]. Infiltration of these inflammatory cells can lead to increased levels of proteases, particularly matrix metalloproteinases (MMPs), which leads to degradation of the extracellular matrix. This decreases the levels of collagen and elastin within the aortic wall, weakening its structure, and leading to progressive dilatation.

1.1.8 Natural History of AAA

The natural course of an AAA is to gradually expand until it reaches the point of rupture, which the majority of patients do not survive[65]. However, as AAAs occur in an increasingly elderly population, many patients have concomitant disease, and therefore succumb to other disease rather than their AAA, and the aneurysm has therefore remained asymptomatic throughout life. The rate of expansion of AAAs has been studied, showing growth rates of between 2.21mm/year[24] and 2.5mm/year[66], although there is significant variability between individuals, with one study showing the rate of expansion to be greater than 10mm/year in 12% of patients[67]. AAA growth rates are also increased in smokers, and decreased in patients with, diabetes[24].

The risk of aneurysm rupture correlates with the size of the aneurysm. Aneurysms with a diameter less than 50mm have a very low risk of rupture, while aneurysms with

a diameter between 55mm-59mm have a 1-year incidence of rupture of 9.4%, rising to 32.5% with a diameter ≥70mm in unfit patients[68]. Female sex, smoking, hypertension, and chronic obstructive pulmonary disease (COPD) are all associated with an increased risk of aneurysm rupture[24,69], although COPD may be confounded by its correlation with smoking. The presence of an AAA is also seen to result in a lower long-term survival even in patients who undergo successful repair of their aneurysm[70], which is secondary to the increased risk of cardiovascular disease in this patient group[71].

1.1.9 AAA Symptoms

An AAA is typically a quiescent disease causing minimal or no symptoms prior to rupture. However, AAA rupture is a significant cause of death, responsible for 5251 deaths per annum in the UK (2010)[72]. Rupture occurs when the pressure within the aneurysmal sac exceeds that of the aneurysm wall, causing blood to leak into the retroperitoneal cavity (80%) or anteriorly into the peritoneal cavity (20%). AAA rupture carries with it an overall mortality rate of 90%, and a peri-operative mortality rate of 48%[65]. Patients presenting with a ruptured AAA are typically in severe pain, tachycardic, with moderate to severe hypotension. The extremely rare Grey-Turner's sign of bilateral flank ecchymosis due to extravasation of blood into the subcutaneous tissues may be present, with evidence of a palpable pulsatile abdominal mass, along with abdominal distension and tenderness. The sensitivity of abdominal palpation as a diagnostic tool for AAA has an overall sensitivity of 68% and specificity of 75%, however, this increases with aneurysm diameter, and nears 100% for aneurysms >50mm when the patients waistline is less than 40 inches[73]. There may also be weak or absent distal pulses.

Although rupture is the most common complication of an AAA, AAA may also very rarely lead to lower limb ischaemia, or an aorto-enteric fistula. Lower limb ischaemia typically occurs due to an embolism of thrombus from the atherosclerotic debris from the aneurysm[74], or rarely due to acute aortic thrombosis[75]. Thrombus formation occurs within the aneurysm sac as the aneurysm expands, which may become disrupted due to rapid aortic expansion, instrumentation, or trauma[76]. One review identified that 5% of patients presented with distal embolisation as their first presentation of an AAA, with 1% of patients presenting with critical limb ischaemia[76]. An aorto-enteric fistula occurs due to a direct communication between the aorta and any part of the gastrointestinal tract[77]. It is most common after aortic surgery, however it may occur as a primary presentation, with the incidence ranging from 0.04-0.07% per year at autopsy[78,79]. Primary presentation of an aorto-enteric fistula may be due to either repeated mechanical trauma between the aorta and the gastrointestinal tract, or due to low-grade infection forming an abscess which erodes into the bowel wall[80]. Typical presentation of an aorto-enteric fistula is with gastrointestinal bleeding, abdominal pain, and sepsis.

1.1.10 AAA Treatment

Treatment for any disease can be divided into prevention, drug treatment, or surgical intervention. Although AAAs can be detected early through screening programmes, which are discussed later, there is currently no means of aneurysm prevention. The probability of developing an AAA can be decreased through risk factor modification such as stopping smoking, however this does not prevent aneurysm formation in all cases as AAA is a multifactorial disease.

Currently, medical treatment for AAAs has not yielded any significant results other than decreasing cardiovascular risk as this is the major cause of death in patients with AAA. Although screening can detect an AAA early, the method of halting or slowing the progress of the disease remains unknown. Current guidelines focus on the modification of risk factors for AAA development, such as hypertension, hypercholesterolaemia, and smoking cessation. Recent reviews have deduced that non-steroidal anti-inflammatory drugs, diuretics, calcium-channel blockers, antiplatelet agents and beta-blockers do not alter aneurysm growth rates, however statins, doxycycline, roxithromycin, cyclo-oxygenase-2 inhibitors and angiotensin II receptor blockers suggest a possible benefit[81-85]. Evidence is currently conflicting regarding the use of angiotensin-converting enzyme (ACE) inhibitors and an evaluation of the effect of angiotensin-converting enzyme (ACE) inhibitors on small aneurysm growth rates (AARDVARK trial) is being conducted (NCT01118520) to evaluate the effects of perindopril against a placebo and amlodipine on the growth rate of small AAAs (although recruitment is poor).

Current research is focusing on the use of physical conditioning to improve aortic haemodynamics and reduce inflammation, therefore decreasing AAA development. Early results have shown that this is well tolerated, and a significant reduction in C-reactive protein and waist circumference has occurred, however analysis of aneurysm growth rates are awaited[86].

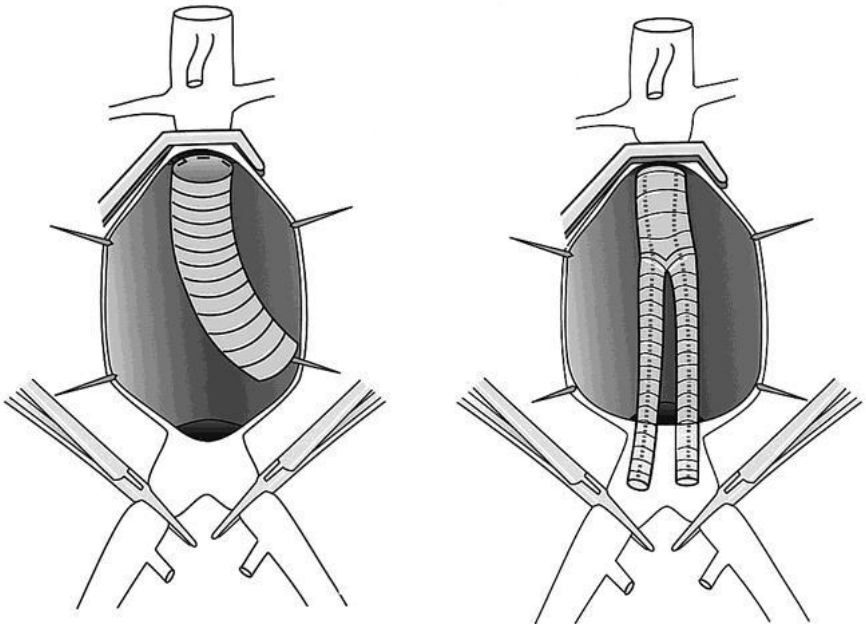
Surgical intervention for AAA has been conclusively found to decrease mortality if performed when aneurysm size reaches 55mm in otherwise healthy individuals[87-89]. Two options are available for the surgical treatment of AAAs, either open surgical

repair (OSR) or endovascular aneurysm repair (EVAR). OSR requires a midline or transverse abdominal incision, clamping above and below the aneurysm, opening the aneurysm sac, and suturing a prosthetic replacement in situ. EVAR utilises a minimally invasive approach by placing a guide-wire into the femoral artery, and inserting a prosthesis into the aneurysm under X-ray guidance. Once the position has been optimised the graft is expanded, and is held in place through the radial force, and in some cases either spikes, barbs, hooks or stents on the outer surface of the graft, to secure it in place (Figure 1-4).

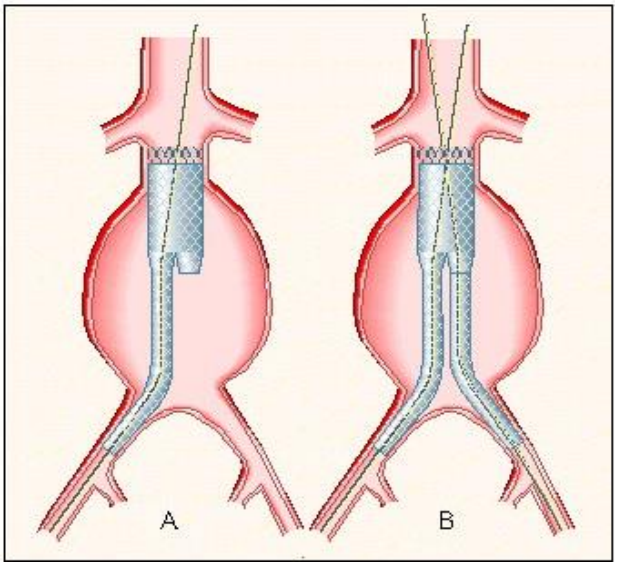
EVAR has become established as the treatment of choice in many vascular centres. Four similar randomised controlled trials have been undertaken to determine both the short and long-term outcomes of EVAR compared to OSR. Although some of these trials are not yet complete, with true long-term ten-year outcomes awaited, these trials have highlighted a significant reduction in 30-day operative mortality and length of stay in favour of EVAR. However, medium and long term follow up from these trials varies with some showing the early survival benefit of EVAR may be lost over time. A number of concerns have arisen regarding the long-term durability of EVAR, and this in addition to a need for lifelong surveillance, and the added cost of EVAR[90] may negate any early survival advantage gained[91]. A recent meta-analysis looked at the results of the EVAR trials and 2 large validated databases[92-103] revealing that there is no long-term survival benefit for patients undergoing EVAR compared to OSR for AAA. It also identified a significantly higher risk of reintervention and aneurysm rupture post EVAR, challenging the long-term durability of EVAR[104].

Although the evidence for EVAR over OSR for AAA repair shows no long-term benefit, one reason for choosing OSR over EVAR lies with aneurysm anatomy. Most manufacturers' instructions for use (IFU) state that EVAR should be performed in patients with an aortic neck greater than 15mm length, less than 28mm diameter, and with an angulation less than 60 degrees. However, as stent-graft designs have improved, and clinicians have become more adept at inserting endovascular devices, standard stent-grafts have been inserted for shorter, more angulated, and wider aortic necks, outside of manufacturer's IFU. Studies have shown that performing EVAR outside of manufacturers' IFU have resulted in poorer short and long-term outcomes[105,106]. A recent meta-analysis combined 16 studies of patients undergoing EVAR outside of IFU, revealing that this increases the technical difficulty, and results in poorer short-term outcomes. In addition there was an increase in the rates of <30-day and >30-day type I endoleaks, along with secondary interventions. There was however no significant difference in >30-day mortality or rupture, which suggests that robust monitoring combined with early intervention can ensure satisfactory mid to long-term outcomes when performing EVAR outside of manufacturer's IFU[107].

Figure 1-4 – Diagram of a) open and b) endovascular aneurysm repair



a) The aorta is clamped proximally and distally. The aneurysmal sac is incised and laid open. The aortic graft is then sutured in proximally and distally, with the aortic sac sutured over the graft. Either a straight or bifurcated graft may be used depending on suitability of the distal anatomy (Reproduced with permission from Moore *et al*. Abdominal Aortic Aneurysm: A 6-year comparison of endovascular versus transabdominal repair. Ann Surg. 1999;230(3):298 Copyright Lippincott Williams and Wilkins Inc)[108].



b) The endovascular stent-graft is inserted through a groin incision and passed up through the femoral artery and expanded in place (A). A second limb is inserted through the opposite femoral artery and expanded in place (B). It is secured in place by spikes and barbs in the graft. (Reproduced with permission from Blum U *et al*. Endoluminal Stent-Grafts for Infrarenal Abdominal Aortic Aneurysms. N Engl J Med. 1997;336:13-20 Copyright Massachusetts Medical Society)[109].

1.1.11 AAA Screening

Since the mortality rate of AAA repair is reduced by a factor of 10 when performed electively rather than as an emergency, detecting and treating AAA prior to rupture is clinically beneficial. The Multicentre Aneurysm Screening Study (MASS) Group undertook a randomised controlled trial demonstrating that screening asymptomatic men over 65 by the use of a single abdominal ultrasound scan for AAA reduces AAA-related mortality, with these results remaining significant at both 7-year[110] and 10-year follow-up[111]. A NIHR Health Technology Assessment Project is also currently undertaking an examination of the cost-effectiveness of AAA screening in the UK, taking the decreasing incidence of AAA into account. Their results suggest that screening remains cost-effective at the current reported incidence in 65 year old men[112]. The evidence therefore supports AAA screening as a cost-effective strategy to prevent deaths. Despite this only a handful of developed countries worldwide have invested in national screening programmes.

Internationally, 7 countries (Sweden, England, Scotland, Wales, Northern Ireland, New Zealand and the USA) have implemented or are in the process of implementing nationwide screening programmes for AAA. Both Denmark (ClinicalTrials.gov NCT00662480) and Norway (NCT01248533) have on-going randomised controlled trials (RCTs) looking at the impact of screening on all-cause mortality with both studies simultaneously screening for hypertension and lower limb atherosclerosis. The Netherlands also started a trial in July 2012 (NCT01643317), and Italy is running a trial in Genoa.

Although it is accepted that screening for AAA is both beneficial for patients and cost-effective, there is marked variability in screening programmes. All countries perform screening for men, with the USA, New Zealand and Italy also screening women. All countries screen individuals in their 65th year or older, however New Zealand only screen subjects with high cardiovascular risk, and the USA only screening subjects with a history of smoking. All screening programmes use a single ultrasound scan to determine aortic diameter and define the minimum diameter for an aneurysm as ≥30mm. Some Swedish counties, Oslo and USA also include subjects with subaneurysmal aortic dilatation (25-29mm) in their surveillance programme, offering them a repeat scan after 5 years. This is due to over two-thirds of patients with subaneurysmal aortic dilatation going on to develop AAA within 5 years, and 26% of patients developing an aneurysm of 55mm or greater at 10-year follow-up[113]. There is marked variation in surveillance frequency between countries, with the most common regimen being to vary the surveillance interval depending upon aneurysm size, however, there is an agreement that the optimum surveillance interval in terms of decreasing mortality and cost effectiveness remains uncertain[114]. In the UK, patients undergo routine surveillance yearly with an aortic diameter between 30-44mm, and 3-monthly when 45mm and greater.

1.2 Conclusions

Abdominal aortic aneurysms are a significant cause of morbidity and mortality worldwide, tending to occur in an ever aging population. The implementation of aneurysm screening programmes has enabled patients with AAA to be detected at a smaller diameter, when there is a significantly lower risk of rupture. Along with risk

factor modification, the development of medical therapy to slow or halt the rate of aneurysm growth would dramatically decrease the need for invasive surgical intervention to prevent aneurysm rupture. Investigating the biological mechanisms underlying aneurysm formation will enable targeted therapies to be developed for their treatment, and permit blood based tests to be used to identify patients at risk of aneurysm formation.

Chapter 2

The Biology of Abdominal Aortic Aneurysms

A large number of studies have attempted to elucidate candidate pathways associated with AAA, in an attempt to understand the pathological processes involved in AAA formation. Modern approaches to understanding disease pathophysiology can be divided into hypothesis free studies such as genome wide association studies (GWAS), proteomics, genomics, transcriptomics, and metabolomics, and hypothesis driven studies where a specific gene or biomarker is analysed. In AAA five GWAS have been published thus far which will be detailed in Chapter 2.1. Thus far there is limited published reproducible data from proteomics, genomics, transcriptomics and metabolomics in AAA, therefore these will not be further discussed. A large number of candidate gene studies and biomarker studies (analysing a specific protein or set of proteins) have been published. Although candidate gene studies often have conflicting results a recent meta-analysis has been conducted which shall be further detailed in Chapter 2.2. Additionally biomarker studies often have conflicting results, therefore I have undertaken a systematic review, meta-analysis and meta-regression of biomarker studies in order to consolidate the published literature and direct my experimental work.

2.1 Genome wide association studies

Genome wide association studies (GWAS) examine a wide variety of common genetic variations in individuals in order to determine if any of these are associated with a specific condition. They assess multiple single nucleotide polymorphisms (SNPs – variations of a single nucleotide at a particular base position) throughout the genome, initially in a group of cases and controls, with significant SNPs then taken forward into a large scale validation cohort.

A GWAS of AAA by Elmore *et al* (2009)[115] identified 4 SNPs on chromosome 3p12.3 to be associated with AAA, with rs7635818 being the most significant, however a subsequent larger validation cohort did not validate this association. A further GWAS by Gretarsdottir *et al* (2010)[116] identified rs7025486-A, located within the DAB2 interacting protein (*DAB2IP)* gene to be associated with AAA, but also with myocardial infarction, peripheral arterial disease, and pulmonary embolism. Bown *et al* (2011)[117] identified rs1466535, found within the second intron of the low density lipoprotein receptor related protein (*LRP1)* gene to be associated with AAA, and this was independent of other cardiovascular risk factors. Jones *et al* (2013)[118] identified the rs599839-G allele, found within the Sortilin 1 (*SORT1)* gene to be associated with AAA. This had previously been associated with dyslipidaemia and coronary artery disease, however modelling for confounding factors found this variant to be independently associated with AAA. Lastly low density lipoprotein receptor (*LDLR)* rs6511720-A was found to be significantly associated with AAA, consistent with its known effects on coronary artery disease[119].

2.2 Candidate gene analyses

Candidate gene analyses focus on genetic variation within specific genes of interest, and are therefore more focused than GWAS. The SNP rs10757278-G on chromosome 9p21 was studied by Helgadottir *et al* (2008)[120] following its previous associations with both coronary artery disease and type II diabetes. In addition this SNP was found to be associated with AAA, as well as myocardial infarction, and intracranial aneurysm.

Thompson *et al* (2008)[121] undertook a meta-analysis of candidate gene studies in AAA, and identified a significant association between matrix metalloproteinase 9 (*MMP9)*,

angiotensin converting enzyme (*ACE)*, and methylenetetrahydrofolate reductase (*MTHFR)* in patients with AAA.

2.3 Biomarkers

2.3.1 Introduction

A biomarker is a naturally occurring molecule (or protein) which can be used to try to identify a particular disease. These are different to genetic studies as biomarkers represent the total expression of a molecule rather than assessing differences in genetic loci. Determining biomarker levels represents total expression of a gene, or a combination of genes, and therefore can be used to correlate variations in genetic loci with gene expression. The difficulty in interpreting alterations in biomarker levels however is in determining whether alterations in biomarker levels are the cause of disease, or are caused by the disease process.

Many studies have investigated the systemic and local levels of biomarkers in patients with AAA, often with conflicting results. The aim of this section was therefore to perform a systematic review, and where possible meta-analysis and meta-regression of studies comparing these markers in patients with and without AAA, in order to ascertain the current knowledge of AAA pathophysiology, and direct my experimental work towards logical microRNAs.

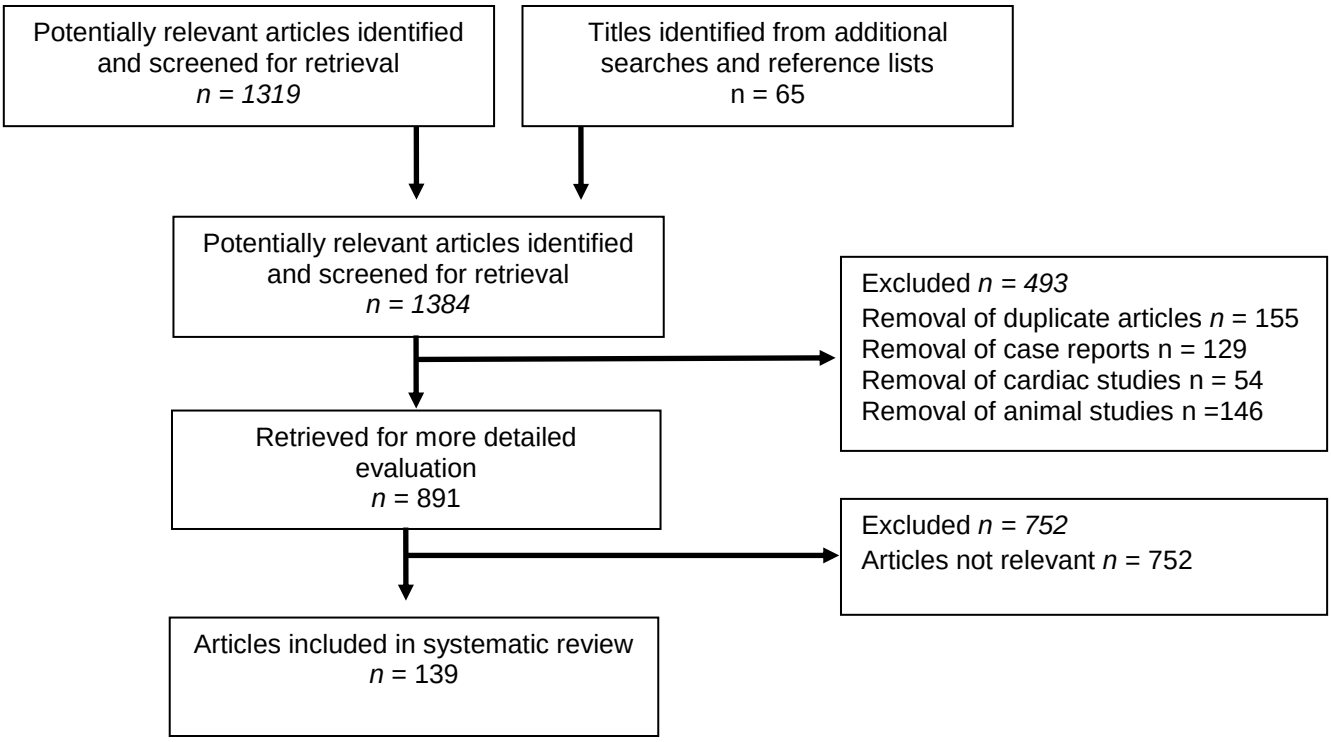
2.3.2 Methods

Search Strategy

A systematic review comparing biomarkers in patients with and without AAA was conducted. Study titles and abstracts were searched according to PRISMA guidelines[122]

(Figure 2-1) using Medline and Embase databases through OVID Online (Version: OvidSP_UI03.04.02.112, Ovid Technologies, Inc.) by myself and Mr David Sidloff, in February 2013 using the mesh terms “aneurysm”, “aorta”, “abdominal”, “miRNA”, “microRNA”, “mRNA”, “RNA, “protein”, “biomarker”, “human”, NOT “thoracic” yielding 1319 studies. Further literature searches for each individual biomarker (matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases, cathepsins, cystatins, c-reactive protein, D-dimer, interleukins, interferon, intercellular adhesion molecule, vascular cell adhesion protein, osteoprotegerin, osteopontin, apolipoproteins, insulin-like growth factors, α1 antitrypsin, cholesterol, high density lipoprotein, low density lipoprotein, triglycerides, ceruloplasmin, fibrinogen, tissue plasminogen activator, plasminogen activator inhibitor, and tumour necrosis factor) were undertaken using PubMed in August 2013, along with manual searching of reference lists, for additional articles. These additional searches identified a further 65 studies to include within this analysis. Following removal of duplicates (155), case reports (129), cardiac studies (54) and animal studies (146) a total of 891 studies were obtained. A total of 139 studies were identified reporting biomarkers in subjects with AAA compared to either controls, subjects with aorto-occlusive disease (AOD), or comparing aortic diameter. Studies were limited to English language. Review articles and those pertaining to thoracic aneurysms were excluded.

Figure 2-1 – PRISMA diagram showing the literature search (conducted by myself and Mr David Sidloff)



Study selection and data extraction

Studies included within this analysis were either case-control studies, or studies comparing biomarker levels to aortic diameter. Data extracted included mean or median and standard deviation (SD), interquartile range (IQR), range, standard error of the mean (SEM), 95% confidence intervals (CI), and/or a P value. In addition, the following data were extracted for each article; year of publication, author, journal, and mean aneurysm diameter.

Statistical Analysis

A meta-analysis of summary statistics from the individual biomarker studies was conducted. For each study, data relating to biomarker levels in both the AAA and

control groups were used to generate standardised mean differences (SMDs) and 95% CI's using Review Manager version 5.2[123]. Forest plots representing the SMDs are shown with a positive SMD representing an increase in subjects with AAA. Studies were combined using the inverse variance method with random effects due to the heterogeneity between studies included. Between study heterogeneity is assessed through a combination of Tau^2, Chi^2 and its P value, and I^2. Tau^2 represents the between study variance in a random effects meta-analysis, with + or – 2 x Tau^2 roughly estimating the range of the mean difference. Chi^2 represents the test for heterogeneity, with its associated P value representing significant heterogeneity if the $P<0.05$. I^2 represents the percentage of variance in a meta-analysis that is attributable solely to study heterogeneity, with >50% deemed to be significant heterogeneity. The summary effect for a meta-analysis is determined by the Z score, with its associated P value representing a significant difference if <0.05[124].

Individual circulating biomarkers (measured either in blood, serum or plasma), and aortic tissue studies were analysed separately. Biomarker concentrations were each converted to the same SI unit (depending on the predominant unit for each biomarker), and converted to mean and SD as per the Cochrane Handbook for Systematic Reviews Version 5.1.0 updated March 2011. Standard conversion charts were used to convert mg/dl to nmol/l. For articles reporting the median and IQR, we took the median to be representative of the mean, and divided the IQR by 1.35 to generate the SD. For studies reporting the SEM, this was multiplied by the square root of the number of subjects within that group to calculate the SD. For studies reporting 95% CI's the SD was calculated by multiplying the difference between the upper and

lower intervals by the square root of the number of subjects within the group, then dividing this by 3.92. In studies where a P value had been given but no SD or IQR or SEM, a SD was calculated from the P value using the standard formula from the Cochrane Handbook for Systematic Reviews Version 5.1.0 updated March 2011. Where studies had reported more than 1 subgroup of patients with AAA, a combined mean and SD was obtained through standard formulae. Publication bias was assessed through visual inspection of funnel plots[125].

To determine whether biomarker concentrations were associated with AAA diameter, meta-regression was performed using n-weighted linear regression (IBM SPSS Version 20). Biomarker concentrations were compared to the reported mean/median aneurysm size from each study. In cases where biomarker concentrations were reported stratified by AAA diameter, each size sub-group was entered into the regression as a separate outcome. Where reported diameter was greater than or less than a specified diameter, the specified diameter was used. Where reported diameter was given as a range, the central point of the range was used. Sensitivity analyses for all outcomes were performed to assess the influence of each study to the standardised mean difference by exclusion of individual studies one at a time.

2.3.3 Results

Literature review identified a total of 139 studies evaluating the levels of proteases, inflammatory markers, acute phase reactants, coagulation markers, and lipids.

<u>Proteases</u>

<u>Matrix Metalloproteinases</u>

Initial work by Busuttil *et al*[126] identified a significant increase in elastase activity in the aortic wall of subjects with AAA. This was then further refined by Cohen *et al*[127] to be attributed to one or more serine proteases. Several early studies suggested that metalloenzymes may participate in the pathogenesis of AAA[128-132] , then in 1995 Thompson *et al*[133] examined whether a 92-kD gelatinase (now known as MMP9) and a 72-kD gelatinase (now known as MMP2) participated in the pathobiology of AAA. Conditioned aortic tissue from control, atherosclerotic (AOD) and aneurysmal subjects underwent gelatin zymography, revealing that all tissues produced MMP2, however MMP9 was significantly increased in subjects with AOD, but more so in AAA (10 fold compared to control and 2 fold compared to AOD). MMP9 was also found to be localised to macrophages within the aortic media. Matrix metalloproteinases (MMPs) degrade extracellular matrix proteins including collagen and elastin and several studies have further quantified their expression in AAA. Five studies have examined MMP2 in the circulation, all except one[134] finding no difference in MMP2 levels[135-138]. Meta-analysis of these studies[134-138] found no difference in MMP2 between patients with and without AAA (P=0.67) (Figure 2-2). This is in contrast to aortic tissue MMP2 which has been found to be significantly increased in seven studies[139-145], with no difference found in only one study[143]. Circulating MMP2 activity, measured by zymography, however is not conclusive[146,142]. Five studies of circulating MMP2 were suitable for meta-regression, finding no correlation between MMP2 levels and aneurysm diameter[135-137,147,148] (P=0.92), with one additional study (unsuitable for meta-analysis due to lack of published data), also finding no correlation between MMP2 and aneurysm diameter[149], however in aortic tissue samples, MMP2 was positively correlated with aortic diameter[150].

Circulating MMP9 levels were significantly increased in patients with AAA in eight studies[134,135,138,151-155] and significantly decreased in another[137], with no difference found in three studies[148,156,157]. Meta-analysis revealed a significant increase in circulating MMP9 in patients with AAA (Std Mean Diff 0.67 ng/mL (95% CI 0.24-1.10); P=0.002) (Figure 2-2). This finding is supported by five studies using aortic tissue MMP9 levels[139,140,143,158,159], although two studies did not find a significant increase[160,141]. Meta-regression of circulating MMP9 studies[135,136,146-148,154,156,157] demonstrated no correlation with aneurysm diameter (P=0.69), which is consistent with three additional studies[138,149,161], however a correlation has been identified in aortic tissue between MMP9 and AAA diameter[162]. Circulating MMP9 activity[142,146,163], has been measured by zymography, showing no clear relationship with AAA, however MMP9 activity within aortic tissue has been found to be significantly increased in six studies[142-145,159,164].

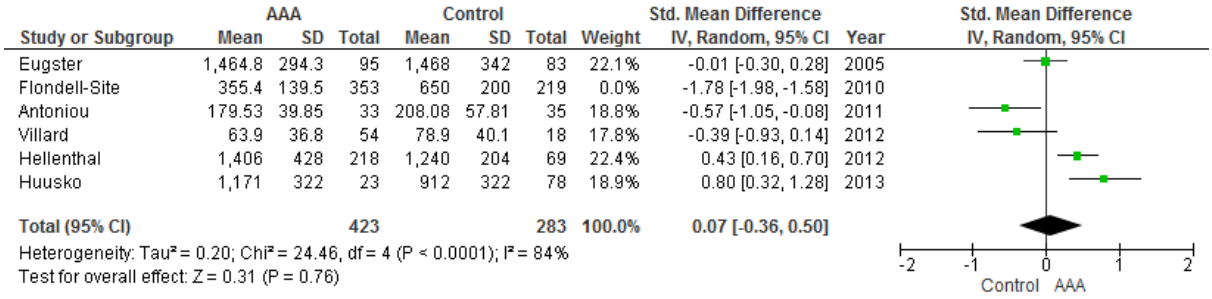
Two studies comparing MMP3 levels in AAA compared to healthy controls found a significant increase in MMP3[152,154], however one study comparing AAA to AOD found no significant difference[165] suggesting these changes may reflect atherosclerotic disease. In aortic tissue, MMP3 levels have not been found to be significantly different[141].

A limited number of studies have reported on other MMPs in aortic tissue, however the evidence is not consistent. No significant difference in the levels of MMP1[141,160] have been found between patients with and without AAA. MMP8 levels[160,166] and activity[166] have been found to be significantly increased in AAA, as has MMP12[167]. MMP13[168] and MMP14[169] have been significantly increased in single studies comparing

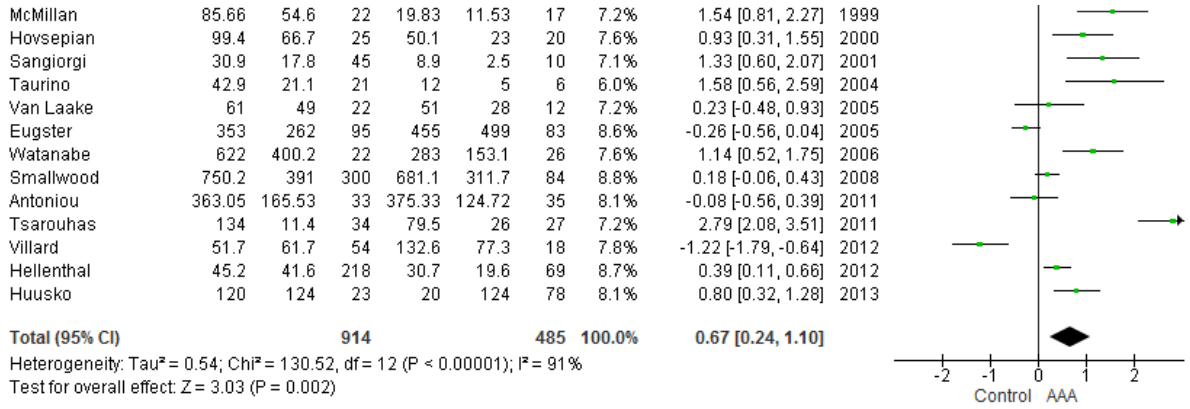
AAA with controls, however no difference was found in additional studies into MMP13[160] and MMP14 levels respectively[160].

Figure 2-2 – Forest plot comparing expression of a) MMP2 and b) MMP9 in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

a) MMP2



b) MMP9



Tissue Inhibitors of Matrix Metalloproteinases

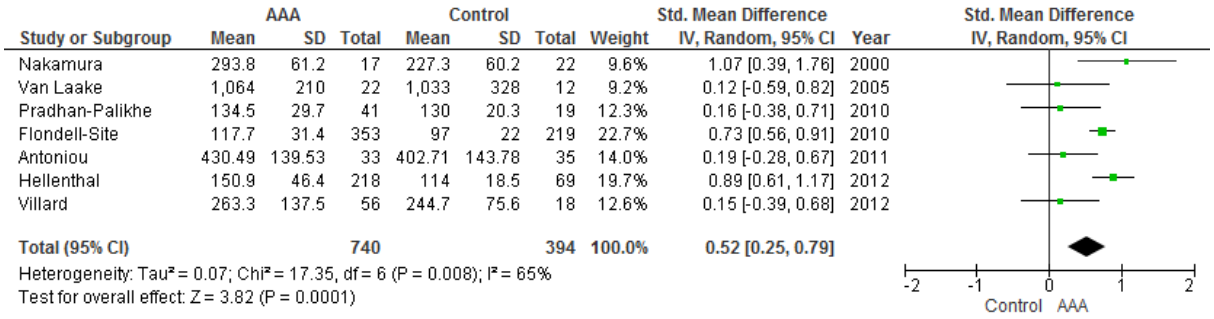
Tissue inhibitors of matrix metalloproteinases (TIMPs) have been extensively studied with regards to AAA pathophysiology. Circulating levels of TIMP1 were significantly elevated in patients with AAA in 3 studies[135,146,170], with no difference found in 5 studies[134,136,148,157,165]. Meta-analysis revealed a significant increase in TIMP1 in patients

with AAA (Std Mean Difference 0.52 ng/mL [95% CI 0.25-0.79]; P<0.0001) (Figure 2-3) however meta-regression revealed no correlation with aortic diameter [134,136,148,157,165] (P=0.93) which is consistent with previous findings[149]. TIMP1 levels in aortic tissue have been found to be significantly downregulated in only a single study[166], with no significant difference found in 3 studies[141,150,160].

Circulating levels of TIMP2 have been found to be significantly decreased in AAA[148], however they have not been found to correlate with AAA growth rates or diameter[149]. Similarly TIMP2 levels in tissue were found to be significantly downregulated in a single study[166], however no significant difference was found in 3 studies[141,150,160].

Circulating levels of both TIMP3 and TIMP4 were not significantly different between AAA and AOD subjects[163], and there has also been no significant difference found in TIMP3 levels in aortic tissue[160].

Figure 2-3 – Forest plot comparing expression of TIMP1 in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.



Cystatins and Cathepsins

Cathepsin L (CatL), Cathepsin K (CatK), Cathepsin V (CatV), Cathepsin S (CatS) and Cathepsin B (CatB) are proteases that act as potent elastases and collagenases. They are highly expressed in human arterial lesions[171] and have been implicated in human

arterial wall remodelling[172]. Cystatins are potent intracellular inhibitors of Cathepsin activity and Cystatin C is the most abundant and potent within the family. In addition to inhibiting Cathepsins, studies have shown that Cystatins may interact directly with MMP9, potentiating its activity[173].

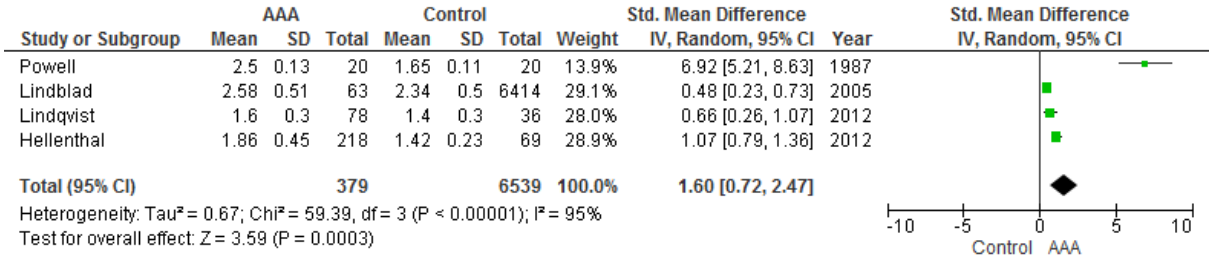
Three studies[158,172,174] analysed CatL levels in either blood or aortic tissue, however only one[172] demonstrated a significantly higher concentration in patients with AAA compared to those without. Three studies analysed CatS[158,172,175], with two in blood finding a significantly higher concentration[172,175] in those with AAA, whilst the study on aortic tissue found no difference[158]. One study analysed CatV[172] in plasma demonstrating a significant increase in patients with AAA. Two studies[158,174] analysed CatB finding no difference. Cystatin C[135,158] has been found to be lower in patients with AAA in two studies however, of the three studies assessing Cystatin C with aortic diameter, two have demonstrated an inverse correlation with aneurysm size [176,177], and one[135] found no significant difference. One study analysed Cystatins A and B[158] in aortic tissue finding no difference and another studied Cystatin S in plasma revealing a significant increase in those with AAA[172]. Cathepsins can degrade the extracellular matrix and may contribute to aneurysm formation by promoting inflammatory responses. A deficiency of Cystatin C may be associated with increased aneurysm size and expansion rate[177] however a review of the literature does not present a clear picture of their respective roles.

Alpha-1 antitrypsin

α-1 antitrypsin (α1AT) is a protease inhibitor acting on a variety of serine proteases including elastase and therefore may play a role in maintaining the integrity of the

aortic wall. Although there is no correlation between α1AT deficiency and AAA[178], several studies have looked at α1AT levels in patients with AAA. Three studies[179-181] found an upregulation of α1AT in AAA, with three finding no difference[135,182,183]. Meta-analysis of these studies[135,180-182] revealed a significant upregulation of α1AT in AAA (Std Mean Diff 1.60 g/L (95% CI 0.72-2.47); P=0.0003) (Figure 2-4). Three studies[135,149,184] found α1AT had no correlation with aneurysm size.

Figure 2-4 – Forest plot comparing expression of α1-antitripsin in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.



Acute Phase Reactants

D-dimer

Plasma concentrations of D-dimer reflect the extent of fibrin turnover in the circulation[185]. Eleven studies reported on concentrations of plasma D-Dimer from which nine studies[186-194] found a significantly increased concentration of plasma D-Dimer in patients with AAA while two[195,196] found no significant difference. Meta-analysis of this data revealed a significant increase in D-dimer in patients with AAA (Std Mean Diff 1.11 ng/mL (95% CI 0.87-1.35); P<0.00001) (Figure 2-5). Meta-regression was possible in six studies[186,188,190,191,197,198] revealing a significant correlation between D-dimer and AAA diameter (r^2 = 0.94, P<0.00001) (Figure 2-6). Whilst it is possible that

markers of haemostasis are involved in the development of AAA as D-Dimer has been shown to stimulate the release of pro-inflammatory cytokines and proteolytic enzymes[199], plasma markers of coagulation have been demonstrated to fall following aortic aneurysm repair[198] suggesting that the aneurysm itself drives the changes observed across these studies.

Figure 2-5 – Forest plot comparing expression of D-dimer in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

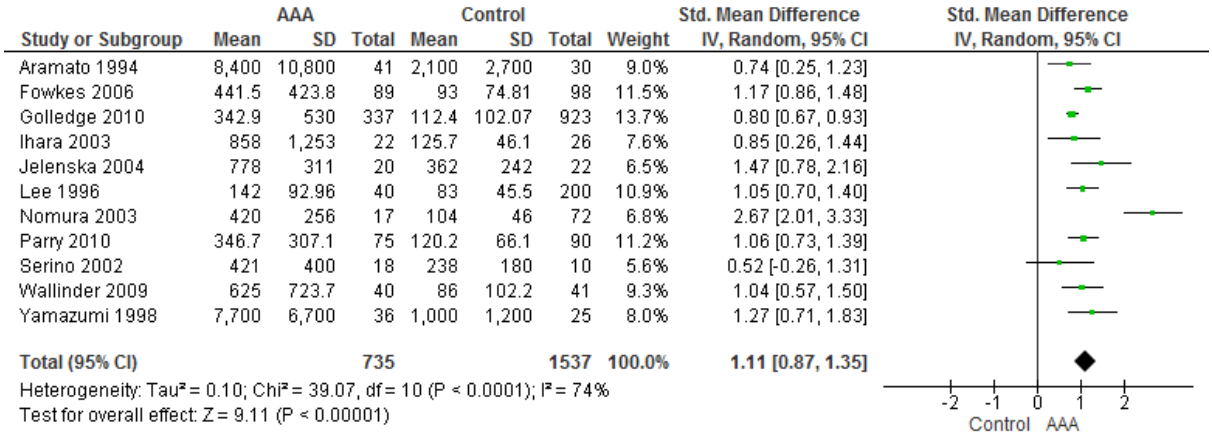
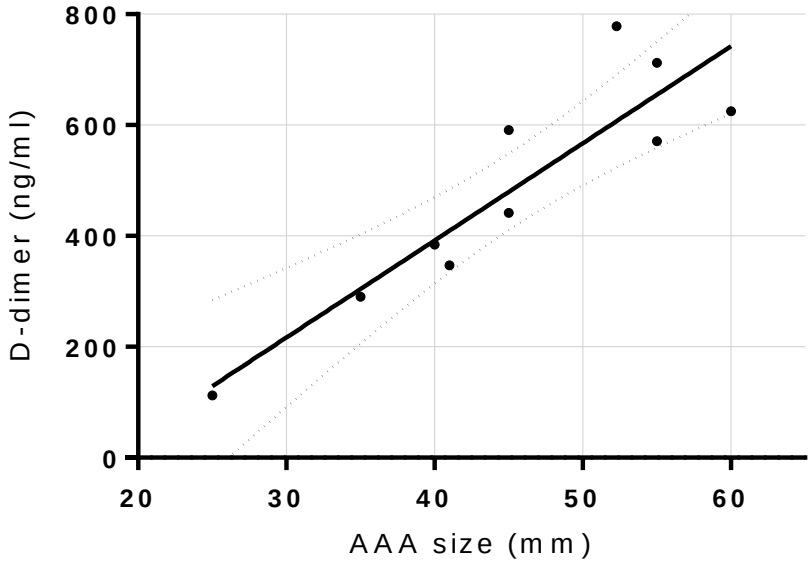


Figure 2-6 – Scatter plot demonstrating the association between D-dimer and aneurysm diameter. P<0.00001) R^2 = 0.94.



C-reactive protein

C-reactive protein (CRP) is an acute phase protein rising in response to acute or chronic inflammation. Inflammation is thought to be a key driver of elastin destruction and AAA development[200] therefore a rise in baseline CRP may be associated with AAA. Both ELISA and turbidimetric assays have been used to quantify CRP levels, however both are deemed equivalent[201] and can therefore be combined through meta-analysis. Seventeen studies have analysed plasma CRP levels between AAA and controls, with ten[135,175,181,182,194,202-206] finding a significant increase, and seven[136,165,207-211] finding no significant difference. Meta-analysis[135,136,165,175,181,182,194,202-209,211] revealed a significant increase in CRP in AAA (Std Mean Diff 0.58 mg/L (95% CI 0.37-0.79); P<0.00001) (Figure 2-7). All studies[175,206,208,212-214] except one[177] found a positive association between CRP and aortic diameter. Meta-regression [135,136,184,194,202,204,205,207,208,215], revealed a significant positive linear correlation between CRP and aortic diameter (P=0.006, adjusted R^2=0.161) (Figure 2-8). No association has been demonstrated between CRP and aneurysm growth rates[161,216-218].

Figure 2-7 – Forest plot comparing expression of C-reactive protein in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

	AAA			Control				Std. Mean Difference		Std. Mean Difference
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year	IV, Random, 95% CI
Powell	56	10	20	28	8	20	3.2%	3.03 [2.10, 3.97]	1987	
Wanhainen	2.4	3.5	35	2.3	7.2	140	6.7%	0.02 [-0.36, 0.39]	2005	
Dawson	4.77	0.93	25	3.62	1.01	12	4.1%	1.18 [0.43, 1.92]	2007	
Badger	3.85	5.32	248	1.8	1.47	400	8.0%	0.59 [0.43, 0.75]	2009	
Vega-De Ceniga	4.1	4	35	1.9	3.3	35	5.9%	0.59 [0.11, 1.07]	2009	
Parry	3.31	0.44	75	3.1	0.51	90	7.1%	0.44 [0.13, 0.75]	2010	
Pradhan-Palikhe	1.9	5.85	41	1.9	3.7	19	5.4%	0.00 [-0.54, 0.54]	2010	
Golledge~	2.55	2.83	312	1.9	2.19	1046	8.2%	0.28 [0.15, 0.40]	2010	
Golledge	2.5	2.7	337	1.7	1.9	923	8.2%	0.37 [0.25, 0.50]	2011	
Pan	4.76	10.8	45	11.07	20.73	49	6.4%	-0.37 [-0.78, 0.03]	2011	
Villard	2.97	3.46	54	2.9	5.11	18	5.5%	0.02 [-0.52, 0.55]	2012	
Lindqvist	3.1	3.6	78	1.5	1.7	36	6.5%	0.51 [0.11, 0.91]	2012	
Ramoz-Mozo	4.2	3	30	2.7	3.5	30	5.6%	0.45 [-0.06, 0.97]	2012	
Hellenthal	4.49	6.19	218	1.3	1.45	69	7.4%	0.58 [0.31, 0.86]	2012	
Kadoglou	5.9	2.05	108	2.96	1.02	41	6.5%	1.60 [1.20, 2.00]	2012	
Qin	6.54	4.93	31	0.89	0.58	32	5.2%	1.60 [1.03, 2.17]	2013	
Total (95% CI)			**1692**			**2960**	**100.0%**	**0.58 [0.37, 0.79]**		

Heterogeneity: Tau² = 0.14; Chi² = 118.26, df = 15 (P < 0.00001); I² = 87%
Test for overall effect: Z = 5.33 (P < 0.00001)

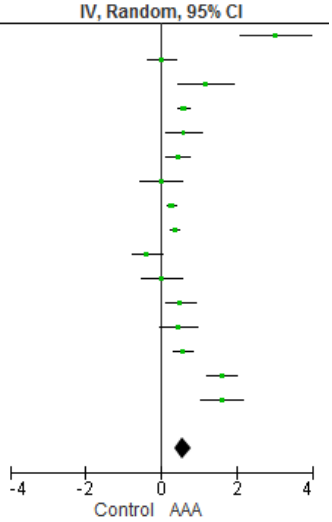
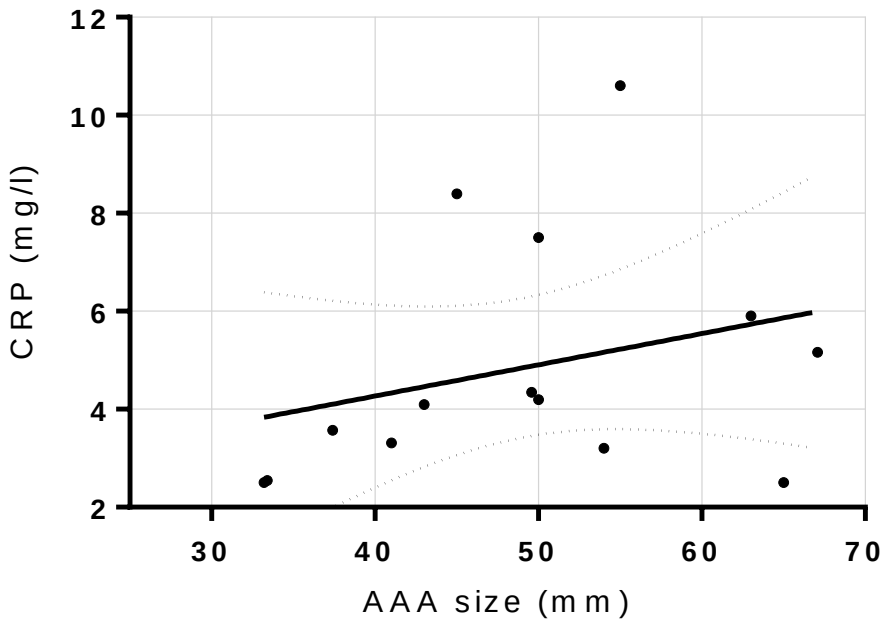


Figure 2-8 – Scatter plot demonstrating the association between C-reactive protein and aneurysm diameter. P=0.006, r^2=0.081

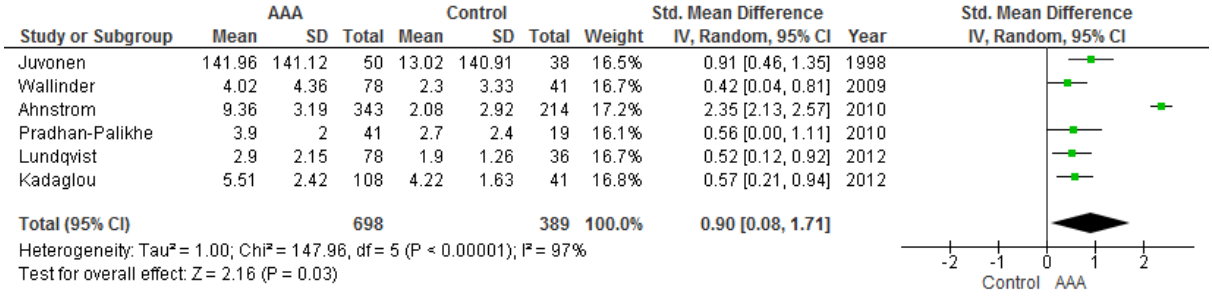


Markers of Inflammation

Interleukins

One of the key features of AAA presence and progression is inflammation[135]. The proinflammatory cytokine IL-6 is a key component in driving the systemic inflammatory response and IL-10 is a potent anti-inflammatory cytokine therefore an imbalance between these mediators could help explain differences between those with and without AAA[219]. Circulating levels of IL-6 were assessed in six studies[165,182,190,202,220,221], with four finding a significant increase[182,202,220,221] and two finding no significant difference[165,190]. Meta-analysis of these studies[165,182,190,202,220,221] found a significant increase in circulating IL-6 in patients with AAA (Std Mean Diff 0.90 pg/ml (95% CI 0.08-1.71); P=0.03) (Figure 2-9). This finding is supported by one study of aortic tissue IL-6 levels[222] finding a significant increase in patients with AAA. Two studies investigated differences in IL-10 levels in plasma, one finding a significant decrease in those with AAA[202], while the other showed no difference[190]. IL-1β, like IL-6, acts as a driver of inflammation[223]. Two studies have compared concentrations of circulating IL-1β[220,224] and one compared aortic tissue IL-1β[225], with all showing IL-1β to be significantly raised in those with AAA. These results demonstrate an increase in IL-1β and IL-6 concentration in those with AAA, and are supported by tissue studies analysing IL-1α, IL-1β, IL-4, IL-5 and IL-6 levels[222,226,227] most of which demonstrated an up-regulation within aneurysmal tissue, although one[228] found a downregulation of IL-1β and IL-6. One limitation of these studies is that levels of inflammatory markers are known to depend on age and smoking habits[219,220], which were only controlled for in one of these studies[190].

Figure 2-9 – Forest plot comparing expression of interleukin-6 in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.



Tumour necrosis factor α

Tumour necrosis factor (TNF) is an inflammatory cytokine causing smooth muscle cell death and release of proteases, leading to degradation of structural proteins in the aortic wall[229]. Eight studies[145,220,221,224-226,230,231] have shown a significant upregulation of TNF in aortic tissue[145,224-226,230], plasma[221] and serum[220,231] from patients with AAA compared to both controls, and subjects with AOD, with only one study showing significant downregulation of TNF in AAA compared to AOD[228]. Hamano *et al*[230] found TNF levels to be highest in patients with small AAAs, with Satoh *et al*[232] identifying the source of TNF to be the media and adventitia of the aortic wall, especially at the transition point between aneurysmal and non-aneurysmal tissue, therefore TNF may be involved in the early pathogenesis of AAA formation, with other factors causing aneurysm expansion. Interestingly, TNF mRNA levels have been reported in three[224,232,233] articles, with two finding significant upregulation[232,233] in patients with AAA, lending further evidence to the role of TNF in AAA pathophysiology.

Osteoprotegerin and Osteopontin

Osteoprotegerin (OPG) is a member of the TNF-related family expressed by vascular smooth muscle cells (VSMCs), and is associated with decreased VSMC proliferation, arterial calcification, and an increase in MMP secretion from monocytes and VSMCs[234,235]. Moran *et al*[235] were the first to identify an 8 to 12 fold increase in OPG levels in aortic tissue from patients with AAA, with this elevation also seen compared to patients with AOD, however these results were not replicated in aortic tissue by Liu *et al*[236]. More recent work has revealed that aortic tissue OPG levels increase with aortic diameter[237]. In the circulation, both blood[238] and plasma[202] samples have demonstrated an increase in OPG levels with AAA, and OPG levels have been correlated with aneurysm growth rates[235], however no correlation has been found with aortic diameter[147]. Therefore OPG is a plausible marker of AAA shown to be upregulated in blood, plasma, and aortic tissue compared to controls, and is potentially correlated with growth rates.

Osteopontin (OPN) is a multifunctional protein which is highly expressed in bone, however it is also expressed in macrophages, endothelial cells, and smooth muscle cells. OPN increases the expression of MMPs and is involved in collagen synthesis, angiogenesis, neovascularisation and calcification. It has been found to be significantly increased in both aortic tissue[139] and the circulation[134,202,239] of patients with AAA, however OPN was found to have a receiver operator characteristic (ROC) area under the curve of 0.65, which shows insufficient sensitivity and specificity for use as an independent marker of AAA.

Interferon γ

IFN-γ is an inflammatory cytokine which interacts with macrophages. Its production is controlled by interleukins[240]. IFN-γ has been found to be elevated in the circulation[220] of women (levels in males were not significantly different), and aortic tissue[222,224,226].

Intercellular Cell Adhesion Molecule 1 and Vascular Cell Adhesion Molecule 1

ICAM-1 and VCAM-1 are members of the immunoglobulin superfamily mediating the adhesion of leukocytes to endothelial cells[241]. They play a role in atherosclerosis and have been found to be increased in AAA. ICAM-1 has been found to be increased in both the circulation[231,242] and aortic tissue[243,244], whereas VCAM-1 has been found to be significantly increased in the circulation[242,245] but not in aortic tissue[243].

Ceruloplasmin

Ceruloplasmin is an acute phase reactant linked with inflammatory disease and is an independent marker of cardiovascular risk[246]. One study has quantified serum ceruloplasmin levels in subjects with AAA vs AOD finding no significant difference[181], however a later study found a small but significant upregulation of ceruloplasmin in subjects with AAA vs controls[180]. There is however no correlation between aneurysm diameter and ceruloplasmin levels[184].

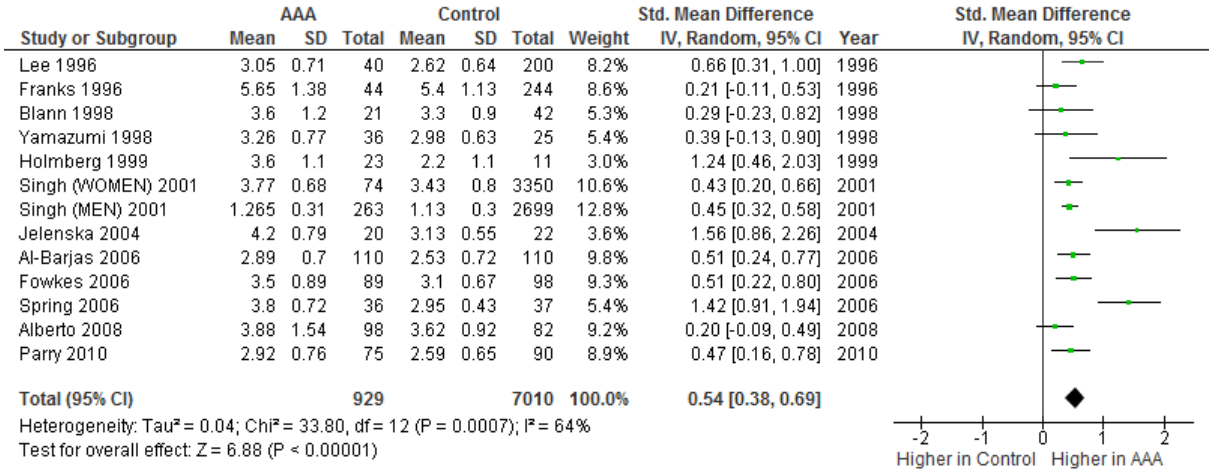
Markers of Coagulation

Fibrinogen

A total of 12 studies reported on concentrations of plasma fibrinogen in patients with and without AAA. Methods were similar between studies in that they all utilised blood samples with the majority comparing ultrasound diagnosed AAA against healthy aged

matched controls however two studies included patients with peripheral arterial disease[242,247]. Nine studies[31,186-188,242,247-250] revealed a significantly higher level of plasma fibrinogen in patients with AAA with three studies[189,242,251] finding no difference. Meta-analysis revealed a significant increase in fibrinogen in patients with AAA (Std Mean Diff 0.54 g/L (95% CI 0.38-0.69); P<0.00001) (Figure 2-10), which remained on exclusion of studies with PAD subjects (Std Mean Diff 0.48 g/L (95% CI 0.35-0.61); P<0.00001). One study[184] analysed fibrinogen concentration in different sized AAA finding no significant association with size. Whilst it is possible that fibrinogen is involved in the development of AAA these differences are likely to reflect the continual remodelling of the intraluminal thrombus[187] with the true importance possibly lying in the association of a raised fibrinogen concentration with cardiovascular risk[252].

Figure 2-10 – Forest plot comparing expression of fibrinogen in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.



Tissue plasminogen activator and Plasminogen activator inhibitor

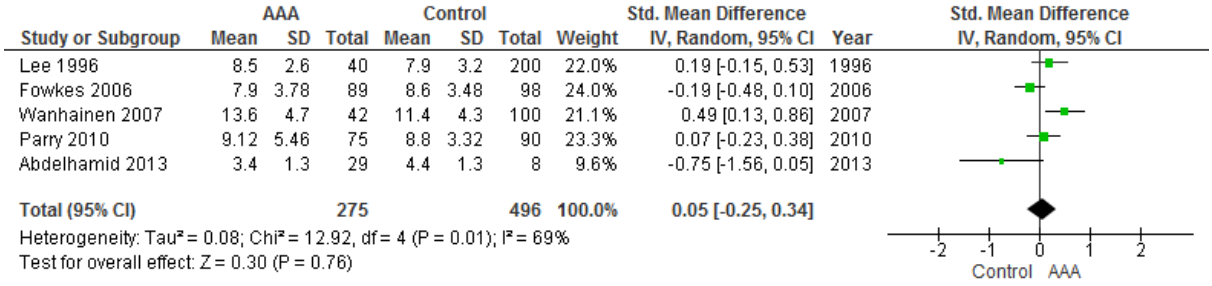
Fibrinolysis is initiated by plasmin, which plays an important role in the degradation of the extracellular matrix by directly digesting matrix proteins and activating proteolytic

enzymes[253]. In the presence of fibrin, tissue plasminogen activator (tPA) converts the proenzyme plasminogen within the thrombus into its active form, plasmin[254]. Plasminogen activator inhibitor type 1 (PAI-1) regulates plasminogen activation by inhibiting free tPA and forming an enzymatically inactive tPA/PAI-1 complex, which results in a loss of plasminogen activation potential and thereby a decreased level of proteolytic and fibrinolytic activity[255]. Plasmin is therefore regulated by tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1).

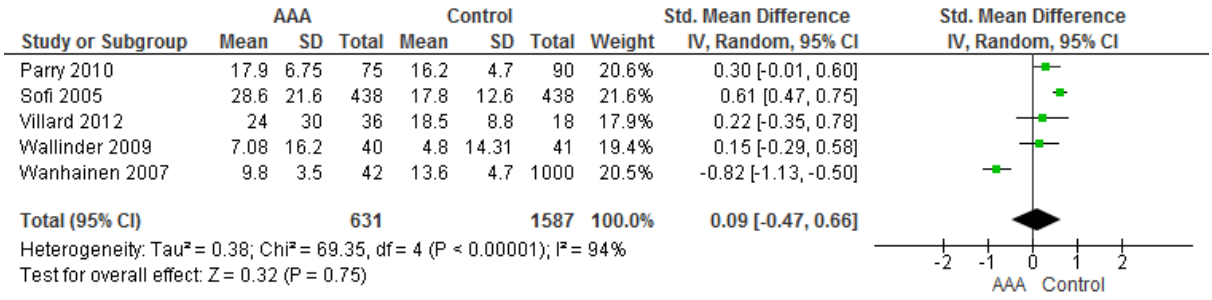
Five studies analysed tPA concentration in blood in those with and without AAA with four[187,191,205,256] finding no significant difference while one[257] found a significantly higher tPA in one group but not in another. Meta-analysis of these results did not demonstrate any significant difference (P=0.76) (Figure 2-11). There was also no significant difference found in aortic tissue tPA levels[258]. Five studies[136,190,205,257,258] analysed PAI concentration between patients with and without AAA finding no difference and only one study found PAI to be significantly higher in patients with AAA[259]. Meta-analysis of the PAI data revealed no significant difference (P=0.75) (Figure 2-11). Three studies analysed PAI activity, one[256] finding it to be significantly elevated in patients without AAA and two[205,258] found no difference. A review of the literature does not demonstrate a clear difference between tPA and PAI-1 concentrations in patients with and without AAA.

Figure 2-11 – Forest plot comparing expression of a) tissue plasminogen activator and b) plasminogen activator inhibitor in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

tPA



PAI



Lipids

Cholesterol levels have been widely analysed in patients with AAA. Five studies have found a significant increase in cholesterol levels in AAA[25,31,180,211,260] whilst four have reported a significant decrease[205,221,261,262], and twelve have reported no difference[135,175,202,203,210,242,251,263-267]. Meta-analysis of these studies revealed no significant difference in cholesterol levels between patients with and without AAA (P=0.39) (Figure 2-12). Meta-regression of seven studies[135,184,202,205,221,263,264], demonstrated no correlation with AAA diameter (P=0.93).

Triglyceride levels were significantly increased in six studies[31,180,205,211,265,266], with no difference found in nine [175,202,203,242,260,261,263,264,267], however meta-analysis of these studies has revealed a significant increase in triglycerides in patients with AAA compared to those without (Std Mean Diff 0.28 mmol/L (95% CI 0.18-0.37); P<0.00001) (Figure 2-12). Meta-regression of 4 studies[202,205,263,264] demonstrated no correlation with aortic diameter (P = 0.60).

High density lipoprotein (HDL) levels were significantly increased in two studies[260,263], decreased in eight studies[31,202,211,221,242,261,265,266], with no difference found in seven [25,175,203,242,262,264,267]. Meta-analysis of this data revealed a significant decrease in HDL levels in patients with AAA (Std Mean Diff -0.29 (95% CI -0.50 to -0.08); P<0.00001) (Figure 2-12), however meta-regression revealed no correlation with aortic diameter[202,205,221,263,264] (P = 0.63).

Low density lipoprotein (LDL) levels have been found to be significantly increased in three studies[202,211,264], significantly decreased in two [205,262], and have no difference in eight studies[175,203,260,261,263,265-267]. Meta-analysis revealed no significant difference in LDL concentration (P=0.47) (Figure 2-12), and no correlation with aneurysm diameter[202,205,263,264] (P=0.75).

Apolipoproteins (Apo) are proteins that bind lipids. ApoA-I is the major protein component of HDL, and has been found to be significantly downregulated in three studies in patients with AAA[221,261,265]. Meta-analysis of these studies found a significant decrease in ApoA-I levels (Std Mean Diff -0.75 g/L (95% CI -1.20 to -0.30); P = 0.001) (Figure 2-13). ApoB is the major protein component of LDL, and no clear relationship appears to exist with AAA, which is supported by this meta-analysis[221,251,261,262,265]

revealing no significant difference (P=0.41) (Figure 2-13). A single study[221] found a significant decrease in ApoM in AAA, with another study finding no difference in levels of ApoC-III or ApoE[261].

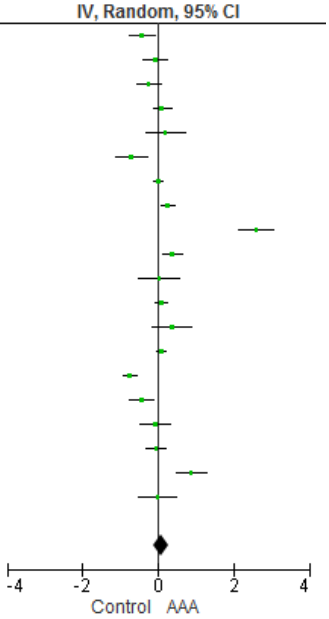
Lipoprotein(a) is a lipoprotein subclass consisting of an LDL-like particle and ApoA. Three studies have found a significant increase in lipoprotein(a) in AAA[259,267,268], with two finding no significant difference[251,265]. Meta-analysis of these studies revealed a significant increase in lipoprotein(a) in patients with AAA (Std Mean Diff 0.41 mg/dL (95% CI 0.03-0.79); P=0.03) (Figure 2-13).

Figure 2-12 – Forest plot comparing expression of a) cholesterol b) triglycerides c) high density lipoprotein d) low density lipoprotein in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

a) Cholesterol

	AAA			Control				Std. Mean Difference	
Study or Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI	Year
McConathy	5.5	1.7	51	6.1	1.2	86	5.0%	-0.42 [-0.77, -0.07]	1989
Louwrens	6.61	1.26	114	6.7	1.26	57	5.1%	-0.07 [-0.39, 0.25]	1993
Franks	5.2	1.3	44	5.5	1.19	244	5.1%	-0.25 [-0.57, 0.07]	1996
Simoni	6.15	1.07	69	6.03	1.14	1460	5.4%	0.11 [-0.14, 0.35]	1996
Blann	5.8	0.9	21	5.6	1.1	42	4.2%	0.19 [-0.33, 0.72]	1998
Naydeck	5.68	0.66	25	6.15	0.66	241	4.7%	-0.71 [-1.13, -0.29]	1999
Singh	6.73	1.2	337	6.72	1.3	6049	5.7%	0.01 [-0.10, 0.12]	2001
Hobbs	5.96	1.1	206	5.76	0.27	252	5.5%	0.26 [0.08, 0.45]	2003
Wanhainen	7.3	0.3	35	6.3	0.4	140	4.5%	2.60 [2.14, 3.07]	2005
Lindblad	6.3	1.1	126	5.9	1	126	5.3%	0.38 [0.13, 0.63]	2005
Forester	5.2	7.1	48	5	7.78	19	4.2%	0.03 [-0.50, 0.56]	2006
Jones	5.6	1.3	425	5.5	1.4	230	5.6%	0.07 [-0.09, 0.24]	2007
Rizzo	4.47	0.8	30	4.22	0.48	26	4.2%	0.37 [-0.16, 0.90]	2009
Golledge~	4.79	0.91	245	4.71	0.92	3082	5.7%	0.09 [-0.04, 0.22]	2010
Ahnstrom	4.98	1.22	343	5.85	1.07	214	5.6%	-0.75 [-0.92, -0.57]	2010
Parry	4.6	0.9	75	5	0.9	90	5.1%	-0.44 [-0.75, -0.13]	2010
Pan	4.61	1.17	45	4.69	0.85	49	4.7%	-0.08 [-0.48, 0.33]	2011
Hellenthal	5.07	1.16	218	5.13	1.33	69	5.3%	-0.05 [-0.32, 0.22]	2012
Kadoglou	5.65	0.8	63	4.77	1.24	41	4.7%	0.88 [0.47, 1.29]	2012
Qin	4.46	4.9	31	4.51	5.66	32	4.4%	-0.01 [-0.50, 0.48]	2013
Total (95% CI)			**2551**			**12549**	**100.0%**	**0.09 [-0.12, 0.30]**	

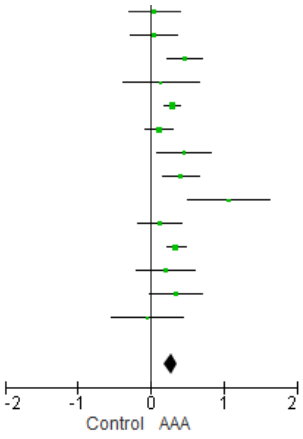
Heterogeneity: Tau² = 0.19; Chi² = 257.79, df = 19 (P < 0.00001); I² = 93%
Test for overall effect: Z = 0.87 (P = 0.39)



b) Triglycerides

McConathy	1.73	1.11	51	1.68	0.86	86	5.5%	0.05 [-0.29, 0.40]	1989
Louwrens	1.48	0.79	114	1.45	0.56	57	6.2%	0.04 [-0.28, 0.36]	1993
Simoni	1.73	0.93	69	1.46	0.56	1460	8.7%	0.46 [0.22, 0.71]	1996
Blann	2	3.04	21	1.6	2.74	42	2.8%	0.14 [-0.39, 0.66]	1998
Singh	1.95	1.15	337	1.64	1.03	6049	15.9%	0.30 [0.19, 0.41]	2001
Hobbs	1.97	1.5	206	1.8	1.5	252	11.5%	0.11 [-0.07, 0.30]	2003
Wanhainen	2.3	1.66	35	1.7	1.21	140	4.9%	0.46 [0.08, 0.83]	2005
Lindblad	1.9	1.2	126	1.5	0.7	126	8.4%	0.41 [0.16, 0.66]	2005
Rizzo	1.74	0.66	30	1.13	0.42	26	2.5%	1.07 [0.51, 1.63]	2009
Parry	1.5	0.88	75	1.4	0.73	90	6.5%	0.12 [-0.18, 0.43]	2010
Golledge~	1.31	0.59	245	1.13	0.52	3082	14.6%	0.34 [0.21, 0.47]	2010
Pan	1.29	0.46	45	1.16	0.76	49	4.3%	0.20 [-0.20, 0.61]	2011
Kadoglou	1.67	0.63	108	1.47	0.44	41	5.1%	0.34 [-0.02, 0.70]	2012
Qin	0.001	0.003	31	0.0011	0.0005	32	3.1%	-0.05 [-0.54, 0.45]	2013
Total (95% CI)			**1493**			**11532**	**100.0%**	**0.28 [0.18, 0.37]**	

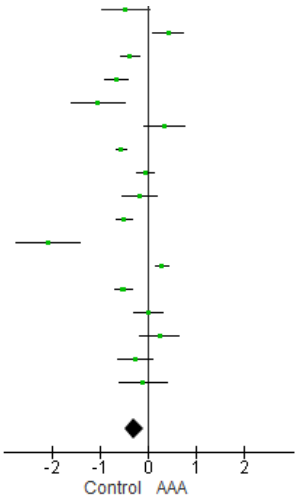
Heterogeneity: Tau² = 0.01; Chi² = 22.74, df = 13 (P = 0.04); I² = 43%
Test for overall effect: Z = 5.71 (P < 0.00001)



c) High Density Lipoprotein

McConathy	0.93	0.53	24	1.13	0.34	44	4.9%	-0.48 [-0.98, 0.03]	1989
Louwrens	1.22	0.32	114	1.08	0.36	57	6.0%	0.42 [0.10, 0.74]	1993
Pleumeekers	1.24	0.21	112	1.38	0.38	5194	6.6%	-0.37 [-0.56, -0.18]	1995
Simoni	1.32	0.31	69	1.58	0.4	1460	6.4%	-0.66 [-0.90, -0.41]	1996
Blann	1.1	0.5	21	1.5	0.3	42	4.6%	-1.05 [-1.60, -0.49]	1998
Naydeck	1.39	0.36	25	1.27	0.36	241	5.5%	0.33 [-0.08, 0.75]	1999
Singh	1.32	0.39	337	1.56	0.43	6049	6.9%	-0.56 [-0.67, -0.45]	2001
Hobbs	1.13	0.48	206	1.16	0.45	252	6.6%	-0.06 [-0.25, 0.12]	2003
Wanhainen	1.2	0.3	35	1.3	0.6	140	5.7%	-0.18 [-0.55, 0.19]	2005
Jones	1.1	0.4	425	1.3	0.4	230	6.7%	-0.50 [-0.66, -0.34]	2007
Rizzo	0.83	0.18	30	1.21	0.18	26	4.1%	-2.08 [-2.74, -1.42]	2009
Golledge~	1.4	0.34	245	1.3	0.35	3082	6.8%	0.29 [0.16, 0.42]	2010
Ahnstrom	1.06	0.35	343	1.24	0.34	214	6.7%	-0.52 [-0.69, -0.35]	2010
Parry	1.3	0.52	75	1.3	0.33	90	6.1%	0.00 [-0.31, 0.31]	2010
Pan	1.04	0.27	45	0.96	0.38	49	5.5%	0.24 [-0.17, 0.65]	2011
Kadoglou	1.09	0.28	108	1.17	0.34	41	5.8%	-0.27 [-0.63, 0.09]	2012
Qin	0.0011	0.0011	31	0.0017	0.007	32	5.0%	-0.12 [-0.61, 0.38]	2013
Total (95% CI)			**2245**			**17243**	**100.0%**	**-0.29 [-0.50, -0.08]**	

Heterogeneity: Tau² = 0.16; Chi² = 200.39, df = 16 (P < 0.00001); I² = 92%
Test for overall effect: Z = 2.76 (P = 0.006)



d) Low Density Lipoprotein

McConathy	3.31	1.2	24	4.18	0.82	44	5.8%	-0.89 [-1.41, -0.37]	1989
Louwrens	4.5	1.26	114	4.88	1.39	57	7.5%	-0.29 [-0.61, 0.03]	1993
Simoni	3.85	1.06	69	3.75	1.01	1460	8.1%	0.10 [-0.14, 0.34]	1996
Blann	3.9	0.7	21	3.4	1.1	42	5.7%	0.50 [-0.03, 1.03]	1998
Naydeck	3.52	1.01	25	4.24	1.01	241	6.7%	-0.71 [-1.13, -0.29]	1999
Hobbs	4.05	0.99	206	3.71	0.97	252	8.5%	0.35 [0.16, 0.53]	2003
Wanhainen	4.2	1.06	35	3.5	1.21	140	7.0%	0.59 [0.21, 0.97]	2005
Jones	3.4	1.2	425	3.3	1.2	230	8.7%	0.08 [-0.08, 0.24]	2007
Rizzo	2.84	0.77	30	2.49	0.54	26	5.7%	0.51 [-0.02, 1.05]	2009
Golledge~	2.79	0.81	245	2.81	0.81	3082	8.8%	-0.02 [-0.15, 0.11]	2010
Parry	2.7	0.7	75	3	1	90	7.6%	-0.34 [-0.65, -0.03]	2010
Pan	2.87	0.88	45	2.87	0.88	49	6.8%	0.00 [-0.40, 0.40]	2011
Kadoglou	3.55	0.49	108	2.93	0.57	41	7.0%	1.20 [0.82, 1.59]	2012
Qin	0.0029	0.004	31	0.003	0.003	32	6.0%	-0.03 [-0.52, 0.47]	2013
Total (95% CI)			**1453**			**5786**	**100.0%**	**0.08 [-0.13, 0.29]**	

Heterogeneity: Tau² = 0.12; Chi² = 94.78, df = 13 (P < 0.00001); I² = 86%
Test for overall effect: Z = 0.73 (P = 0.47)

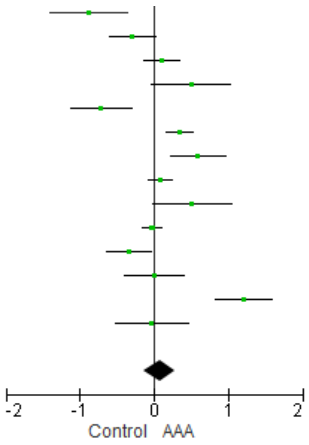
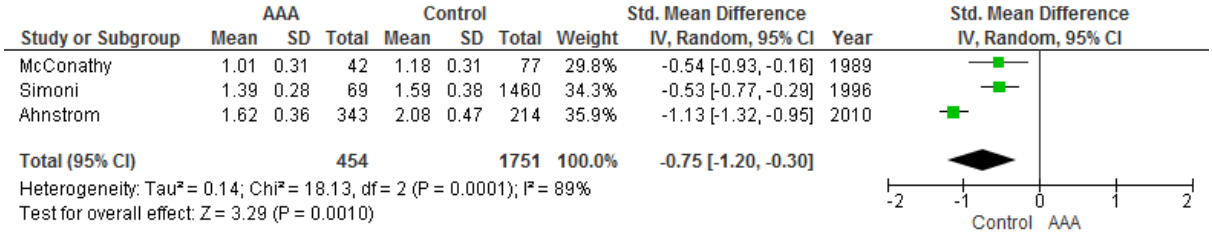
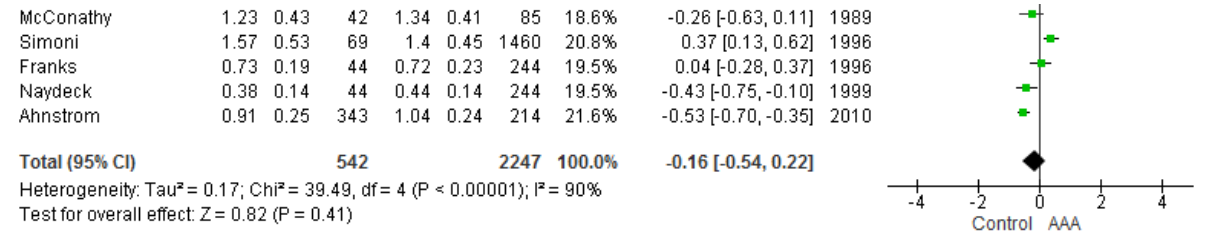


Figure 2-13 – Forest plot comparing expression of a) apolipoprotein A-I b) apolipoprotein B c) lipoprotein(a) in patients with abdominal aortic aneurysm versus controls. An inverse-variance random effects model was used. Standardised mean differences are shown with 95% confidence intervals.

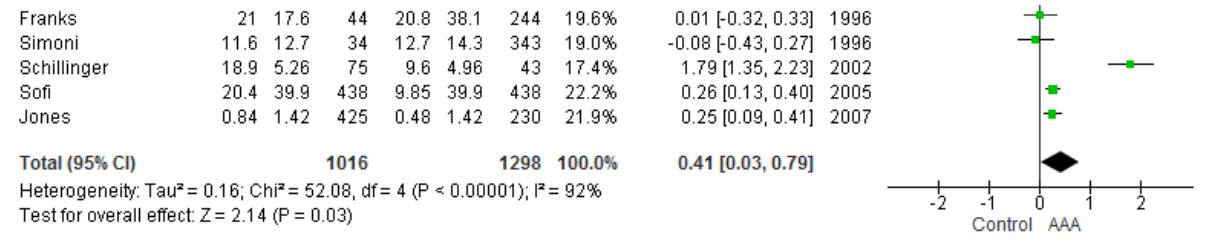
a) ApoA-I



b) ApoB



c) Lp(a)



In conclusion, triglycerides and Lp(a) appear to be increased in patients with AAA, and HDL and ApoA appear to be decreased, however whether this is causative or AAA, or as a result of aneurysm formation is debatable.

Insulin-like growth factors

IGF is involved in cell proliferation, collagen synthesis and matrix degradation. Circulating levels of IGF-1 have been found to be elevated in patients with AAA[269]. Studies looking at IGF-1 levels within the aortic wall itself however have yielded conflicting results[226,270]. In contrast IGFBP-1 levels have been shown to be elevated in both the plasma and aortic wall of patients with AAA[207,270], and elevated levels of IGFBP-3 have been found in patients with AAA[270]. The variability between studies makes the significance of these findings unclear but it is possible that the differences between circulating and tissue levels of IGF axis components are due to a reciprocal relationship that exists between these two body compartments. Furthermore changes in IGF-1 levels may also have a role in mediating the protective effect of diabetes on AAA since diabetes is known to dysregulate circulating IGF-1 levels[271].

2.3.4 Conclusion

An abundance of markers of AAA have been studied, with this review finding an increase in several proteases (MMP2, MMP9, TIMP1), inflammatory markers (IL-6, TNFα, OPG, OPN, IFNγ, ICAM-1 and VCAM-1), acute phase reactants (D-dimer, CRP, α1AT), coagulation markers (fibrinogen), and lipids (triglycerides, Lp(a), with ApoA and HDL decreased). In addition, meta-regression analysis has identified a significant positive linear correlation between aortic diameter and levels of D-dimer and CRP, with MMP2 and MMP9 levels correlated with AAA diameter in aortic tissue, but not in the circulation.

AAA is a multifaceted disease, associated with a wide variety of biomarkers, therefore understanding its true pathophysiology is complex. This study has summarised

differences in biomarker levels between patients with and without AAA, however their individual sensitivity and specificity remains low, as each of these biomarkers have been associated with a wide variety of clinical pathologies, particularly acute inflammatory and atherosclerotic processes. This is often further complicated by a lack of control for smoking or other causes of raised inflammatory markers. An ideal method of reporting for biomarkers studies would include reporting of receiver operator characteristics (ROC). ROC curve analysis has been sparsely reported in studies into AAA biomarkers, however Golledge *et al*[239] reported an area under the curve (AUC) in OPN to be 0.65, and in CRP to be 0.61, with a combination of OPN and CRP leading to an AUC of 0.66. An additional study by Golledge *et al*[194] reported an AUC for D-dimer of 0.83. Although these results show promise, they remain insufficient for use as a screening tool for AAA, however utilising an array of biomarkers such as those identified through this review may potentially provide a sufficiently sensitive and specific panel to accurately screen for patients with AAA. This would require a large scale study to determine the usefulness of this approach.

2.4 Overview of biology of AAA

The studies outlined and conducted within this chapter have identified 4 genes/loci from GWAS (DAB2IP, LRP1, SORT1 and LDLR), and 3 candidate genes from meta-analysis (MMP9, ACE, MTHFR). In addition, 16 biomarkers (MMP2, MMP9, TIMP1, IL-6, TNFα, OPG, OPN, IFNγ, ICAM-1, VCAM-1, D-dimer, CRP, α1AT, fibrinogen, triglycerides, and Lp(a)) were found to be increased in patients with AAA, and 2 biomarkers were found to be decreased (ApoA and HDL). Meta-regression analysis also identified a significant positive linear correlation between aortic diameter and levels of D-dimer

and CRP, with MMP2 and MMP9 levels correlated with AAA diameter in aortic tissue, but not in the circulation.

Although each of these genes and biomarkers show promise, none have yet proven to be useful as part of aneurysm screening, or in the development of a treatment for AAA. The majority of biomarkers for AAA have been associated with atherosclerotic disease, however very few studies were identified which examined whether differences in biomarker levels were specific to AAA or due to generalised atherosclerosis, and none of these determined any biomarkers specific to AAA. Therefore, I decided to focus my research in a relatively new field which had not previously been studied with regards to human AAA, and determine whether any findings were specific to AAA by including a cohort of patients with peripheral arterial disease (PAD). This led me to investigate the expression of microRNA (miRNA) in patients with AAA.

Chapter 3

Introduction to MicroRNAs

3.1 Overview

Micro-RNA's (miRNAs) are short (19-26) nucleotide non-coding RNA sequences, which are transcribed from DNA, however they are not translated into proteins. They are involved in human gene expression by binding to messenger RNA (mRNA) through complimentary base pairing, and preventing gene expression by the inhibition of protein synthesis[272]. Individual miRNAs are capable of binding to hundreds of mRNAs, and a single mRNA can be regulated by several miRNAs[273]. miRNAs therefore play an important role in the regulation of many biological processes.

3.2 Discovery of miRNAs

miRNAs were first discovered in 1993 in the *lin-4* gene in *Caenorhabditis elegans* when genetic screens were used to identify regulators of developmental timing[274]. The *lin*-4 gene was found to be necessary for development beyond an early larval stage, however examination of its sequence revealed a 22 nucleotide RNA sequence with no open reading frame. This was found to interact with the 3'UTR (untranslated region) of its negatively regulated protein target, *lin*-14, thereby inhibiting protein synthesis[274]. In humans, *let-7* was the first miRNA identified, which is involved in the control of developmental timing[275], and this was found to be well conserved between species[276] as well as highly conserved among plants, micro-organisms and animals, suggesting that they represent an important regulatory pathway[277]. In humans, there are currently over 1500 miRNAs in the microRNA database (www.mirbase.org), which are thought to have been shown to target approximately 60% of genes[278].

3.3 miRNA Biogenesis and Maturation

The regulation of miRNA expression is not fully understood. Regulation is thought to be at the transcriptional level, however studies have revealed that precursor miRNAs (pre-miRNAs) can be expressed throughout all tissue types, whereas mature miRNAs have a tissue specific expression, which suggests that there is a degree of post-transcriptional regulation[279].

miRNA biogenesis begins in the cell nucleus with transcription. miRNAs are coded for in regions located in the introns of both coding and non-coding genes[280], within exons[281], and also in intergenomic repeats, but many coding regions remain unidentified[282]. They may have their own promoters, and are therefore independently expressed, or they may occur in clusters (42% of known cases), with each cluster sharing the same transcriptional regulator[283]. Transcription occurs through either RNA polymerase II[284] or RNA polymerase III[285] to produce primary miRNA (pri-miRNA), which are between one hundred and a thousand nucleotides in length[286]. Pri-miRNA has a hairpin structure (Figure 3-1) with the mature miRNA sequence contained within the stem-loop head. Pri-miRNA is cleaved by two members of the RNase III family, Drosha and Dicer, to form mature miRNA. There are three classes of RNase III which specifically cleave double stranded RNA (dsRNA), each containing ribonuclease domains and a dsRNA binding domain. The three isoforms differ in the number of nuclease domains, and in the composition of the N-terminal domain (Figure 3-2).

Drosha is an enzyme belonging to Class 2 of the RNase III endonucleases. It is located within the nucleus and takes part in the processing of pri-miRNA into pre-miRNA. The enzyme is a multiprotein complex, comprising of Drosha and its dsRNA binding protein

Pasha. Within the nucleus the Drosha Pasha complex cleaves end tails of the stem-loop of the pri-miRNA in a process known as cropping[287] (Figure 3-1), to form a 70 nucleotide long structure termed precursor miRNA (pre-miRNA)[288]. This is then transported into the cytoplasm via a nuclear membrane transporter Exportin 5, and Ran-GTP[289]. Once pre-miRNA enters the cytoplasm it is further processed by Dicer, a class 3 RNase III enzyme.

Figure 3-1 – miRNA biogenesis. miRNA genes are initially transcribed into pri-miRNA with a hairpin like structure. They then undergo slicing by Drosha into pre-miRNA, followed by export into the cytoplasm. Pre-miRNAs then undergo dicing to a mature miRNA duplex followed by uncoupling into single-stranded miRNA. This then binds with the RNA-induced silencing complex, which can then bind to mRNA to regulate translation. DGCR8 – DiGeorge Syndrome Critical Region Gene 8, TRBP – Transactivation Response RNA Binding Protein. (Reproduced with permission from Oxford University Press: Chang S, Wen S, Chen D, Jin P. Small regulatory RNAs in neurodevelopmental disorders. Human Molecular Genetics. 2009;18:R18-26)[290].

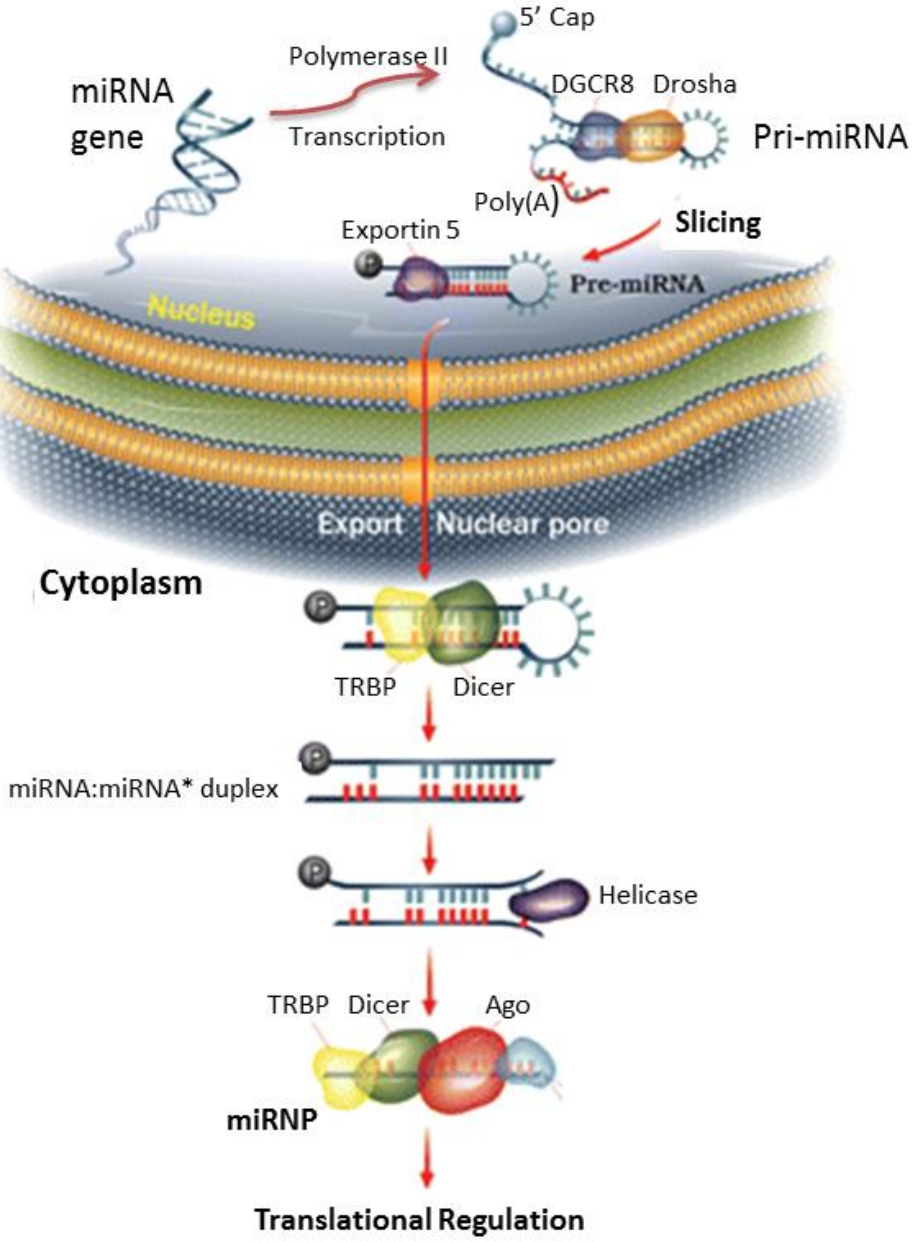
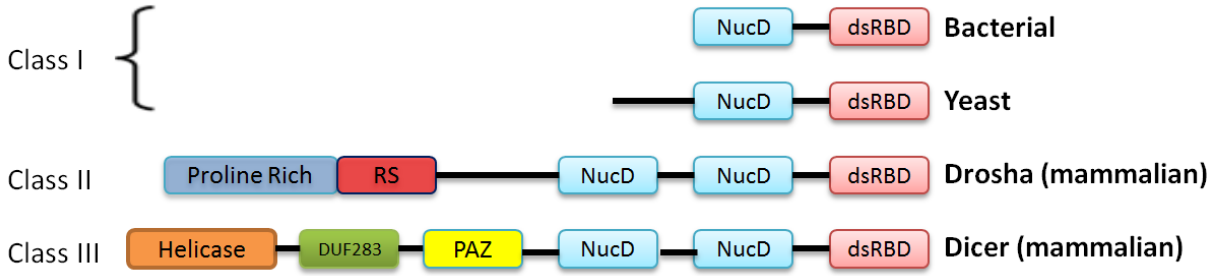


Figure 3-2 – Three classes of RNase III polypeptides and their functional domains. Class 1 orthologues include bacterial and yeast RNases. The class II orthologues are represented by Drosha, whereas class III orthologues are represented by Dicer. DUF – domain of unidentified function, PAZ – Pizi-Ago-Zwille domain, RS – arginine-serine-rich region, dsRBD – double stranded RNA binding domain, NucD – Nuclease domain. (Reproduced with permission from Portland Press Ltd: Meng W, Nicholson AW. Heterodimer-based analysis of subunit and domain contributions to double-stranded RNA processing be Escheria Coli RNase III in vitro. Biochemical Journal. 2008;410:39-48)[291].



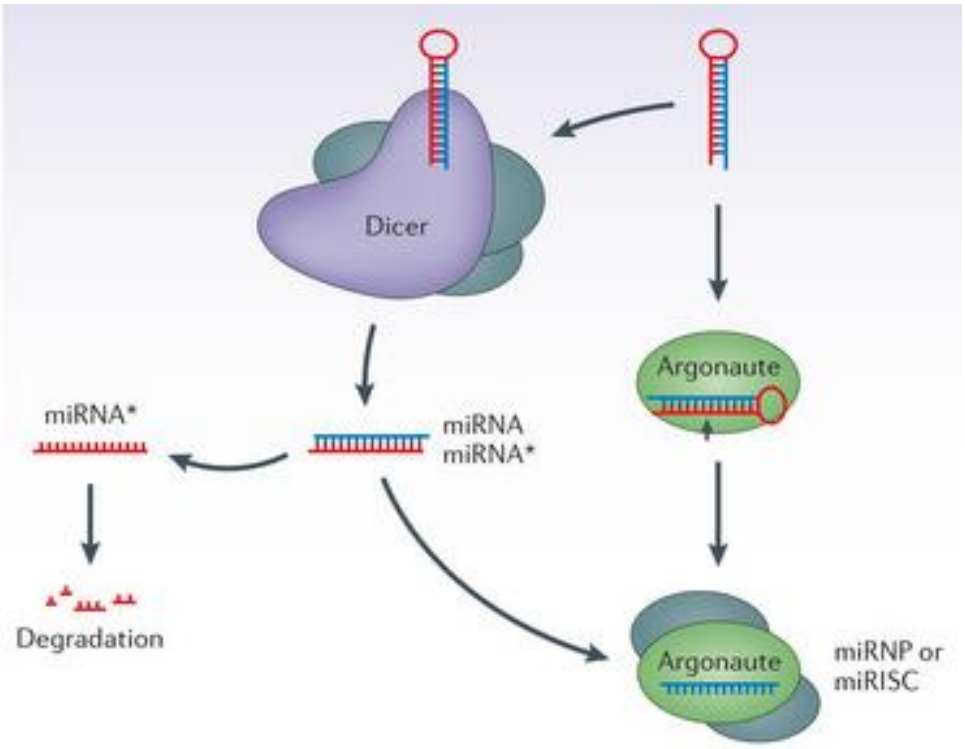
Dicer (Figure 3-2) recognises the base of the pre-miRNA hairpin and cleaves approximately 20 base pairs from the hairpin end (Figure 3-1). This leaves an imperfect double-stranded RNA duplex (Figure 3-1) (with a 2 nucleotide 3' overhang), known as mature miRNA. Drosha and Dicer are essential components of miRNA processing, with their knockdown in endothelial cells through siRNA transfection leading to downregulation of approximately 60% of human miRNAs (with the further 40% thought to have remained non-significant due to the transient nature of the knockdown, and miRNA stability)[292]. Mature miRNA must be incorporated (Figure 3-3) into a RNA-induced silencing complex (RISC) in order to exert an effect on its target mRNA. It does this through the mature miRNA binding to a protein (Argonaute 2) as a part of the RISC. The complementary strand is most commonly released and degraded[293], although in some instances both strands are functional.

Although the processing pathway described above is the most common pathway for mature miRNA formation, several other mechanisms have been described. A single class of miRNAs known as mirtrons generate pre-miRNAs directly from a splicing event[294,295]. MiR-451 has been found to be processed independent of Dicer, instead being cleaved by Drosha into an 18 base pair duplex system, which is too short to serve as a Dicer substrate, and therefore binds to Argonaute 2 and is spliced on the 3' hairpin arm guided by the 5' end resulting in a 23 nucleotide mature miRNA[296,297]. Despite the difference in processing, the resulting mature miRNA sequences are typically 19-26 nucleotides in length, and are able to exert an effect on their target mRNA.

3.4 Mechanisms of miRNA Function

Following its synthesis and incorporation into the RISC complex, the mature miRNA become capable of interacting with its target mRNAs. miRNAs target mRNAs through the 2^{nd}-7^{th} bases of the mature miRNA sequence, known as the miRNA seed. This miRNA seed has complementarity to target sites in the 3' UTR of mRNA transcripts[298-300]. The binding of miRNA to mRNAs through this mechanism allows miRNAs to regulate hundreds of genes, as the miRNA is capable of binding to any mRNA with sufficient seed region complementarity, as described later[301,302].

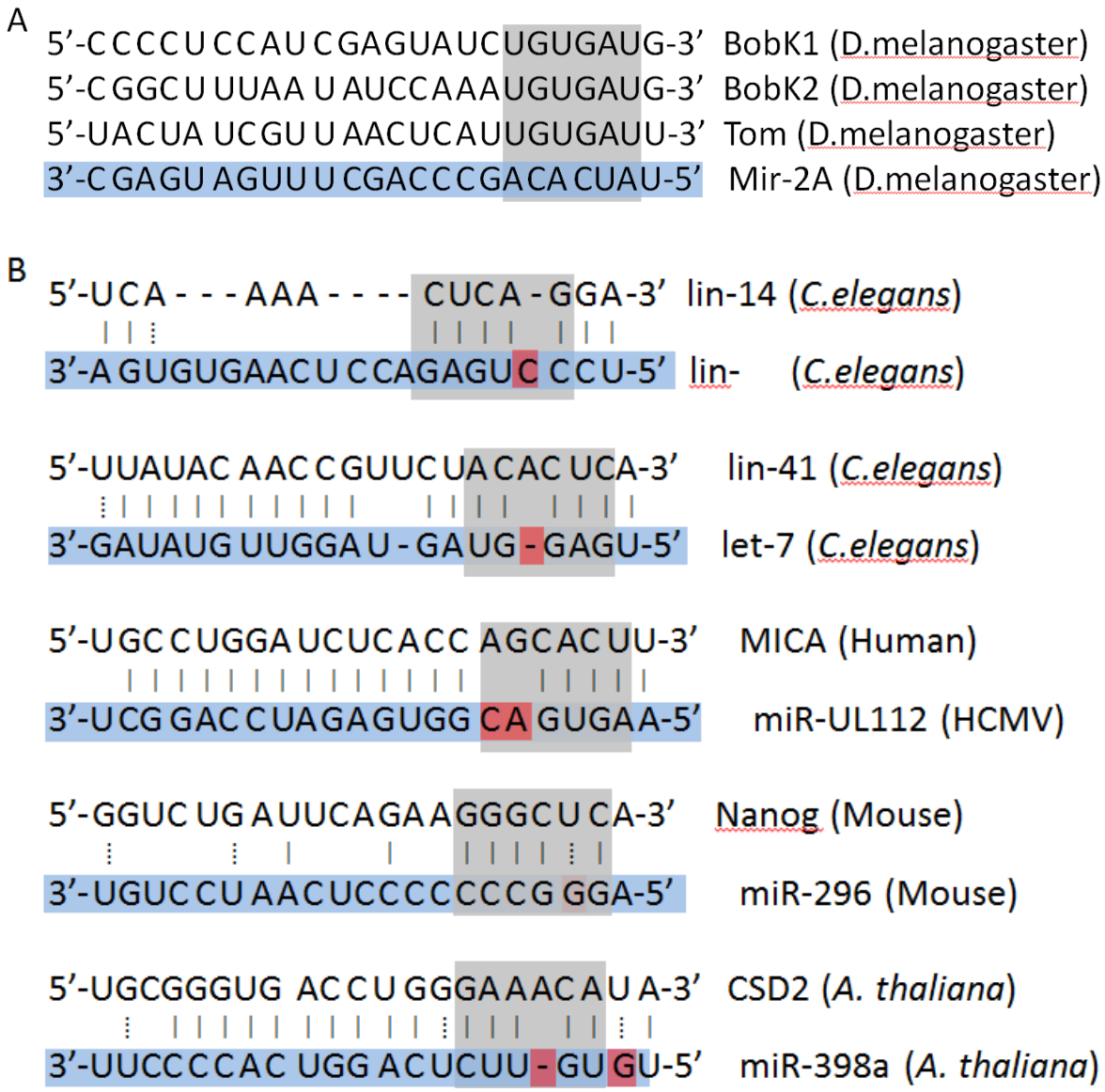
Figure 3-3 –Dicer cleaves off the loop of the miRNA hairpin and generates a double-stranded RNA. Mature miRNA is incorporated into the RNA-induced silencing complex (RISC, or miRISC for miRNA-containing RISC), where it directly binds to a member of the AGO protein family. The other strand is referred to as miRNA* and is normally degraded. It has also been shown that miRNAs can be processed independently of Dicer. AGO2, for example, can cleave miRNA precursors and produce mature miRNAs (Reproduced with permission from Macmillan Publishers Ltd: Meister *et al.* Argonaute proteins: functional insights and emerging roles. Nature Reviews Genetics. 2013;14:447-459. Copyright 2013).



MiRNAs have been shown to silence gene expression through initiating endonuclease cleavage of mRNA, mRNA degradation, translational repression, or deadenylation. The mechanism is in part determined by the level of complementarity between the miRNA seed and the mRNA sequence, however alternative methods have been described. mRNA cleavage or degradation, which is seen with almost perfect complementarity, is typically seen in plants.

In animals[303-305], the most common method of miRNA:mRNA interaction is first through translational repression followed by deadenylation of the mRNA, leading to mRNA degradation and a decrease in protein expression[306]. Translational repression is where a decrease in protein product is found that is greater than the observed decrease in mRNA, therefore the mRNA is not being transcribed. This occurs initially through miRNAs binding to mRNAs in a complex with Argonaute proteins 1-4, forming the initial translational repression at the 3' UTR. This complex then recruits one of the trinucleotide repeat-containing proteins and binds to a secondary structure in the 5' UTR causing deadenylation. mRNAs are formed with the addition of a 3' poly(A) tail (additional Adenine nucleotides) which increase the stability of the mRNA structure, thus limiting degradation. miRNAs cause degradation of this poly(A) tail (deadenylation), and therefore destabilisation of the mRNA. Mismatches in the seed region can be compensated for in some instances with additional pairing at the 3' end with bases 13-16 of the miRNA[307]. Non-seed matched pairing may occur in cases of centered site pairing, where 11 continuous nucleotides between the 4^{th}-15^{th} base pairs of the mature miRNA bind with the mRNA[308]. Additionally, Argonaute 2 mediated target cleavage may occur opposite positions 10 and 11 of the mature miRNA when near perfect complementarity exists[303,309-311] (Figure 3-9).

Figure 3-9 – mRNA target sequences are shown in black and miRNAs are highlighted in blue. Connecting solid lines indicate a Watson–Crick base pair, and connecting interrupted lines indicate a GU wobble pair. The seed region is shaded in grey and positions that violate the seed rule are shown in red. A) Several Notch target genes in Drosophila melanogaster are regulated by miRNAs through target sites that have perfect seed matches but minimal complementarity elsewhere. B) Examples of non-seed matches. Caenorhabditis elegans lin-14–lin-4 and lin-41–let-7 contain non-seed target sites with single bulges in the seed match region (shown in red). The human cytomegalovirus miR-UL112 targets human MICA despite the occurrence of a double mismatch in the seed region, and several miR-296 sites in murine Nanog contain GU wobbles in the seed region. Similarly, the Arabidopsis thaliana CSD2–miR398a interaction contains both a bulge and GU wobble in the seed region. (Reproduced with permission from MacMillan Publishers Ltd: Brodersen P, Voinnet O. Revisiting the principles of microRNA target recognition and mode of action. Nature Reviews Molecular Cell Biology. 2009;10(2):141-8 Copyright 2009)[312]



3.5 Tissue-Specific miRNAs

There is a clear difference in mature miRNA signature between cell and tissue types. Several studies have looked at the abundance of miRNAs in specific tissues, identifying distinct patterns of miRNA expression in human skeletal muscle, heart, prostate, brain, liver, spleen and colon[313-315]. Specific miRNAs are also only found in certain tissues, such as miR-122 in liver[316], and miR-1, miR-208 and miR-133 in muscle or heart[317]. This is of particular use in acute conditions such as myocardial infarction, where cardiac specific miRNAs are released into the circulation. It can also be of use in states of increase cell proliferation, such as cancer, where high levels of circulating miRNAs can be identified in plasma, which are not normally present[318,319].

3.6 miRNA Nomenclature and Annotation

miRNAs are named in three parts. The initial classification relates to the species of origin of the miRNA, with hsa relating to human miRNAs, and mmu referring to mouse miRNAs. miRNAs are then named as such with the prefix miR, followed by a number. The number relates to the miRNAs time of discovery, with more recently discovered miRNAs receiving a higher number. miRNAs with a nearly identical sequence are distinguished by a lower case letter following the number, for example miR-29a, miR-29b, and miR-29c are very similar in sequence. Some miRNAs are transcribed from two different genetic loci, and are then annotated by a dash and a second number, for example miR-250-1 and miR-250-2 are identical. Finally, two miRNAs may arise from the same pre-miRNA, but originate from opposite arms of the pre-miRNA. These miRNAs are distinguished by a 3p or 5p suffix[320], or alternatively with a *.

3.7 Target Predictions and Algorithms

As the mechanism of miRNA:mRNA interactions is known to primarily occur through complimentary or near-complimentary base pairing to the 3' UTR of the mRNA many algorithms have been developed in order to predict the mRNAs targeted by each miRNA species. Several groups of features are used for prediction software. The first miRNA alignment algorithm was miRANDA[321]. This awards points through base pair matching, employs greater weight to interactions within the first 11 base pairs, and has 4 empirical rules: No mismatches in bases 2-4, <5 mismatches in bases 3-12, at least 1 mismatch in bases 9 – 5 from the end, and <2 mismatches in the final 5 base pairs. It then incorporates the energy required for the specific miRNA:mRNA interaction to occur from a completely dissociated state, therefore determining the probability of an interaction. Finally the conservation of the binding site within the 3' UTR is incorporated to determine miRNA:mRNA interactions.

Since the introduction of miRANDA, several prediction algorithms have been developed (many of which are based on miRANDA), such as miRWalk, miRecords, TargetScan, RNA22, MicroRNA.org, microCosm, PITA, PicTar, Diana-microT, miRDB, and RNAhybrid. The newer programs incorporate several of the more modern theories of miRNA:mRNA interactions into their algorithms. Seed sequence match is used by all prediction algorithms, and looks for a high degree of complementarity between the 5' end of the miRNA and the 3' end of the mRNA target. Particular attention is paid to the seed region, however base-pair mismatches and G:U wobbles (where a G aligns opposite a U rather than a C) are allowed for[322]. Several prediction algorithms take into account the conservation of the miRNA binding sequence within the mRNA target, as a

highly conserved target is thought to produce a more reliable prediction[323]. As well as binding site conservation, the mRNA binding site must be unfolded to permit the miRNA to access it, and refold into a duplex allowing an interaction to occur[324]. Many algorithms incorporate a greater probability of an interaction if several binding sites are possible in the 3' UTR of a particular mRNA reasoning that a dose-dependent effect may be exerted on target expression. In addition, mRNA binding sites may be for a single miRNA or multiple different but closely related miRNAs, resulting in synergistic gene expression[325]. For example, milk production in goats is synergistically upregulated by 3 miRNA pairs (miR-23a and miR-27b, miR-103 and miR-200a, and miR-27b and miR-200a), with overexpression of these miRNAs in tandem further decreasing milk production[326]. Several studies have shown that the ideal inter-binding site distance falls between 8-40 base pairs[327,328].

The local mRNA sequence surrounding the binding site may be taken into account within algorithms as this can affect binding capability, and site accessibility. In addition, it is known that the 15 nucleotides after the stop codon in the 3' UTR form poor miRNA binding sites that show little ability to repress expression, due to the mRNA entering the ribosome 15 nucleotides downstream of the decoding site[329]. This therefore blocks additional binding until the ribosome dissociates from the messenger RNA (the ribosomal shadow)[328]. Studies have shown that an enrichment of A or U base pairs in the 30 nucleotides surrounding the miRNA binding site tends to associate with gene repression[328,330]. Some prediction algorithms reward miRNA target sites that fall within the first or final quartile of the 3' UTR as these sites are more selectively maintained[328]. Finally, other than a strong association with the seed site, many

algorithms also require nucleotide binding between the 12th-17th base pairs of the miRNA and the mRNA[328].

In addition to tools for predicting miRNA targets, databases are kept of experimentally validated miRNA:mRNA interactions such as miRWalk, miRTarBase, and miRecords which all link to PubMed, enabling an additional level of strength to pathway analysis compared to prediction software. However, as several miRNAs interact with a single mRNA, and a single mRNA can be affected by several miRNAs, the number of potential interactions identified can be extremely high. Two prediction algorithms (miRANDA and PITA) detail over 7 million interactions between miRNA and mRNAs, therefore, due to the laborious nature of assessing every predicted interaction, the use of prediction tools remains essential. A modern way of combating prediction software inaccuracies is to use multiple prediction algorithms simultaneously, as can be done with miRWalk. One can then ensure that the interaction of interest is predicted by several different algorithms, thereby improving specificity of the results[323].

3.8 MicroRNAs as therapeutic targets

The modulation of miRNAs has the potential to evolve as a new branch of therapeutics designed to treat specific conditions. Although there are currently no commercial drugs in use which work through targeting miRNAs, in-vitro and animal trials are currently being undertaken to explore this potential use. Several preclinical trials are utilising miRNA replacements, such as let-7 for lung cancer, and miR-34 for lymphoma, with others antagonising miRNAs such as miR-208/499 for chronic heart failure, and miR-195 for post myocardial infarction remodelling. Miravirsen (Santaris Pharma) is the first drug to enter human clinical trials. It is a specific inhibitor of miR-122, a liver

specific miRNA, which is required by the Hepatitis C virus (HCV) for replication[331]. In preclinical trials miravirsen produced a potent, dose-dependent and long lasting inhibition of miR-122 in mice, monkeys[332,333], and chimpanzees[334], with no evidence of viral resistance in long-term studies. Phase 1 dose safety studies in healthy volunteers showed that miravirsen was well tolerated[334], and initial data from Phase 2a clinical trials, where miravirsen was given as a 4 week therapy to Hepatitis C patients showed a significant reduction in viral load with 4 out of 9 patients having undetectable levels of HCV RNA. These effects were also maintained for more than 4 weeks beyond the end of therapy[335]. This suggests that miRNA inhibition could potentially be of use in a wider variety of diseases, with repeated doses over time slowing the progress of chronic disease. Therefore determining miRNAs dysregulated in subjects with AAA could potentially lead to a long-term therapy to slow AAA growth.

Chapter 4

MicroRNAs in Abdominal Aortic Aneurysms

AAA is a degenerative condition of the aneurysm wall characterised by a combination of local chronic inflammation, apoptosis leading to a decrease in the number of smooth muscle cells, fragmentation of the extracellular matrix through proteolysis, and oxidative stress as previously described (Chapter 1.1.7). As such, miRNAs associated with vascular smooth muscle cells (VSMCs) or the extracellular matrix (ECM), are candidate miRNAs for aneurysm pathogenesis.

4.1 MicroRNAs in Vascular Smooth Muscle Cells

VSMCs play an important role in the pathogenesis of AAA. Increased VSMC remodelling occurs in AAA formation through a process of increased apoptosis in the medial layer of the aorta[336], and de-differentiation of VSMCs[337], which permits increased VSMC proliferation[338,339]. Several miRNAs have been identified which increase VSMC proliferation (upregulation of miR-21, miR-221/222, miR-146a, miR-31, miR-26a, and miR-208, downregulation of miR-1), and increase VSMC de-differentiation (mir-143 and miR-145), therefore miRNA regulation of VSMCs is a plausible pathway leading to aneurysm formation.

Ji *et al* [340] undertook the first functional studies into the role of miRNAs in VSMCs, through experimental balloon angioplasty in rat carotid arteries, revealing significant upregulation of miR-21 following carotid angioplasty. Downregulation of miR-21 inhibited proliferation, thereby inhibiting neointima formation. miR-21 has been extensively studied in cancer, being shown to both promote and inhibit cell proliferation in different tumours[341], and it has also been found to be essential for the differentiation of VSMCs through downregulation of programmed cell death protein 4[342].

miR-143 and miR-145 (co-transcribed miRNAs), which have been shown to be downregulated in thoracic aortic aneurysms by Elia *et al*[343], are involved in the regulation of VSMC phenotype[344] with their knockout shown to alter the contractile phenotype of VSMC to a dedifferentiated, synthetic SMC phenotype that leads to neointima formation in aged mice[345]. Chen *et al*[346] reported that myocardin overexpression in VSMCs induces the expression of miR-1, resulting in downregulation of Pim-1 (a serine/threonine kinase), thus inhibiting VSMC proliferation.

Dedifferentiation and proliferation of VSMCs has also been found to be associated with miR-221, miR-222, and miR-146a[347]. miR-221 exerts this effect through its interaction with platelet derived growth factor, causing VSMC proliferation, migration, and reduced expression of contractile genes[348]. The expression of miR-146a has been found to be elevated in proliferative VSMCs, possibly through its interaction with Kruppel-like factor 4[349]. These miRNAs have been found to be elevated in rat balloon injured arteries compared to uninjured controls[344], suggesting a role in VSMC proliferation and neointima formation.

Other miRNAs affecting VSMC pathophysiology include miR-26a, miR-31, and miR-208. miR-26a directly targets SMAD1 and SMAD4 (mothers against decapentaplegic homologue 1 and 4), thus promoting VSMC differentiation[350]. miR-31 is an abundant miRNA in VSMCs, with its expression increased both in the proliferative state, and alongside neointimal growth[351]. Furthermore, upregulation of miR-31 has a pro-proliferative effect on VSMCs through the large tumour suppressor homolog 2 (LATS2), however miR-31 may be inhibited by mitogen-activated protein kinase (MAPK), suggesting the possibility of a MAPK/miR-31/LATS2 pathway[347]. miR-208 increases

basal and insulin-mediated VSMC proliferation through increasing the progression of the cell cycle from G0/G1 phase to S phase[352].

A total of 10 miRNAs have been identified in the above studies to be associated with VSMC pathophysiology. Despite these studies being conducted in non-human models and at anatomical locations distant from the infrarenal aorta where AAAs usually occur, this association between miRNAs and VSMC regulation is a potential target for future AAA research.

4.2 MicroRNAs in the Extracellular Matrix

Destruction of the ECM is an established contributing factor for AAA formation, with evidence that the balance between MMPs and their inhibitors is altered leading to matrix degradation[353]. There are currently no studies determining miRNA involvement in the ECM in AAA disease specifically, however plausible biological candidates can be identified.

The miR-29 family (miR-29a, miR-29b, miR-29c) are all key regulators of the ECM. Evidence from the ECM of both trabecular meshwork cells[354] and hepatic stellate cells[355] have revealed that the miR-29 family causes downregulation of fibrillins, elastin, collagens[355], and laminin[356] with work on murine aortic development by Ott *et al*[357] replicating these results in the aortic ECM. miR-29b has also been found to directly target MMP2[358], which has been linked to AAA formation[359], along with the mRNAs for bone morphogenetic protein 1, disintegrin and metallopeptidase domain 12 and NF-Kappa-B inhibitor-interacting Ras-like protein 2[359]. Results by Boon *et al*[360] demonstrated that vascular aging and human thoracic aneurysms both have upregulated miR-29 expression. As miR-29 has been shown to target Mcl-1 and p53,

which induce apoptosis in cancer cells[361,362], and miR-29 is highly expressed in VSMCs[363] as well as the ECM, Boon *et al* hypothesised that miR-29 may cause VSMC apoptosis, which is believed to promote aneurysm formation[364]. Recent work by Maegdefessel *et al*[365] has contradicted this theory, by identifying decreased levels of miR-29b in both mouse models of AAA, and human AAA tissue, however this was performed in aortic smooth muscle cells and aortic adventitial fibroblasts, rather than the ECM (full details of this study discussed in Chapter 4.4).

There is also evidence miR-7 causes downregulation of MMP2 and MMP9 in glioblastoma cell invasion[366], and miR-18a, miR-19a, and miR-19b decrease are associated with an increase in connective tissue growth factor (CTGF) and thrombospondin-1 with age-related heart failure, causing alteration of collagens type 1 and 3[367].

Decreased levels of miR-133 and miR-30 have also been found to cause upregulation of CTGF in heart disease and left ventricular hypertrophy, leading to collagen synthesis[368], showing their role in the control of structural change in the extracellular matrix of the myocardium.

Although there is no direct evidence that these miRNAs are differentially expressed in the ECM of the aneurysmal aorta the 9 miRNAs above have not been directly assessed in AAA.

4.3 MicroRNAs in Animal Models of Abdominal Aortic Aneurysm

Two approaches to investigating the role of miRNAs in AAA are using a miRNA transcriptome-wide (miRNome) approach, or looking directly at specific miRNAs.

The first study into miRNA expression in the aorta was undertaken by Boon *et al*[369], who identified 18 miRNAs which were dysregulated with age in mice aortas by microarray (upregulated miR-146, miR-142-3p, miR-29b, miR-223 and downregulated miR-299*, miR-181c, miR-127, miR-154, miR-337-5p, miR-379, miR-136, miR-329, miR-31, miR-322, miR-377, miR-434-3p, miR-411, miR-181d). The upregulation of miR-29a, miR-29b, and miR-29c were validated using PCR. The targets of the miR-29 family were also found to be downregulated with age in murine aortic tissue (including elastin, collagen, fibrillins, MMP16, MMP14, MMP2, MMP9, MMP13, and TIMP4).

A single study by Liu *et al*[370] used a miRNome approach, identifying fifteen differentially expressed miRNAs in a rat model of AAA, where aortic tissue was treated with either collagenase or saline, identifying upregulation of miR-146a, miR-221, miR-92a, miR-19b, miR-222, miR-34b, miR-142-3p, miR-188, miR-132, and miR-19a, and downregulation of miR-301a, miR-152, miR-497, miR-148b-3p, and miR-181d. Four miRNAs (miR-19a, miR-19b, miR-132, and miR-222) were selected randomly for quantitative PCR validation using the same aortic tissue samples, confirming that these miRNAs were upregulated. Although this study used small sample sizes (3 cases vs 3 controls), it determined significant results with stringent criteria, including a 2 fold change in expression, $p<0.001$ and a false discovery rate <0.05.

Leeper *et al*[371] identified 135 dysregulated miRNAs in serum-starved human aortic smooth muscle cells (hASMCs), and went on to investigate the effects of miR-26a in aneurysmal disease. Two murine AAA development models of $ApoE^{-/-}$ mice were utilised (Elastase and AngII infusion), identifying miR-26a to be significantly downregulated in the aneurysm wall after 7 days. The level of miR-21 was also found

to be increased in the elastase AAA model. Of 535 predicted targets of miR-26a, 124 were found to be increased during AAA development in this model, with many of these genes involved in the cell cycle, apoptosis, proliferation, migration, and cytokine production.

Maegdefessel *et al*[365] used a targeted approach to evaluate the levels of miR-29a, miR-29b, and miR-29c in mouse models of AAA. They identified that decreased levels of miR-29b were associated with AAA formation in two mouse models of AAA induction; porcine pancreatic elastase (PPE) infused mice and Angiotensin II (AngII) infused mice, however miR-29a and miR-29c were not significantly altered. They determined that AAA induced mice had significantly decreased levels of miR-29b, and went on to show that addition of anti-miR-29b increased collagen and elastin, but decreased MMP2 and MMP9, whereas addition of pre-miR-29b normalised collagen and elastin, and increased MMP2 and MMP9. The authors then used hASMC and human aortic adventitial fibroblasts (hAFB) treated with transforming growth factor β1 (TGF-β1), a known regulator of miR-29b to determine downstream regulation of mRNA levels. TGF-β1 treatment significantly decreased miR-29b expression in hAFBs but not hASMCs, and the inhibition of miR-29b increased levels of collagen type 1 alpha 1 (COL1A1), collagen type 3 alpha 1 (COL3A1) and elastin (ELN). In the mouse models, *in vivo* addition of pre-miR-29b augmented abdominal aortic diameter growth, leading to rupture in several cases, whereas inhibition with anti-29b reduced abdominal aortic diameter expansion and increased levels of COL1A1 and COL3A1. Total MMP activity was lower in anti-29b treated mice, and higher in pre-miR-29b mice at 14 days, as was MMP2 and MMP9 expression using polymerase chain reaction (PCR). This study also

correlated their results with human samples, comparing 15 human AAA tissue specimens and 5 organ donor control samples. This revealed miR-29b to be significantly downregulated in human AAA, with these samples also containing significantly increased levels of collagen and elastin. This study contradicts the association between MMP9 and AAA, identified in a meta-analysis by Thompson *et al*[121] to be upregulated in AAA, however this may solely be an effect of AAA induction in mice, as MMP levels were not assessed in human aortic tissue.

An additional study by Maegdefessel *et al*[372] investigated whether nicotine accelerated AAA progression in PPE infused mice, finding a significantly increased aortic diameter from days 7-28 in those undergoing nicotine infusion compared to controls. Aortic tissue miR-21 expression was measured using PCR at days 7, 14 and 28, and was found to be significantly higher in the nicotine group than in the placebo group at each time-point. The miR-21 target PTEN was also found to be significantly downregulated in nicotine supplemented mice $P<0.05$, and the PDCD4 and SPRY1 genes were significantly downregulated at day 14 only. The pro-apoptosis gene BCL2 was also significantly upregulated after 28 days. The authors then used primary human aortic endothelial cells, hASMCs, and hAFBs treated with nicotine, finding that miR-21 expression was upregulated in all cell types. Modulation of miR-21 expression using antagomir-21 (antagomirs are synthetic molecules that bind to the mRNA at the same binding site to miRNAs, thus silencing miRNA effects) and pre-miR-21 in hASMCs and hAFBs was found to significantly modulate the expression of PTEN, PDCD4 and SPRY1, but not BCL2, highlighting the potential for the use of miRNAs as treatments. Therefore, using a PPE infusion mouse model the authors infused antagomir-21 and

pre-miR-21 and demonstrated that antagomir-21 increased PTEN expression at days 7 and 14, but not 28 (possibly due to single injection at day 0 wearing off), and pre-miR-21 treatment led to further downregulation of PTEN at days 7, 14, and 28. Inhibition of miR-21 was also shown to increase AAA growth, and treatment with pre-miR-21 decreased AAA growth. In order to validate these results, the $ApoE^{-/-}$ AngII mouse model was also used, revealing similar outcomes. Finally, human aortic tissue was obtained from 8 smokers and 5 never smokers with AAA, and 5 controls, finding that miR-21 expression was upregulated in AAA, and more so in smokers with AAA than controls. In the same population PTEN was downregulated in AAA, and further downregulated in smokers.

Damrauer *et al*[373], in a recent abstract, looked at the role of the homeostatic protein A20 in a mouse model. Mice with a single functional copy of the A20 gene were bred, with comparisons made between haploninsufficient mice (with decreased gene product) and haplonsufficient mice (with sufficient gene product to bring about a wild-type condition). The authors investigated the expression of miR-143 and miR-145 as part of a pathway analysis and demonstrated a 1.6 fold increase in the expression of miR-143 and miR-145 in A20 haplonsufficient mice who did not form AAA, compared to control animals which developed AAA enlargement.

Maegdefessel *et al*[374], in a further abstract, investigated the role of miR-24 in AAA in both PPE and AngII models. In both models miR-24 downregulation was accompanied by an increase in the expression of YKL-40, a regulator of inflammation and calcification in vascular disease. Lentiviral overexpression of miR-24 inhibited YKL-40 induction, leading to a decrease in AAA expansion, and also significantly decreased

inflammatory activity and macrophage infiltration, as well as stimulated calcification within the aortic wall. Treatment with antagomiR-24 further increased YKL-40 expression, leading to augmentation of inflammation and decreased calcification with subsequent acceleration of AAA progression. Similar results were also found in human aortic tissue samples, hASMCs, hAFBs and endothelial cells.

The results from animal studies show marked variability in miRNAs investigated, with 10 validated miRNAs identified in a variety of mouse models, only some of which have been replicated in human aortic tissue (Table 4-1).

Table 4-1 – Summary of miRNAs found to be associated with abdominal aortic aneurysms, identified in animal studies. Only miRNAs validated in an additional cohort are included, in order to decrease the false discovery rate.

microRNA	Tissue type	Dysregulation	Study
miR-19a	Sprague Dawley rat aortic tissue	Upregulated	Liu *et al*[370]
miR-19b	Sprague Dawley rat aortic tissue	Upregulated	Liu *et al*[370]
miR-21	PPE and ApoE$^{-/-}$/AngII mice aortic tissue, hAECs, hASMCs, hAFBs, and human aortic tissue	Upregulated	Maegdefessel *et al*[372]
miR-24	PPE and ApoE$^{-/-}$/AngII mice aortic tissue, hAECs, hASMCs, hAFBs, and human aortic tissue	Downregulated	Maegdefessel *et al*[374]
miR-26a	ApoE$^{-/-}$ mice aortic tissue (Elastase and AngII infusion models)	Downregulated	Leeper *et al*[371]
miR-29b	PPE and ApoE$^{-/-}$/AngII mice aortic tissue, hAFBs, and human aortic tissue	Downregulated	Maegdefessel *et al*[365]
miR-132	Sprague Dawley rat aortic tissue	Upregulated	Liu *et al*[370]
miR-143	Wild-type C57BL/6 AAA mouse model aortic tissue	Downregulated	Damrauer *et al*[373]
miR-145	Wild-type C57BL/6 AAA mouse model aortic tissue	Downregulated	Damrauer *et al*[373]
miR-222	Sprague Dawley rat aortic tissue	Upregulated	Liu *et al*[370]

4.4 MicroRNAs in Human Abdominal Aortic Aneurysms

Although animal studies provide great insight into the mechanisms of disease, analysing the role of miRNAs in human AAA is essential, as animal models require experimental aneurysm initiation which may limit interpretation and translation of results into human disease.

The first studies to incorporate human tissue looking at miRNA expression in AAA, described above by Maegdefessel *et al*[365,372,374] were used to validate their animal models, revealing that miR-29b and miR-24 are significantly downregulated in AAA, whereas miR-21 is significantly upregulated in AAA. Recently, two miRNome studies (microRNA transcriptome i.e. investigating all possible miRNAs) have been conducted revealing further insights into aneurysmogenesis.

Pahl *et al*[375] looked at the expression of 847 miRNAs in human aortic tissue. An initial microarray discovery study comparing 5 AAA samples vs 5 control samples identified upregulation of miR-181*, miR-146, and miR-21, and downregulation of miR-133b, miR-133a, miR-331-3p, miR-30c-2*, and miR-204. Validation work was undertaken using 25 elective AAA samples, 11 ruptured AAA samples, and 7 control samples, using PCR methodology. PCR replicated the downregulated results, but not the upregulated, with an additional finding of no significant difference between elective AAA and ruptured AAA samples.

Kin *et al*[376] also used a miRNome approach analysing the expression of 384 miRNAs in aortic tissue from 13 AAA samples vs 7 control samples. Initial microarray was conducted identifying 77 upregulated miRNAs (Table 4-2). Validation work was performed for the miRNAs reproducibly and reliably altered in the microarray analysis

(no further statement was available as to how these were selected) finding a significant difference in the levels of let-7f, miR-21, miR-20a, miR-27a, miR-92a, miR-126, miR-221, miR-222, miR-29b, miR-124a, miR-146a, miR-155, and miR-223. However validation work for miR-1, miR-133a, miR-499-3p, miR-143, and miR-145 were not significant. The authors then looked at miRNA expression in plasma samples from 23 patients with AAA, 17 with CAD, and 12 controls. The expression of miR-15a, miR-15b, miR-124a, miR-126, miR-223, miR-21, miR-29b, miR-155, and miR-146a was analysed. miR-126, miR-124a, miR-146a, miR-155, miR-223, miR-29b, miR-15a, and miR-15b were significantly downregulated in AAA compared to controls, and in addition, miR-124a, miR-155, miR-223, and miR-29b were downregulated in AAA v CAD.

The above studies have shown inconsistent results between identified miRNAs, with only miR-21 being consistently upregulated in two studies, and miR-29b has been found to be both upregulated and downregulated in different studies (Table 4-3). This may be partly due to the differences in miRNA expression in aortic tissue and plasma, or due to the methodology used, however a similar set of miRNAs was compared within both miRNome studies. When comparing human and animal studies (excluding replication of results within the same article), only miR-21 and miR-222 are found to be consistently upregulated in AAA cases compared to controls. Therefore, more work is required to fully assess miRNA expression in subjects with AAA, and in addition determining circulating miRNAs associated with AAA would permit a blood test to be taken to determine at risk patients, without the requirement of aortic biopsy, which would be very invasive. I therefore decided that due to the discrepancies in previous

studies, and the lack of miRNome studies using blood from subjects with AAA I should initially evaluate all miRNAs in the circulation of patients with AAA compared to screened controls, in order to determine differentially expressed miRNAs, and then use the results from the initial study combined with the literature to undertake a validation study. The discovery study is to be undertaken using a greater number of cases and controls than previous discovery studies with an aim to decrease sampling error. The validation study has also been designed to determine miRNAs dysregulated not only in blood, but also in plasma and aortic tissue in order to assess whether circulating miRNA expression correlates to that at the disease site. Finally, previous validation studies have used either the same samples with a different technique, or an additional cohort of subjects with relatively few samples. I therefore designed the validation study to include 5 cohorts of patients, including small AAA, large AAA, post surgical repair (either open or endovascular), controls, and subjects with peripheral arterial disease in order to determine whether miRNAs were dysregulated in patients with AAA, whether these miRNAs were associated with AAA diameter, remained dysregulated post repair, and whether they were specific to AAA or due to generalised atherosclerosis. More detail can be found in Chapter's 6 and 7.

Table 4-2 – Dysregulated microRNAs identified by Kin *et al* in aortic tissue samples, all of which were downregulated.

Discovery Study Results	Validated miRNAs
let-7a, let-7c, let-7e, let-7f, miR-10a, miR-10b, miR-18a, miR-20a, miR-21, miR-23b, miR-25, miR-27b, miR-29b, miR-30b, miR-32, miR-92a, miR-93, mmiR-99, miR-101, miR-103, miR-107, miR-124, miR-126, miR-127-3p, miR-128, miR-130b, miR-132, miR-134, miR-135a, miR-135b, miR-139-5p, miR-142-3p, miR-146a, miR-146b-3p, miR-148a, miR-152, miR-181c, miR-183, miR-187, miR-195, miR-199a-5p, miR-199a-3p, miR-200b, miR-210, miR-216a, miR-217, miR-221, miR-222, miR-223, miR-296-5p, miR-301b, miR-302c, miR-324-5p, miR-361-5p, miR-374a, miR-379, miR-410, miR-423-5p, miR-425, miR-431, miR-451, miR-455-3p, miR-495, miR-502-3p, miR-502-5p, miR-532-3p, miR-532-5p, miR-545, miR-590-5p, miR-629, miR-642, miR-655, miR-708, miR-886-3p.	let-7f, miR-21, miR-20a, miR-27a, miR-92a, miR-126, miR-221, miR-222, miR-29b, miR-124a, miR-146a, miR-155, and miR-223

Table 4-3 – Summary of miRNAs found to be associated with abdominal aortic aneurysms, identified in human studies. Only miRNAs validated in an additional cohort are included.

microRNA	Tissue type	Dysregulation	Study
miR-15a	Plasma	Downregulated	Kin *et al*[376]
miR-15b	Plasma	Downregulated	Kin *et al*[376]
miR-20a	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-21	hAECs, hASMCs, hAFBs, and human aortic tissue	Upregulated	Maegdefessel *et al*[372]
	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-24	hAECs, hASMCs, hAFBs, and human aortic tissue	Downregulated	Maegdefessel *et al*[374]
miR-27a	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-29b	hAECs, hASMCs, hAFBs, and human aortic tissue	Downregulated	Maegdefessel *et al*[365]
	Human aortic tissue and plasma	Upregulated in tissue, downregulated in plasma	Kin *et al*[376]
miR-30c-2*	Human aortic tissue	Downregulated	Pahl *et al*[375]
miR-92a	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-124a	Human aortic tissue and plasma	Upregulated in tissue, downregulated in plasma	Kin *et al*[376]
miR-126	Human aortic tissue and plasma	Upregulated	Kin *et al*[376]
miR-133a	Human aortic tissue	Downregulated	Pahl *et al*[375]
miR-133b	Human aortic tissue	Downregulated	Pahl *et al*[375]
miR-146a	Human aortic tissue and plasma	Upregulated in tissue, downregulated in plasma	Kin *et al*[376]
miR-155	Human aortic tissue and plasma	Upregulated in tissue, downregulated in plasma	Kin *et al*[376]
miR-204	Human aortic tissue	Downregulated	Pahl *et al*[375]
miR-221	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-222	Human aortic tissue	Upregulated	Kin *et al*[376]
miR-223	Human aortic tissue and plasma	Upregulated in tissue, downregulated in plasma	Kin *et al*[376]
miR-331-3p	Human aortic tissue	Downregulated	Pahl *et al*[375]
Let-7f	Human aortic tissue	Upregulated	Kin *et al*[376]

4.5 Summary of miRNAs Associated with Abdominal Aortic Aneurysms

The current studies investigating the miRNAs associated with VSMCs and the ECM, as well as animal and human studies investigating the association between miRNAs and AAA, are far from conclusive. A total of thirty-five potential miRNAs have been identified, with only ten of these found in more than one experimental model. In addition, only one study has looked at circulating miRNAs in human plasma, one study undertaking a discovery study in human aortic tissue, and three studies validating animal work with human aortic tissue. Therefore additional work is required to fully elucidate the underlying pathophysiological changes in miRNA expression in human AAA, and to determine whether miRNAs can be used as biomarkers of disease, prognostic indicators or potential future treatments.

Chapter 5

Predicting miRNAs in Abdominal Aortic Aneurysm using Genomic Data

5.1 Introduction

As discussed in the previous chapter, the expression of miRNAs in AAA has not been well defined, although 35 potential miRNAs, 10 of which have been replicated, have been found. In order to determine which of the 35 miRNAs were biologically plausible to be involved in the pathophysiology of AAA, the validated and predicted miRNA databases discussed in Chapter 3.7 were used to determine their interactions with known biomarkers of AAA. As described in Chapter 2, there is an abundance of studies looking at AAA genetics and biomarkers, and the aim of this chapter therefore is to determine whether there is any interaction between the miRNAs identified in previous studies (Chapter 4), and the genes and biomarkers previously discussed (Chapter 2). This will aim to further guide the validation study by selecting the most plausible miRNAs for further investigation.

5.2 Methods

Validated and predicted miRNA targets were searched using miRWalk. Validated targets have been experimentally defined and published. Target prediction using the MirWalk server allows several different prediction programmes to be searched for miRNA:gene interactions. The servers Diana-microT, miRANDA, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22 and TargetScan were searched. Due to the variability in prediction tools and potential inaccuracies between algorithms, gene targets predicted by 5 or more servers were included to improve specificity.

5.3 Results and Discussion

As previously discussed in Chapter 2, a total of 4 loci/genes from GWAS (DAB2IP, LRP1, SORT1, LDLR), and 3 candidate genes from meta-analysis (MMP9, ACE, MTHFR) have

been associated with AAA. In addition, 16 biomarkers (MMP2, MMP9, TIMP1, IL-6, TNFα, OPG, OPN, IFNγ, ICAM-1, VCAM-1, D-dimer, CRP, α1AT, fibrinogen, triglycerides, and Lp(a)) were found to be increased in patients with AAA, and 2 biomarkers were found to be decreased (ApoA and HDL).

From Chapter 4, a total of 35 miRNA's were found to be associated with either VSMC's, ECM, murine, or human AAA studies (let-7f, miR-1, miR-7, miR-15a, miR-15b, miR-18a, miR-19a, miR-19b, miR-20c, miR-21, miR-24, miR-26a, miR-27a, miR-29a, miR-29b, miR-29c, miR-30, miR-30c-2*, miR-31, miR-124a, miR-126, miR-132, miR-133, miR-133a, miR-133b, miR-143, miR-145, miR-146a, miR-155, miR-204, miR-208, miR-221, miR-222, miR-223, miR-331-3p).

Both predicted (those interactions determined through the prediction databases rather than experimentally validated) and validated (those interactions which have been proven in previous studies) interactions were identified between these miRNAs and the majority of genes/biomarkers associated with AAA (Table 5-1) providing further insight into potential pathways for AAA formation, and improving the plausibility of these miRNAs. Validation and prediction software however is relatively new, with validation databases requiring constant updating and relying on published experimental data, therefore over time it is likely that more interactions will be identified or validated. As can be seen by comparing the predicted and validated miRNAs, there are several validated targets which were not predicted by 5 or more of the prediction algorithms, highlighting the potential inaccuracies in prediction methodology. For example, lipoprotein(a) was predicted to interact with miR-21 by DIANA-microT, miRanda, miRDB, miRWalk, PICTAR5 and TargetScan, but not by

RNAhybrid, PITA or RNA22. In addition, miRanda predicted an interaction between Lp(a) and miR-143, but this was not predicted by any other algorithms. Although prediction methodology is likely to become useful in the future to guide focused research, it currently remains underdeveloped for this purpose.

Table 5-1 – Validated and predicted interactions between genes/biomarkers associated with AAA and miRNAs associated with AAA (from Chapter 4).

Gene/Biomarker	Validated miR's	Predicted miR's
Interleukin-6	Let-7f, miR-15a, miR-15b, miR-18a, miR-19b, miR-21, miR-26a, miR-31, miR-132, miR-146a, miR-155, miR-223	
Tumour necrosis factor α	Let-7f, miR-15b, miR-21, miR-27a, miR-31, miR-126, miR-132, miR-143, miR-146a, miR-155, miR-221, miR-222, miR-223	
Osteoprotegerin		miR-21, miR-145
Osteopontin	miR-146a	miR-1
Interferon γ	Let-7f, miR-21, miR-26a, miR-29a, miR-145, miR-146a, miR-155, miR-221, miR-223	miR-24, miR-26a, miR-27a, miR-29a, miR-29b, miR-29c, miR-143
Intercellular adhesion molecule-1	miR-31, miR-221, miR-222	miR-223
Vascular cell adhesion molecule-1	miR-126, miR-155, miR-221, miR-222	Let-7f, miR-204
C-reactive protein	miR-1, miR-30c-2*, miR-155	Let-7f, miR-7, miR-15a, miR-15b, miR-27a, miR-133a, miR-133b, miR-145
A1-antitrypsin		
Matrix metalloproteinase 2	miR-29b, miR-146a	miR-29a, miR-29b, miR-29c, miR-143
Matrix metalloproteinase 9	miR-1, miR-21, miR-26a, miR-29b, miR-132, miR-143, miR-145, miR-155	miR-133a, miR-133b, miR-204
Tissue inhibitor of Matrix metalloproteinase 1		
High density lipoprotein	miR-145, miR-222	
Lipoprotein A	miR-21	miR-21, miR-223
Apolipoprotein A	miR-21	miR-21, miR-223
Fibrinogen	miR-29a, miR-29b, miR-29c	
Methylenetetrahydrofolate reductase		Let-7f, miR-1, miR-15a, miR-15b, miR-18a, miR-24, miR-26a, miR-31, miR-223, miR-331-3p
Disabled homologue 2-interacting protein		miR-7, miR-15a, miR-15b, miR-29a, miR-29b, miR-29c, miR-221, miR-222
Low density lipoprotein receptor 1	miR-1, miR-30c-2*, miR-155	miR-1, miR-27a, miR-204
Sortilin 1		miR-204
Angiotensin converting enzyme	miR-27a, miR-29c, miR-132, miR-143, miR-155, miR-222	miR-24

Chapter 6

Aims and Objectives

6.1 Project Background

Abdominal aortic aneurysms are a major cause of morbidity and mortality in the elderly. Elucidating the underlying mechanism behind aneurysm formation may lead to the development of therapeutic agents to slow or halt aneurysm growth, thus decreasing the need for surgical intervention. The discovery of miRNAs, which play a significant role in the processing of the majority of proteins, and the recent advancements in utilising miRNAs as therapeutic agents, makes miRNAs an important part of aneurysm pathogenesis which remains poorly understood.

6.2 Project Aims

This study aims to elucidate the expression profiles of miRNAs in patients with AAA compared to controls, then validate those miRNAs found to be differentially expressed in a larger and more varied cohort of patients as outlined below. Due to the paucity of research into miRNAs in AAA this stepwise approach is required in order to initially identify potential miRNAs and then validate those identified. In addition to this strategy, selected plausible miRNAs from the literature are to be included within the validation study.

6.3 Hypothesis

I postulate that:

1. The peripheral blood miRNA expression profile of individuals with large and small AAA is significantly different from each other.
2. The peripheral blood miRNA expression profile of individuals with large and small AAA is significantly different from those with a normal calibre aorta.

3. The peripheral blood miRNA expression profile of individuals with large and small AAA is significantly different from those with a normal calibre aorta and with peripheral arterial disease.
4. The peripheral blood miRNA expression profile of individuals with large AAA is significantly different from those patients who are post either open or endovascular AAA repair.

Chapter 7

Study design

The studies within this thesis were conducted in 2 parts. Primarily a discovery study was undertaken to determine which miRNAs were differentially expressed between aneurysm cases and controls, followed by a large scale validation study. The methods will be discussed in more detail in the following chapter.

7.1 Discovery Study

The discovery study was undertaken using whole blood samples. Samples were collected from 10 control individuals and 15 participants with AAA >30mm diameter, limited to this size due to cost. All participants were male and aged over 55 years. The differential expression of 754 miRNAs were analysed. This number of miRNAs was chosen due to the methodology. TaqMan Low Density Array cards were used, which consists of two pre-designed 384 well cards, totalling 754 different miRNAs. Although over 1500 human miRNAs are known to exist, array card technology is not yet designed to analyse all of these miRNAs, therefore the decision was based on a combination of the total number of miRNAs, cost, and previous experience with the technology within the department. Results from the discovery study along with miRNAs identified in the literature were taken forward into the validation study.

7.2 Validation study

Following identification of differentially expressed miRNAs from the discovery study, the 12 miRNAs with a combination of the best fold change, P value, and PCR curve (as determined by cut off threshold value), and 4 miRNAs identified within the literature were evaluated in a further cohort of patients. The aim of the validation study was not only to determine whether the discovery study findings were replicated in a larger cohort, but also to determine whether miRNA dysregulation was affected by aneurysm size or surgical repair of the aneurysm, and to determine whether these results were

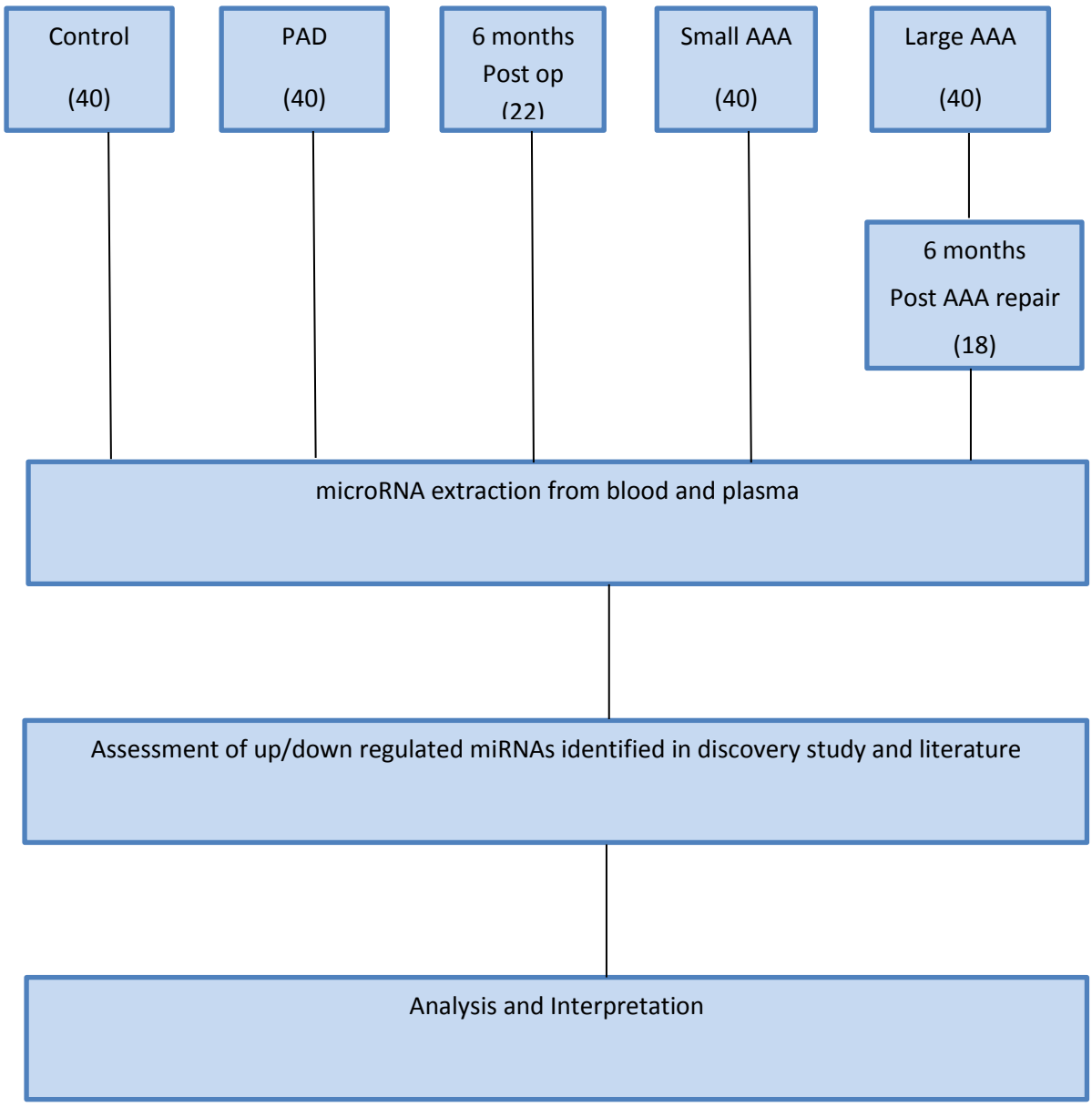
specific to AAA. As well as in whole blood samples, I also aimed to determine whether these miRNAs were also dysregulated in plasma (as plasma miRNAs are thought to be involved in intercellular communications) and aortic tissue. Within whole blood samples the majority of miRNA is located within cellular components (mononuclear cells), which is not contained within plasma samples, therefore comparing both sample types may help to determine any communication between blood cells and aortic tissue through secretion of miRNAs. Therefore in addition to utilising whole blood samples, matched plasma samples from the same patients were also assessed. Aortic tissue samples obtained from AAA specimens and cadaveric donors were used for further validation of the differentially expressed miRNAs identified.

Within the validation study additional cohorts of subjects were collected. These were divided into five groups (Figure 7-1):

1. Patients with small aneurysms (3.0 to 5.4cm) (n=40)
2. Patients with large aneurysms (≥5.5cm) (n=40)
3. Patients with peripheral arterial disease (n=40) with no evidence of AAA on abdominal USS or CT within past 5 years, and both symptomatic (intermittent claudication) and imaging evidence of peripheral arterial disease (arterial narrowing on USS)
4. Controls (n=40) with no evidence of AAA on abdominal USS or CT within past 5 years, no symptoms of intermittent claudication, and scan performed at 65 years of age or beyond
5. 6 month post-operative AAA repair patients (n=40, 18 of which were matched with pre-operative samples). Both open and endovascular repair were included.

All participants, including controls and PAD, had up-to-date aortic imaging to ensure accuracy. This was conducted using abdominal ultrasound scans. This comprehensive study design aimed to permit thorough analysis of each miRNA, the difference between expression and aneurysm size, as well as compensate for confounding factors such as atherosclerosis.

Figure 7-1 – Flow diagram of validation study



Chapter 8

Methods

8.1 Ethical Approval and Recruitment

A previous ethical approval had been obtained (REC: 6819; An investigation into candidate genes and protein profiling for abdominal aortic aneurysms. Chief Investigator Professor Robert Sayers, University of Leicester) permitting the collection of blood and aortic tissue from patients with AAA, and blood from controls. I was successful in amending the ethical approval to permit collection of blood and tissue from patients with AAA, as well as from patients with peripheral arterial disease, and controls. In addition, this ethical approval was expanded to permit analysis of miRNA levels, and to allow the incorporation of additional research sites. From this amendment I obtained site specific ethical approval from Northampton General Hospital to collect aortic tissue from patients undergoing AAA repair, and patients undergoing aortic surgery for aorto-occlusive disease. Control aortic tissue from cadaveric patients was collected under an additional ethical approval (REC: 12/EM/0355).

Consent was obtained for all subjects prior to inclusion within the study. All subjects (except cadaveric donors) underwent a face to face interview with the one of the research fellows (either myself (>95% of blood samples), Mr Ben Wild, Mr David Sidloff, Mr Badri Vijaynagar, or Mr Nikesh Dattani (the majority of aortic tissue samples) during which relevant clinical information including medical and family history was obtained. Clinical information included age, smoking history, history of hypertension, hypercholesterolaemia, myocardial infarction, angina, cerebrovascular accident, diabetes, chronic obstructive pulmonary disease, peripheral vascular disease and any history of malignancy or autoimmune/inflammatory disease. Medication

history was also taken, including aspirin, beta-blockers, statin, clopidogrel, digoxin, warfarin, diuretics and ACE inhibitors.

All subjects underwent an abdominal ultrasound scan to measure their aortic diameter using the inner wall to inner wall measurements in both antero-posterior and lateral planes, with all peripheral vascular disease patients also having ultrasound imaging of their lower limbs to assess for arterial stenosis or occlusion. A small AAA was defined as 30-54mm diameter, with a large AAA ≥55mm diameter (chosen due to this being considered the minimum aortic diameter for elective AAA repair). Peripheral arterial disease was defined as both symptoms of intermittent claudication and arterial stenosis/occlusion identified on USS.

Patients were recruited from ward attendance, patients attending for angioplasty, vascular clinic, the vascular screening programme, aneurysm surveillance and EVAR surveillance clinics. Men are invited to their health centre for aneurysm screening in the year of their 65th birthday. Permission was obtained to attend the local aneurysm screening programme and from local general practitioners to consent patients following aneurysm screening in order to obtain control participants. Patients with peripheral arterial disease were obtained prior to angioplasty and from vascular clinic. Patients with small and large aneurysms were obtained from aneurysm surveillance and vascular clinics. Patients post AAA repair were obtained from EVAR surveillance clinic and vascular clinics. In order to obtain matched post operative samples patients follow up clinic appointments were obtained to ensure their post operative sample was obtained during their appointment.

Sample collection was obtained for whole blood and plasma for all subjects (except cadaveric donors), and aortic tissue samples were obtained from any subject undergoing open surgery on their abdominal aorta, and from cadaveric donors.

8.2 Inclusion and Exclusion criteria

The following inclusion criteria were implemented in order to permit standardisation and reduce bias when comparing cases and controls: Males > 55 years; Caucasian (due to variations in AAA incidence in different ethnicities); No systemic illness which could confound miRNA expression profiling results e.g. malignant disease process, systemic autoimmune or inflammatory disease.

8.3 Sample Collection and Storage

8.3.1 Peripheral Blood Cells

Due to the instability of RNA within cells, which rapidly degrades within hours of blood collection, whole blood samples were collected directly into PAXgene® Blood RNA tubes (PreAnalytiX GmbH, Switzerland) containing an additive to lyse blood cells and immediately stabilise the transcriptome by reducing *in vitro* RNA degradation and minimizing gene induction. Following blood collection into the PAXgene® Blood RNA tube the tube is inverted 8-10 times then stored at room temperature for 2-72 hours (according to manufacturer's protocol) prior to freezing at -20˚C. Prior to use the PAXgene® Blood RNA tube was left to defrost overnight to reach room temperature prior to RNA extraction.

8.3.2 Plasma

Plasma samples were obtained by taking whole blood directly into EDTA tubes. These tubes were kept at room temperature and processed immediately following sample collection. The samples were spun at 2,000g for 10 minutes. The plasma was then

collected in 600ul aliquots into 1.5ml processing tubes. These were then spun at 10,000g for 10 minutes to remove the platelets. Plasma was then pipetted off taking care to avoid the platelet pellet, and stored at -80˚C. Plasma samples remained frozen until thawed on ice prior to RNA extraction.

8.3.3 Aortic Tissue

Aneurysmal aortic tissue samples were obtained from theatre at Leicester Royal Infirmary and Northampton General Hospital. A full thickness section of aortic tissue from the aneurysm wall was excised and the sample dissected into approximately 50mg pieces and stored in 10 times the volume of RNA later® solution overnight. This RNA later® solution was then decanted and the tissue sample was stored at -80˚C. These were thawed on ice prior to RNA extraction.

Control aortic tissue samples were collected from cadaveric kidney donors. Organs are sent on ice and in organ preservation solution (Soltran, Baxter International Inc, Illinois) from the donor unit to the recipient unit (in this case Leicester General Hospital). On arrival, the kidney with the accompanying renal artery and aorta is transferred to theatre if deemed suitable for transplantation or to the transplantation laboratory if deemed unsuitable for use in transplantation (and therefore used for research purposes only). The transplant/research team then prepares the kidney for transplantation at which time a section of aortic tissue is collected by either Nikesh Dattani or myself for further study. This tissue is further kept in Soltran and on ice until arrival at the vascular research laboratory where the tissue is dissected into 6 equal sections, and one of these is placed in RNALater for future RNA extraction. The usual time between the tissue being placed in organ preservation solution at the donor unit, and the tissue being placed in RNALater is about 18 – 24 hours. Thereafter cadaveric

aortic tissue specimens are treated in exactly the same fashion as aneurysmal aortic samples as described above.

8.4 RNA Extraction

High quality RNA is required for further downstream applications such as polymerase chain reaction (PCR) as contamination within samples or degradation will affect results; therefore stringent standards were required for RNA extraction.

i) Prior to undertaking RNA extraction all surfaces and pipettes were disinfected with RNase Zap to remove any RNases. RNases are enzymes which degrade RNA samples, with long and low abundance transcripts particularly vulnerable. RNase free water was used throughout all experiments, and disposable pipette tips were used throughout. A clean lab coat and gloves were worn, and changed frequently to prevent contamination with RNases, and contamination between samples.

ii) The time from sample collection to freezing/processing was minimised. Storage solutions such as RNA later® were used to help limit RNA degradation in tissue samples, blood samples were collected directly into PAXgene tubes, and plasma samples were processed as rapidly as possible.

iii) RNA purity can be affected by contamination with proteins or solvents, which can interfere with downstream RNA amplification. RNA extraction was undertaken on spin columns to minimise this effect, with DNA digestion used in all blood and tissue RNA extraction (this was not required in plasma due to the typically low DNA concentrations). All samples were

also checked for purity through a 3-step process, including quantification, RNA integrity, and 260/280nm absorbance ratios (described below).

8.4.1 Peripheral Blood Cells

Total RNA from whole blood was extracted from samples taken in PAXgene® Blood RNA tubes. This was accomplished through the use of the PAXgene® Blood miRNA Kit, which enables purification of RNA >18 nucleotides in length. Due to copyright laws the contents of specific buffers are not published, therefore their action has been included instead. Both manual and automated protocols were used:

Manual protocol – Following defrosting, PAXgene® Blood RNA tubes were centrifuged at 5000g for 10 minutes. The supernatant was removed by decanting, taking care not to disturb the pellet, thus separating the plasma and retaining the cellular components. 4ml of RNase free water was then added, the pellet vortexed until visibly dissolved, followed by centrifuging at 3000-5000g for a further 10 minutes to further purify the pellet. The entire supernatant was again discarded taking care not to disturb the pellet. 350uL of buffer BM1 (resuspension buffer) was then added followed by vortexing until visibly dissolved.	 Spin Wash Spin Resuspend BM1
The sample was then transferred into a 1.5ml processing tube, followed by addition of 300uL of buffer BM2 (binding buffer) and 40uL of proteinase K. This was mixed by vortexing, and incubated at 55°C for 10 minutes in a shaker incubator to digest the protein, leaving nucleic acids intact.	 Add BM2 and proteinase K Vortex and incubate

The sample was then transferred into the PAXgene shredder spin column and centrifuged for 3 minutes at 10,300g to collect the nucleic acids in the flow through, and remove residual cell debris and digested proteins on the shredder column.		Shredder column Centrifuge
The supernatant from the flow through was transferred to a new 1.5ml processing tube without disturbing the pellet, and 700uL of 100% isopropanol was added and mixed by vortexing, in order to precipitate the nucleic acids.		Processing tube Add isopropanol
The solution was then transferred to the PAXgene spin column in 700uL aliquots and centrifuged for 1 minute at 10,300g, with the flow through discarded. This allows the precipitated nucleic acids to bind to the spin column membrane.		Spin column Centrifuged RNA binds membrane
The spin column was placed in a new processing tube, and 350uL of buffer BM3 (wash buffer) was added to wash the nucleic acids, removing any residual salts or debris. The sample was centrifuged for 15 seconds at 10,300g, and the flow through discarded.		Wash with BM3
A DNase incubation mix was then made, by adding 10uL of DNase 1 stock solution to 70uL of buffer RDD, and gently mixing. The DNase incubation mix was then pipetted directly onto the PAXgene RNA spin column membrane and incubated at room temperature for 15 minutes. This removed any DNA contamination, leaving only RNA.		Digest DNA
To remove salts, chemicals, and proteins from the previous enzymatic reactions (i.e. DNAse treatment) and impurities the membrane was then washed with 350uL of buffer BM3 (wash buffer). Buffer BM3 was added to the spin column and centrifuged for 15 seconds at 10,300g, with the flow through discarded.		Wash with BM3

The membrane was then washed twice with 500uL of wash buffer BM4 and centrifuged at 10,300g for 15 seconds for the first spin, and 2 minutes for the second. The spin column was then spun for a further 1 minute at 10,300g to dry the membrane.		Wash with BM4
The spin column was transferred to a new RNAse free collection tube and 40uL of buffer BR5 (elution buffer) was applied directly to the spin column membrane. The sample was centrifuged at 10,300g for 1 minute to elute the RNA, and this step was then repeated with a further 40uL of buffer BR5 (elution buffer). The eluate was then incubated for 5 minutes at 65°C to denature the RNA strands, then immediately chilled on ice, prior to freezing.		Elute with BM5 Heat to denature

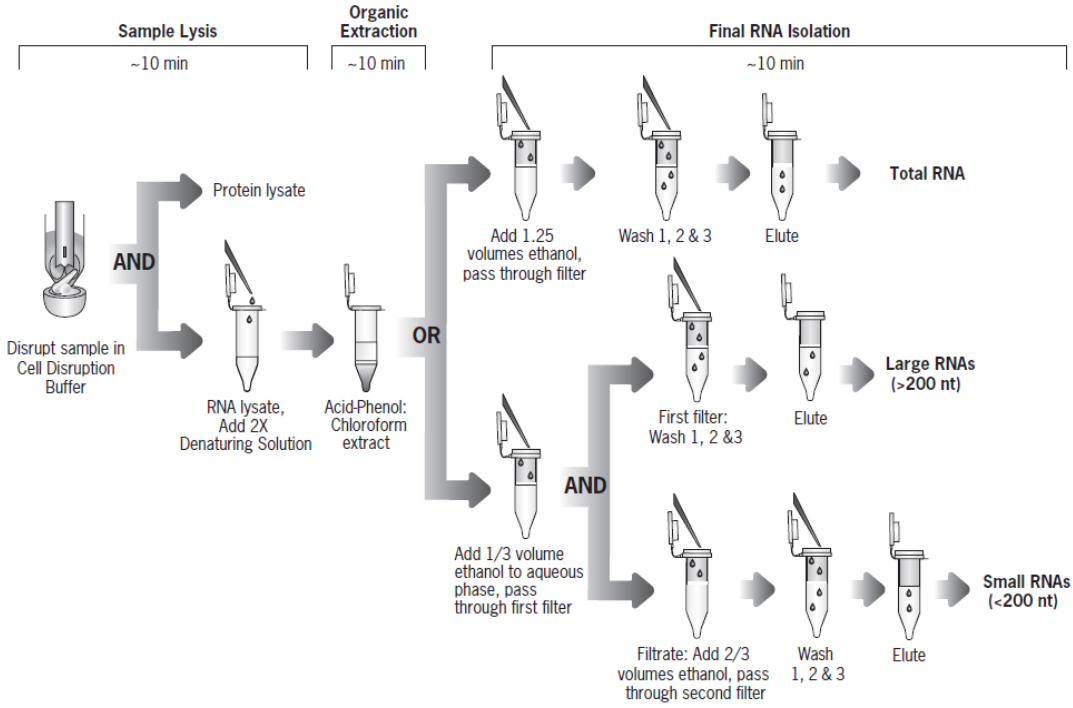
Automated protocol – Automated RNA extraction was also undertaken using the QiaCube® (Qiagen, Germany). The same manual protocol is used up until the addition of buffer BM1 (resuspension buffer) and vortexing, however all additional buffers, proteinase K, DNase incubation mix and shredder and spin columns were then added to the QiaCube®. There were no differences in buffers or volumes between the automated and manual protocols (see Chapter 9.1.1 for comparison of samples extracted through manual and automated methods).

8.4.2 Plasma

Total RNA was extracted from plasma using the mirVana™ Paris Kit™ (Ambion®, Life Technologies, California) (Figure 8-1). 500uL of plasma was mixed with an equal volume of 2X denaturing solution and stored on ice for 5 minutes to denature the protein. An equal volume of Acid-Phenol:Chloroform was then added to the solution to partition the nucleic acids, and the sample vortexed for 1 minute. The sample was then centrifuged at 10,000g for 5 minutes, to separate the 3 phases of the mixture.

The aqueous upper phase containing the nucleic acids was transferred to a fresh processing tube and the volume measured. The lower phase (denatured proteins) and interphase (cellular debris) were discarded. 100% ethanol was then added to precipitate the nucleic acids, at a volume of 1.25 x the volume of the lysate, and the lysate ethanol mix was then transferred to a filter. The sample was centrifuged at 10,000g for 30 seconds, with the flow through discarded, until all of the lysate ethanol mix had passed through the filter. The nucleic acids were then bound to the filter. 700uL of miRNA wash solution 1 was then applied to the filter and centrifuged at 10,000g for 15 seconds with the flow through discarded. 500uL of wash solution 2 was then applied to the filter and centrifuged at 10,000g for 15 seconds with the flow through discarded, and this step was repeated to ensure that any salts and chemicals were removed. Finally, a further centrifugation at 10,000g for 1 minute was carried out to remove any residual fluid. The filter cartridge was then transferred to a fresh collection tube and 100uL of 95˚C RNase free water was applied directly to the filter membrane. The sample was centrifuged for 30 seconds, with the eluate collected and frozen at -20˚C.

Figure 8-1 – Flow diagram of RNA extraction from plasma, using the MirVana Paris Kit (reproduced from manual).



8.4.3 Aortic Tissue

Extraction of RNA from aortic tissue was performed initially using the RNeasy mini kit, however this yielded poor RNA concentrations and integrity. Therefore 4 different RNA isolation kits were compared for optimisation (Appendix 1). Results identified the miRNeasy mini kit to yield the highest RNA concentration and best RNA integrity number, therefore this kit was used for further RNA extraction. Two separate aortic tissue studies were carried out, using slightly different methodology. The initial validation study was carried out using aortic tissue samples which were not treated with DNAse, and the second validation study utilised samples which had been treated with DNAse. This was undertaken following analysis of the initial aortic tissue samples in order to further validate the results and ensure accuracy.

50mg of full thickness aortic tissue was homogenised using the Precellys® tissue homogeniser (Bertin Technologies, France) with CK14 beads in 700uL of Phenol-guanidine based QIAzol lysis buffer (Qiagen, Germany) for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 8500g. The lysis buffer serves to lyse the cell membranes whilst also denaturing DNase and RNase enzymes. The sample was then left at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes.	 	Lyse and homogenise
140uL Chloroform was then added to the sample, vortexed for 15 seconds and left at room temperature for 3 minutes to separate the aqueous and organic phases of the solution. The sample was then centrifuged for 15 minutes at 12,000g at 4˚C.		Add chloroform and centrifuge
The upper aqueous phase containing the nucleic acids was then transferred to a new 1.5ml processing tube and 1.5 x the volume of the sample of 100% ethanol was added and mixed using a pipette, to precipitate the nucleic acids.		Separate upper phase Add ethanol
The sample was then placed onto the RNeasy mini spin column in 700uL aliquots and centrifuged at 8,000g for 15 seconds to bind the nucleic acids to the membrane.		Bind RNA
350uL RWT buffer was added to the spin column and centrifuged at 8,000g for 15 seconds to wash the precipitate.		Add RWT Buffer
DNase mix was made by mixing 10uL DNase 1 stock solution with 70uL of buffer RDD, and this was placed directly onto the spin column membrane and incubated for 15 minutes at room temperature.		DNA digestion

A further 350uL of RWT buffer was added to the spin column and centrifuged at 8,000g for 15 seconds, and the flow through discarded, to wash away the digested DNA.		Add RWT Buffer
Further washes to remove salts and impurities were then undertaken, using 500uL of buffer RPE added to the spin column and centrifuged at 8,000g for 15 seconds, then a further 500uL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 2 minutes.		Wash with RPE Buffer
The spin column was then transferred to a new 2ml processing tube and centrifuged at 8,000g for 1 minute to dry the membrane. The spin column was then transferred to a new 1.5ml collection tube and 30uL of RNase free water was added directly to the spin column membrane to elute the RNA. The sample was then centrifuged at 8,000g for 1 minute and the eluate immediately cooled on ice prior to storing at -80°C.		Elute with water

8.5 RNA Quantification and Integrity

Quality control was performed for isolated RNA. Initially all samples underwent quantification and purity check using a Nanodrop (Thermo Fisher Scientific, Massachusetts). Secondly the RNA quality was analysed using the Agilent® 2100 Bioanalyser (Agilent Technologies, California) for a selection of samples. The small RNA quantity was also checked for a selection of samples.

8.5.1 Nanodrop

The total RNA concentration was measured using the Nanodrop. This uses ultraviolet light absorbance at 260nm to determine RNA concentration, and at 280nm for protein concentration. Poor 260/280 ratios are indicative of protein contamination, with a

ratio between 1.8 and 2.1 indicating that the RNA is sufficiently pure to be used for downstream processing.

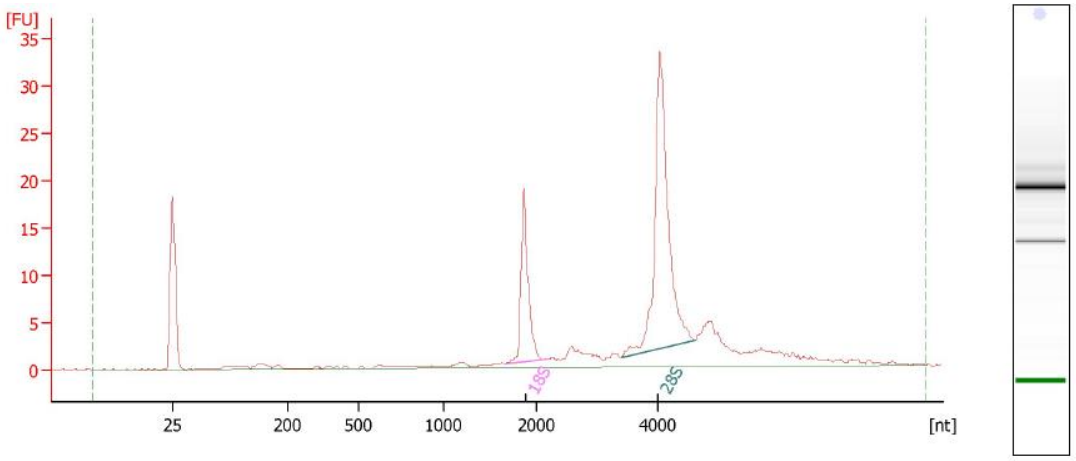
The Nanodrop was initially normalised to the eluent solution (Buffer BM5 for whole blood, RNase free water for plasma or tissue). 1uL of sample was placed on the pedestal, and formed a channel on the measuring column. Ultraviolet light is then shone through the sample with absorption spectra measured. Only blood and tissue samples with a concentration >30ng/uL, and 260/280 ratios between 1.8-2.1 were utilised for downstream processing. Plasma samples typically have a low abundance of miRNA and therefore a concentration >1ng/uL was required for these samples.

8.5.2 Agilent® RNA 6000 Nano Kit

RNA samples are susceptible to degradation, with longer transcripts more likely to degrade. It is therefore important to assess the integrity of these samples prior to further processing. The Agilent® RNA 6000 Nano Kit (Agilent Technologies, California) was used to determine the RNA integrity number (RIN) using the Bioanalyzer 2100 for a limited number of samples to prove methodology. This works by using a software algorithm analysing the shape of the curve in the electropherogram (Figure 8-2) and the amplitudes of the 18S and 28S bands, compared to the degree of noise in the baseline. RNA integrity is graded on a scale of 1 to 10, with 10 being no degradation.

Figure 8-2 – Electropherogram showing the RNA integrity using the Agilent RNA 6000 Nano Kit. The RNA integrity number is calculated through comparison of the 18S/28S ratio compared to an internal ladder run on the same card. A) This sample shows an RIN of 10, meaning there is no RNA degradation. B) This sample shows a RIN of 5.8 showing considerable degradation with low 18S and 28S peaks.

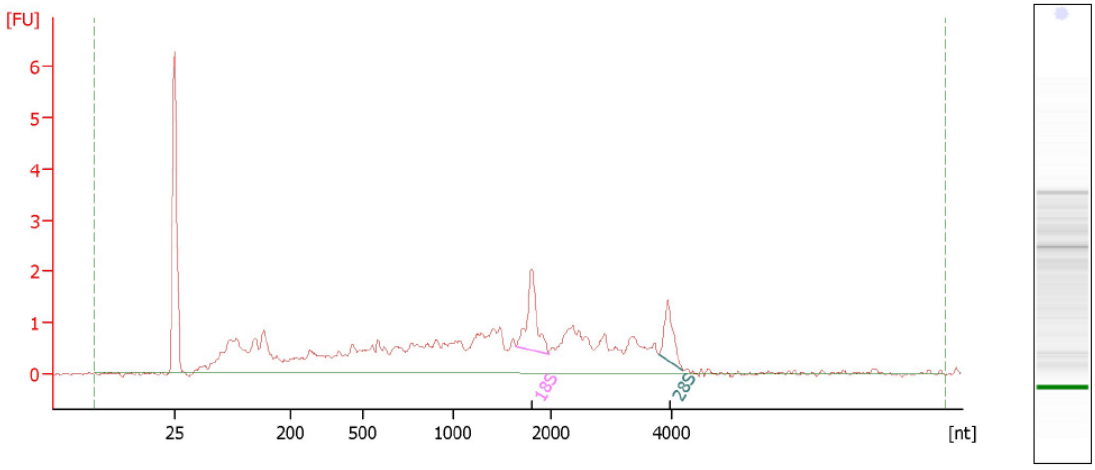
A)



Overall Results for sample 1 : Phil C1265

RNA Area:	135.1	RNA Integrity Number (RIN):	10 (B.02.07)
RNA Concentration:	89 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2.7	Result Flagging Label:	RIN:10

B)



Overall Results for sample 3 : A1464

RNA Area:	39.8	RNA Integrity Number (RIN):	5.8 (B.02.07)
RNA Concentration:	38 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	0.6	Result Flagging Label:	RIN: 5.80

8.5.3 Agilent® Small RNA Kit

The Agilent® Small RNA Kit (Agilent Technologies, California) was used to determine the concentration of small RNAs within each sample using the Bioanalyzer 2100 for a limited number of samples to prove methodology. This works using a similar method to the Agilent® RNA 6000 Nano Kit however it specifically determines the quantity of small RNAs up to 200 nucleotides, and is capable of detecting miRNA quantities as low as 100 picograms.

8.5.4 RNA Concentration, 260/280 ratios and RNA Integrity Numbers

The accuracy of PCR results is dependent upon the purity and quality of RNA, therefore samples were checked for their concentration, purity and integrity.

8.5.4.1 Blood Samples (Tables 8-1 and 8-2)

The initial step for quality control was to analyse the total RNA concentration for each sample, as described, using the Nanodrop. This was done for each individual sample, with only samples with > 40ng/uL being used for downstream processing (low concentration samples were discarded). Comparing the manual and the automated RNA extraction protocols revealed a significant increase in total RNA concentration in those undergoing automated extraction (n=17, mean manual concentration 60.6ng/uL, Standard Deviation 18.7ng/uL, n=11, mean automated concentration 103.4ng/uL, Standard Deviation 33.3ng/uL; $P<0.001$).

The 260/280 ratios were also obtained through the use of the Nanodrop as previously described, with the vast majority of samples having an excellent 260/280 ratio. Comparing samples extracted using the manual and automated protocol revealed no significant difference (n=17, mean 260/280 ratio 2.01, Standard Deviation 0.13, n=11,

mean 260/280 ratio 2.02, Standard Deviation 0.11; P=0.93). Only those samples with a 260/280 ratio between 1.80-2.10 were used for further downstream processing.

Table 8-1 – Results from Agilent 2100 Bioanalyser for RNA integrity, small RNA concentration, and miRNA concentration for total RNA extracted from whole blood using the manual protocol (not all samples were used in studies if they did not meet RNA quality control criteria outlined in Chapter 7.5). RIN = RNA integrity number.

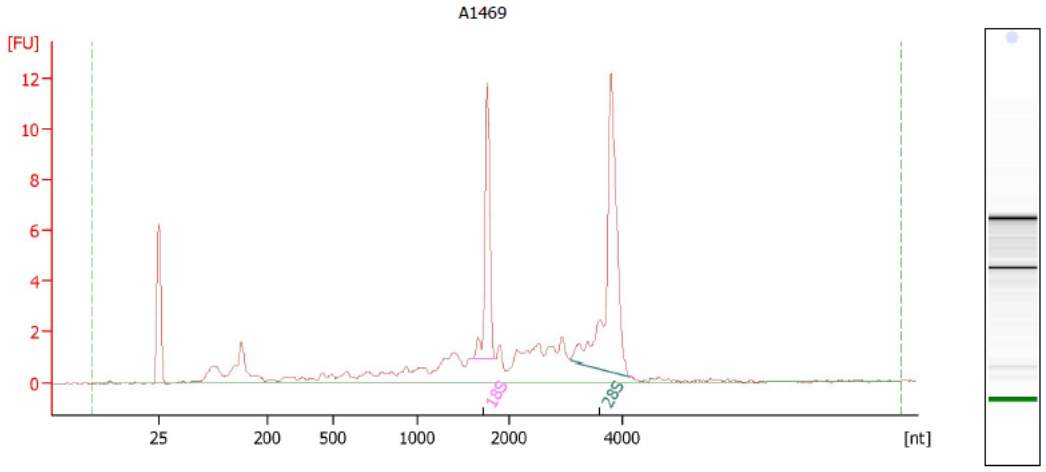
Sample name	RNA conc^{n} (ng/uL)	260/280 ratio	RIN	Small RNA Concentration (pg/uL)	miRNA concentration (pg/uL)
LA1522	42.5	2.07	8.3	4712.4	202
LA1529	56.7	2.03	8.5	7598.7	481.8
SA1143	71.7	2.12	7.9	4316.4	335
SA1466	55.9	1.85	7.5	3245.4	148.9
SA1467	51	1.69	7.8	5344.6	220.7
SA1470	64.4	2.12	6.9	8034.2	935.3
SA1475	59.3	2.09	8	6028.1	250
LA1474	49.3	2.04	8.5	4701.1	649.1
LA1484	54.2	1.96	7.9	3841.3	210.8
SA1469	26.5	2.19	8.3	4239.3	378.4
LA1496	53.2	2.13	8.1	5378.1	384.2
C1225	89.3	2.00	7.7	14957.8	833.7
C1226	68.3	2.01	8.3	6621.4	636.7
C1227	94	2.07	8.8	7509.1	419.3
C1217	94.9	2.07	8.1	7055.4	1084.7
C1232	39.2	1.96	8.4	4788	595.3
C1241	59	1.84	8.2	3799.6	651

Table 8-2 – Results from Agilent 2100 Bioanalyser for RNA integrity for total RNA extracted from whole blood using the automated protocol (not all samples were used in studies if they did not meet RNA quality control criteria outlined in Chapter 7.5). RIN = RNA integrity number. The small RNA and miRNA concentrations were not assessed due to the same extraction methodology being used, and downstream processing based on total RNA rather than small RNA components.

Sample name	RNA concentration (ng/uL)	260/280 ratio	RIN
C1245	86.1	2.13	9.3
P1302	96.8	2.09	9.1
P1293	128.0	2.03	8.9
C1247	78.5	2.19	9.2
P1299	80.5	2.02	9.1
P1295	163.9	1.97	6.7
P1304	145.3	1.91	8.6
P1306	112.4	2.10	8.6
P1294	79.4	1.93	8.8
P1289	50.9	1.83	8.9
P1292	115.6	2.02	7.4

In order to validate the RNA extraction methods, RNA integrity was determined for a subgroup of samples (Figure 8-3). There was a small but significant improvement in RIN for RNA extracted through the automated method (manual RIN 8.07, automated RIN 8.6; P=0.005), however sufficient RIN's were obtained with each method. RNA integrity was not assessed for every sample due to the consistently high RIN found through both methods, and cost, and it was therefore felt that the extraction methodology was proven. The automated protocol was however used for further RNA extraction from whole blood samples to obtain the highest quality RNA.

Figure 8-3 - Electropherogram for total RNA integrity from blood samples. This shows the rRNA (18S/28S) ratio to be 2.0, with an RNA integrity number of 8.3.



Overall Results for sample 6 : A1469

RNA Area:	66.2	RNA Integrity Number (RIN):	8.3 (B.02.07)
RNA Concentration:	63 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2.0	Result Flagging Label:	RIN: 8.30

Fragment table for sample 6 : A1469

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,589	1,847	8.8	13.3
28S	3,088	4,136	17.6	26.6

8.5.4.2 Plasma Samples

In plasma samples, due to the lack of cellular component, miRNA is present due to release from necrotic cells, apoptosis, or intracellular communication, therefore is extracellular rather than intracellular. This results in a deficiency of large RNA, and as such the RNA integrity number is not possible to determine. As such standard analysis is performed using a set volume of sample rather than a set mass of RNA. Quality control for plasma samples was therefore performed purely through ensuring the presence of RNA using the nanodrop, with all samples requiring >1ng/uL for use in downstream processing.

8.5.4.3 Aortic Tissue Samples

As with blood and plasma samples, the initial step for quality control was to analyse the total RNA concentration for each sample. Mean total RNA concentration for aortic tissue samples was 205ng/uL (standard deviation 86ng/uL), with all samples having a 260/280 ratio between 1.8 to 2.2 (mean 260/280 ratio 1.98, SD 0.08).

The additional quality control for tissue samples can be seen in Appendix 1 through the discussion of different RNA extraction methods, however the mean RIN for the selected aortic tissue samples tested was 6.75.

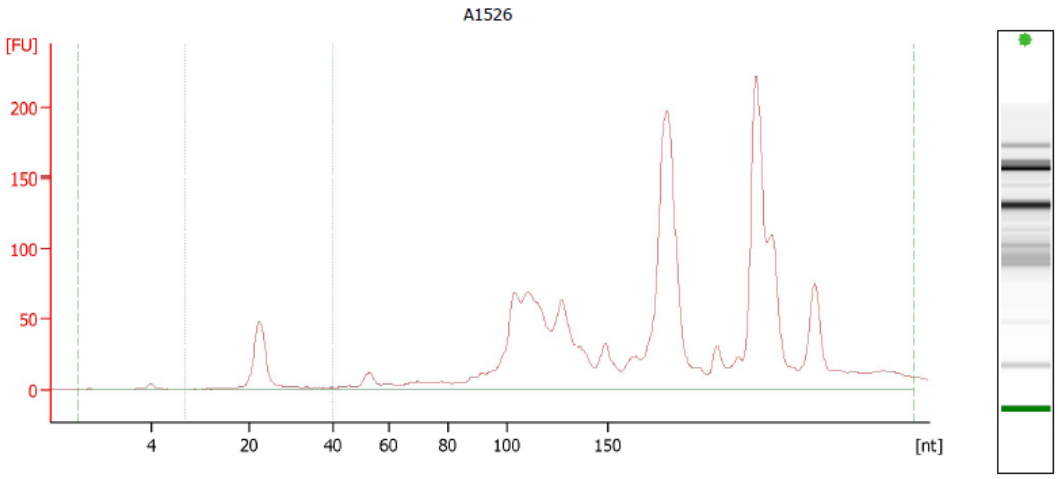
8.5.4.4 Small RNA and microRNA Concentration

The quantity of small RNAs and miRNAs was only tested in RNA extracted from whole blood samples. This was not possible for plasma samples due to low levels of long RNA strands within the samples making an 18S/28S ratio inaccurate.

Small RNA concentration and miRNA concentration were quantified using the Agilent® 2100 Bioanalyser. All samples were shown to contain both small RNAs and miRNAs at good concentrations as outlined in Table 8-1, and Figure 8-4. As good concentrations

were obtained through the manual protocol these were not assessed in the automated protocol due to the same extraction methodology. In addition, downstream processing is based on total RNA concentration rather than miRNA concentration, so it was felt unnecessary to repeat this test.

Figure 8-4 - Example electropherogram for small RNA concentration and miRNA concentration. Small RNA concentration is the total RNA between 0-300 nucleotides, with miRNA concentration between 10 and 40 nucleotides.



Overall Results for sample 6 : A1526

Small RNA Concentration [pg/µl]:	144,470.9	Result Flagging Color:	
miRNA Concentration [pg/µl]:	14,905.4	Result Flagging Label:	10 % miRNA; Concentration: 14905.40 pg/µl
miRNA / Small RNA Ratio [%]:	10		

Region table for sample 6 : A1526

Name	From [nt]	To [nt]	Average Size [nt]	Size distribution in CV [%]	Conc. [pg/µl]	% of Total	Color
Small RNA	0	300	173	36.8	144,470.9	100	
miRNA	10	40	23	18.5	14,905.4	10	

8.6 Reverse Transcription and Pre-amplification

Prior to polymerase chain reaction (PCR) RNA must first be converted into its complementary DNA (cDNA) which can then be used as a template for exponential amplification. This process is known as reverse transcription. Pre-amplification is undertaken due to the low abundance of miRNAs within samples. This permits the cDNA complementary to the primers to be increased within the sample, thus

decreasing the background noise from other miRNAs (i.e. when amplifying with a primer for only miR-29b this will be increased compared to all other miRNAs, and therefore more reliably quantified in RT-qPCR).

8.6.1 Discovery Study

Total RNA was diluted in RNase free water to 100ng in 3uL, prior to reverse transcription. Reverse transcription, pre-amplification and RT-qPCR was undertaken using pool A and pool B reagents separately for each sample. As the discovery study was undertaken across two 384 well plates, a different set of miRNAs was examined within each 384 well plate. Therefore reagents specific to the miRNAs examined within each plate were required, hence pool A (containing primers for the miRNAs within card A) and pool B (containing primers for the miRNAs within card B). These permitted analysis of a total of 754 miRNAs in total.

Reverse transcription was undertaken using TaqMan MegaPlex RT Primers. 6uL of RT Primer Pool was mixed with 0.3uL dNTPs with dTTP (100mM), 3.00uL MultiScribe reverse transcriptase (50U/uL), 1.50uL 10X RT buffer, 0.19uL RNase Inhibitor (20U/uL), and 1.01uL nuclease free water per sample. This was mixed in each individual well of a 96 well plate with 3uL of diluted sample. This was mixed by pipetting and incubated on ice for 5 minutes. Reverse transcription was run using the Bioer® Thermal Cycler (Bioer Technology Co, China) at 16˚C for 30 minutes, 42˚C for 30 minutes, then 85˚C for 5 minutes.

The pre-amplification reaction mix consisted of 12.5uL TaqMan PreAmp Master Mix (2X), 3.75uL Megaplex Custom PreAmp Primers (10X), and 6.25uL RNase free water. 22.5uL of pre-amplification reaction mix was pipetted into each well of a 96 well plate, and 2.5uL of RT product added and mixed by pipetting. The pre-amplification reaction

was then run using the Bioer® Thermal Cycler (Bioer Technology Co, China) under the following conditions; hold at 95˚C for 10 minutes, hold at 55˚C for 2 minutes, hold at 72˚C for 2 minutes, 12 cycles of 95˚C for 15 seconds then 60˚C for 4 minutes, then a final hold at 99.9˚C for 10 minutes.

Following pre-amplification 175uL of RNase free water was added to each well of the 96 well plate to make a total volume of 200uL. The plate was then spun down and frozen at -20˚C prior to RT-qPCR.

8.6.2 Validation Study

For the validation study the quantity of total RNA used for downstream processing varied according to the tissue type. For RNA extracted from whole blood and aortic tissue a total of 100ng RNA in 3uL was used, as it is the relative expression of individual miRNAs compared to a control, rather than total miRNA expression (which would be confounded by number of cells), required for analysis. Plasma samples contain a very small quantity of total RNA, which is therefore difficult to quantify. In addition there is no cellular component within plasma, therefore the total concentration of RNA will not be related to the total number of cells, as is usually the case with whole blood or tissue samples. miRNAs will only be present in plasma due to intercellular communications, cell apoptosis or necrosis, therefore a total volume of RNA extracted from plasma is used for downstream applications rather than an RNA concentration. In total, 3uL of extracted plasma was used.

Reverse transcription was undertaken using TaqMan Custom Megaplex RT primer. The following components were combined in a 96 well plate along with 3uL of sample; 0.75uL Megaplex RT primers (10X), 0.15uL dNTPs with dTTP (100mM), 1.50uL MultiScribe reverse transcriptase (50U/uL), 0.75uL 10X RT buffer, 0.90uL MgCl2

(25mM), 0.09uL RNase Inhibitor (20U/uL), 0.35uL nuclease free water. The components were mixed by pipetting up and down, the plate sealed, spun down, and incubated on ice for 5 minutes. The reverse transcription reaction involved 40 cycles of 16˚C for 2 minutes, 42˚C for 1 minute and 50˚C for 1 second, followed by a 5 minute hold at 85˚C using the Bioer® Thermal Cycler (Bioer Technology Co, China).

The pre-amplification reaction mix consisted of 12.5uL TaqMan PreAmp Master Mix (2X), 2.5uL Megaplex Custom PreAmp Primers (10X), and 7.5uL RNase free water. For blood and tissue samples 22.5uL of pre-amplification reaction mix was pipetted into each well of a 96 well plate, and 2.5uL of RT product added and mixed by pipetting. The pre-amplification reaction was then run using the Bioer® Thermal Cycler (Bioer Technology Co, China) under the following conditions; hold at 95˚C for 10 minutes, hold at 55˚C for 2 minutes, hold at 72˚C for 2 minutes, 12 cycles of 95˚C for 15 seconds then 60˚C for 4 minutes, then a final hold at 99.9˚C for 10 minutes. For plasma samples additional pre-amplification reaction mix was used per sample, to total 32.5uL per well, and 7.5uL of RT product was used. In addition the pre-amplification reaction required 16 cycles instead of 12. This was due to the lower abundance of miRNAs in plasma, thus increasing their cDNA concentration to an appropriate level for RT-qPCR.

Following pre-amplification 156uL of RNase free water was added to each well of a 96 well plate, and 4uL of pre-amplification reaction was added to this and pipetted up and down. The plate was then spun down and frozen at -20˚C prior to RT-qPCR.

8.6.3 Additional Aortic Tissue Validation Study

The additional validation study into aortic tissue used the same methodology as the discovery study described above, however specific miRNA primers were used instead of pool A and pool B (Pool A and pool B contain all primers required for reverse

transcription, pre-amplification and PCR for their respective TaqMan Low Density Array cards, however as these cards were not used for the additional aortic tissue validation study specific primers for the individual miRNAs of interest were more cost effective).

8.7 Real-Time Quantitative PCR

Polymerase chain reaction (PCR) is a technique that allows amplification of a few copies of DNA over several orders of magnitude, generating thousands of copies of the same DNA sequence, which can then be detected. The reaction works by thermal cycling allowing enzymatic replication of the DNA. Initially DNA is denatured by heat, causing single-stranded DNA. Primers which are complimentary to the target DNA sequence along with DNA polymerase are then able to bind to their target sequence, and replicate the section of DNA. Following initial amplification, the replicate then becomes a template for further replication, causing an exponential increase in PCR product. It is the number of thermal cycles prior to detection of the PCR product that is used to quantify the initial input DNA.

8.7.1 Discovery study

For the discovery study the expression of 754 miRNAs was analysed using TaqMan® Low Density Array (TLDA) microRNA A+B cards set v3.0 (Life Technologies, California) (Figure 8-3). Each well contains a specific PCR primer corresponding to a single miRNA. For example, the sequence for miR-411 is:

UGGUACUUGGAGAGAUAGUAGACCGUAUAGCGUACGCUUUAUCUGUGACGUAUGUAAC ACGGUCCACUAACCCUCAGUAUCAAAUCCAUCCCCGAG

A specific complementary primer for miR-411 is placed on card A row 13 (position highlighted on Figure 8-5). Only an exact match between primer and miRNA will result

in amplification, therefore there is a specific primer for each miRNA spread across the TLDA cards. Cards A and B therefore contain different sets of miRNA primers to enable the analysis of 754 miRNAs.

The PCR reaction mix was prepared by mixing PCR Master Mix 2X with an equal volume of pre-amplified cDNA for a single sample. This was then pipetted into the fill ports of the TLDA card followed by centrifuging twice for 1 minute at 1,200 rpm. The TLDA card was then sealed, fill reservoirs excised, and RT-qPCR started using the ABI 7900HT instrument (Applied Biosystems, California). Each TLDA card contained a single sample only.

Figure 8-5 – Schematic of TaqMan Low Density Array Cards A and B. PCR reaction mix was pipetted directly into each of the 8 reservoirs, and transferred into each well during centrifugation.

a)

b)

8.7.2 Validation study

For the validation study a high throughput system was required to analyse a large number of samples in a cost effective manner. This was used for the whole blood, plasma and initial aortic tissue studies. TaqMan OpenArray technology was used with the QuantStudio 12K Flex Real-Time PCR machine (Life Technologies, California). This system can be custom designed to analyse either 12, 16, 24, 48, 96 or 144 samples per slide (with a minimum purchase of 10 slides), with either 256, 192, 128, 64, 32 or 16 miRNAs per sample respectively (including 2 internal controls). Therefore as the validation study included 200 blood samples, 200 plasma samples and 29 aortic tissue samples the optimum number of samples per card was 48, resulting in 16 miRNAs per sample. A custom made TaqMan MicroRNA OpenArray Card was designed so that each 8 by 8 grid within the card was specific to an individual sample (Figure 8-7). Specific miRNAs identified from the discovery study and literature were located in specific wells within the 8 by 8 grid, allowing a spreadsheet of samples and miRNAs to be uploaded into the QuantStudio 12K Flex Real-Time PCR machine. The diluted pre-amplified samples were thawed on ice and were mixed by gently vortexing then centrifuging the plate. For each sample 2.5uL of TaqMan Custom OpenArray Real-Time Master Mix was mixed with 1.3uL of RNase free water, and 1.2uL of pre-amplified cDNA. This was then transferred to the TaqMan Custom MicroRNA OpenArray Card using a standard AccuFill method. The corresponding miRNA and sample panel was loaded into the QuantStudio and RT-qPCR was run, with miRNA levels displayed immediately post run.

8.7.3 Additional Aortic Tissue Validation Study

RT-qPCR for the second aortic tissue validation study was undertaken using 96 well plates on the Applied Biosystems 7900HT (Applied Biosystems, California). This was used due to the smaller number of samples and miRNAs analysed, and was therefore more cost effective. Each sample was analysed in triplicate. The PCR reaction mix was prepared by mixing PCR Master Mix 2X with an equal volume of pre-amplified cDNA. This was then pipetted into the corresponding wells of the 96 well plate along with the specific miRNA primer, followed by centrifuging twice for 1 minute at 1,200 rpm. The 96 well card was then sealed and PCR run.

8.8 Raw Data Analysis

8.8.1 Discovery study

PCR curves were visually inspected using RQ manager (Applied Biosystems, California) to ensure a normal curve was present for each miRNA, with poor quality miRNAs excluded (Figure 846). The CT (threshold cycle) and baseline for each miRNA was optimised prior to analysis through visual inspection of PCR curves. The baseline is the initial cycles of PCR during which there is little change in the signal. The CT is the cycle number through which the fluorescence passes a fixed threshold, which is determined as the start point of the increase in fluorescence. As you can see from Figure 8-6, when comparing ΔRT v cycle this is the most vertical point on the curve, whereas on the RT v cycle this is the point at which the curve starts to rise. For the discovery study, CT values were calculated using SDS 2.4 (Applied Biosystems, California) through manual insertion of baseline and threshold levels according to visual inspection of the PCR plots for each miRNA (i.e. the curves for miR-29b from each sample were inspected simultaneously, and the same baseline and threshold was added for each sample). CT

values were normalised to the geometric mean of two internal controls, RNU48 and Mammalian U6 (MammU6). Analysis was undertaken using the relative standard curve method for fold change (see chapter 8.9.1).

8.8.2 Validation Study

For the validation study each miRNA was analysed in triplicate, normalised to the geometric mean of RNU48 and MammU6, and analysed using the comparative CT (ΔΔCT) method for fold change (see chapter 8.9.2). Initially the OpenArray cards were visually inspected to ensure complete coverage within each well (Figure 8-7). Where incomplete coverage occurred the sample was repeated or excluded as necessary. Expression of each individual miRNA and the internal controls was then assessed for each sample. Those with poor PCR curves were repeated or excluded as necessary. A geometric mean CT value was calculated for each miRNA within each sample.

8.8.3 Additional Aortic Tissue Validation Study

The same methods as the discovery study were used for the additional aortic tissue validation study, however each miRNA was analysed in triplicate using the geometric mean CT value.

Figure 8-6 – a) example of a good PCR curve as shown be the clear S-shape in the Rn v cycle view; b) example of a bad PCR curve as shown by a lack of clear S-shape in the Rn v cycle view; c) example of a good PCR curve in the ΔRn v cycle view, with the green line being the threshold value; d) example of a bad PCR curve in the ΔRn v cycle view, with no threshold value possible.

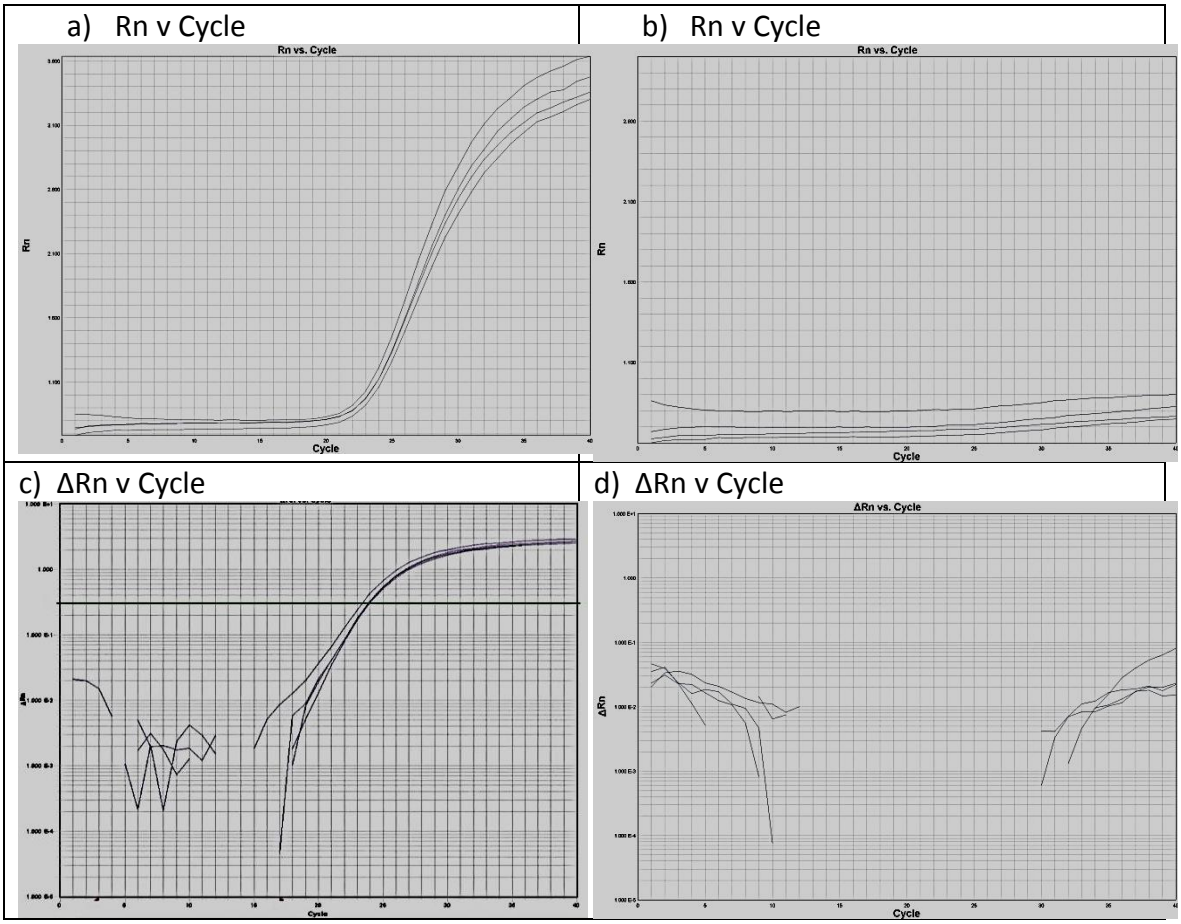
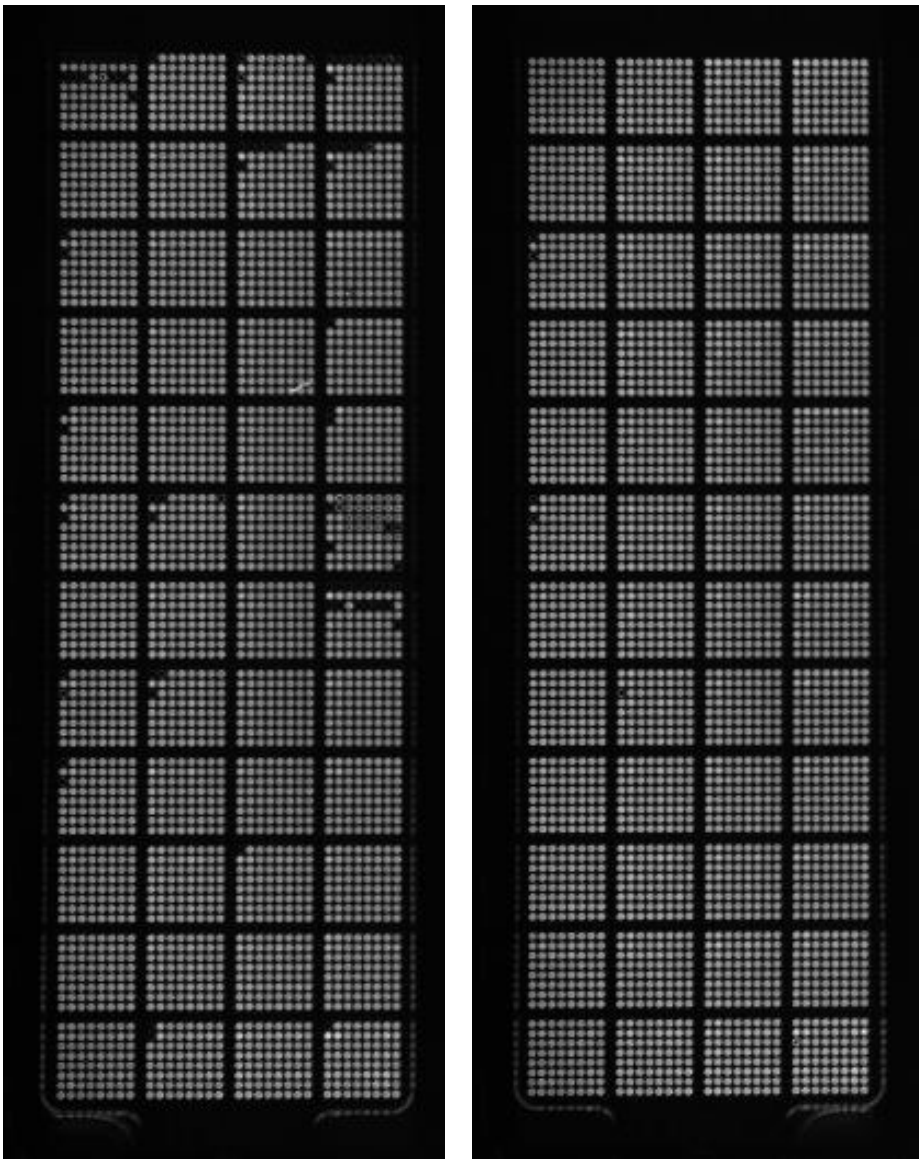


Figure 8-7 – Image of two of the OpenArray cards. The card of the left shows incomplete coverage within several well, as represented by darker or absent wells. The card on the right shows better coverage. Each 8 by 8 square represents the internal controls and triplicates for each sample, i.e. 1 square = 1 patients sample, therefore those with incomplete coverage were repeated or excluded as necessary.



8.9 Statistical Analysis

8.9.1. Discovery Study Fold Change

Fold change was calculated using the relative standard curve method. CT values were determined from the PCR curve, as described in Chapter 8.8, and 2 internal controls (Mammalian U6 and RNU48) were used to normalise the miRNA expression (thus decreasing variation between samples). Initially the logarithmic scale for the CT values was removed using the formula $2^{-(CT)}$. The value for each miRNA was then normalised to the geometric mean of the 2 internal controls (MammU6 and RNU48) within that sample to calculate a relative expression value. The geometric mean is a measure of the central tendency of a set of numbers, calculated by multiplying the values of RNU48 and MammU6 (each of which were in contained within the card template in triplicate for each sample), and the 'n'th root of their product. The geometric mean gives a better estimate than the arithmetic mean when using 2 different exogenous controls as it gives equal weighting to each control. For example we will calculate the relative expression value for miR-196b in sample LA1484:

miR-196b expression = $2^{-(CT)} = 2^{-(25.9)} = 1.60 \times 10^{-8}$

Geomean (MammU6 + RNU48) = 1.57×10^{-5}

Relative expression = expression miRNA / expression internal controls

Relative expression = miR-196b / GeoMean MammU6 + RNU48

Relative expression = $1.60 \times 10^{-8} / 1.57 \times 10^{-5}$

Relative expression = 1.02×10^{-3}

The fold change between cases and controls was then calculated using the arithmetic mean of the cases divided by the arithmetic mean of the controls. For example we will calculate the fold change in miR-196b between patients with aneurysms and controls:

Fold Change = relative expression cases / relative expression controls

Fold Change = 0.233 / 0.440

Fold Change = 0.53

A fold change of 1 is equivalent to no change in expression, therefore less than 1 is classified as a down-regulated miRNA. Fold changes of less than 1 were therefore converted to a negative integer to permit better visualisation of down-regulated miRNAs compared to up-regulated miRNAs. For example:

Fold change = -1 / 0.53

Fold change = -1.89

8.9.2 Validation Study Fold Change

Fold change within the validation study was calculated using the comparative CT method. The CT value for each miRNA was calculated by Expression Suite® (Applied Biosystems, California), with the geometric mean of the 3 replicates calculated for each miRNA. This was then normalised to the internal controls within the sample, taken as the geometric mean of Mammalian U6 (MammU6) and RNU48.

The change in CT value (ΔCT) was calculated by the formula:

ΔCT = CT target (miRNA) – CT reference (RNU48+MammU6)

The comparative CT value (ΔΔCT) was calculated using the formula:

ΔΔCT = ΔCT cases - ΔCT control

The standard deviation of the ΔΔCT value was taken as the standard deviation of the case. Fold differences calculated using the ΔΔCT method are expressed as a range, incorporating the standard deviation. The range of fold change for the case compared to the control sample is calculated by the formula:

$2^{-(\Delta\Delta CT +/- SD)}$

For example the ΔΔCT of miR-196b in plasma from the validation study gave mean a ΔCT value of 2.15 in controls, and 4.06 (SD 2.76) in cases.

The comparative CT value (ΔΔCT) was then calculated:

$$\Delta\Delta CT = CT\ Cases - CT\ Controls$$

$$\Delta\Delta CT = 4.06 - 2.15$$

$$\Delta\Delta CT = 1.91$$

The fold difference was calculated using the formula:

$$Fold\ Change\ miR\text{-}196b = 2^{-(\Delta\Delta CT +/- SD)}$$

$$Fold\ Change\ miR\text{-}196b = 2^{-(1.91)} = -3.75$$

$$Minimum\ Fold\ Change\ miR\text{-}196b = 2^{-(1.91+2.76)} = -25.44$$

$$Maximum\ Fold\ Change\ miR\text{-}196b = 2^{-(1.91-2.76)} = 1.81$$

Therefore the fold change for miR-196b in plasma is -3.75 (Range -25.44 – 1.81).

8.9.3 Additional Aortic Tissue Validation Study

The relative standard curve method as described above was used for the additional aortic tissue validation study.

8.9.4 Calculation of P values

For the discovery study a Mann-Whitney U test was performed using the normalised CT values and the geometric mean of RNU48 and MammU6 using SPSS version 20. For the validation study statistical analysis was undertaken using the geometric mean of the CT values for each miRNA, and internal controls using SPSS version 20. For the validation study initial analysis was undertaken using an ANOVA to determine any significant difference in miRNA expression in blood between controls, patients with AAA, and patients with PAD. Further post-HOC analysis was then undertaken using t-tests and binary logistic regression to determine whether significant differences were

specific to AAA, or whether they were due to generalised atherosclerosis. Additional analysis was undertaken to determine whether differences in miRNA expression were maintained post AAA repair, as this would determine whether miRNA expression is altered due to strain on the aneurysmal sac, or is an underlying predisposition to aneurysm formation. The correlation between aneurysm size and miRNA expression was also analysed using bivariate correlation.

Further validation work was then carried out in plasma samples with the same analysis as described above. Additional validation work was carried out comparing aortic tissue samples from patients undergoing AAA repair and control samples from cadaveric donors using the Mann-Whitney U test for the initial and additional aortic tissue cohorts.

8.10 Graphical Representation of Differential Gene Expression

Graphpad Prism 6 was used for graphical representation of data.

8.11 Bioinformatic Analysis of miRNA data

The MiRWalk software described in chapters 3.7 and 5.2 was used to determine both validated and predicted interactions between miRNAs and aneurysm biomarkers/genes. Validated targets had been previously experimentally verified and published, whilst predicted targets had to be identified through at least 5 out of the 9 prediction algorithms searched. This cut off was determined to balance the sensitivity and specificity of the prediction software.

Chapter 9

Results 1 – Discovery study

9.1 Background Comparison

For the discovery study 10 control and 15 subjects with AAA were used. There was no significant difference in total RNA concentration between groups (median RNA concentration in controls 57.7 ng/uL (IQR 50.1 to 84.1), median RNA concentration in aneurysms 54.2 ng/uL (IQR 48.9 to 58.0); P=0.311).

Analysis of subjects' previous medical history revealed a significant increase in age in patients with AAA, and a significant increase in the use of statins in patients with AAA (Table 10-1).

Table 9-1 – Background comparison between control and AAA samples used in discovery study. Total number of patients with that factor positive and percentage given. Mann-Whitney U and Fisher's Exact test used.

	Control (10)	**Aneurysm (15)**	**P Value**
Age	65.0 (IQR 65.0-65.0)	77.0 (IQR 69.5-79.0)	<0.01
Ever Smoker	6 (60%)	14 (93.3%)	0.12
Myocardial Infarction	2 (20%)	4 (26.7%)	1.00
Hypertension	5 (50%)	8 (53.3%)	1.00
Hypercholesterolaemia	6 (60%)	14 (93.3%)	0.12
Angina	0 (0%)	2 (13.3%)	1.00
Cerebrovascular Accident	1 (10%)	1 (6.7%)	1.00
Diabetes	2 (20%)	2 (13.3%)	1.00
Chronic Obstructive Pulmonary Disease	0 (0%)	2 (13.3%)	0.50
Aspirin	3 (30%)	9 (60%)	0.23
Beta Blocker	3 (30%)	7 (46.6%)	0.68
Statin	6 (60%)	14 (93.3%)	0.02
Clopidogrel	1 (10%)	1 (6.7%)	1.00
Digoxin	1 (10%)	0 (0%)	0.40
Warfarin	1 (10%)	2 (13.3%)	1.00
Diuretic	1 (10%)	3 (20%)	0.63
ACE Inhibitor	4 (40%)	3 (20%)	0.38

9.2 Data Analysis

The expression curves of all 754 miRNAs were visually inspected, with miRNAs with suboptimal amplification PCR curves (absence of clear S-shaped curves) leading to their exclusion (Figure 8-4, Chapter 8.8.2). This led to the exclusion of 395 miRNAs from further analysis. The remaining 359 miRNAs were compared using a Mann-Whitney U test (Figure 9-1 and Table 9-2) identifying 29 miRNAs to be differentially expressed in patients with AAA with a P value < 0.1 and fold change > 1.5 (For full table of P values and fold changes see Appendix 2). In addition, the miRNAs highlighted as associated with AAA through the literature review detailed in Chapter 4 are also represented in Table 9-3.

Figure 9-1 – Volcano plot showing the distribution of fold change and P value for each miRNA expression in the discovery study. 13 miRNAs had a fold change greater than 3 or -3 (with 1 of these being significantly differentially expressed), and are therefore not included within this plot). The upper outer quadrants (shaded) represent the miRNAs with both a significant P value and a significant fold change.

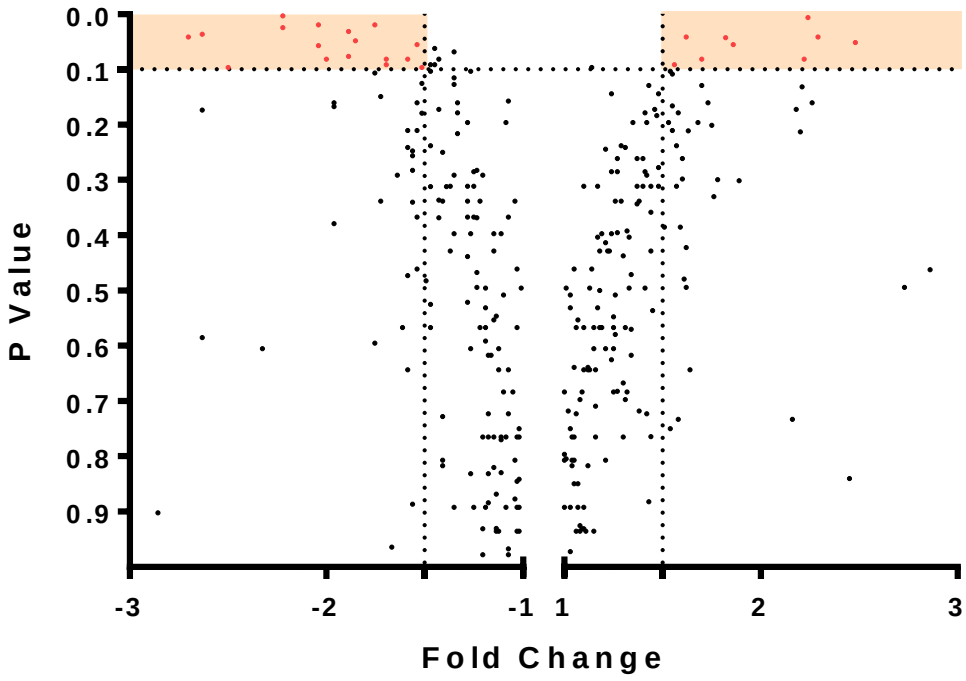


Table 9-2 – Differentially expressed miRNAs from the discovery study. Those with a P value less than 0.1 and fold change greater than 1.5 were deemed to be statistically significant. P values calculated using Mann-Whitney U test.

microRNA	Fold Change	P Value
miR-672	-2.22	<0.01
miR-19a*	2.24	<0.01
miR-15a	-2.04	0.02
miR-16-1*	-1.75	0.02
miR-9*	-2.22	0.02
miR-125a-3p	69866.67	0.03
miR-196b	-1.89	0.03
miR-134	-2.63	0.04
miR-181a	1.62	0.04
miR-942	-2.70	0.04
Let-7e	2.29	0.04
miR-431	1.82	0.04
miR-1291	-1.85	<0.05
miR-27b	2.48	0.05
miR-1249	-1.54	0.06
miR-483-5p	1.86	0.06
miR-624	-2.04	0.06
miR-411	-2.04	0.06
miR-589	-1.51	0.07
miR-29b	-1.89	0.08
miR-1179	-2.00	0.08
miR-1227	1.70	0.08
miR-138	2.22	0.08
miR-26a-1*	-1.59	0.08
miR-374a	-1.69	0.08
miR-125b	1.56	0.09
miR-628-5p	-1.69	0.09
miR-148b*	-1.52	<0.10
miR-15b*	-2.50	<0.10

Table 9-3 – Summary of discovery study results for all miRNAs identified as associated with VSMCs, the ECM, animal AAAs or human AAAs. Those miRNAs meeting the criteria for differential expression within the discovery study are highlighted in grey.

miRNA	Study type identifying association with AAA	Fold Change	P value
let-7f	Human	-1.14	0.43
miR-1	VSMC	-1.29	0.37
miR-7	ECM	-1.94	0.38
miR-15a	Human	-2.06	0.02
miR-15b	Human	-1.34	0.22
miR-18a	ECM	1.48	0.14
miR-19a	ECM	2.24	<0.01
	Animal		
miR-19b	ECM	-1.60	0.24
	Animal		
miR-20c	Human	Not expressed	
miR-21	VSMC	1.03	
	Animal		
	Human		0.53
miR-24	Animal and Human (same study)	1.33	0.40
miR-26a	VSMC	1.40	0.26
	Animal		
miR-27a	Human	-1.03	0.77
miR-29a	ECM	1.04	1
miR-29b	ECM	-1.90	
	Animal and Human (same study)		0.08
miR-29c	ECM	-1.21	0.57
miR-30	ECM	Not expressed	
miR-30c-2*	Human	Not expressed	
miR-31	VSMC	1.70	0.13
miR-124a	Human	1.37	0.34
miR-126	Human	1.24	0.14
miR-132	Animal	1.53	0.20
miR-133	ECM	-1.21	0.93
miR-133a	Human	-1.05	0.81
miR-133b	Human	Not expressed	
miR-143	VSMC		
	Animal	1.25	0.55
miR-145	VSMC	1.40	0.26
	Animal		
miR-146a	VSMC	-1.47	0.09
	Human		
miR-155	Human	1.15	0.61
miR-204	Human	Not expressed	
miR-208	VSMC	Not expressed	
miR-221	VSMC	1.14	0.46
	Human		
miR-222	VSMC	1.04	0.81
	Animal		
	Human		
miR-223	Human	1.25	0.61
miR-331-3p	Human	1.09	1

9.3 Determining which microRNAs for the validation study

For the validation study I wanted to combine the data from the discovery study with that from the literature to determine the best 16 miRNAs to take forward. 16 miRNAs were chosen as this was the most cost-effective number to assess using the validation study methodology described in Chapter 8.7.2. However, due to the reverse transcription and pre-amplification process I initially had to choose between the miRNAs assessed in pool A and pool B. This is because the primers for reverse transcription, pre-amplification and PCR are produced in separate pools, and each pool must be analysed on a different array card. Therefore combining miRNAs from each pool or evaluating all differentially expressed miRNAs would have doubled the cost.

The miRNAs analysed in pool A are the earlier miRNAs identified, and as such more has been discovered about their function. In addition, 17 miRNAs differentially expressed in the discovery study were from pool A, and 12 were from pool B. Within the literature, 31 miRNAs were within pool A, and 4 were within pool B. I therefore decided to use pool A for the validation study.

Four miRNAs differentially expressed within the discovery study had also previously been identified in the literature as associated with AAA (miR-15a in human AAA, miR-29b in human AAA, animal models and VSMC's, miR-138 and miR-181a), therefore these were selected for further analysis.

From the literature I decided to select 4 further miRNAs for the following reasons. MiR-145 had a fold change of 1.40 in the discovery study, and has been associated with VSMC pathophysiology and identified in animal studies as associated with AAA. Mir-155 has been previously identified in a human AAA study. MiR-221 has been associated with VSMC pathophysiology and previously been identified in a human AAA

study. MiR-31 had the highest fold change of 1.70, and has been associated with VSMC pathophysiology.

From the remaining 13 miRNAs in the discovery study I next looked at the reliability of their expression. 5 miRNAs did not produce PCR curves for all samples (miR-672 (missed 3 PCR curves), miR-125a-3p (missed 4 PCR curves), miR-134 (missed 1 PCR curve), miR-624 (missed 4 PCR curves), miR-628-5p (missed 2 PCR curves).

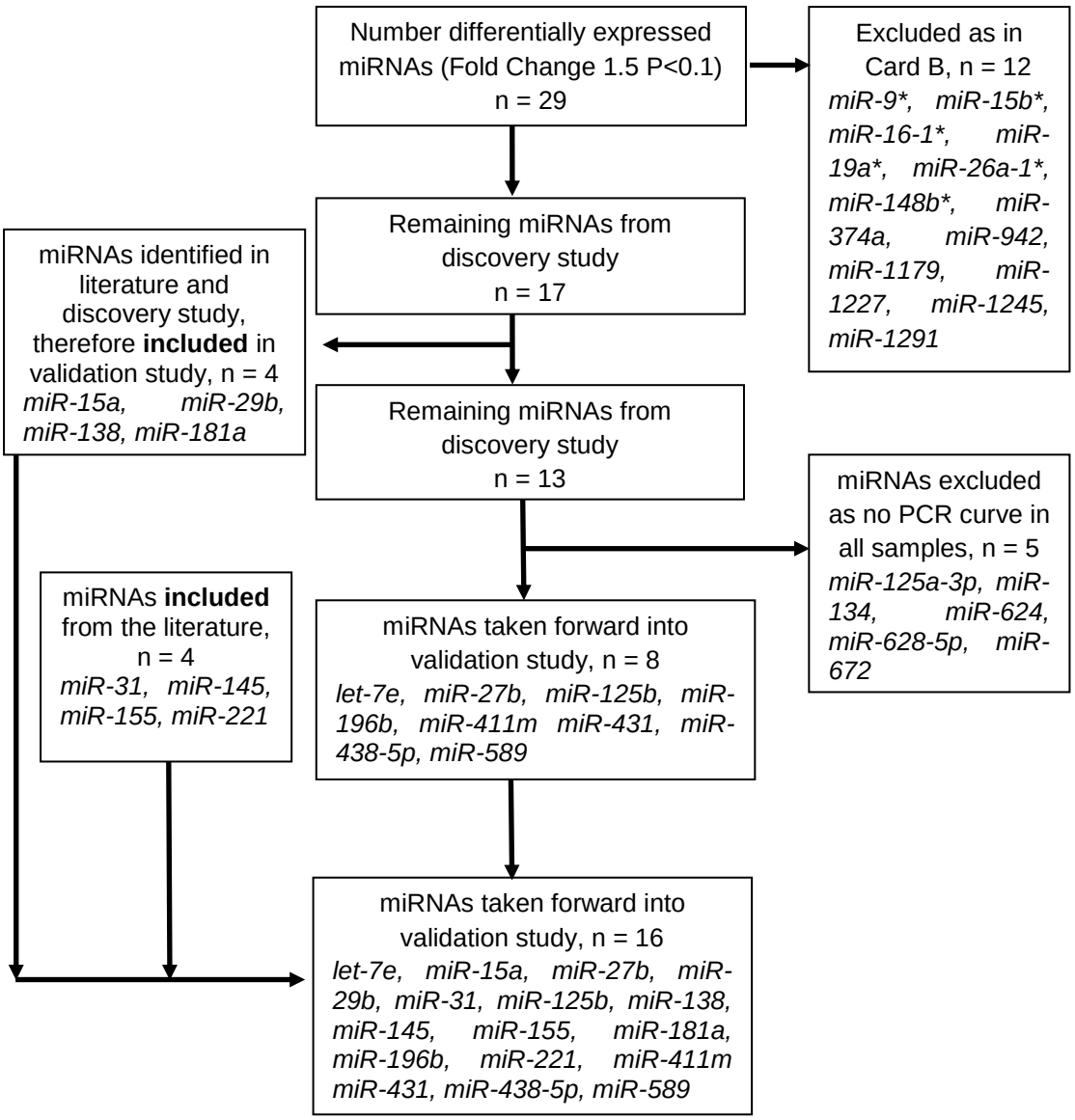
For the remaining 8 miRNAs I determined their mean CT value. miRNAs with a CT value greater than 30 are thought to be unreliable as they are expressed late in the run and therefore of very low concentrations. All 8 miRNAs had a CT value <30 (Table 9-4).

Table 9-4 – The 8 miRNAs taken from the discovery study to validation study showing mean fold change, p value and mean CT value.

microRNA	Fold Change	P Value	Mean CT value
miR-196b	-1.89	0.03	27.4
Let-7e	2.29	0.04	19.5
miR-431	1.82	0.04	29.8
miR-27b	2.48	0.05	25.3
miR-483-5p	1.86	0.06	28.3
miR-411	-2.04	0.06	28.3
miR-589	-1.51	0.07	28.0
miR-125b	1.56	0.09	23.8

Combining the miRNAs from the literature, those from the literature and discovery study, and those from the discovery study alone, I took let-7e, miR-15a, miR-27b, miR-29b, miR-125b, miR-138, miR-181a, miR-196b, miR-411, miR-431, miR-483-5p, and miR-589 forward into the validation study (Figure 9-2).

Figure 9-2 – Flow diagram showing the methods for determining which miRNAs to take through to the validation study



9.4 Conclusion

The discovery study compared the expression levels of 754 miRNAs between 15 subjects with AAA and 10 controls, identifying a total of 29 differentially expressed miRNAs with a fold change > 1.5 and a P value < 0.1. Four of these miRNAs had been previously associated with AAA through either genes known to be involved in aneurysmogenesis, their interaction with VSMC's or the ECM, or through animal or human studies into AAA. Unfortunately due to technical and monetary constraints I was not able to take every miRNA through for further validation, however 16 miRNAs, 4 from the literature, 8 from the discovery study, and 4 from both the literature and discovery study have been selected to take forward into further validation work.

Chapter 10

Results 2 - Validation Study

10.1 Background comparison

10.1.1 Blood samples

Whole blood samples were collected as previously described for 5 cohorts of subjects, namely those with large AAA (≥5.5cm), small AAA (3.0-5.4cm), post open or endovascular aneurysm repair, control subjects, and those with PAD. A total of 40 samples for each cohort were used for the validation study, with 18 of the 40 post-operative samples being matched, i.e. collected from the same patients as those within the large AAA cohort. All whole blood samples showed excellent PCR curves and as such all 200 samples were used for analysis.

For the validation study there was no significant difference in total RNA concentration between groups (P=0.282; Table 10-1).

Comparison of cardiovascular risk factors revealed a significant difference in age and smoking status between the cohorts, and in previous medical history of hypertension and hypercholesterolaemia. Comparison of medication use within each cohort revealed a significant difference in the use of aspirin, statins, clopidogrel, warfarin and diuretics (Table 10-1). Due to these significant differences, binary logistic regression was used for further analysis, henceforth referred to as adjusted P values, to take these confounding factors into consideration.

Table 10-1 – Background analysis of subjects included in whole blood sample validation study. Statistical analysis performed using ANOVA for continuous data and Chi Squared for categorical data.

	Control (n=40)	PAD (n=40)	SA (n=40)	LA (n=40)	PO (n=40)	P Value
Mean RNA concentration ng/uL (SD)	105.4 (42.6)	122.8 (47.7)	115.6 (48.9)	101.8 (57.4)	116.0 (41.9)	0.282
Age (median + range)	64 (64-65)	67 (55-89)	73 (59-89)	73 (55-90)	71 (55-87)	0.01
Ever Smoker	29 (72.5%)	38 (95%)	35 (87.5%)	36 (90%)	34 (85%)	0.04
Myocardial Infarction	6 (15%)	8 (20%)	11 (27.5%)	9 (22.5%)	10 (25%)	0.52
Hypertension	21 (52.5%)	17 (42.5%)	31 (77.5%)	27 (67.5%)	24 (60%)	0.02
Hypercholesterolaemia	16 (40%)	22 (55%)	30 (75%)	33 (82.5%)	34 (85%)	<0.01
Angina	4 (10%)	2 (5%)	9 (22.5%)	6 (15%)	5 (12.5%)	0.21
Cerebrovascular Accident	1 (2.5%)	4 (10%)	5 (12.5%)	3 (7.5%)	3 (7.5%)	0.56
Diabetes	4 (10%)	0 (0%)	4 (10%)	3 (7.5%)	3 (7.5%)	0.39
Chronic Obstructive Pulmonary Disease	1 (2.5%)	7 (17.5%)	5 (12.5%)	6 (15%)	6 (15%)	0.28
Aspirin	7 (17.5%)	21 (52.5%)	29 (72.5%)	26 (65%)	30 (75%)	<0.01
Beta Blocker	9 (22.5%)	10 (25%)	15 (37.5%)	18 (45%)	12 (30%)	0.17
Statin	12 (30%)	21 (52.5%)	34 (85%)	30 (75%)	34 (85%)	<0.01
Clopidogrel	0 (0%)	6 (15%)	2 (5%)	0 (0%)	1 (2.5%)	<0.01
Digoxin	0 (0%)	1 (2.5%)	1 (2.5%)	1 (2.5%)	1 (2.5%)	0.91
Warfarin	0 (0%)	3 (7.5%)	0 (0%)	6 (15%)	4 (10%)	0.03
Diuretic	6 (15%)	2 (5%)	9 (22.5%)	12 (30%)	11 (27.5%)	0.03
ACE Inhibitor	12 (30%)	11 (27.5%)	15 (37.5%)	13 (32.5%)	8 (20%)	0.52

PAD = peripheral arterial disease, SA = small aneurysm (30-54mm), LA = large aneurysm (>55mm), PO = post-operative case

10.1.2 Plasma samples

Plasma samples were collected as previously described. For the validation study, plasma samples used were from the same patients as in the whole blood analysis. Unfortunately 30 samples showed poor PCR curves and were therefore excluded from the analysis.

Total RNA concentration across the cohorts was not analysed due to the inaccuracy in quantifying RNA in plasma as previously described. Each sample met the inclusion criteria of > 1ng/uL.

Comparison of cardiovascular risk factors revealed a significant difference in age but not smoking status between the cohorts, and in previous medical history of hypertension and hypercholesterolaemia. Comparison of medication use within each cohort revealed a significant difference in the use of aspirin, statins, warfarin and diuretics (Table 10-2). Due to these significant differences, binary logistic regression was used for further analysis, henceforth referred to as adjusted P values, to take these confounding factors into consideration.

Table 10-2 – Background analysis of subjects included in plasma sample validation study. Statistical analysis performed using ANOVA for continuous variables and Chi Squared for categorical variables.

	Control (28)	PAD (35)	SA (36)	LA (36)	PO (35)	P Value
Age (median + range)	64 (64-65)	65 (55-89)	73 (58-89)	74 (55-90)	71 (55-84)	<0.01
Ever Smoker	24 (85.7%)	33 (94.3%)	32 (88.9%)	32 (88.9%)	31 (88.6%)	0.77
Myocardial Infarction	3 (10.7%)	7 (20%)	10 (27.8%)	8 (22.2%)	8 (22.9%)	0.47
Hypertension	13 (46.4%)	14 (40%)	29 (80.6%)	24 (66.6%)	22 (62.9%)	<0.01
Hypercholesterolaemia	10 (35.7%)	19 (54.2%)	27 (75%)	30 (83.3%)	30 (85.7%)	<0.01
Angina	2 (7.1%)	2 (5.7%)	8 (22.2%)	6 (16.7%)	5 (14.3%)	0.16
Cerebrovascular Accident	1 (3.6%)	3 (8.6%)	4 (11.1%)	3 (8.3%)	2 (5.7%)	0.84
Diabetes	3 10.7%)	0 (0%)	4 (11.1%)	1 (2.8%)	3 (8.6%)	0.25
Chronic Obstructive Pulmonary Disease	1 (3.6%)	6 (17.1%)	4 (11.1%)	6 (16.7%)	6 (17.1%)	0.44
Aspirin	4 (14.3%)	19 (54.2%)	25 (69.4%)	24 (66.6%)	25 (71.4%)	<0.01
Beta Blocker	5 (17.6%)	8 (22.9%)	12 (33.3%)	18 (50%)	9 (25.7%)	0.06
Statin	6 (21.4%)	18 (51.4%)	30 (83.3%)	28 (77.8%)	29 (82.9%)	<0.01
Clopidogrel	0 (0%)	3 (8.6%)	2 (5.5%)	0 (0%)	1 (2.9%)	0.26
Digoxin	0 (0%)	1 (2.9%)	1 (2.8%)	1 (2.8%)	1 (2.9%)	0.94
Warfarin	0 (0%)	2 (5.7%)	0 (0%)	6 (16.7%)	3 (8.6%)	0.02
Diuretic	4 (14.3%)	1 (2.9%)	9 (25%)	12 (33.3%)	11 (31.4%)	<0.01
ACE Inhibitor	6 (21.4%)	8 (22.9%)	13 (36.1%)	13 (36.1%)	6 (17.1%)	0.29

PAD = peripheral arterial disease, SA = small aneurysm (30-54mm), LA = large aneurysm (>55mm), PO = post-operative case

10.1.3 Tissue samples

Tissue samples were collected as previously described. For this initial validation study aortic tissue was collected from 19 AAA subjects and 10 cadaveric donors. For the additional validation study aortic tissue was collected from 22 subjects undergoing AAA repair, 3 subjects undergoing aortobifemoral surgery due to atherosclerosis, and

17 cadaveric donors. All samples showed good PCR curves and were therefore used for the analysis. Background data for both the initial and additional aortic tissue cohorts are presented in tables 10-3 and 10-4.

Table 10-3 – Background analysis of subjects included in initial aortic tissue study. CAD – cadaveric donor, AAA – abdominal aortic aneurysm

	CAD (10)	**AAA (19)**
Mean RNA concentration ng/uL (SD)	193 (47.8)	199 (94.8)
Age (median + range)	58 (36-68)	68 (50-77)
Ever Smoker	8 (80%)	18 (94.7%)
Ischaemic Heart Disease	0 (0%)	6 (31.6%)
Hypertension	2 (20%)	9 (47.4%)
Hypercholesterolaemia	1 (10%)	15 (78.9%)
Cerebrovascular Accident	0 (0%)	0 (0%)
Diabetes	0 (0%)	2 (10.5%)
Chronic Obstructive Pulmonary Disease	1 (10%)	4 (21.1%)
Aspirin	2 (20%)	13 (68.4%)
Beta Blocker	0 (0%)	6 (31.6%)
Statin	1 (10%)	12 (63.2%)
Clopidogrel	0 (0%)	0 (0%)
Digoxin	0 (0%)	0 (0%)
Warfarin	0 (0%)	1 (5.3%)
Diuretic	1 (10%)	2 (10.5%)
ACE Inhibitor	1 (10%)	7 (36.8%)

Table 10-4 – Background analysis of subjects included in additional aortic tissue study. AOD - aorto-occlusive disease, CAD – cadaveric donor, AAA – abdominal aortic aneurysm

	AOD (3)	CAD (17)	AAA (22)
Mean RNA concentration ng/uL (SD)	170 (27.4)	166 (56.5)	248 (94.7)
Age (median + range)	57 (53-65)	58 (32-77)	66 (50-77)
Ever Smoker	3 (100%)	11 (64.7%)	21 (95.5%)
Ischaemic Heart Disease	0 (0%)	3 (17.6%)	6 (27.3%)
Hypertension	2 (66.7%)	3 (17.6%)	12 (54.5%)
Hypercholesterolaemia	3 (100%)	1 (5.9%)	15 (68.2%)
Cerebrovascular Accident	0 (0%)	0 (0%)	0 (0%)
Diabetes	0 (0%)	0 (0%)	3 (13.6%)
Chronic Obstructive Pulmonary Disease	0 (0%)	3 (17.6%)	2 (9.1%)
Aspirin	2 (66.7%)	2 (11.8%)	13 (59.1%)
Beta Blocker	0 (0%)	1 (5.9%)	6 27.3%)
Statin	3 (100%)	3 (17.6%)	14 (63.6%)
Clopidogrel	0 (0%)	0 (0%)	0 (0%)
Digoxin	0 (0%)	0 (0%)	0 (0%)
Warfarin	0 (0%)	0 (0%)	1 (4.5%)
Diuretic	1 (33.3%)	1 (5.9%)	4 (18.2%)
ACE Inhibitor	1 (33.3%)	2 (11.8%)	10 (45.5%)

10.2 Data Analysis

10.2.1 Whole Blood Results

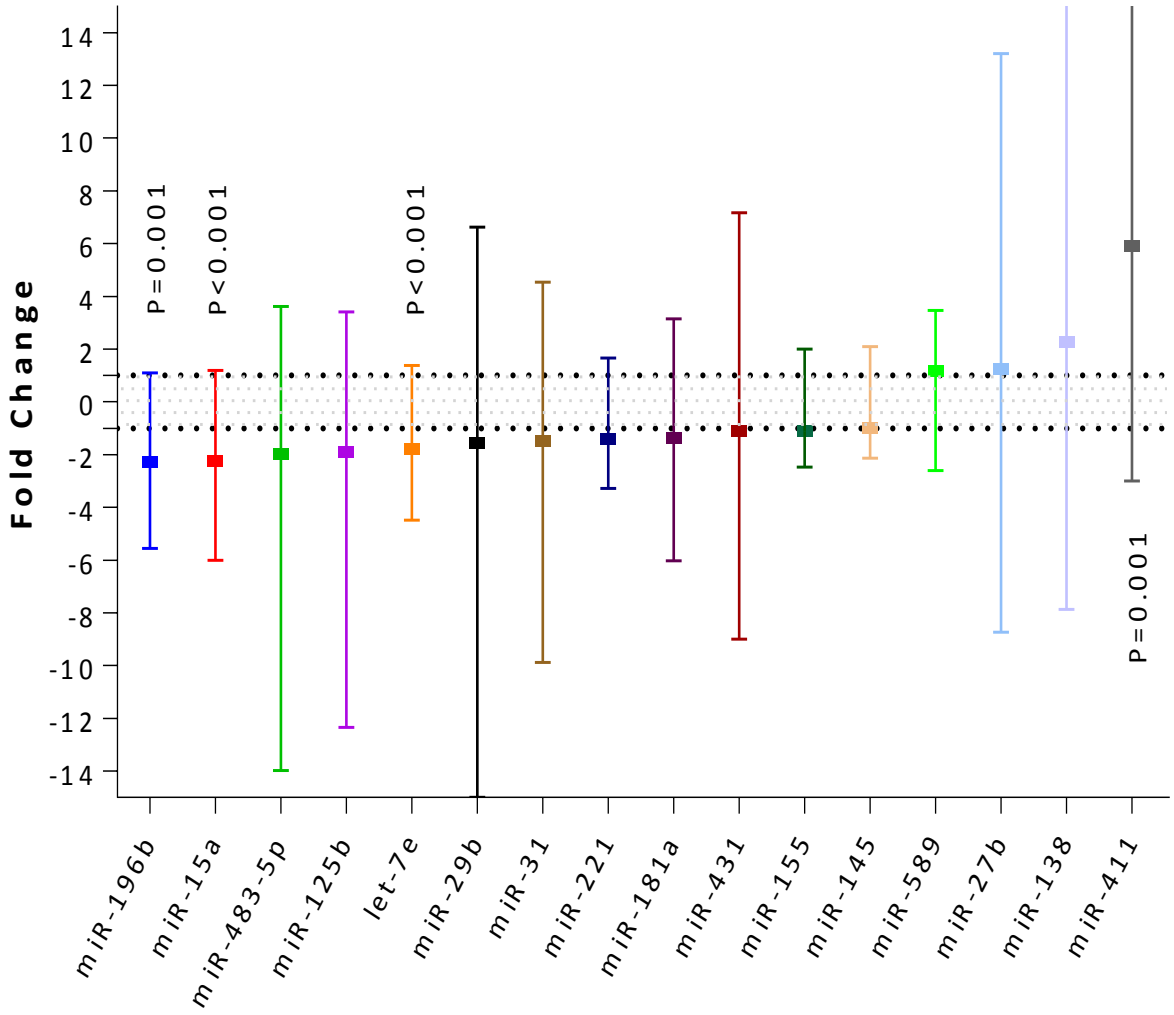
10.2.1.1 ANOVA

The relative expression (CT miRNA – CT reference gene) was used to determine any significant differences between cohorts. This was initially undertaken using a one-way ANOVA test to compare all 80 subjects with AAA, 40 subjects with PAD and 40 controls. A significant difference was identified between the cohorts in let-7e (P=0.001), miR-15a (P=<0.001), miR-196b (P<0.001) and miR-411 (for full results see Table 10-3 and Figure 10-1).

Table 10-3 – Results of one-way ANOVA test comparing relative miRNA expression in whole blood samples between all 80 AAA subjects, 40 PAD subjects and 40 controls.

miRNA	P Value
Let-7e	0.001
miR-125b	0.218
miR-138	0.266
miR-145	0.994
miR-155	0.726
miR-15a	<0.001
miR-181a	0.684
miR-196b	<0.001
miR-221	0.083
miR-27b	0.310
miR-29b	0.706
miR-31	0.258
miR-411	0.002
miR-431	0.383
miR-483-5p	0.117
miR-589	0.320

Figure 10-1 – Whole blood validation results showing the fold change (and range as described in Chapter 8.9.2) for each miRNA compared to controls. A fold change greater than 1 represents an upregulated miRNA, whereas a fold change less than -1 represents a downregulated miRNA. A fold change of 1 (or -1) is equivalent to no difference in miRNA expression.



10.2.1.2 Post-Hoc Analysis

For each miRNA identified as differentially expressed in the initial ANOVA I then undertook further analyses. As the ANOVA included subjects with small AAA, large AAA, PAD and controls, I wanted to determine whether there was a significant difference between any of these cohorts individually, and in addition whether there was any difference between the pre and post surgical repair cohorts. This was

conducted using the relative CT values (relative CT values are used as fold change is calculated on a cohort v cohort basis rather than for each individual data point therefore CT values are required to compare multiple data points) as follows:

1 – AAA v Control

2 – PAD v Control

3 – AAA v PAD

4 – Post-op v Control

5 – Pre-op v Post-op

6 – Matched Pre-op v post-AAA repair

I undertook both a Student's t-test (P value) and binary logistic regression (adjusted P value) taking all significant differences in background into account to determine an adjusted P value for comparisons 1-5. For comparison 6 I used a Mann-Whitney U test due to the smaller sample size requiring non-parametric statistical analysis. I also calculated the fold change for each cohort compared to the control where there was a significant difference.

The analysis of Let-7e (Figure 10-2a) revealed a significant downregulation in subjects with AAA compared to controls (Fold change -1.80 (-4.49 to 1.38); P=0.001; adjusted P = 0.026). There was also a significant downregulation of let-7e in patients with PAD (Fold Change -1.71 (-3.59 to 1.22); P=0.003; adjusted P=0.017) but no significant difference in let-7e expression between AAA and PAD cohorts (adjusted P=0.610). There was no significant difference in let-7e expression between post-operative

subjects and controls (adjusted P=0.111), and no significant difference between pre and post-operative let-7e expression when comparing all samples (adjusted P=0.214) or when comparing matched samples (P=0.385).

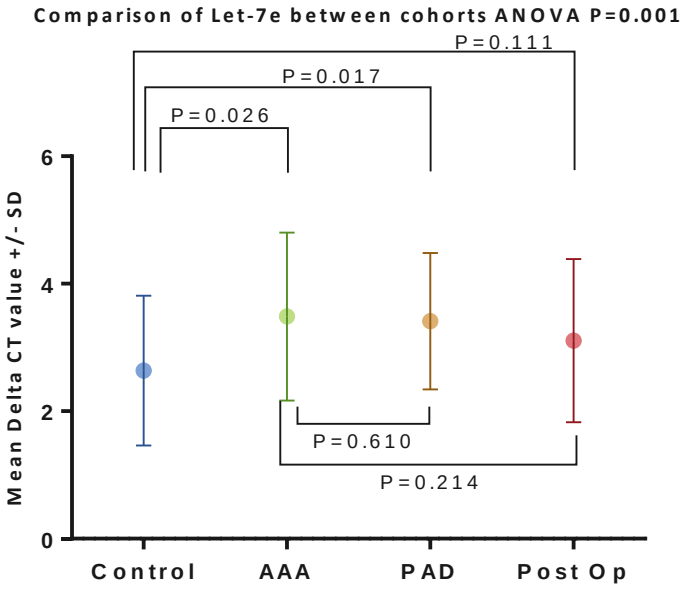
The analysis of miR-15a (Figure 10-2b) revealed a significant downregulation in subjects with AAA compared to controls (Fold change -2.24 (-6.00 to 1.19); P<0.001; adjusted P = 0.022). There was also a significant downregulation of miR-15a in patients with PAD (Fold Change -2.19 (-4.87 to 1.01); P<0.001; adjusted P=0.001) but no significant difference in miR-15a expression between AAA and PAD cohorts (adjusted P=0.727). There was a significant difference in miR-15a expression between post-operative subjects and controls (Fold Change -1.80 (-4.53 to 1.40); P=0.003; adjusted P=0.063), however there was no significant difference between pre and post-operative miR-15a expression when comparing all samples (adjusted P=0.290) or when comparing matched samples (P=0.287).

The analysis of miR-196b (Figure 10-2c) revealed a significant downregulation in subjects with AAA compared to controls (Fold change -2.26 (-5.56 to 1.09); P<0.001; adjusted P = 0.003). There was also a significant downregulation of miR-196b in patients with PAD (-2.11 (-3.96 to -1.12); P<0.001; adjusted P<0.001) but no significant difference in miR-196b expression between AAA and PAD cohorts (adjusted P=0.643). There was a significant difference in miR-196b expression between post-operative subjects and controls (Fold Change -1.74 (-4.52 to 1.49); P=0.004; adjusted P=0.042), however there was no significant difference between pre and post-operative miR-196b expression when comparing all samples (adjusted P=0.182) or when comparing matched samples (P=0.602).

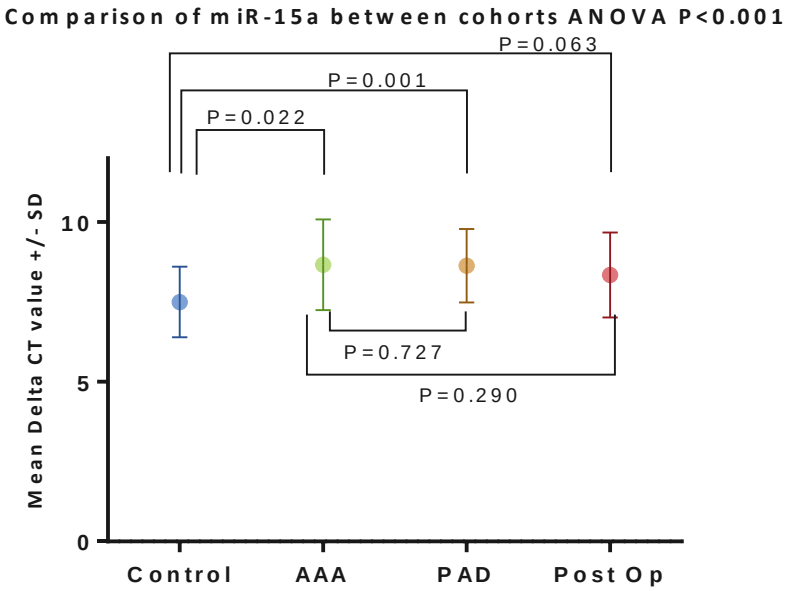
The analysis of miR-411 (Figure 10-2d) revealed a significant upregulation in subjects with AAA compared to controls, however this did not remain on binary logistic regression (Fold change 5.90 (-3.00 to 104.39); P=0.001; adjusted P = 0.079). There was also a significant downregulation of miR-411 in patients with PAD (4.52 (-3.94 to 80.54); P=0.005; adjusted P=0.018) but no significant difference in miR-411 expression between AAA and PAD cohorts (adjusted P=0.636). There was a significant difference in miR-411 expression between post-operative subjects and controls (Fold Change 9.30 (-1.81 to 156.50); P<0.001; adjusted P=0.038), however there was no significant difference between pre and post-operative miR-411 expression when comparing all samples (adjusted P=0.652) or when comparing matched samples (P=0.748).

Figures 10-2a-d – Comparison of mean delta CT values between AAA, PAD, post-operative and control subjects. Graph shows mean delta CT value and Standard Deviation. Adjusted P values on Binary Logistic Regression are shown. Figure a) let-7e, b) miR-15a, c) miR-196b, d) miR-411.

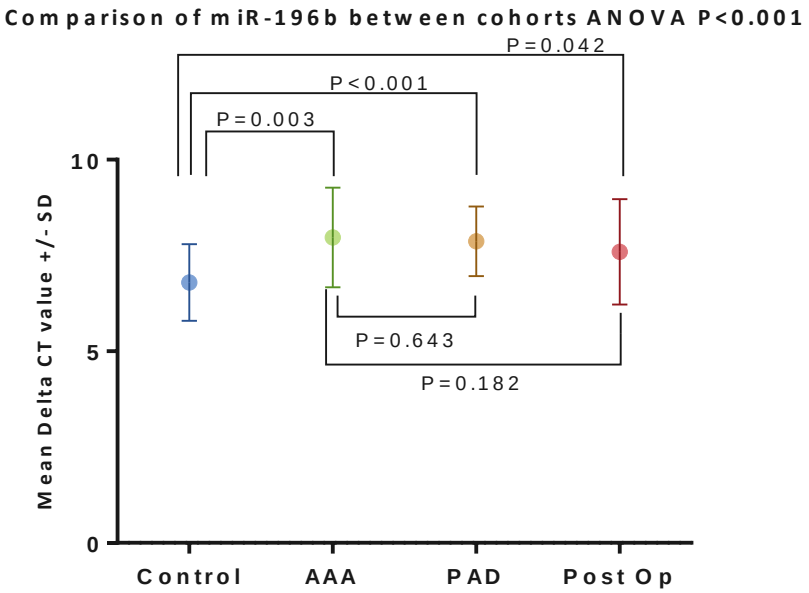
a)



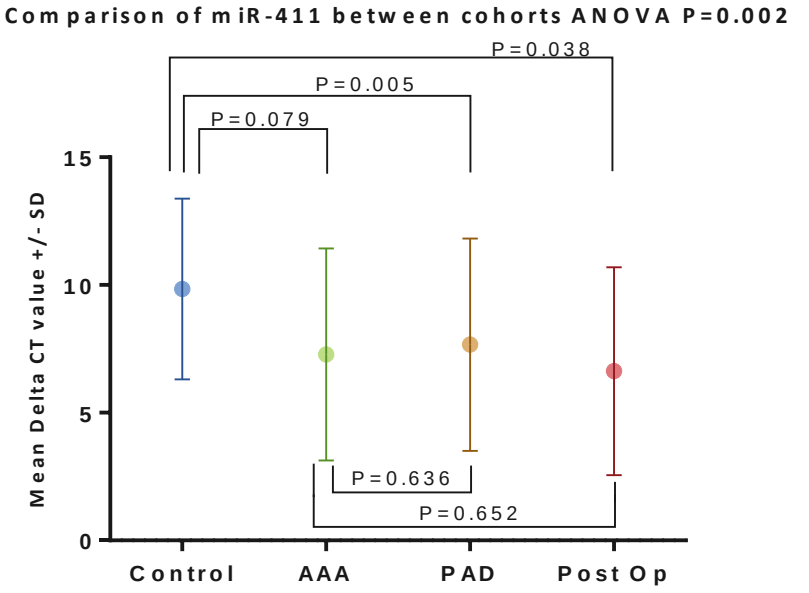
b)



c)


Comparison of miR-196b between cohorts ANOVA P<0.001
P=0.042
P<0.001
P=0.003
10
5
0
Mean Delta CT value +/- SD
P=0.643
P=0.182
Control
AAA
PAD
Post Op

d)

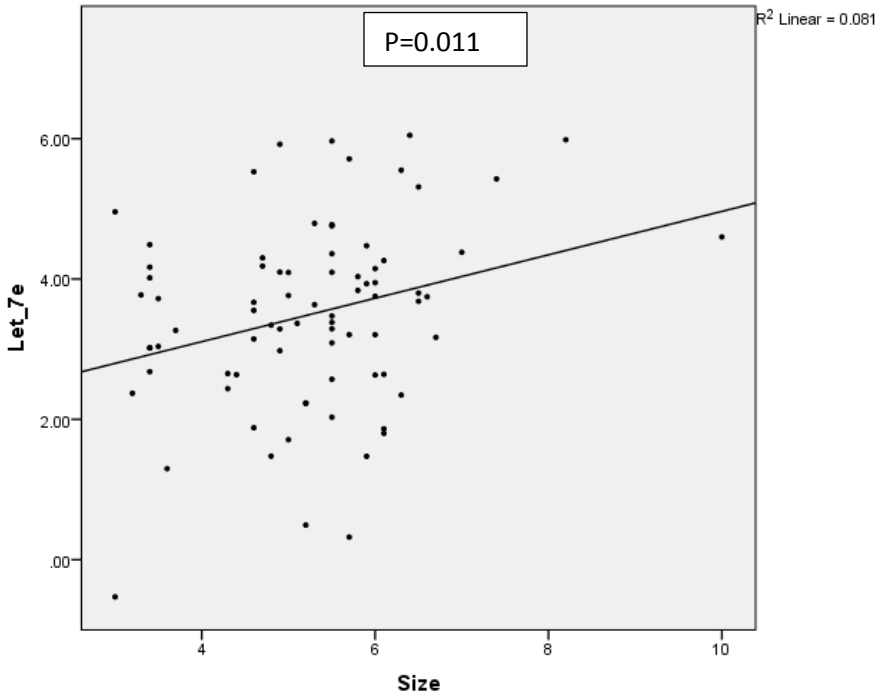

Comparison of miR-411 between cohorts ANOVA P=0.002
P=0.038
P=0.005
P=0.079
15
10
5
0
Mean Delta CT value +/- SD
P=0.636
P=0.652
Control
AAA
PAD
Post Op

10.2.1.3 Correlation Analysis

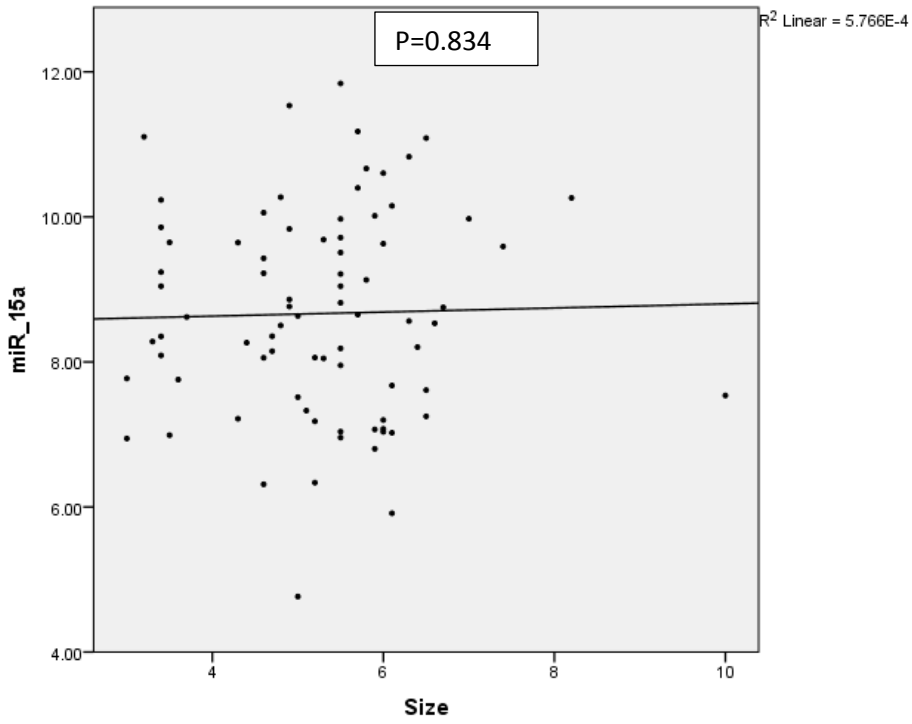
In addition to comparing differences in miRNA expression between cohorts, I also aimed to determine whether aneurysm size affected miRNA expression. I therefore compared the relative CT values (CT values are used rather than fold change as fold change is only calculated for a cohort v cohort comparison, and not for individual samples) for let-7e, miR-15a, miR-196b and miR-411 to aneurysm diameter in all pre-operative AAA subjects, revealing a significant correlation between miRNA expression and size for let-7e (P=0.011; R^2=0.081) (Figure 10-3), but not for miR-15a (P=0.834), miR-196b (P=0.724) or miR-411 (P=0.450). Although let-7e was found to be significantly correlated with AAA diameter, this correlation was weak (R^2=0.081), and therefore not of clinical use.

Figure 10-3 – Comparison of mean delta CT values for a) let-7e b) miR-15a, c) miR-196b, d) miR-411, and aneurysm size for all pre-operative subjects with AAA.

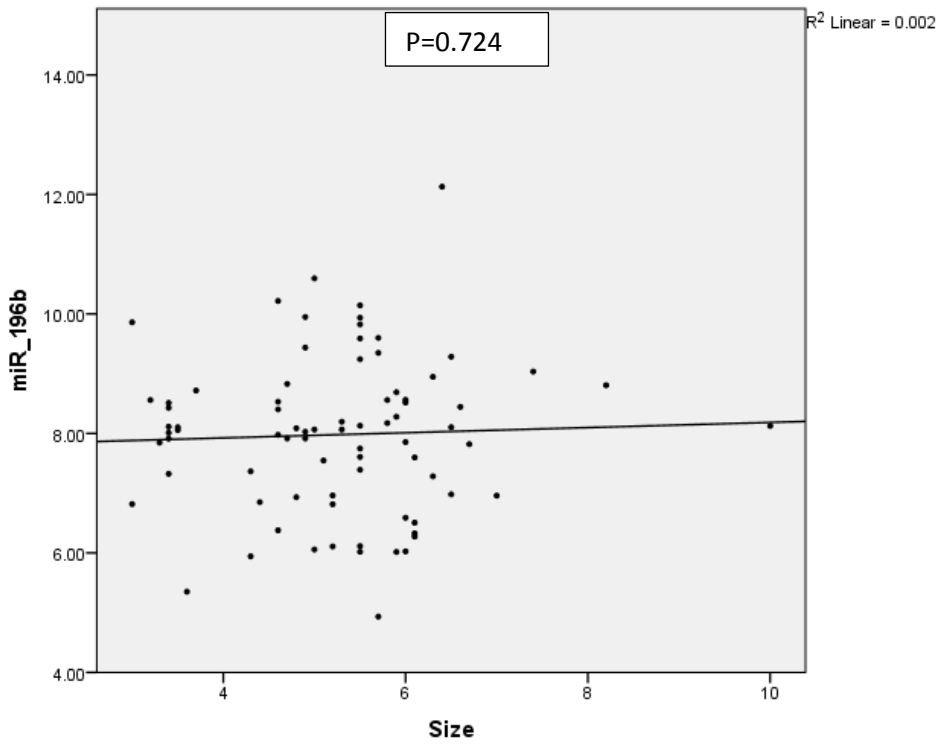
a)



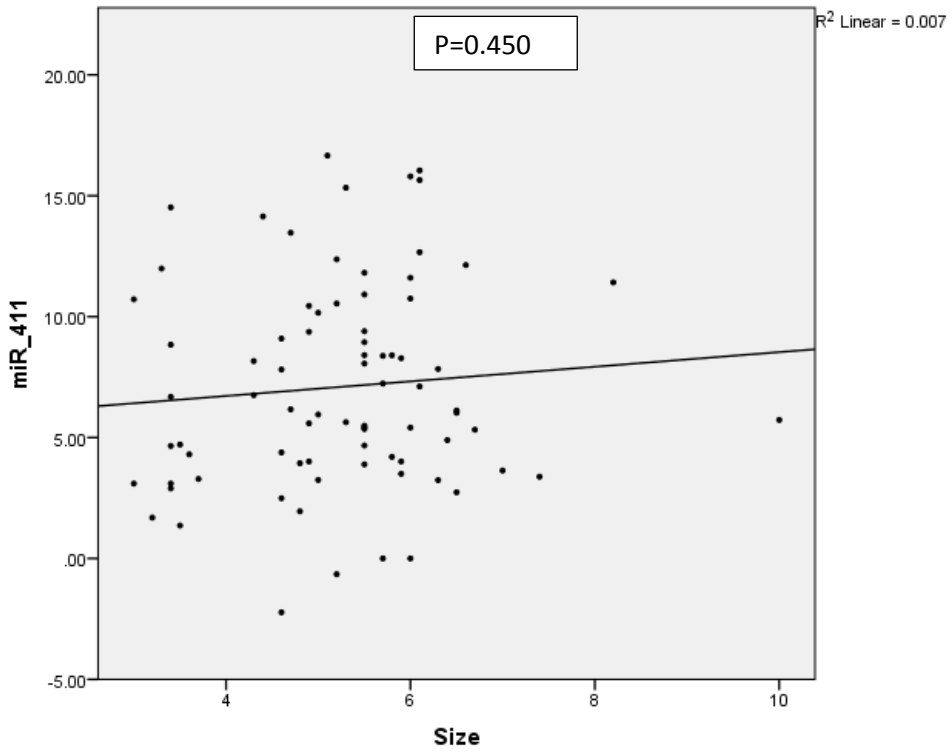
b)



c)


P=0.724
R² Linear = 0.002
miR_196b
Size
14.00
12.00
10.00
8.00
6.00
4.00
4
6
8
10

d)


P=0.450
R² Linear = 0.007
miR_411
Size
20.00
15.00
10.00
5.00
.00
-5.00
4
6
8
10

10.2.1.4 Conclusion

The results of the validation study in whole blood samples has revealed 3 miRNAs to be significantly downregulated in patients with AAA compared to controls (let-7e, miR-15a, miR-196b), and 1 miRNA (miR-411) to be significantly upregulated, with all except miR-411 remaining significant on binary logistic regression. miR-15a, miR-196b and miR-411 were also found to be differentially expressed in patients post-operatively compared to controls, with miR-196b and miR-411 remaining so following binary logistic regression, however there were no differences found in miRNA expression between pre and post-operative subjects, therefore exclusion of the aneurysm sac did not alter miRNA expression. Additionally, let-7e has been found to be significantly, but weakly correlated to aneurysm size. Although these results are significant, it should be noted that both miR-411 and let-7e have been found to be dysregulated in the opposite direction to the discovery study.

An important aim of this study was to determine whether the miRNAs found to be differentially expressed in AAA subjects were specific to AAA or due to generalised atherosclerosis, as it has been previously hypothesised that these conditions are due to a similar underlying aetiology. The validation cohort found the differentially expressed miRNAs to also be dysregulated in patients with PAD, giving further weight to these conditions having a similar genetic basis. Therefore the reason why certain patients develop atherosclerotic disease and others develop AAA remains to be elucidated.

10.2.2 Plasma Results

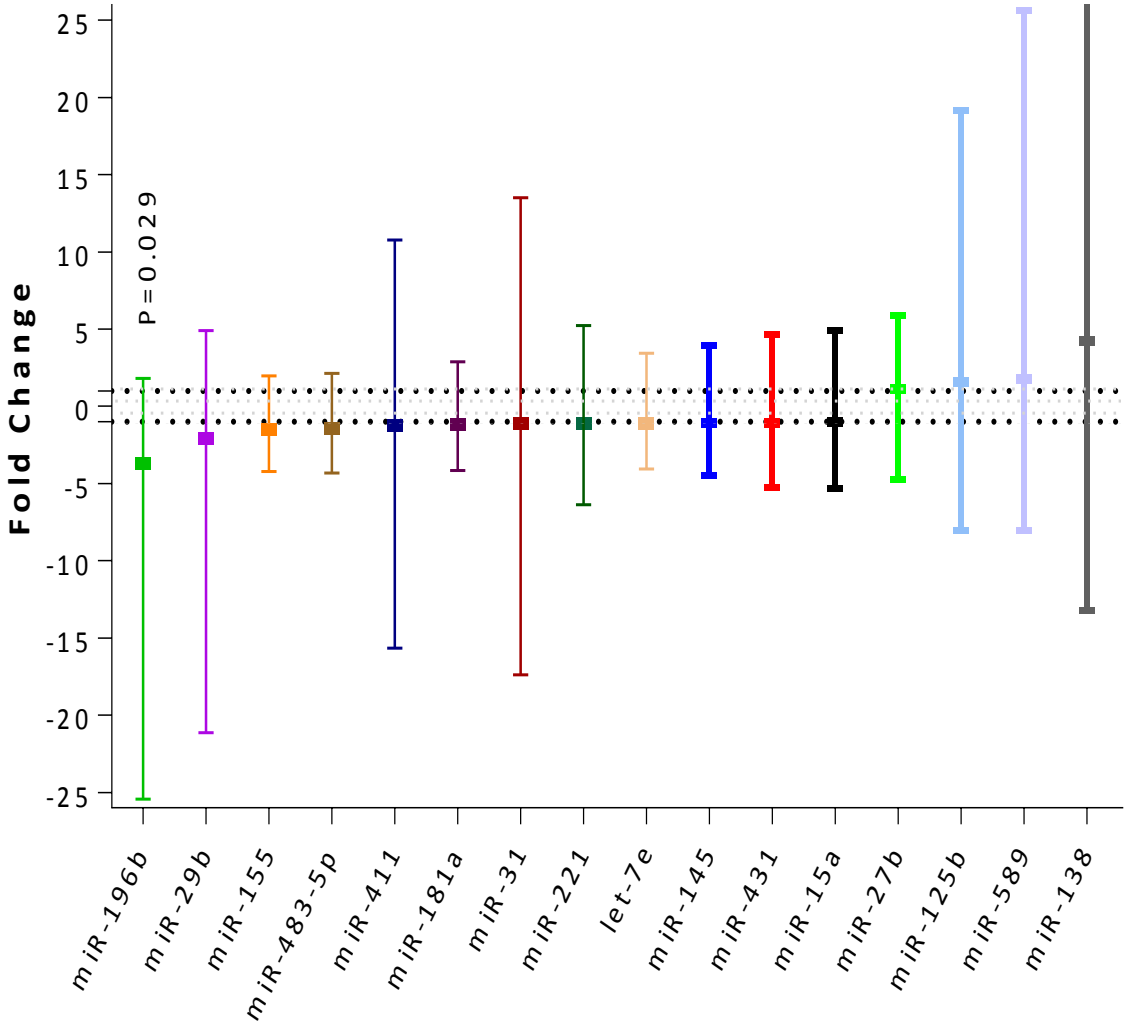
10.2.2.1 ANOVA

The same methods were used for analysis of the plasma samples as with the whole blood samples. Relative expression (CT miRNA – CT reference gene) was used to determine any significant differences between cohorts. This was initially undertaken using a one-way ANOVA test to compare all 80 subjects with AAA, 40 subjects with PAD and 40 controls. A significant difference was identified between the cohorts in miR-196b (P=0.010) only (for full results see Table 10-4 and Figure 10-4).

Table 10-4 – Results of one-way ANOVA test comparing relative miRNA expression in plasma samples between all 80 AAA subjects, 40 PAD subjects and 40 controls.

miRNA	P Value
Let-7e	0.793
miR-125b	0.695
miR-138	0.217
miR-145	0.616
miR-155	0.327
miR-15a	0.632
miR-181a	0.812
miR-196b	0.010
miR-221	0.378
miR-27b	0.754
miR-29b	0.144
miR-31	0.816
miR-411	0.695
miR-431	0.616
miR-483-5p	0.325
miR-589	0.438

Figure 10-4 – Plasma validation results showing the fold change (and range) for each miRNA compared to controls. A fold change greater than 1 represents an upregulated miRNA, whereas a fold change less than -1 represents a downregulated miRNA. A fold change of 1 (or -1) is equivalent to no difference in miRNA expression.



10.2.2.2 Post-HOC Analysis

In order to determine where the significant difference was I then analysed miR-196b using the relative CT values as follows:

1 – AAA v Control

2 – PAD v Control

3 – AAA v PAD

4 – Post-op v Control

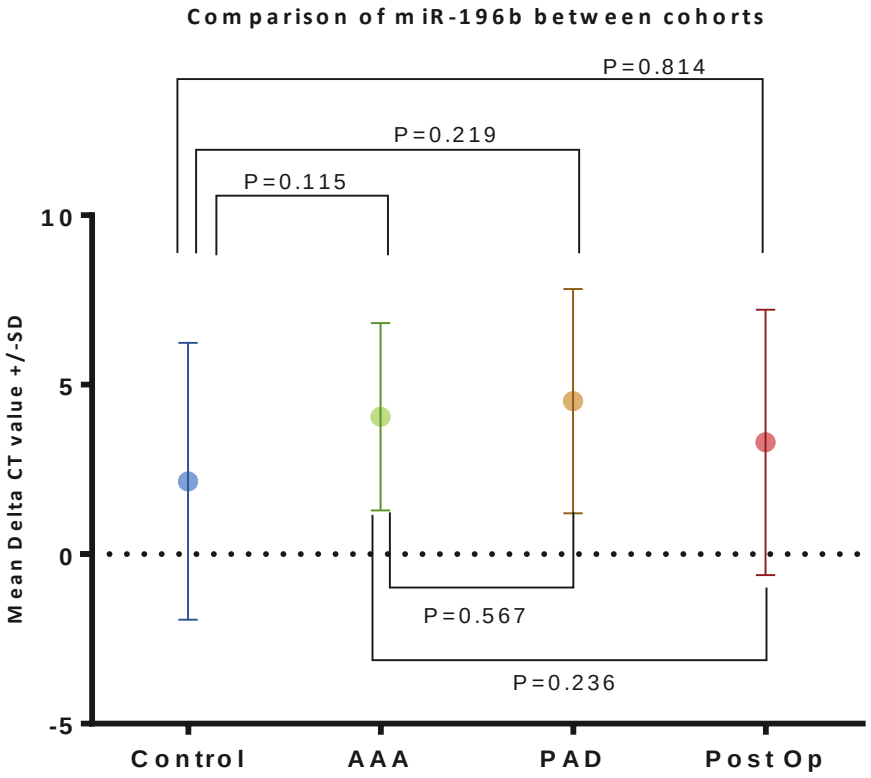
5 – Pre-op v Post-op

6 – Matched pre-op v post-op

I undertook both a Student's t-test and binary logistic regression taking all significant differences in background into account to determine an adjusted P value for comparisons 1-5. For comparison 6 I used a Mann-Whitney U test due to the smaller sample size requiring non-parametric statistical analysis. I also calculated the fold change for each cohort compared to the control where there was a significant difference.

The analysis of miR-196b (Figure 10-5) revealed a significant downregulation in subjects with AAA compared to controls, although this was not maintained on binary logistic regression (Fold change -3.75 (-25.44 to 1.81); P=0.029; adjusted P = 0.115). There was also a significant downregulation of miR-196b in patients with PAD compared to controls, although again this was not maintained on binary logistic regression (Fold Change -5.15 (-50.97 to 1.92); P=0.014; adjusted P=0.219). There was no significant difference in miR-196b expression between AAA and PAD cohorts (adjusted P=0.567), and there was no significant difference between post-operative subjects and controls (adjusted P=0.814). There was also no significant difference between pre and post-operative miR-196b expression when comparing all samples (adjusted P=0.236) or when comparing matched samples (P=0.146).

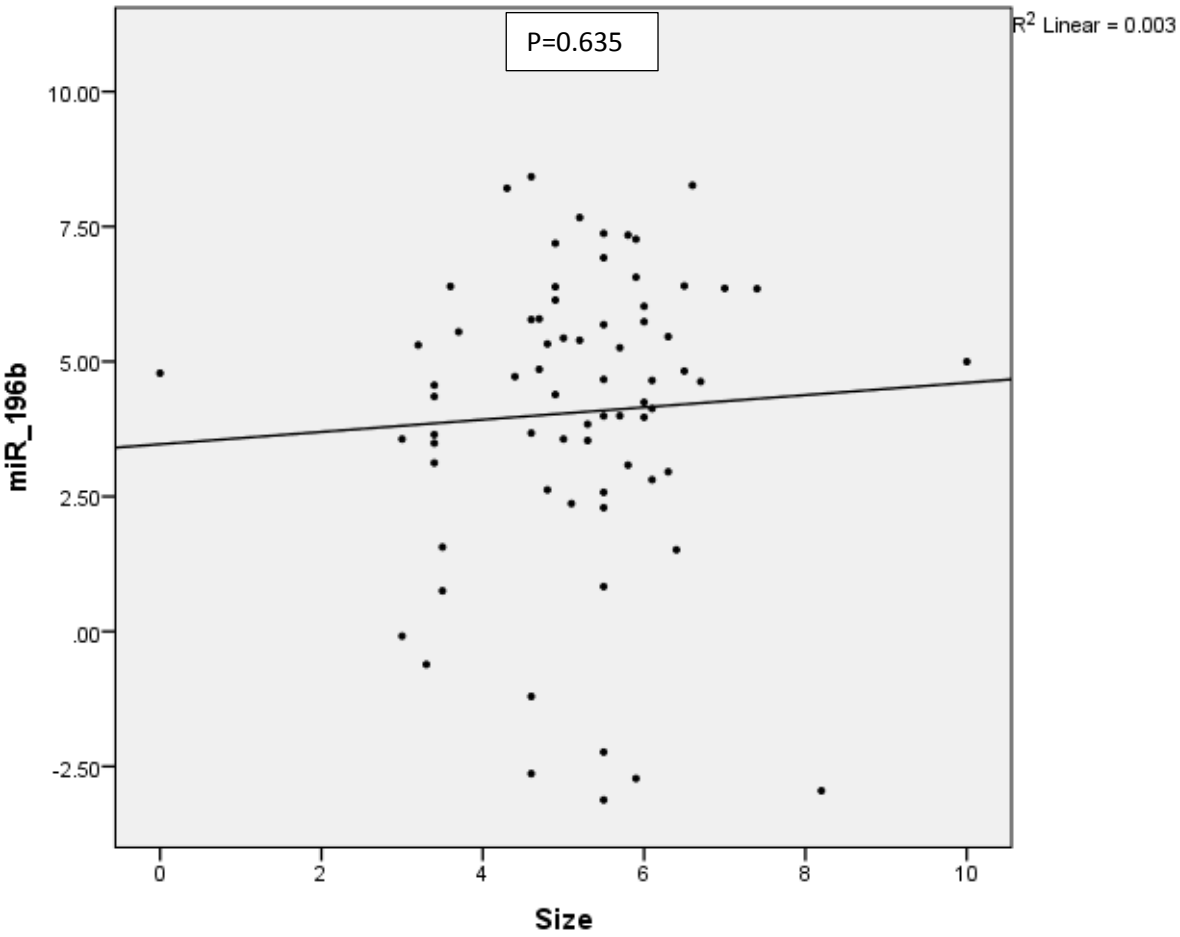
Figure 10-5 – Comparison of mean delta CT values between AAA, PAD, post-operative and control subjects. Graph shows mean delta CT value and Standard Deviation. Adjusted P values on Binary Logistic Regression are shown.



10.2.2.3 Correlation Analysis

In addition to comparing differences in miR-196b expression between cohorts, I also aimed to determine whether aneurysm size affected miR-196b expression. I therefore compared the relative CT values aneurysm diameter in all pre-operative AAA subjects. There was no correlation between miR-196b expression and size (P=0.635; R^2=0.003) (Figure 10-6).

Figure 10-6 – Comparison of mean delta CT values for miR-196b and aneurysm size for all pre-operative subjects with AAA.



10.2.2.4 Conclusions

The results of the validation study in plasma samples has revealed miR-196b to be significantly differentially expressed between patients with AAA, PAD and control subjects on ANOVA. Although miR-196b was found to be significantly downregulated in subjects with AAA and subjects with PAD using Students t-test, neither of these remained significant following binary logistic regression. There were no differences found in miR-196b expression between pre and post-operative subjects, therefore exclusion of the aneurysm sac did not alter miRNA expression. Additionally, miR-196b was not correlated to aneurysm diameter.

The results from the plasma study reinforce the likelihood of miR-196b playing a role in the pathogenesis of abdominal aortic aneurysms, however they also give further weight to AAA and PAD sharing a similar aetiology.

10.2.3 Aortic Tissue Results

10.2.3.1 Initial Aortic Tissue Results

The same methods were used for analysis of the aortic tissue samples as with the whole blood samples. Relative expression (CT miRNA – CT reference gene) was used to determine any significant differences between cohorts. This was undertaken using a Mann-Whitney U test to compare all 19 subjects with AAA to all 10 cadaveric donors. No significant difference was identified between the cohorts (for full results see Table 10-5).

Table 10-5 - Mann Whitney U test comparing miRNA expression in aortic tissue from 19 AAA samples v 10 cadaveric donors

microRNA	Fold Change	P Value
Let-7e	-1.19	0.804
miR-125b	1.06	0.910
miR-138	1.21	0.456
miR-145	1.06	0.910
miR-155	-1.31	0.769
miR-15a	1.34	0.456
miR-181a	2.42	0.512
miR-196b	1.03	0.875
miR-221	1.22	0.668
miR-27b	1.20	0.910
miR-29b	-1.22	0.308
miR-31	1.67	0.604
miR-411	1.13	0.403
miR-431	-1.07	0.484
miR-483-5p	1.21	0.769
miR-589	-1.36	0.164

10.2.3.2 Additional Aortic Tissue Results

Due to the aortic tissue samples used within the initial validation study having not undergone DNAse digestion, and therefore having potential contamination, the aortic tissue study was repeated using new samples. The expression of let-7e, miR-15a, miR-196b and miR-411 was analysed in a total of 22 aneurysm samples, 3 samples from subjects with peripheral arterial disease, and 17 cadaveric donor samples. The relative expression (CT miRNA – CT reference gene) was used to determine any significant differences between cohorts. This was undertaken using a Kruskal-Wallis test to compare all subjects due to the small number of samples requiring non-parametric tests. There was no significant difference identified in miRNA expression between the cohorts (Table 10-6).

Table 10-6 - Kruskal-Wallis comparing AAA v PAD v CAD. Fold change represented here compares AAA v CAD samples.

microRNA	Fold Change	P Value
Let-7e	-1.15	0.218
miR-15a	1.41	0.235
miR-196b	-1.52	0.155
miR-411	-1.33	0.313

10.2.3.3 Conclusions

The results of the validation study in aortic tissue samples has revealed no significant difference in expression levels of any of the miRNAs in the validation study. This may be due to a type 2 error caused by insufficient numbers of samples, however as these results have been replicated in 2 independent cohorts it is likely that there is no correlation between circulating miRNA expression and aortic tissue expression of these miRNAs.

Chapter 11

Conclusions

In summary, this thesis has initially identified a significant association between several biomarkers and AAA (upregulation of MMP2, MMP9, TIMP1, IL-6, TNFα, OPG, OPN, IFNγ, ICAM-1, VCAM-1, D-dimer, CRP, α1AT, fibrinogen, triglycerides, and Lp(a), and downregulation of ApoA and HDL) through meta-analysis, although their sensitivity and specificity is poor. It has then gone on to undertake a discovery and validation study into miRNAs in AAA, identifying a significant dysregulation of 29 miRNAs within the discovery study, 4 of which were validated in blood (let-7e, miR-15a, miR-196b, miR-411), and miR-196b being further validated in plasma. There was however no miRNA dysregulation found in aortic tissue.

The 4 miRNAs identified within this thesis were not only differentially expressed in AAA, but also similarly dysregulated in patients with peripheral arterial disease, therefore the specificity of these miRNAs is poor, and as such ROC curve analysis was not conducted. These miRNAs may therefore be due to generalised atherosclerosis, and these results must be interpreted with caution.

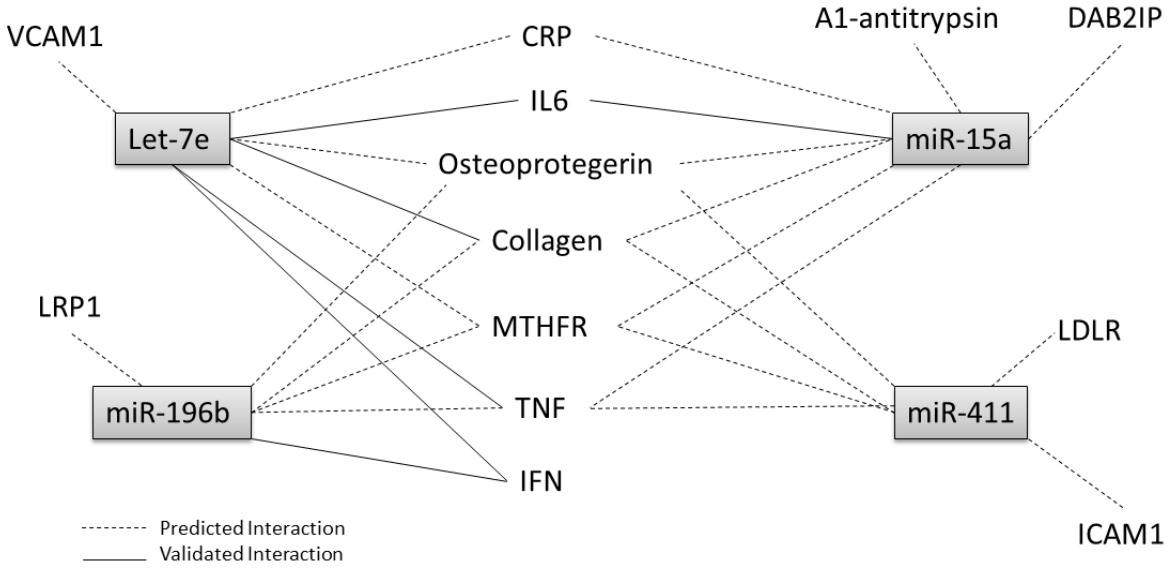
In order to determine the plausibility of these miRNAs in the context of known genes and biomarkers of AAA, the MiRWalk server was used as previously described in Chapter 5 to identify both validated and predicted targets of each miRNA. Validated and predicted miRNA targets were searched using miRWalk. Validated targets have been experimentally defined and published and these are elaborated on below. The servers Diana-microT, miRANDA, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22 and TargetScan were searched for predicted targets. Due to the variability in prediction tools and potential inaccuracies between algorithms, gene targets predicted by 5 or more servers were included to improve specificity.

Several validated interactions were identified with let-7e (IL-6, TNFα, osteoprotegerin), miR-15a (IL-6, osteoprotegerin, α1-antitrypsin), miR-196b (osteoprotegerin) and miR-411 (osteoprotegerin). There was also several predicted interactions with let-7e (VCAM-1, CRP, MTHFR), miR-15a (IL-6, TNFα, CRP, MTHFR, DAB2IP), miR-196b (MTHFR) and miR-411 (TNFα, ICAM-1, MTHFR) (Figure 11-1).

Let-7e has been found to interact with IL-6 by Meng *et al*[377], who demonstrated that knocking down IL-6 in hepatocellular cancer stem cells leads to a significant reduction in let-7 expression. Let-7e has also been found to be upregulated in response to osteoblast differentiation by targeting collagens COL1A2, COL4A2, and COL5A1 in a murine model[378], showing that it may have direct effects on the extracellular matrix. Let-7e has been found to induce translation upregulation of TNF on cell cycle arrest, and represses translation in proliferating cells[379].

miR-15a has been found to interact with IL-6 in a single study in multiple myeloma[380], where bortezomib (a monoclonal antibody acting as a proteasome inhibitor) was found to increase miR-15a expression. miR-15a expression was then found to significantly inhibit the secretion of VEGF (vascular endothelial growth factor), which in turn led to a decrease in IL-6 secretion.

Figure 11-1 – Diagram showing the validated and predicted interactions between the miRNAs differentially expression in blood in patients with AAA, and known genes and biomarkers found to be differentially expressed in subjects with AAA.



Several validated and predicted interactions between the miRNAs found to be differentially expressed in whole blood in subjects with AAA and known genes or biomarkers of AAA have been identified. This highlights the plausibility of let-7e, miR-15a, miR-196b and miR-411 being dysregulated in subjects with AAA, however these genes and biomarkers, as previously discussed, are not specific to AAA. Therefore although these interactions add credibility to the miRNAs identified, the results should be viewed with caution, as the majority of these genes/biomarkers are also dysregulated in subjects with generalised atherosclerosis. One limitation of this thesis was that data regarding claudication distance and degree of stenosis was not documented for all patients with PAD, therefore no data regarding PAD disease severity and miRNA expression was possible. Future work would also benefit from including a cohort of patients with coronary artery disease and carotid stenosis to further investigate the role of miRNAs in atherosclerosis and determine whether

miRNA expression varies depending on atherosclerotic location. In addition, obtaining samples from patients with popliteal, thoracic and intracranial aneurysms would permit one to determine any difference in miRNA expression between anatomical location of the aneurysm.

One major limitation of this thesis is that only 12 miRNAs dysregulated in the discovery study were analysed within the validation study. In particular those within Pool B were not analysed and it may be that those miRNAs (which are less well known) that are more specific to AAA. The primary step in any future work must therefore be to analyse the remaining miRNAs within the validation cohort to determine their potential for use as biomarkers of AAA. In addition the sample size was limited to 40 participants, for the blood and plasma studies, in each cohort due to the validation study methodology. Although this is the largest sample size to date analysing miRNAs in AAA, enough samples were collected for 60 subjects within each cohort to analysed.

Although the aortic tissue studies did not identify any difference in miRNA expression between AAA and control samples, this may be due to using the full thickness of the aortic wall. Further studies to determine miRNA expression separately in the aortic intima, media and adventitia may produce different results as aneurysmal disease primarily causes VSMC depletion within the media.

Another limitation of this thesis is that no miRNAs were dysregulated across blood, plasma and aortic tissue specimens. An alternative methodology would be to undertake discovery studies within all three samples and look for miRNAs dysregulated in each discovery study to take forward into a validation study. This could

help to determine a miRNA pathway and would identify the most likely candidate miRNAs for further research.

Although this thesis has studied a wide range of miRNAs, it was only possible to take a subset of those identified within the discovery study through to the validation study. Future work therefore must initially evaluate the expression of these remaining miRNAs in blood, plasma and aortic tissue samples. In addition, taking these positive findings forward into experimental models to determine whether dysregulation of the miRNAs in cell culture and in vivo models alters expression of AAA-related biomarkers such as MMP9 and LRP1, and whether their dysregulation leads to AAA development in animal models.

Appendix 1 - Determining RNA extraction methods from aortic tissue

RNA extraction from aortic tissue was optimised by initially comparing four different RNA extraction protocols, namely ThermoScientific® GeneJET RNA purification kit, the Qiagen® RNeasy micro Kit, the Qiagen® miRNeasy mini kit, and the Ambion® miRVana Paris kit. Below is a brief outline of the methods for each kit. Following identification of the optimum RNA extraction kit, an additional comparison between inclusion or exclusion of DNA digestion was undertaken.

Aortic tissue samples were obtained from Leicester Royal Infirmary. A full thickness section of aortic tissue from the aneurysm wall was excised and the sample dissected into approximately 50mg pieces and stored in 10 times the volume of RNA later® solution overnight. This RNA later® solution was then decanted and the tissue sample was stored at -80˚C. This was thawed on ice prior to use.

Control aortic tissue samples were collected from cadaveric kidney donors. Organs are sent on ice and in organ preservation solution (Soltran, Baxter International Inc, Illinois) from the donor unit to the recipient unit (in this case Leicester General Hospital). On arrival, the kidney with the accompanying renal artery and aorta is transferred to theatre if deemed suitable for transplantation or to the transplantation laboratory if deemed unsuitable for in vivo use (and therefore used for research purposes only). The transplant/research team then prepares the kidney for transplantation at which time a section of aortic tissue is collected by our team for further study. This tissue is further kept in Soltran and on ice until arrival at the vascular research laboratory where after the tissue is dissected into 6 equal sections, one of these being placed in RNALater for future RNA extraction. The usual time

between the tissue being placed at the donor unit in organ preservation solution and the tissue being placed in RNALater is about 18 – 24 hours. Thereafter cadaveric aortic tissue specimens are treated in exactly the same fashion as aneurysmal aortic samples as described above.

For optimisation, the same 3 aortic tissue samples (2 from aneurysmal tissue, 1 cadaveric) were used for each kit to enable direct comparison. To compare with and without DNA digestion 6 aortic tissue samples (3 from aneurysmal tissue, 3 cadaveric) were used.

Methods for ThermoScientific® GeneJET RNA purification kit

30mg of frozen whole aortic tissue was placed in 300uL of propriety lysis buffer supplemented with beta-mercaptoethanol. The lysis buffer breaks down the cell membrane with the beta-mercaptoethanol added to rapidly inactivate any intracellular RNases by reducing disulphide bonds and destroying the enzyme conformation, thus retain high RNA integrity. The sample, lysis buffer and beta-mercaptoethanol were homogenised using the Precellys® tissue homogeniser (Bertin Technologies) with CK14 beads for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 8500g. Regular pauses are required due to the rapid generation of heat within the homogenisation process which could denature the homogenate. Thereafter 600uL of diluted proteinase K was added to the homogenate due digest contaminating proteins, and this was vortexed for 15 seconds then incubated for 10 minutes at room temperature. The homogenate was then centrifuged at 12,000g for 10 minutes, and the supernatant was then transferred to a new RNase free processing tube. 450uL of

100% ethanol was added to the supernatant and mixed using a pipette. The sample was then passed through the GeneJET RNA purification column in 700uL aliquots by centrifuging the column for 1 minute at 12,000g, with all flow through discarded. The column was then washed with 700uL of wash buffer 1 for 1 minute at 12,000g, followed by washing with 600uL of wash buffer 2 for 1 minute at 12,000g, then a further 250uL of wash buffer 2 for 2 minutes at 12,000g. The total RNA was then eluted in 80uL of RNase free water by centrifuging for 1 minute at 12,000g and collected in a 1.5ml processing tube and the elute immediately cooled on ice.

Methods for Qiagen® RNeasy micro kit

5mg of full thickness aortic tissue was homogenised using the Precellys® tissue homogeniser (Bertin Technologies) with CK14 beads in 350uL of lysis buffer RLT (containing beta-mercaptoethanol) for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 8500g. The lysate was then centrifuged for 3 minutes at 15,000g, and the supernatant was removed and transferred to a fresh processing tube. An equal volume of 70% ethanol was added to the clear lysate and mixed by pipetting. The sample was then transferred to the RNeasy spin column in 700uL aliquots and centrifuged at 8,000g, with the flow through discarded. 700uL of buffer RW1 was added to the spin column and centrifuged at 8,000g for 15 seconds. 500uL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 15 seconds. 500uL of 80% ethanol was then added to the spin column and centrifuged at 8,000g for 2 minutes. The spin column was then transferred to a new 2ml processing tube and centrifuged at 8,000g for 5 minute to dry the membrane. The spin column was then transferred to a new 1.5ml

collection tube and 14uL of RNase free water was added directly to the spin column membrane. The sample was then centrifuged at 8,000g for 1 minute and the eluate immediately cooled on ice.

Methods for Qiagen® miRNeasy mini kit

50mg of full thickness aortic tissue was homogenised using the Precellys® tissue homogeniser (Bertin Technologies) with CK14 beads in 700uL of QIAzol lysis buffer for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 8500rpm. The sample was then left at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. 140uL chloroform was then added to the sample, shaken vigorously for 15 seconds and left at room temperature for 2-3 minutes. The sample was then centrifuged for 15 minutes at 12,000g at 4°C. The upper aqueous phase of the sample was then transferred to a new 1.5ml processing tube and 1.5 x the volume of the sample of 100% ethanol was added and mixed using a pipette. The sample was then placed onto the RNeasy minelute spin column in 700uL aliquots and centrifuged at 8,000g for 15 seconds at room temperature, and the flow through discarded.

Initial samples were extracted without DNA digestion by adding 700uL of buffer RWT to the spin column and centrifuged at 8,000g for 15 seconds.

For samples extracted with DNA digestion (for further optimisation studies) 350uL RWT buffer was added to the spin column and centrifuged at 8,000g for 15 seconds. DNase mix was made by mixing 10uL DNase 1 stock solution with 70uL of buffer RDD, and this was placed directly onto the spin column membrane and incubated for 15

minutes at room temperature. A further 350uL of RWT buffer was added to the spin column and centrifuged at 8,000g for 15 seconds, and the flow through discarded. Following the addition of RWT the protocol was the same whether DNA digestion was performed or not. 500uL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 15 seconds. 500uL of buffer RPE was then added to the spin column and centrifuged at 8,000g for 2 minutes. The spin column was then transferred to a new 2ml processing tube and centrifuged at 8,000g for 1 minute to dry the membrane. The spin column was then transferred to a new 1.5ml collection tube and 40uL of RNase free water was added directly to the spin column membrane. The sample was then centrifuged at 8,000g for 1 minute and the eluate immediately cooled on ice.

Methods for Ambion mirVana® Paris Kit

50mg of full thickness aortic tissue was homogenised using the Precellys® tissue homogeniser (Bertin Technologies) with CK14 beads in 300uL of ice cold cell disruption buffer for 10 seconds followed by a 10 second pause, a further 10 seconds followed by a 10 second pause, and a final 15 seconds at 8500g. The homogenate was mixed with an equal volume of 2X denaturing solution and stored on ice for 5 minutes. An equal volume of Acid-Phenol:chloroform was then added to the solution, and the sample vortexed for 1 minute. The sample was then centrifuged at 10,000g for 5 minutes, to compact the interphase, and the aqueous upper phase was transferred to a fresh processing tube and measured. 100% ethanol was then added at a volume of 1.25 x the volume of the aqueous upper phase. The lysate ethanol mix was then transferred to a filter cartridge in 700uL aliquots and centrifuged at 10,000g for 30 seconds, with the flow through discarded. 700uL of miRNA wash solution 1 was then applied to the

filter and centrifuged at 10,000g for 15 seconds with the flow through discarded. 500uL of wash solution 2/3 was then applied to the filter and centrifuged at 10,000g for 15 seconds with the flow through discarded, and this step was repeated. Finally, a further centrifugation at 10,000g for 1 minute was carried out to remove any residual fluid. The filter cartridge was then transferred to a fresh collection tube and 100uL of 95°C RNase free water was applied directly to the filter membrane. The sample was centrifuged for 30 seconds, with the eluate collected and immediately cooled on ice.

RNA Quantification and Integrity

Quality control was performed for isolated RNA. All samples underwent quantification as described in section 3.4. The Nanodrop (ThermoScientific®) was used to determine RNA concentration, RNA purity was assessed using the 260/280 ratio, and the RNA integrity was analysed using the Agilent® 2100 Bioanalyser.

Results

The total RNA concentrations, 260/280 ratios, and RNA integrity numbers are shown below (Table A1 and Figure A1). The mean RNA concentration was highest when using the Qiagen® miRNeasy mini kit. The RNA integrity number (RIN) was consistently highest using the Qiagen® miRNeasy mini kit. The 260/280 ratio ranged from 1.96-2.08 when using the Qiagen® miRNeasy mini kit, therefore this protocol was taken forward for further optimisation.

Although the Qiagen® miRNeasy mini kit yielded the best results, in view of the modest RIN compared to those achieved following RNA extraction from blood, it was decided that DNA digestion would be attempted to improve the RNA quality. Initially

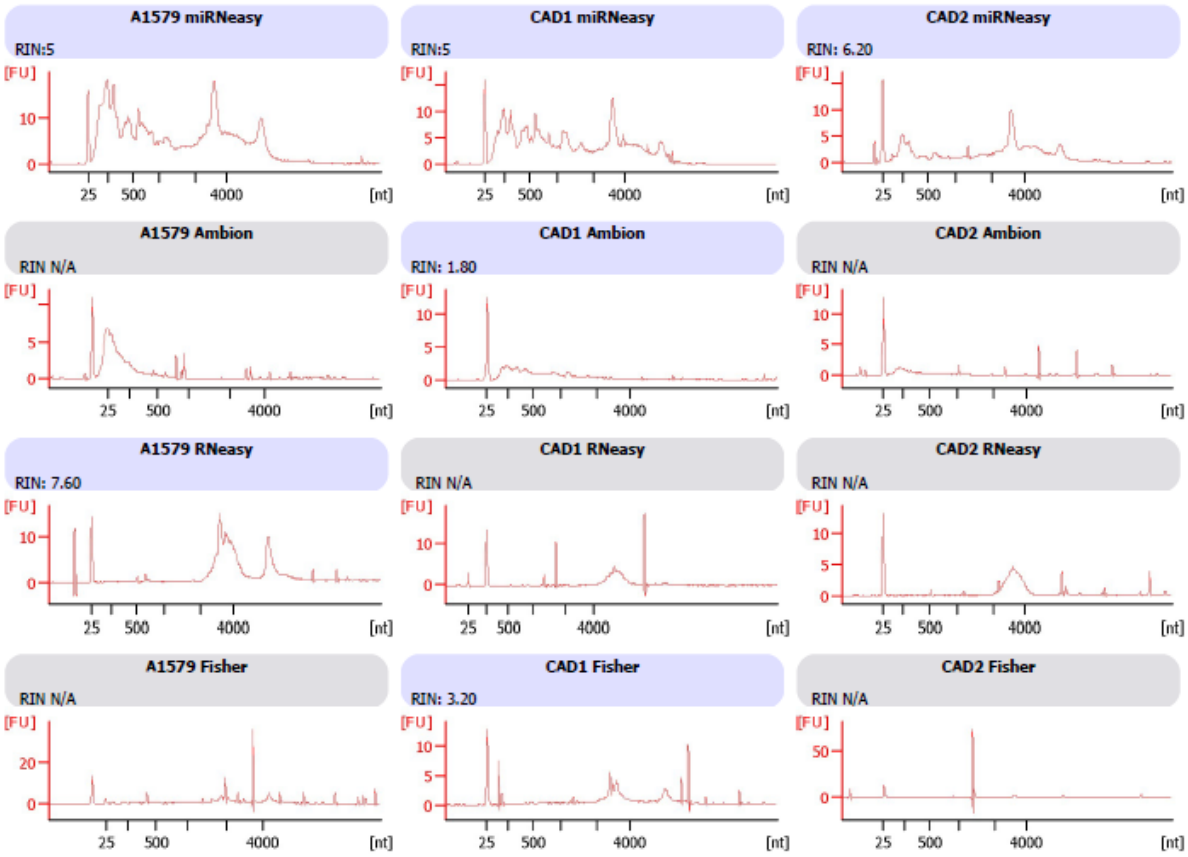
DNase was added to the eluted RNA, however this did not improve the RIN (Sample 1 No RIN number, Sample 2 RIN 1.7, Sample 3 RIN 2.2). Therefore DNase digestion was performed within the RNA extraction according to the Qiagen® miRNeasy mini kit protocol. This further improved the RIN by a small but significant amount (Mean RIN without DNA digestion 5.95, mean RIN with DNA digestion 6.75; P value 0.02 Mann-Whitney U test) (Table A2 and Figure A2).

Table A1 – Results from total RNA extraction using the ThermoScientific® GeneJET RNA purification kit, the Qiagen® RNeasy micro Kit, the Qiagen® miRNeasy mini kit, and the Ambion® miRVana Paris kit.

Sample	Kit	Concentration (ng/ul)	260/280	RIN
Sample 1	Qiagen® mRNeasy micro kit	17.59	1.53	N/A
Sample 2	Qiagen® mRNeasy micro kit	7.095	1.79	N/A
Sample 3	Qiagen® mRNeasy micro kit	30.83	2.17	7.6
Sample 1	ThermoScientific® GeneJET RNA purification kit	3.978	6.71	N/A
Sample 2	ThermoScientific® GeneJET RNA purification kit	15.95	2.26	3.2
Sample 3	ThermoScientific® GeneJET RNA purification kit	25.79	2.21	N/A
Sample 1	Qiagen® miRNeasy mini kit	113.8	2.07	6.2
Sample 2	Qiagen® miRNeasy mini kit	194	2.08	5
Sample 3	Qiagen® miRNeasy mini kit	367.7	1.96	5
Sample 1	Ambion® miRVana Paris kit	34.21	2.62	N/A
Sample 2	Ambion® miRVana Paris kit	82.21	2.25	1.8
Sample 3	Ambion® miRVana Paris kit	155.1	2.16	N/A

RIN = RNA integrity number

Figure A1 – Electropherogram of RNA integrity using Agilent® RNA 6000 Nano Kit on the 2100 Bioanalyser. Comparison of different RNA extraction protocols from ThermoScientific® GeneJET RNA purification kit, the Qiagen® RNeasy micro Kit, the Qiagen® miRNeasy mini kit, and the Ambion® miRVana Paris kit.

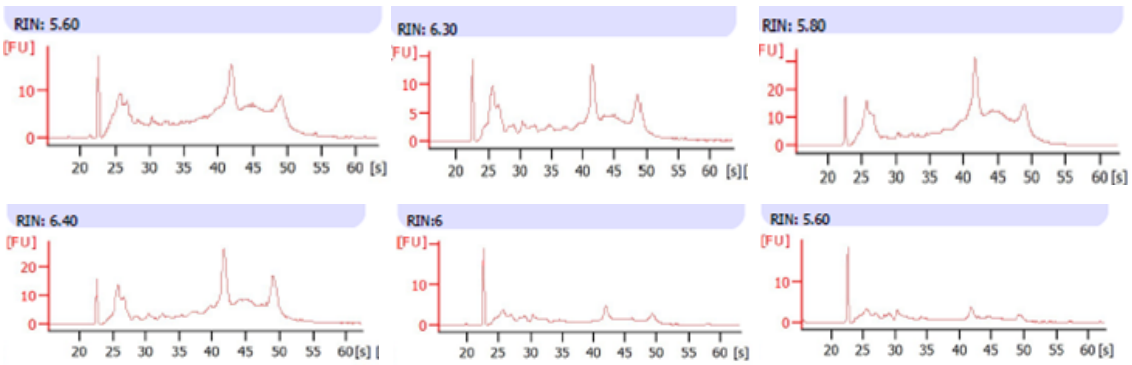


Supplemental Table A2 - Results from total RNA extraction using the Qiagen® miRNeasy mini kit, with and without DNA digestion.

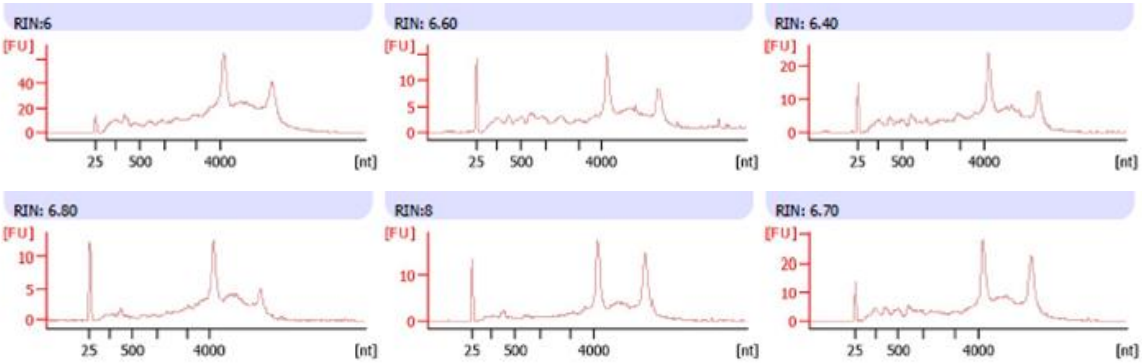
Sample	Concentration (ng/ul) without DNA digestion	Concentration (ng/ul) with DNA digestion	260/280 ratio without DNA digestion	260/280 ratio with DNA digestion	RIN without DNA digestion	RIN with DNA digestion
Sample 1	285	166.6	2.08	2.01	5.6	6.4
Sample 2	84.7	93.9	1.68	2.04	6.3	6.6
Sample 3	288.3	455.4	1.99	1.97	5.8	6.0
Sample 4	246.7	173.5	2.00	2.04	6.4	6.7
Sample 5	95.4	98.5	1.92	2.04	6.0	8.0
Sample 6	86.7	158.3	1.91	1.88	5.6	6.8

Supplemental Figure X – Electropherogram of RNA integrity using Agilent® RNA 6000 Nano Kit on the 2100 Bioanalyser for total RNA extracted from samples using Qiagen® miRNeasy mini kit a) without DNA digestion b) with DNA digestion.

a)



b)



Conclusion

The above results showed that RNA extraction using the Qiagen® miRNeasy mini kit with DNA digestion and CK14 beads yielded the best results in terms of RNA concentration, quality, and integrity, and this was therefore used for RNA extraction from all aortic tissue samples.

Appendix 2 – Table showing all microRNAs expressed in whole blood samples from the discovery study, along with their associated fold change and P value, calculated using Mann-Whitney U test. Those miRNAs highlighted in grey met both inclusion criteria to be termed differentially expressed, i.e. fold change >1.5, P value <0.1.

microRNA	Fold Change	**P Value**
miR-672	-2.22	**0.003**
miR-19a*	2.24	**0.006**
miR-15a	-2.04	**0.019**
miR-16-1#	-1.75	**0.019**
miR-9#	-2.22	**0.024**
miR-125a-3p	69866.67	**0.031**
miR-196b	-1.89	**0.031**
miR-134	-2.63	**0.036**
miR-181a	1.62	**0.041**
miR-942	-2.70	**0.041**
Let-7e	2.29	**0.0413**
miR-431	1.82	**0.042**
miR-1291	-1.85	**0.048**
miR-27b	2.48	**0.051**
miR-1249	-1.54	**0.055**
miR-483-5p	1.86	**0.055**
miR-624	-2.04	**0.057**
miR-411	-2.04	**0.058**
miR-1274A	-1.45	**0.062**
miR-589	-1.51	**0.068**
miR-29b	-1.89	**0.076**
miR-1179	-2.00	**0.081**
miR-1227	1.70	**0.081**
miR-138	2.22	**0.081**
miR-26a-1#	-1.59	**0.081**
miR-28-5p	-1.43	**0.081**
miR-374a	-1.69	**0.081**
miR-125b	1.56	**0.091**
miR-146a	-1.47	**0.091**
miR-194	-1.45	**0.091**
miR-628-5p	-1.69	**0.091**
miR-148b#	-1.52	**0.096**
miR-15b#	-2.50	**0.096**
miR-55	1.14	**0.096**
miR-183#	-1.47	**0.103**
miR-197	-1.27	**0.103**
miR-618	1.54	**0.103**
Let-7a#	-1.75	**0.106**
miR-495	1.55	**0.108**
miR-617	-14.29	**0.114**
miR-323-3p	-1.35	**0.115**
miR-28b	-1.52	**0.125**
miR-3e-3p	-1.35	**0.127**

miR-129	1.43	**0.129**
miR-31	1.70	**0.129**
miR-95	2.21	**0.131**
miR-126	1.24	**0.144**
miR-18a	1.48	**0.144**
miR-515-3p	-1.72	**0.149**
miR-638	-1.08	**0.157**
Let-7b	-1.33	**0.16**
miR-146b-3p	1.73	**0.16**
miR-191#	2.26	**0.16**
miR-32	-1.54	**0.16**
miR-34#	-1.96	**0.16**
miR-13	1.55	**0.166**
Let-7e#	-1.96	**0.167**
miR-127-3p	1.46	**0.172**
miR-19b-1#	-1.43	**0.172**
miR-362-3p	2.18	**0.172**
miR-519d	-2.63	**0.173**
miR-1271	1.41	**0.178**
miR-23a	1.58	**0.178**
miR-365	-1.33	**0.178**
miR-16b#	-1.52	**0.179**
miR-941	1.47	**0.183**
miR-519a	-6.25	**0.186**
miR-127	1.42	**0.196**
miR-132	1.53	**0.196**
miR-139-3p	1.68	**0.196**
miR-14-5p	1.35	**0.196**
miR-182	-1.28	**0.196**
miR-51-5p	-1.09	**0.196**
miR-648	1.75	**0.201**
miR-192#	-1.54	**0.21**
miR-335#	1.55	**0.21**
miR-625#	-1.59	**0.21**
miR-582-3p	1.63	**0.211**
miR-32c	2.20	**0.213**
miR-15b	-1.33	**0.216**
miR-16b	1.29	**0.238**
miR-28-3p	1.57	**0.238**
miR-339-3p	-1.47	**0.238**
miR-19b	-1.59	**0.241**
miR-424#	1.31	**0.241**
miR-1238	1.21	**0.244**
miR-136#	-1.56	**0.247**
miR-487a	-1.41	**0.25**
miR-224	-1.56	**0.256**
miR-128	1.27	**0.261**
miR-145	1.40	**0.261**
miR-26a	1.40	**0.261**

miR-328	1.37	**0.261**
miR-342-3p	1.60	**0.261**
miR-54	1.48	**0.277**
miR-151-3p	-1.23	**0.282**
miR-26	-3.45	**0.282**
miR-628-3p	-1.56	**0.282**
Let-7c	-1.25	**0.285**
miR-1178	1.24	**0.285**
miR-184	1.27	**0.285**
miR-324-5p	1.24	**0.285**
miR-361-5p	1.41	**0.285**
miR-337-5p	-1.64	**0.291**
miR-367	-1.20	**0.291**
miR-383	-1.35	**0.291**
miR-576-5p	1.42	**0.291**
miR-144	1.60	**0.298**
miR-218	1.78	**0.299**
miR-92a-1#	1.89	**0.301**
miR-181c	1.10	**0.311**
miR-183	1.57	**0.311**
miR-18b	1.40	**0.311**
miR-192	-1.37	**0.311**
miR-376a	1.48	**0.311**
miR-636	1.44	**0.311**
miR-652	1.17	**0.311**
miR-664	1.37	**0.311**
miR-72	1.17	**0.311**
miR-886-3p	-1.28	**0.311**
miR-93	-1.25	**0.311**
miR-199a-3p	-1.39	**0.312**
miR-454#	-1.47	**0.312**
miR-938	72855.29	**0.313**
miR-654-5p	1.76	**0.33**
miR-338-3p	-1.43	**0.336**
miR-11	-1.28	**0.338**
miR-118	1.38	**0.338**
miR-1248	-1.04	**0.338**
miR-125a-5p	1.29	**0.338**
miR-1296	-1.22	**0.338**
miR-181a-2#	-1.72	**0.338**
miR-212	1.26	**0.338**
miR-361-3p	-1.41	**0.338**
miR-381	-1.72	**0.338**
miR-135a	-1.56	**0.34**
miR-124	1.37	**0.343**
miR-548d-3p	1.44	**0.358**
miR-1	-1.28	**0.367**
miR-142-3p	-1.25	**0.367**
miR-195	-1.08	**0.367**

miR-25#	-1.54	**0.367**
miR-129#	-6.67	**0.368**
miR-149#	-1.25	**0.368**
miR-214#	-1.43	**0.368**
miR-425#	-1.23	**0.368**
miR-61	-1.43	**0.368**
miR-7-2#	-1.96	**0.379**
miR-581	1.51	**0.385**
miR-597	1.59	**0.385**
miR-34b	1.32	**0.392**
miR-53	1.27	**0.395**
miR-142-5p	-1.35	**0.397**
miR-151-5p	-1.15	**0.397**
miR-16	-1.11	**0.397**
miR-17	1.19	**0.397**
miR-27a#	1.24	**0.397**
miR-645	3.15	**0.397**
miR-886-5p	-1.27	**0.397**
miR-24	1.33	**0.403**
miR-758	1.17	**0.403**
miR-872	1.21	**0.413**
miR-337-3p	1.62	**0.422**
Let-7f	-1.15	**0.428**
miR-1275	1.22	**0.428**
miR-215	1.23	**0.428**
miR-66	-1.37	**0.428**
miR-744#	1.18	**0.428**
miR-92a	1.44	**0.428**
miR-65	3.49	**0.432**
miR-34a	1.30	**0.437**
miR-483-3p	-1.28	**0.438**
miR-1276	3.58	**0.461**
miR-221	1.14	**0.461**
miR-425	-1.03	**0.461**
miR-433	-1.54	**0.461**
miR-59-5p	1.05	**0.461**
miR-99b#	-3.33	**0.461**
miR-147	2.86	**0.462**
miR-13b#	-1.23	**0.467**
miR-627	1.34	**0.471**
miR-511	-1.59	**0.473**
miR-579	1.61	**0.479**
miR-21#	-1.49	**0.482**
Let-7f-1#	-1.23	**0.494**
miR-141	1.62	**0.494**
miR-52c-3p	2.73	**0.494**
miR-121	1.01	**0.495**
miR-1282	-1.19	**0.495**
miR-33-3p	1.41	**0.495**

miR-342-5p	1.13	**0.495**
miR-375	1.33	**0.495**
miR-49-3p	-1.01	**0.495**
miR-489	1.18	**0.5**
miR-376c	1.03	**0.508**
miR-494	-1.10	**0.508**
miR-576-3p	1.26	**0.508**
miR-659	-1.28	**0.521**
miR-131	-1.47	**0.525**
miR-1244	-1.19	**0.531**
miR-21	1.03	**0.531**
miR-3c	1.17	**0.531**
miR-654-3p	1.45	**0.536**
miR-487b	-1.14	**0.546**
miR-143	1.25	**0.547**
miR-222#	-1.15	**0.553**
miR-411#	1.07	**0.553**
miR-1254	-1.47	**0.567**
miR-1255B	1.31	**0.567**
miR-1285	-1.19	**0.567**
miR-148b	1.18	**0.567**
miR-191	1.10	**0.567**
miR-22#	1.26	**0.567**
miR-26b	1.25	**0.567**
miR-26b#	1.06	**0.567**
miR-29c	-1.22	**0.567**
miR-335	-1.61	**0.567**
miR-486-5p	1.19	**0.567**
miR-642	-1.03	**0.567**
miR-769-5p	1.15	**0.567**
miR-145#	1.34	**0.57**
miR-1183	-3.33	**0.573**
miR-144#	1.26	**0.579**
miR-516-3p	-2.63	**0.585**
miR-519e	-1.19	**0.591**
miR-548c-3p	-1.75	**0.595**
miR-155	1.15	**0.605**
miR-19a	1.21	**0.605**
miR-211	-1.27	**0.605**
miR-223	1.25	**0.605**
miR-52-3p	-1.12	**0.605**
miR-93#	-2.33	**0.605**
miR-523	-1.16	**0.617**
miR-52f	1.34	**0.617**
miR-655	-1.18	**0.617**
miR-875-5p	7.77	**0.617**
miR-41	1.24	**0.625**
miR-213	1.12	**0.639**
miR-543	1.05	**0.639**

Let-7g	1.64	**0.643**
miR-139-5p	1.10	**0.643**
miR-186	1.16	**0.643**
miR-24-2#	-1.12	**0.643**
miR-2c	-1.08	**0.643**
miR-363	1.13	**0.643**
miR-422a	-1.59	**0.643**
miR-939	1.12	**0.643**
miR-52b	1.30	**0.667**
miR-346	1.27	**0.682**
miR-126#	-1.10	**0.683**
miR-13b	1.00	**0.683**
miR-15	1.25	**0.683**
miR-451	1.09	**0.683**
miR-454	1.00	**0.683**
miR-484	1.32	**0.683**
miR-485-3p	-1.05	**0.683**
miR-486-3p	1.00	**0.683**
miR-29a#	1.31	**0.697**
miR-96	1.08	**0.697**
miR-1a	1.16	**0.709**
miR-29b-2#	1.02	**0.718**
miR-55#	1.38	**0.718**
miR-1274B	-1.08	**0.723**
miR-25	-1.18	**0.723**
miR-52-5p	1.42	**0.723**
miR-532-3p	1.06	**0.723**
miR-574-3p	-1.08	**0.723**
miR-78	-1.41	**0.728**
miR-32b	1.58	**0.733**
miR-548d-5p	2.16	**0.733**
miR-32a	1.03	**0.75**
miR-373	-1.02	**0.75**
miR-888	1.54	**0.75**
Let-7g#	1.44	**0.764**
miR-152	1.16	**0.765**
miR-16a	-1.02	**0.765**
miR-193a-5p	-1.20	**0.765**
miR-27a	-1.03	**0.765**
miR-296-5p	-1.18	**0.765**
miR-324-3p	1.04	**0.765**
miR-326	1.30	**0.765**
miR-37	-1.15	**0.765**
miR-374b	1.05	**0.765**
miR-5	-1.11	**0.765**
miR-532-5p	-1.09	**0.765**
miR-616	-1.11	**0.77**
miR-31a	1.00	**0.796**
miR-363#	1.01	**0.804**

miR-133a	-1.04	**0.807**
miR-148a	1.21	**0.807**
miR-22	-1.41	**0.807**
miR-222	1.04	**0.807**
miR-491-5p	1.00	**0.807**
miR-935	1.05	**0.807**
miR-31b	1.12	**0.817**
miR-378	-1.41	**0.817**
miR-889	1.04	**0.817**
miR-331-5p	-1.15	**0.82**
miR-1253	-1.11	**0.829**
miR-596	-1.27	**0.831**
miR-629	-1.18	**0.831**
miR-548J	2.45	**0.84**
miR-15a#	-1.02	**0.841**
miR-661	-1.02	**0.841**
miR-199a-5p	-1.03	**0.845**
miR-423-5p	1.07	**0.849**
miR-598	1.05	**0.849**
miR-181c#	-1.14	**0.868**
miR-223#	-1.04	**0.877**
miR-3d	-16.67	**0.877**
miR-1256	1.43	**0.882**
miR-199b-5p	-1.18	**0.884**
miR-1236	-1.56	**0.886**
miR-14-3p	-1.03	**0.892**
miR-185	-1.02	**0.892**
miR-18a#	-1.25	**0.892**
miR-23b	1.10	**0.892**
miR-2a	-1.03	**0.892**
miR-2a#	-1.35	**0.892**
miR-339-5p	1.00	**0.892**
miR-345	1.07	**0.892**
miR-362-5p	-1.09	**0.892**
miR-51-3p	1.03	**0.892**
miR-572	-1.19	**0.892**
miR-548c-5p	-2.86	**0.902**
miR-668	1.08	**0.925**
miR-452	-1.14	**0.93**
miR-133	-1.20	**0.931**
miR-1825	1.10	**0.931**
miR-33a#	1.09	**0.931**
Let-7a	-1.02	**0.935**
Let-7d	-1.12	**0.935**
miR-146b-5p	-1.03	**0.935**
miR-193b	-1.14	**0.935**
miR-34	1.08	**0.935**
miR-3a-3p	1.15	**0.935**
miR-3b	1.11	**0.935**

miR-99b	1.06	**0.935**
miR-329	-1.67	**0.964**
miR-372	-1.08	**0.967**
miR-23	1.03	**0.972**
miR-744	-1.20	**0.978**
miR-99a	-1.08	**0.978**
miR-1233	-1.18	**1**
miR-13a	1.00	**1**
miR-148a#	1.10	**1**
miR-29a	1.04	**1**
miR-331-3p	1.09	**1**
miR-33-5p	1.25	**1**
miR-3a-5p	-1.32	**1**
miR-424	1.12	**1**
miR-542-3p	-2.13	**1**
miR-564	1.04	**1**
miR-584	1.07	**1**
miR-625	1.01	**1**
miR-671-3p	-1.05	**1**
miR-766	-1.19	**1**
miR-885-5p	-1.09	1

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