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Exploring the Mechanisms by which Restenosis Develops within the Arteriovenous Fistula

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KING'S COLLEGE LONDON

**“Exploring the Mechanisms by
which Restenosis Develops within
the Arteriovenous Fistula”**

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Faculty of Life Sciences & Medicine

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2020

70,000 words

**A thesis submitted to King's College London in
Candidature of Doctor of Philosophy**

Abstract

The main determinant of the effectiveness of a patient's haemodialysis is the patency of their vascular access. An arteriovenous fistula (AVF) is the preferred method for providing vascular access for haemodialysis. Stenotic lesions caused by neointimal hyperplasia (NIH) commonly occur in fistulas resulting in patients requiring a fistuloplasty procedure, yet restenosis in the AVF occurs in nearly half of patients within the first year of their procedure by poorly defined mechanisms. The need for repeat procedures following a fistuloplasty is both difficult for patients and expensive for the NHS. This thesis sought to investigate some of the biological changes that occur post fistuloplasty in order to better understand how restenosis occurs in the AVF and to suggest potential biomarkers for predicting a poor outcome post fistuloplasty.

Haematoxylin and eosin staining, modified elastin staining and immunohistochemistry for von Willebrand Factor, alpha smooth muscle actin, smooth muscle myosin heavy chain, vimentin, desmin, CD34 and CD68 was carried out on four excised AVF tissues with known NIH. Blood samples were taken from patients before their fistuloplasty procedure and after their fistuloplasty procedure. The leukocyte populations were investigated using flow cytometry, and the plasma proteins were examined using enzyme-linked immunosorbent assays and Luminex assays. A microarray was carried out on CD14 bead isolated cells, alongside reverse transcription polymerase chain reaction (RT-PCR). Univariate and multivariate cox regression were carried out in order to find associations with post fistuloplasty patency.

The primary composition of late stage NIH lesions within AVF tissue are myofibroblasts, but their origin is likely to vary from patient to patient. Whilst there was no increase in monocyte count post fistuloplasty, the proportion of classical monocytes (CD14⁺⁺CD16⁻) decreased, whilst intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺) monocytes increased 1 day post fistuloplasty and this shift did not

occur during the acute inflammation phase and it is associated with patient outcome. Plasma levels of IL-6, TNF- α and myeloperoxidase (MPO) significantly changed 1-2 days post fistuloplasty, with MPO decreasing below baseline levels following an initial peak at 3 hours, IL-6 increasing above baseline levels, but peaking 2-3 hours post fistuloplasty and TNF- α peaking at 1 day post fistuloplasty. Plasma levels of calcium-binding protein A9 (S100A9), platelet factor 4 and MPO are likely to be associated with post-intervention primary and cumulative patency. The relative gene expression of calreticulin is also likely to be associated with post-intervention primary and cumulative patency, with higher levels in peripheral blood mononuclear cells associating with a worse post-intervention primary and cumulative outcome, but higher levels in monocytes associating with a better post-intervention primary outcome.

This thesis highlights the importance of monocyte subsets in the pathophysiology of restenosis in AVFs alongside how the initial inflammatory response to the fistuloplasty has overall long term consequences on the patency of the AVF following a fistuloplasty procedure.

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Acknowledgements

I would like to express my gratitude to my first supervisor, Dr Michael Robson, firstly for providing me with the opportunity to be his student, and secondly for his continuous support during my PhD. I would also like to thank my second supervisor, Prof Anthony Dorling for giving me valuable guidance, and to all of the staff working for the PAVE clinical trial.

I would also like to thank the following people who assisted with key components of this thesis: Dr Emily Robinson suggested the methodology for the multivariate cox regression analysis and Mr Florin-Silviu Paulet showed me how to format the cox regression tables. Dr Paul Lavender ran the microarray chip and carried out some of the relevant statistical analysis. Ms Alessandra Bisquera carried out the principle component analysis and multivariate distance matrix regression analysis for the microarray. Dr Ho-Tzer Ren taught me how to carry out RT-PCR and also provided invaluable support. All flow cytometry and Luminex assays were carried out at the Biomedical Research Centre based at Guy's and St Thomas.

This project could not be carried out without the selfless help of the patients and healthy volunteers who chose to donate research blood and the wonderful NHS staff I worked closely with. This includes the nurses within both the renal day case unit and satellite dialysis units, the radiology and surgery teams at Guy's and St Thomas. I am thankful for the funding of this work through the NIHR/MRC Efficacy and Mechanism Evaluation (EME) programme, a MRC and NIHR partnership.

During the course of this PhD, Dr Reena Popat, Ms El li Tham, Dr Siobhan Bearder, Dr Ferndada-Florez Barros and Dr Lucy Meadler were like my older and wiser laboratory sisters. They provided not only practical help and advice, but also made menial laboratory tasks (even taking out the bins) enjoyable. My friends (including Elif and Yeliz, from my MSc, the hVIVO team and the International Space group) always kept me smiling when I most needed it.

Finally, I would like to thank my family for their unconditional support throughout my thesis and throughout my life.

Declaration

I declare that I have personally prepared for this thesis. The work described herein this thesis is my own and carried out personally unless otherwise stated. All sources of information, including quotations, are acknowledged by means of reference.



Seran Hakki

Date: 30th July 2020

Abbreviations

Abbreviation	Meaning
ACR	Albumin to creatinine ratio
AGEs	Advanced glycosylation end products
ANOVA	Analysis of variance
AVF	Arteriovenous fistulae and
AVG	Arteriovenous graft
BDNF	Brain derived neurotropic factor
BSA	Bovine serum albumin
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CD	Cluster of differentiation
CD40L	CD40 Ligand
CKD	Chronic kidney disease
Cl ⁻	Chloride anion
CRBSI	Catheter-related bloodstream infection
CRP	C-reactive protein
CSF3R	Colony stimulating factor 3 receptor
Ct	Cycle threshold
CTACK	Cutaneous T-cell-attracting chemokine
CVC	Central venous catheter
CVD	Cardiovascular disease
DAB	3'3-Diaminobenzidine
DAMP	Danger associated molecular pattern
DMSO	Dimethyl sulfoxide

Abbreviation	Meaning
DPX	Dibutylphthalate polystyrene xylene
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
EndMT	Endothelial-mesenchymal transition
FAM	6-carboxyfluorescein
FBC	Full blood count
Flt-3	Fms-like tyrosine kinase-3
FPR1	Formyl peptide receptor 1
FSC-A	Forward scatter area
FSC-H	Forward scatter height
FSP-1	Fibroblast-specific protein 1
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Haematoxylin and eosin
H ₂ O ₂	Hydrogen peroxide
HGF	Hepatocyte growth factor
HIF-1 α	Hypoxia-inducible factor 1-alpha
HOCl	Hypochlorous acid
IFN	Interferon
IL	Interleukin
KDOQI	The kidney disease outcomes quality initiative
LDN	Low density neutrophils
Ly6C	Lymphocyte antigen 6 complex
MCP-1	Monocyte chemoattractant protein 1

Abbreviation	Meaning
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony-stimulating factor
MDMR	Multivariate distance matrix regression
MeSH	PubMed medical subject headings
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MMT	Macrophage-myofibroblast transition
MPO	Myeloperoxidase
MRP	Myeloid-related proteins
MRSA	Methicillin-resistant Staphylococcus aureus
n	Number of samples
NDN	Normal density neutrophils
NFκB	Nuclear factor kappa-B
NICE	National Institute of Health and Care Excellence
NIH	Neointima hyperplasia
NK	Natural Killer
NKG2D	Natural killer group 2 membrane D
NLR	Neutrophil-to-lymphocyte ratio
NO	Nitric oxide
OPD	o-phenylenediamine dihydrochloride
PAD	Peripheral arterial disease
PBS	Dulbecco's phosphate buffered saline

Abbreviation	Meaning
PBUT	Protein bound uremic toxin
PBUT	Protein-bound uremic toxins
PCA	Principle component analysis
PCB	Paclitaxel-coated balloon
PDGF	Platelet derived growth factor
PDGF-R β	Platelet derived growth factor receptor- β
PF4	Platelet factor 4
RAGE	Receptor for advanced glycosylation end products
RANTES	Regulated upon activation, normal T cell expressed and presumably secreted
RNA-seq	High-throughput RNA sequencing
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RRT	Renal replacement therapy
RT-PCR	Reverse transcription polymerase chain reaction
S100	Calcium-binding protein
SCGF	Stem cell growth factor
SDF	Stromal cell-derived factor
SMA	α -smooth muscle actin
SMC	Smooth muscle cell
SMMHC	Smooth muscle myosin heavy chain
SSC-A	Side scatter area
SSC-H	Side scatter height
TGF- β	Transforming growth factor beta 1
TNFRSF	Tumour necrosis factor receptor superfamily

Abbreviation	Meaning
TNF- α	Tumour necrosis factor alpha
UK	United Kingdom
UNG	Uracil-DNA glycosylase
VEGF	Vascular endothelial growth factor
VIC	2'-chloro-7'phenyl-1,4-dichloro-6-carboxy-fluorescein
VNN	Vanin
WSS	Wall shear stress

Chapter 1 – Introduction

1.1 Dialysis for Chronic Kidney Disease Patients

1.1.1 Definition of Chronic Kidney Disease

The major function of the kidneys is to filter metabolic and foreign end products from the blood and to regulate blood pressure and electrolyte concentration. A patient is said to have Chronic Kidney Disease (CKD) if they have kidney damage, defined here as functional or structural abnormalities present for more than 3 months with implications for health. In 2014 the severity of CKD was classified into severity stages based on the patient's estimated glomerular filtration rate (eGFR), albumin to creatine ratio (ACR) and haematuria [1]. Table 1-1 depicts these stages.

Table 1-1: Classification of chronic kidney disease based on glomerular filtration rate and albumin to creatine ratio categories.

			Albumin to creatine ratio categories		
			Stage		
			A1	A2	A3
Glomerular filtration rate categories	Stage	eGFR (ml/min)	<3 mg/mmol	3-30 mg/mmol	>30 mg/mmol
	G1	90-100			
	G2	60-89			
	G3a	45-59			
	G3b	30-44			
	G4	15-29			

CKD most often results from damage caused by diabetes mellitus but can also occur due to a multitude of other health problems, including high blood pressure, autoimmune diseases, such as lupus nephritis, and genetic diseases, such as polycystic kidney disease. In England it is estimated that 5.2% of people aged 16 or older have CKD between stages 3-5, and with an ageing population, a rise in diabetes, hypertension and obesity, the prevalence of CKD continues to be an ever growing public health problem [2].

1.1.2 Renal Replacement Therapy

When a patient has a severe loss of kidney function, they may require renal replacement therapy (RRT). RRT replaces the non-endocrine kidney function in patients with renal failure. RRT is used for patients with CKD and it may be delivered in the form of kidney transplantation or dialysis.

Kidney transplantation, in particular live donor kidney transplantation, is the optimal form of RRT due to superior survival outcomes, quality of life benefits and health costs [3] [4]. Indeed, a study by Wu *et al* comparing 1,276 kidney transplant patients with score-matched patients undergoing dialysis found that the transplant patients in their study cohort had a 13% higher ten-year survival rate in comparison to patients undergoing dialysis [5]. Despite this, transplantation is not always an option. Firstly, patients must be healthy enough to withstand a major operation, which renders many patients with late stage CKD as unsuitable. Secondly, there is a significant disparity between the number of patients on the kidney transplantation national waiting list and the number of kidneys available for transplantation. In 2012, patients in the United Kingdom (UK) had to wait on average 1,167 days before being able to receive a transplant [6]. As a result, dialysis is often the reality for most patients with CKD.

The fundamental principle of dialysis is that unwanted solutes and fluid are removed across a semi-permeable membrane through the process of diffusion and ultrafiltration, thereby replicating the filtering process that takes place in the kidneys within the glomerulus. There are two main forms of dialysis for patients with late stage CKD: haemodialysis and peritoneal dialysis. During peritoneal dialysis, dialysate is run through a catheter into the patient's peritoneum in the abdomen to remove excess waste products and fluid from the body. This way the patient's own tissue acts as the partially permeable membrane to filter their blood. In contrast, during haemodialysis, the patient's blood is pumped through a dialysis machine, which is composed of hollow synthetic fibres that act as the semipermeable membrane.

In the UK haemodialysis is the most common form of RRT. Indeed Figure 1.1 shows that 69.4% of patients on RRT at 90 days were carrying out some form of haemodialysis as opposed to only 20.5% of patients carrying out peritoneal dialysis [7].

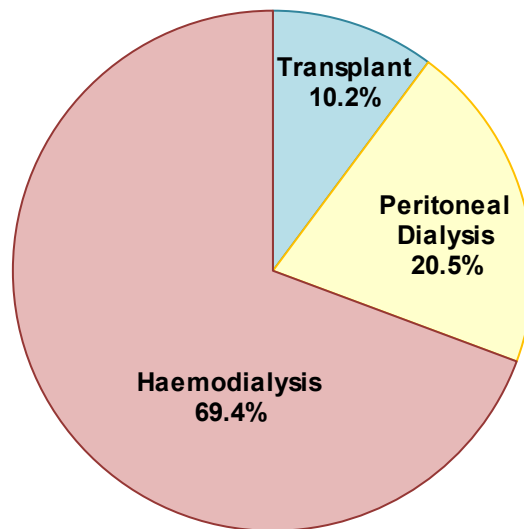


Figure 1.1: RRT modality at 90 days (patient cohort from 1/10/2016 - 30/09/2017).
Adapted from the UK Renal Registry 21st Annual Report [7].

1.1.3 The Types of Vascular Access

For haemodialysis to occur, the dialysis machine needs to have reliable access to the blood circulation through a vessel that will be able to withstand repeated punctures. Vascular access for haemodialysis is provided mainly through three main routes: through an arteriovenous fistula (AVF), arteriovenous graft (AVGs) or through a central venous catheter (CVC).

CVCs consist of a plastic tube containing two lumens. It is usually inserted into a large vein, often in the neck or chest, to allow for large flows of blood to be drawn from one lumen, enter the dialysis circuit and then be returned to the other lumen. Whilst CVCs allow for immediate access to the circulation without the requirement for cannulation, the blood flow they provide is almost always less than that of a well-functioning AVF or AVG. Furthermore, immediately after insertion of a CVC, the normal skin barrier is

disrupted leading to the CVC becoming coated with plasma proteins. This provides a portal for skin flora bacteria to enter the patient's bloodstream and can result in a catheter-related bloodstream infection (CRBSI). CRBSI causes a systemic inflammatory response that can range from a fever to a life-threatening acute illness. Bacteria may deposit at other sites causing endocarditis or discitis. As a result, CVCs should only be used for emergency dialysis, when patient life expectancy is short, when a patient has run out of vascular access options, or when permanent vascular access becomes dysfunctional [8].

Long term vascular access can be created either through an AVF or AVG. An AVF is created through joining an artery and a vein together through anastomosis, as further detailed in section 1.2. When peripheral veins are of poor quality or thrombosed and it is not possible to create an AVF, an AVG can be used instead. An AVG is created by joining an artery and a vein with a graft vessel, usually made from polytetrafluoroethylene. Modern AVGs have the benefit of being able to be used straight away. Whilst an AVF requires significantly more time than this to be used for vascular access, they are less associated with infections than AVGs. Xue *et al* also found that the 1-year crude mortality rate for patients on haemodialysis using a simple AVF for vascular access was 24.9%, whilst for patients using a synthetic AVG this was 28.1% and for patients using a catheter this was 41.5% [9]. Indeed, it is well established that an AVF is the ideal form of vascular access for long term haemodialysis because of its overall superior patency and mortality rate [10].

1.2 Vascular Access through the Arteriovenous Fistula

1.2.1 Native AVF Creation and Maturation

An AVF is created through joining a feeding artery to a nearby vein through anastomosis. The Kidney Disease Outcomes Quality Initiative (KDOQI) lists that the top four arteriovenous anastomotic sites includes the snuff box AVF (in which the cephalic vein is anastomosed to the radial artery at the dorsal aspect of the hand), radiocephalic AVF (in which the cephalic vein is anastomosed to the radial artery in the wrist), the brachiobasilic transposition AVF (in which the basilic vein or median cubital vein is anastomosed to the brachial artery at the centre of the arm), and the brachiocephalic AVF (in which the cephalic vein is anastomosed to the radial artery at the upper arm) [11]. Usually the preferred choice for an AVF is the most distal location available in the upper limb. This would be the snuff box AVF, which reserves the radiocephalic site for future use. However, as this form of AVF is surgically more difficult to create, has a poorer cosmetic appearance and an increased frequency of early failure, surgeons in the UK prefer initially creating a radiocephalic AVF for vascular access, followed by the brachiocephalic AVF if the radiocephalic AVF fails [12].

Once an AVF is created, it takes four to six weeks for the AVF to “mature” enough to be used for haemodialysis. During a successful maturation process, both the artery and the vein undergo vascular outward remodelling. Vascular outward remodelling refers to the process by which the muscular media layer of the venous segment thickens, thereby undergoing arterialisatation, and the lumen of both the artery and the vein dilates. This maturation process is triggered by an adaption to different haemodynamic conditions, namely an increase in blood flow, pressure, shear stress, and to an oxygen-rich arterial environment in the venous segment. On a biological level, the endothelial cells act as mechanotransducers whereby they respond to the change in the haemodynamic stimuli by producing biochemical signals. These signals include the

production of nitric oxide (NO) and prostacyclin, which inhibits thrombosis and platelet coagulation whilst promoting vasodilation [13].

1.2.2 Current Outcomes for Fistula Creation

Complications and hospitalisations associated with AVF placement occur in a significant number of patients. A retrospective report following 317 AVFs in 293 patients over a two year period reported 82 complications arising from AVF creation in 62 patients. The most common complications included bleeding, infection, steal syndrome, aneurysm, thrombosis, seroma, subclavian stenosis and nerve injury [14]

A critically important problem in vascular access planning is the unpredictability of successful AVF maturation. A small retrospective review examining the 178 AVFs in patients with CKD stage 4-5 found that 16.29% of patients had primary failure, defined as “thrombosis or inability for cannulation of AVF within 3 months” [15]. Another, larger study, which assessed 1012 AVFs and considered primary failure as an AVF that could not provide needle cannulation consistently for 1 month within 6 months of its creation, found the primary failure rate to be 40% [16]. Similarly, a study involving 7301 patients between the years 2010 to 2012 found that only for 56% of patients successful AVF maturation occurred without prior intervention [17].

Several studies exploring different populations have identified a variety of factors associated with the maturation success of native AVFs. Examples include age, a history of cardiovascular disease (CVD), obesity, diabetes, hyperinsulinism, thrombophilia, and being female [18-22]. There are also many studies investigating the molecular events which determine successful venous remodelling for AVF maturation. For instance, using an AVF model in CKD induced mice, Nath *et al* described that cells within the vein of a dysfunctional AVF have shortened telomeres and an increase in cell cycle arresting phosphoproteins, resulting in an increase in inflammatory cytokines, such as IL-6 and IL-1 α , and prothrombotic factors. The authors suggest that this

senescent phenotype, which seems to tip the balance from vascular outward remodelling to inward remodelling, may also explain why age negatively correlates with AVF maturation success [23].

1.2.3 Classifications of AVF Vascular Access Failure

Complications in maturation and patency in AVFs remain significant barriers in improving vascular access care in patients undergoing haemodialysis. One of the complications in current vascular access research literature is that an AVF shows distinct phases of vascular access failure throughout its cycle of use of which there is a lack of standardised definitions. Lee *et al* attempted to rectify this by proposing some definitions which this thesis broadly follows [24], but discrepancies within vascular access failure definitions persist.

The various classifications of AVF vascular access failure are summarised in Figure 1.2. If an AVF when first created never sufficiently matures for vascular access, then it is said to have had maturation failure. The time of successful access creation to the time of the access circuit's first intervention, such as a fistuloplasty or surgical revision, used to maintain or restore the blood flow is referred to as 'primary unassisted patency.' The time from when patency is restored following a successful intervention to the access circuit to when the access circuit requires a re-intervention is known as 'post-intervention primary patency.' Finally, 'post-intervention cumulative patency' refers to the time of access intervention until access abandonment (with or without an attempt at surgical or endovascular intervention), or patient censorship. Patient censorship can include the end of study period, the patient receiving a transplant, switching from haemodialysis to peritoneal dialysis, death or being lost to follow-up.

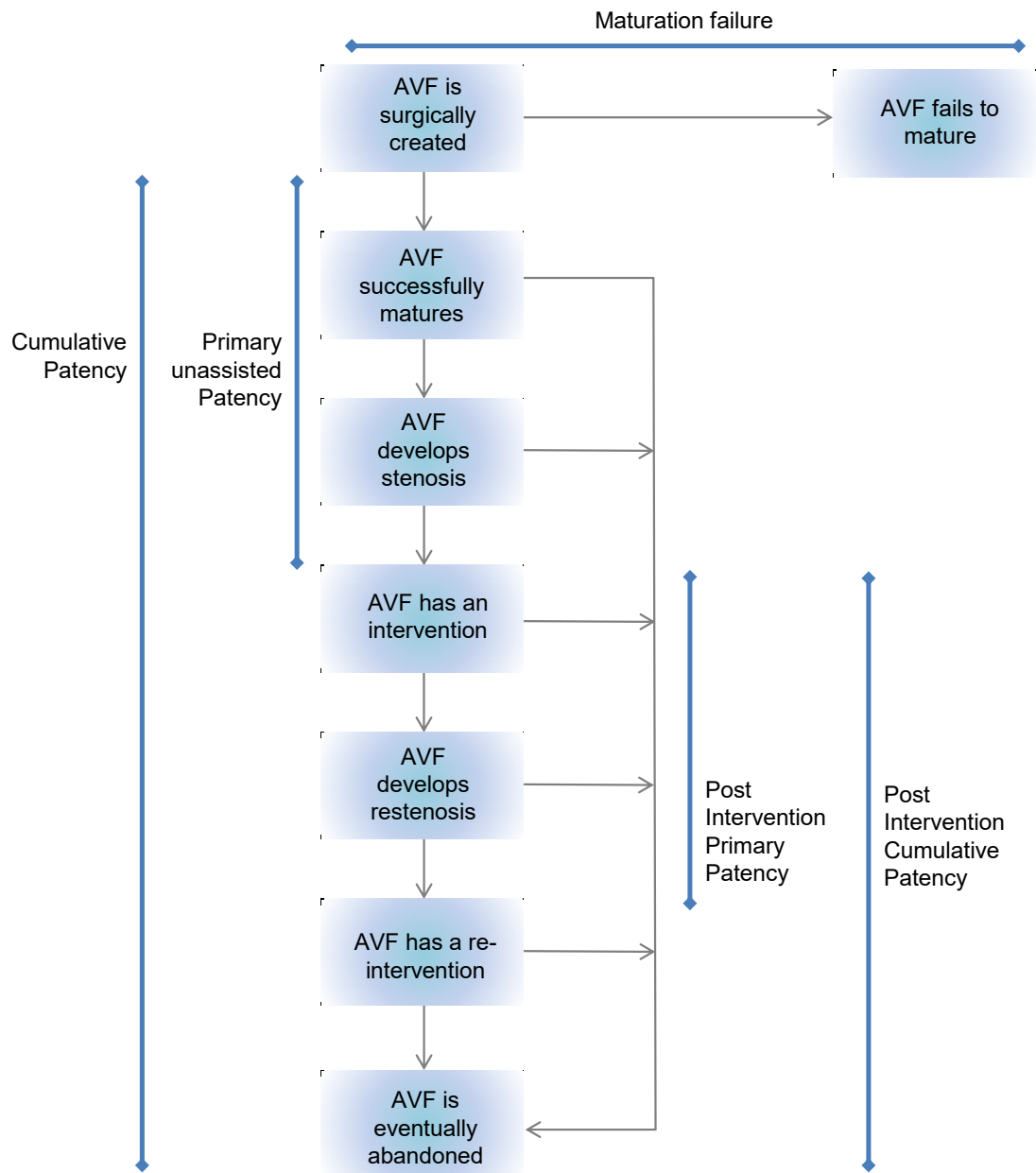


Figure 1.2: Diagram demonstrating the various classifications of AVF vascular access failure.

1.2.4 The Fistuloplasty Procedure for Stenotic Fistulas

Despite the importance of AVF patency, the Dialysis Outcomes and Practice Patterns Study showed that the one year patency for AVFs is only 68% in the US and 83% in Europe [26]. The most observed pathology in both primary and secondary AVF failure are stenotic lesions, which are structures resulting in an abnormal narrowing of the blood vessel lumen, thus limiting blood flow. In the context of fistulas, stenosis is often regarded as a luminal diameter narrowing equal to or exceeding 50% as compared to

the adjacent blood vessel used for vascular access. The initial treatment for this is a fistuloplasty procedure.

During the fistuloplasty procedure, initially the access circuit is examined in a practise known as a fistulogram. Under aseptic conditions, contrast medium is introduced to the fistula through a sheath and a series of x-ray images are taken. Images of the access circuit from the anastomosis with the artery to the central veins are reviewed in order to confirm the number, severity and the location of any stenoses as well as to select the size of the treatment balloon to be used during the fistuloplasty procedure if it is to proceed. If the fistuloplasty procedure is to proceed, then heparin is administered to the patients in order to aid the preservation of blood flow. During this step, an interventional radiologist passes a balloon into the fistula to carefully stretch open the narrowing. A small guide wire is subsequently passed through the fistula and across the stenosis. Then, over the wire, a balloon, usually 6-9mm, is passed into the lesion site and dilated serially to nominal pressure for a minimum duration of 1 minute, deflated and then removed. In order to ensure that the narrowing has been treated, the balloon may need to be inflated and deflated several times. A completion fistulogram is performed in order to confirm that there is no residual stenosis. Finally, the catheter is removed, and the puncture site is sutured or pressed down on in order to prevent further blood loss.

Details regarding the fistuloplasty procedure are subsequently relayed to the dialysis centre where the patient usually undergoes haemodialysis. Procedural success is common, with interventional facilities reporting an average stenosis of 74.1% becoming reduced to an average of 12.9% following a fistuloplasty in over 95% of cases [25].

1.2.5 Expected AVF Patency Rates following a Fistuloplasty Procedure

Despite the initial success rates of fistuloplasty procedures for treating dysfunctional fistulas, the longevity of the procedure's success is questionable. One study found that, within four years, 63 mature fistulas required 209 procedures in order to maintain

patency, with 83% of those procedures being a venous and 9% being an arterial fistuloplasty procedure. This averaged out as each patient requiring 1.75 procedures per access year [26].

Turmel-Rodrigues *et al* examined the outcome of 220 dysfunctional AVF cases following a fistuloplasty treatment for both stenosis and thrombosis between the years 1987 and 1999. They found that the primary patency of the AVFs with no intervention within 12 months and 24 months were 51% and 37% for forearm AVFs, and only 35% and 24% for upper arm AVFs respectively [27]. Heye *et al* found that post-intervention primary patency for the 217 stenoses or segmental occlusions in de novo dysfunctional AVFs at 1 year was 48.5% [28]. Similarly, and more recently, Bountouris *et al* reported the outcomes of 159 fistuloplasties in AVFs as having a post-intervention primary patency rate of 42% and 35% in 12 and 24 months respectively between 2008 and 2009 [29]. Manou-Stathopoulou S *et al* found that in patients with a single lesion, nearly a third of patients that had a single lesion treated had lost post-intervention primary patency within 6 months [30] .

Overall, most studies seem to conclude that the primary patency rates for an AVF is about 50% in one year following an intervention.

1.2.6 Consequences of Fistula Restenosis

The main determinant of the effectiveness of a patient's haemodialysis is the patency of their fistula and so vascular access failure is a crucial cause of morbidity in patients undergoing haemodialysis. Poor vascular access will have direct consequences on a patient's health. Most notably, when the AVF ceases to function effectively, patients often are obligated to dialyse through CVCs for several months. CVCs are plagued with the risk of infection and thrombosis. Indeed, a population analysis from 2005 to 2011 in America found that 60.4% of patients undergoing haemodialysis with an invasive Methicillin-resistant *Staphylococcus aureus* (MRSA) infection were using a CVC for

vascular access and nearly all during this period required hospitalisation [31]. Furthermore, readmission to hospital due to vascular access infections are common with studies reporting readmissions occurring for nearly a third of all the original hospitalisations [32].

It therefore comes as no surprise that vascular access complications lead to high levels of hospital bed occupancy. A survey in the UK found that patients on haemodialysis occupy 320,000 bed days per year, with nearly a third of those admissions being related to vascular access complications [33]. The cost of this is great, with an estimated \$1 billion alone being spent in the US on vascular access and its problems [34]. Hence, problems with vascular access are an important cause of morbidity and mortality in patients on haemodialysis and a huge burden on the NHS.

Despite the frequency of restenosis following a fistuloplasty procedure and the importance of maintaining good vascular access for patients undergoing dialysis, there has been limited research in preserving vascular access, which may be reflective of the general lack of research in nephrology overall. Indeed, Yaseen *et al* found that there were consistently eight times more published articles from grant-funded research with the PubMed Medical subject headings (MeSH) “cardiology” or “cardiovascular diseases” in comparison to the MeSH “nephrology” or “kidney diseases” between the years 2001 to 2015, and goes as far as to claim that nephrology research is in a “stagnant era” [35].

1.2.7 The PAVE Clinical Trial

Despite being in this “stagnant era” of nephrology research, there has been an emergence in some promising vascular access clinical trials. This is largely due to the recent advances in technology stemming from cardiology research that have allowed for angioplasty balloons to be coated with drugs.

The active ingredient of the drug paclitaxel was first isolated from the bark of a pacific yew tree and subsequently reported for having potentially antileukemic and tumour inhibitory properties in 1971 [36]. Since then it has been approved for the treatment of various types of cancer. The suggestion of using much lower than antineoplastic levels of paclitaxel for it to be used as an anti-proliferative agent in the prevention of restenosis was first published in 1995. This was based on systemic paclitaxel delivery on rats undergoing angioplasties on their carotid arteries and smooth muscle cell (SMC) culture experiments [37]. Paclitaxel exerts its anti-proliferative effects by binding to the beta subunit of microtubules and stabilizing them. By doing so, the microtubules are prevented from disassembly, thus blocking mitosis from progressing beyond the G2 phase of the cell cycle. As a result, the stenotic cells are inhibited from proliferating [38]. Using a porcine model of coronary restenosis and paclitaxel coated stents, Heldman *et al* first demonstrated that local paclitaxel delivery directly into the treatment site can be used as an alternative to systemic therapy and that local delivery enables for higher concentrations of the drug to be used whilst minimising systemic toxic effects [39]. Since then, there have been many randomised clinical trials in coronary arteries establishing that paclitaxel coated stents, and paclitaxel coated angioplasty balloons can reduce restenosis [38] [40].

The efficacy of paclitaxel-coated balloons (PCBs) was evaluated in a small trial involving 40 patients with dysfunctional AVFs (35%) or AVGs (65%), with half of the cohort having the PCB and the other half having the plain balloon. Whilst the trial showed that PCBs significantly improved patency and was more cost effective than plain balloons, the study had many limitations, most notably the inclusion of patients with both AVGs and AVFs, which have profoundly different patency rates and the lack of blinding [41]. More recently, a study was carried out on 132 patients with recurrent stenoses in their AVFs at three Australian hospitals and found that PCBs in comparison to plain balloons significantly delayed late lumen loss at six months and this effect persisted at 12 months. Again, however, this trial has important limitations, including

how nearly half of patients had in stent stenoses within a bare metal stent and that blinding was removed after 6 months [42]. Controversially, another recent small trial based in Finland on 39 patients with AVFs found that the PCB group did worse than the placebo group in terms of target lesion revascularization-free survival [43]. This finding, alongside the limitations of the previous studies highlights the need for a good large clinical trial to investigate the efficacy of PCBs.

The Lutonix industry-sponsored large trial investigating the efficacy of PCBs recruited 286 patients with dysfunctional AVFs at 23 centres [44]. The trial found that whilst PCB assisted fistuloplasty did not meet the primary effectiveness end point at 180 days compared with a conventional fistuloplasty, there was a trend suggesting a benefit for PCBs. This study, however, had several limitations, namely the inclusion of patients with multiple lesions that could not be treated with a single drug coated balloon.

The Paclitaxel assisted balloon Angioplasty of Venous stenosis in haemodialysis access (PAVE) clinical trial is a double-blind, phase III, multi-centre randomised control trial with the aim to determining whether the use of PCBs rather than standard high-pressure balloons during the fistuloplasty procedure could reduce the restenosis recurrence rate in patients on haemodialysis. A total of 212 patients with native AVFs were recruited with the follow up finishing in October 2019. A detailed view of the trial protocol is illustrated in Figure 1.3, which summarises the previously published trial protocol [45], whilst Table 1-2 shows the inclusion and exclusion criteria used in the trial. Importantly, patients containing lesions that could not be treated with a single drug coated balloon were not randomised to the trial.

Table 1-2: Table showing the inclusion and exclusion criteria used for including patients in the in the PAVE clinical trial taken from the trial protocol with permission.

Inclusion Criteria	Exclusion Criteria
• Patients aged 18 or over who have a native AVF in the arm. • An indication for fistuloplasty as determined by the local clinical team. • The access circuit is free of synthetic graft materials or stents. • A reduction of vessel diameter of $\geq 50\%$ measured angiographically, and a reference diameter of the outflow vein of at least 4mm and less than the size of the largest available drug-coated balloon. • A residual stenosis of $\leq 30\%$ after plain balloon fistuloplasty. • A treatment segment, containing one or more lesions, which can be treated with a $\leq 120\text{mm}$ of single drug-coated balloon.	• Patient unable to give informed consent. • Patient unwilling or unable to comply with all study-related procedures. • Systemic or local (to the fistula) infection treated for less than 10 days prior to the study procedure. • One or more lesions outside the treatment segment, with a reduction of vessel diameter of $\geq 50\%$ measured angiographically, in the same access circuit. The patient will also be excluded if any lesions outside the treatment segment are treated even if these are $\leq 50\%$. • Location of stenosis central to the thoracic inlet. • Thrombosed (failed) access circuit at the time of treatment. • Women who are breastfeeding, pregnant or are intending to become pregnant, or men intending to father children within two years of study treatment. • Known hypersensitivity or contraindication to contrast medium which cannot be adequately pre-medicated. • Known hypersensitivity or contraindication to paclitaxel.

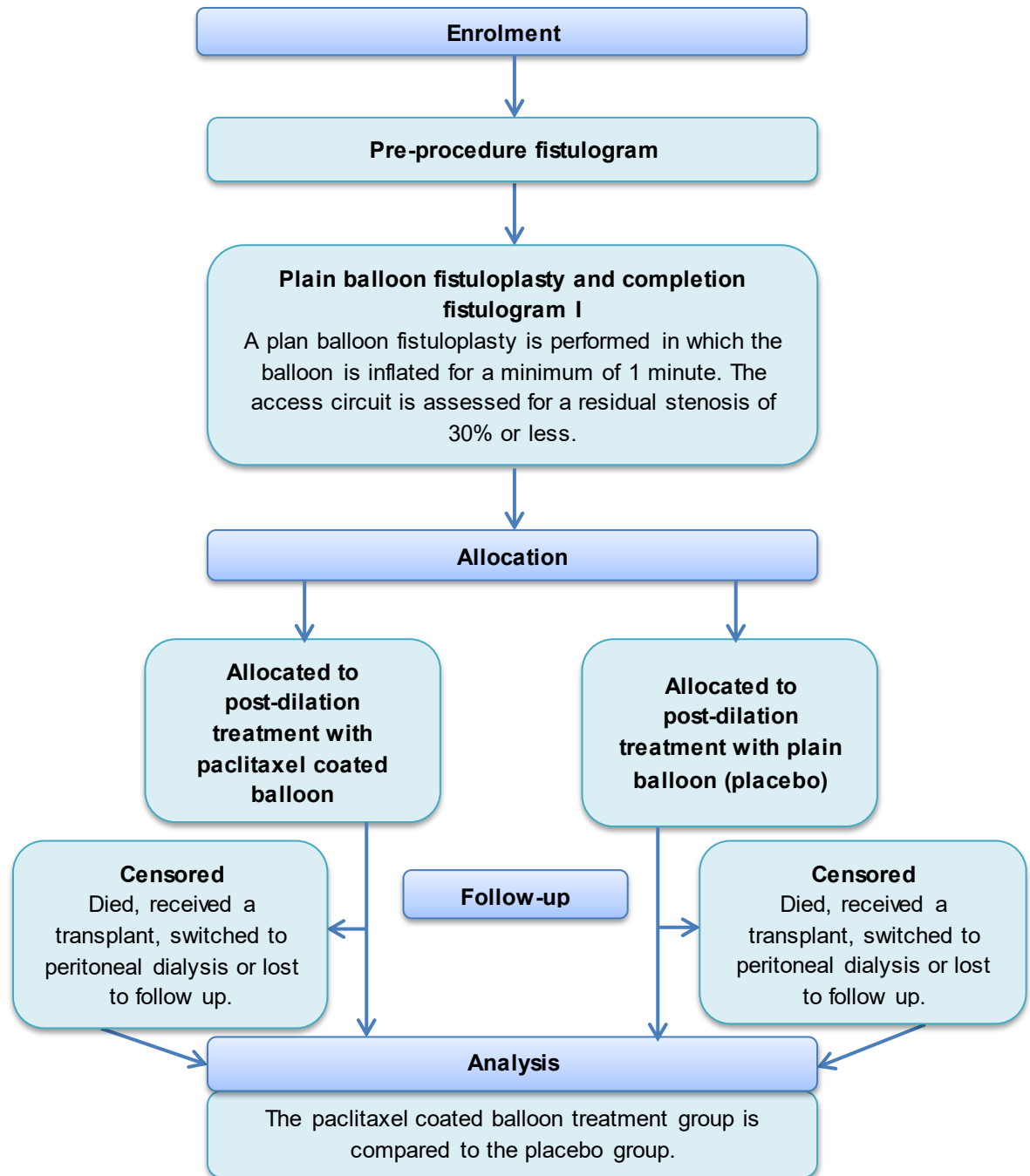


Figure 1.3: Flowchart demonstrating a patient's progress through the PAVE clinical trial protocol. Patients to undergo a fistuloplasty for their stenotic AVF are initially consented. Patients have a fistulogram procedure during which their entire access circuit from anastomosis to their central vein is assessed. They are administered with heparin before having their plain balloon fistuloplasty. Another fistulogram is subsequently performed to assess for a residual stenosis of 30% or less. The patient is then randomised to either receiving post-dilation treatment with the paclitaxel-coated balloon or with the uncoated balloon. The patient is followed up and data is analysed. The figure is adapted from the previously published trial protocol with permission [42].

1.3 Cellular Migration & Phenotypic Changes during NIH Development

1.3.1 Overview to the Conventional Paradigm of Neointima Hyperplasia

Neointimal hyperplasia (NIH) is the pathological process commonly leading to stenosis in AVFs [46]. NIH is defined here as the proliferation and migration of alpha smooth muscle actin (SMA) expressing cells within a proteoglycan-rich extracellular matrix between the internal elastic lamina and the endothelium layer. This process can result in a decrease in vessel lumen space as a result of vessel outward re-modelling not being able to compensate for the increase in intima size. NIH occurs in a variety of different contexts, including in coronary bypass surgery vein grafts, chronically rejected allografts, implantations of metallic stents, prosthetic devices, and in fistulas. Despite this, the pathogenesis of NIH is broadly the same regardless of the context. As first hypothesised by Forrester *et al* in 1991, the pathogenesis of NIH parallels the wound healing process [47]. Forrester described three main stages during the development of NIH based on the general biologic response to tissue injury: Inflammation, granulation and an extracellular matrix remodelling phase, as summarised in Figure 1.4.

The endothelial layer is a dynamic structure that maintains tissue homeostasis. It secretes factors such as NO, which renders SMCs quiescent, inhibits platelet aggregation, and protects cells within the media layer from circulating growth factors by acting as a non-permeable barrier. During the inflammation step, there is damage to the endothelial layer with variable levels of damage to the SMC level below. In the context of restenosis after a fistuloplasty, this is as a direct result of the mechanical trauma caused by the fistuloplasty balloon. Aggregated platelets release elements of the coagulation cascade, potent growth factors, including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) from their alpha granules. These have both mitogenic and chemotactic effects for cells of mesenchymal origin, including SMCs and endothelial cells. When no longer in a confluent monolayer,

endothelial cells rapidly become activated to produce reactive oxygen species (ROS) and growth factors such as angiogenin and resistin. Overall, the actions of activated platelets and endothelial cells results in a complex paracrine release of pro-inflammatory mediators [47].

During the granulation phase, there is a recruitment of leukocytes, namely neutrophils and monocytes as a result of the release of these pro-inflammatory mediators. For instance, during endothelial activation, P-selectin followed by E-selectin, both cell adhesion molecules, traffic to the endothelial cell surface, thus mediating leukocyte capture and rolling. Neutrophils and monocytes recruited to the injury site produce an increase of pro-inflammatory cytokines including interleukin (IL)-1, IL-6 and tumour necrosis factor alpha (TNF- α), which further recruits circulating leukocytes, and induces monocytes to differentiate into macrophages. In addition, the production of macrophage colony-stimulating factor (M-CSF) results in a negative feedback loop that encourages more monocytes to differentiate at the site of injury. Activated macrophages, neutrophils and cells within the vessel produce matrix metalloproteinase (MMP). MMPs catalyse the hydrolysis of major extracellular matrix molecules, including the internal elastic lamina, thus allowing space for SMCs to proliferate and migrate into the vessel's intima layer [48].

The final step in Forrester *et al's* model is the extracellular matrix remodelling phase, which occurs over a longer period. During this step, there is reduced cellularity and an increase fibrosis within the enlarged media layer, with Farb *et al* reporting a 45% reduction in SMCs between 3 and 9 months in NIH lesions within in stent coronary arteries [49]. An increase in extracellular matrix components, including fibronectin and hyaluronic acid, facilitates the migration of endothelial cells from the lesion margins resulting in the reformation of a confluent monolayer of cells [50]. Confluent endothelial cells in turn produce heparin sulphate proteoglycan, which is a potent inhibitor of SMC proliferation. Cell-to-cell contact revert endothelial cells back to their quiescent state to

continue to produce mesenchymal cell mitogen inhibitors. In addition, proliferating SMCs continuously synthesize tropoelastin, which undergoes post translation modifications to become organised into elastin fibres. Eventually, the elastin fibres accumulate to reform the internal elastic lamina. The internal elastic lamina in turn exerts regulatory functions on SMCs via G-protein signalling pathways. These signals instruct SMCs to anchor around the elastic fibres, hence reverting the cells back to their quiescent contractile state [51].

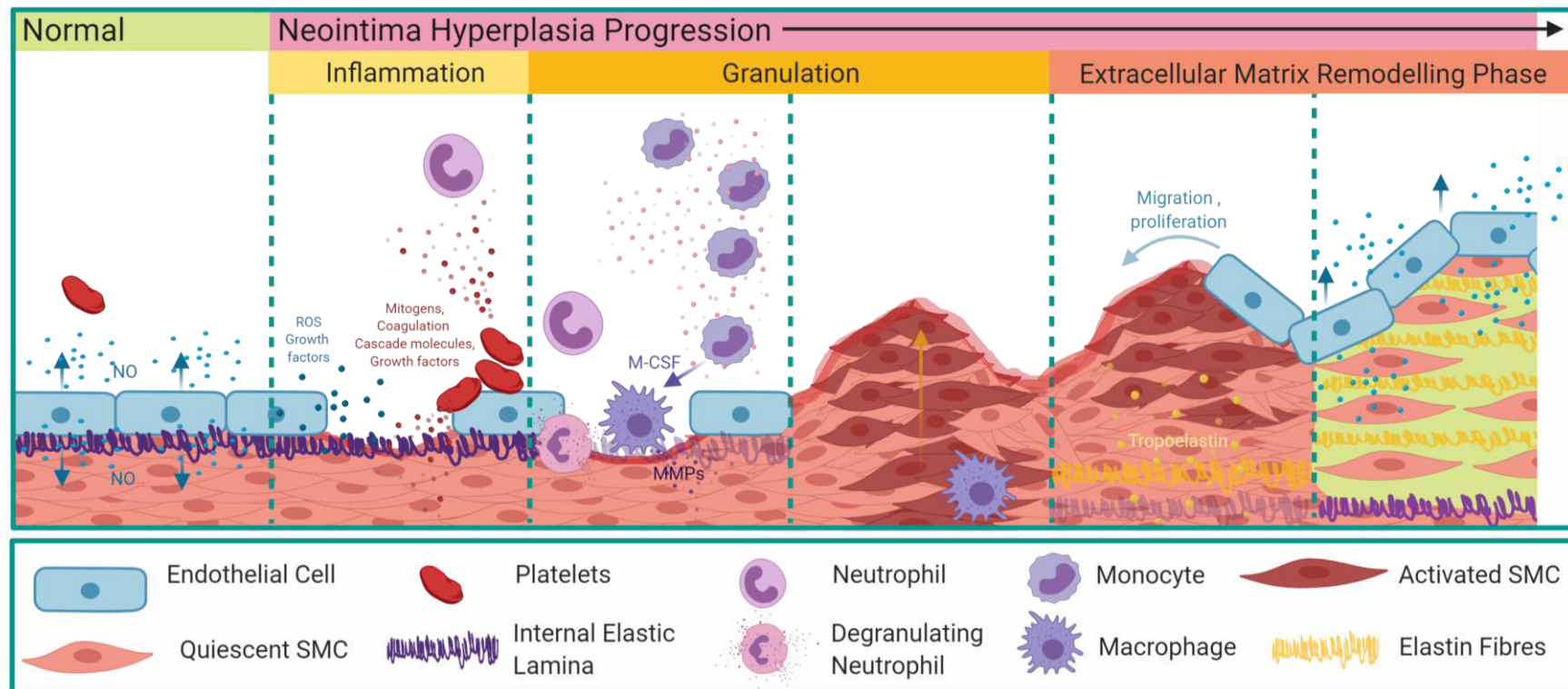


Figure 1.4: An overview to the conventional paradigm of neointima hyperplasia.

Under normal conditions, there is an intact endothelial wall. It secretes factors such as nitric oxide (NO), which renders smooth muscle cells (SMCs) quiescent and inhibits platelet aggregation. During the inflammation step, there is damage to the endothelial layer and some to the SMC layer. Platelets aggregate and release mitogens, coagulation cascade molecules and growth factors which have mitogenic and chemotactic effects SMCs and endothelial cells. The no longer confluent endothelial cells become activated to produce reactive oxygen species and growth factors. The release of these pro-inflammatory mediators results in a recruitment of neutrophils and monocytes during the granulation phase. Neutrophils and monocytes recruited to the injury site produce an increase of pro-inflammatory cytokines, which further recruits circulating leukocytes. Macrophage colony-stimulating factor (M-CSF) induces monocytes to differentiate into macrophages, which, in turn with neutrophils, produce matrix metalloproteinases (MMPs) that catalyse the hydrolysis of the internal elastic lamina. The removal of the internal elastic lamina allows for SMCs to further proliferate and migrate into the vessel's intima layer. The final is the extracellular matrix remodelling phase. Proliferating SMCs continuously synthesize tropoelastin, which undergoes post translation modifications to become organised into elastin fibres. Eventually, the elastin fibres accumulate to reform the internal elastic lamina. Endothelial cells migrate from the lesion margins resulting in the reformation of a confluent monolayer of cells that reverts the endothelial cells back to their quiescent state to continue to produce mesenchymal cell mitogen inhibitors. SMCs undergo apoptosis and there is a reduction in cellularity and an increase in fibrosis within the NIH layer.

1.3.2 The Controversial Origin of Cells that Contribute to Neointima Growth

During NIH there is a progressive accumulation of SMA expressing cells within the intima layer, yet the precise origin of these cells is a matter of debate as summarised by Figure 1.5. According to the traditional paradigm for the pathogenesis of NIH, SMCs migrate from the media layer into the intima. Resting state SMCs have a “contractile” differentiated senescent phenotype in which they have a high expression of contractile apparatus proteins, such as smooth muscle myosin heavy chain (SMMHC). Contractile SMCs have an elongated spindle shaped morphology and a fusiform growth in culture. After exposure to inflammatory growth factors, SMCs revert into a “synthetic” proliferative migratory phenotype with extensive extracellular matrix synthesis capabilities. Morphologically contractile SMCs look larger and in culture grow in a characteristic “hill and valley” morphology. Engelse *et al* found that reverting SMCs into a contractile phenotype, via administering activin A with adenovirus, prevented NIH formation in an *in vitro* saphenous vein model, and in a murine arterial cuff model, treated animals showed a strongly reduced development of neointimal tissue [52].

Tracking the fate of individual SMCs, however, is difficult as no specific markers for migratory SMCs exist. Traditionally studies, including Engelse *et al*, have used SMA as a specific marker for SMCs, which renders the importance of contractile SMCs questionable as many other cells have the plasticity to acquire SMA expression. For instance, Roy-Chaudhury *et al* described that most cells within the venous NIH of fistulas with early failure as myofibroblasts rather than SMCs [53]. Myofibroblasts express SMA and are distinguished from SMCs by their expression of the structural protein vimentin, their lack of desmin and SMMHC expression. The expression of vimentin is thought to demonstrate that these myofibroblasts originated from fibroblasts, spindle shaped vimentin positive cells found within the adventitia, which migrated to the intima layer to differentiate into myofibroblasts. Despite the difference in vimentin expression, whether once in the intima layer myofibroblasts and SMCs form

distinct or similar phenotypic populations is debatable and is often dependent on arbitrary definitions.

Indeed, there is increasing evidence that the adventitia layer can be an important *in vivo* source of myofibroblasts. Fate tracing experiments on pericytes, cells of the microcirculation which wrap around endothelial cells, have revealed that pericytes have the capabilities to transform into myofibroblasts, thus they are mesenchymal stem cells [54]. Kramann *et al* found that there are mesenchymal stem cells within not only within the pericyte niche around the vasavorum, but also within the arterial adventitia distinct from the CD31+ endothelial cells. Using genetic fate tracing, it was found that four weeks after wire injury in the femoral artery in mice over 50% of newly formed SMA expressing cells in the intimal layer were adventitia derived progenitor cells. This data suggests that mesenchymal stem cells within the adventitia can differentiate into SMC like cells and are actively involved in NIH formation after injury [55].

Endothelial-mesenchymal transition (EndMT) is the process of endothelial cell's morphology and cell specific markers de-differentiating to acquire mesenchymal cell characteristics. This includes losing CD31 expression and acquiring a SMA expressing myofibroblast phenotype. Whilst originally described in the 70s as being important for embryonic development [56], more recently it has emerged that EndMT can also be associated with NIH development. Cooley *et al* investigated the origin of vascular intimal cells within the NIH of vein-to-artery cross-transplantation mice. They demonstrated that endothelial lineage-derived cells comprised of most of the intimal cells within the murine model and that early failed human grafts also displayed EndMT with transforming growth factor beta 1 (TGF- β) signalling activation is at the core of triggering EndMT [57]. Controversially, however, the study used Tie2-Cre transgenic mice for endothelial cell lineage analyses with the assumption that Tie2, a gene which encodes for an angiopoietin receptor, is exclusively expressed by endothelial cells throughout their development. For instance, it is now known that Tie2 can be

expressed on progenitors of myeloid precursors [58], monocytes and macrophages [59]. Therefore, the EndMT cells Cooley *et al* detected in the neointima of vein grafts might be in fact myeloid cells.

Indeed, more recently studies have suggested that some NIH cells originate externally to the blood vessel and instead are recruited from haematopoietic stem cells, both bone marrow residing and circulating. These cells then bind to the site of vascular injury, migrate into the lesion and finally differentiate into SMA expressing cells. For instance, it has been proposed that myofibroblasts can originate from monocytes via a process of macrophage-myofibroblast transition (MMT). Using a model of renal fibrosis in chimeric mice with labelled macrophages, Wang *et al*, identified that CD206+ macrophages from the bone marrow can give rise to myofibroblasts, and can constitute over 60% of the myofibroblasts within the fibrosing kidney [60]. During this process, macrophages gain the expression of SMA and collagen type I. This study is, however, is limited by using CD11b as their sole marker for bone marrow derived macrophages, which is also expressed by neutrophils and fibrocytes.

Fibrocytes are cells with the inflammatory features of macrophages and the tissue remodelling properties of fibroblasts, which express the progenitor cell marker CD34, the haematopoietic marker CD45 and stromal markers such as collagen type 1. Fibrocytes comprise only a miniscule fraction of circulating leukocytes, with only an average of 1.69 cells/ μ l in healthy donors' blood, equating to about 0.1% of peripheral blood leucocytes [61]. Despite this, Chen *et al* investigating intimal hyperplasia using a wire injury model on mice describes that most of the cells within the NIH lesion were of fibrocyte origin [62]. Whilst there are many fate tracking studies demonstrating that fibrocytes can originate from the bone marrow [63] [62], it is assumed that the circulating monocyte pool gives rise to much of the fibrocytes found within lesion. Indeed, *in vitro*, these cells are grown by enhancing for monocytes via CD14+ bead isolation followed by exposure to various factors known to promote wound healing and

tissue remodelling, such as TGF- β , semaphorin 7A and endothelin-1. The research efforts to more clearly elucidate the contribution fibrocytes have in NIH formation is greatly hindered by the markers used to identify the cells. In many studies, the identification of fibrocytes was based on just CD34, CD45 and collagen production, which overlaps with several other cell types including macrophages and fibroblasts. Adding to the complexity, many of the cell surface markers fibrocytes express are expressed only transiently. For instance, fibrocytes lose their expression of CD34 during periods of culture alongside various other markers [64].

Most importantly, the type of model used to study NIH plays great importance in which cells contribute to the development of NIH. For instance, likewise to *Chen et al*, Tanaka *et al* found that whilst using a wire injury model, a substantial number of the cells within the NIH lesion were derived from bone marrow, however, these cells were seldom detected when using cuff replacement or carotid ligation models [65]. This, alongside the lack of distinguishing cellular markers, and the remarkable plasticity of cells thought to significantly contribute to the development of NIH is the overall cause of controversy in origin of stenotic cells. Figure 1.5 summarises the possible cellular sources of cells which cause stenosis.

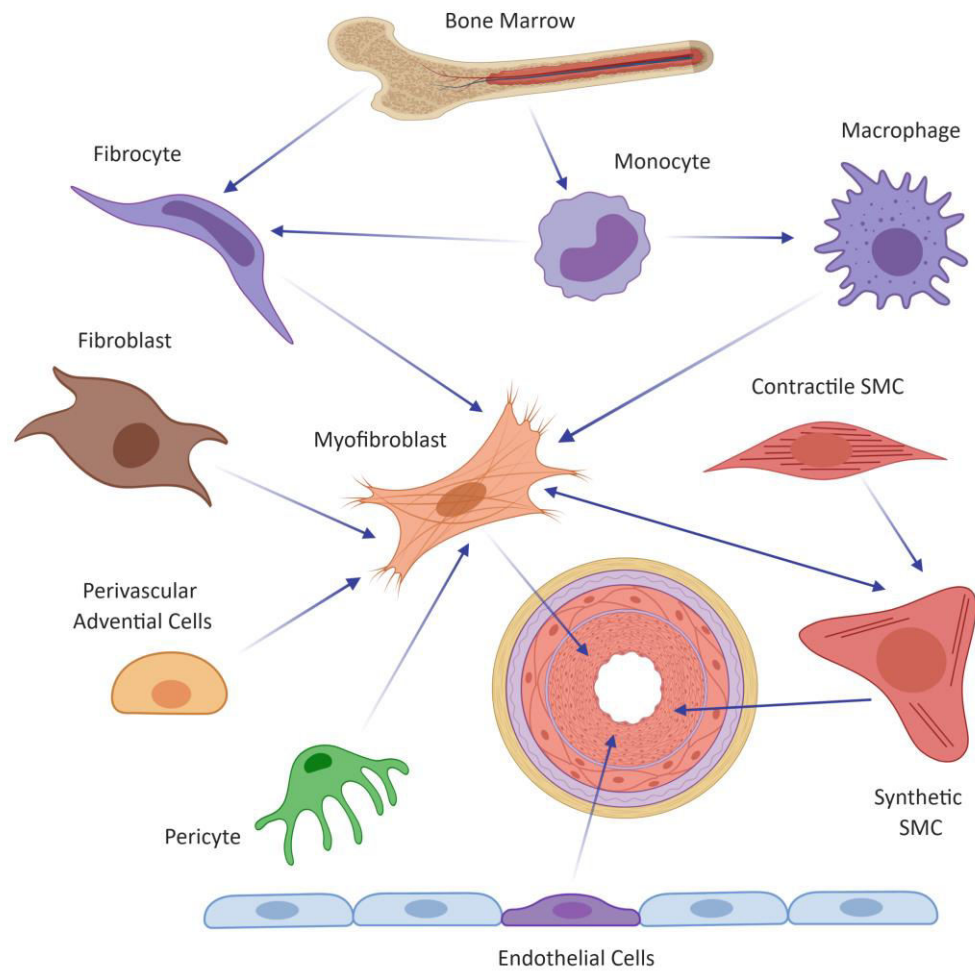


Figure 1.5: Cells contributing to NIH development have been reported to originate from a variety of precursor cell types.

This includes contractile smooth muscle cells differentiating into synthetic smooth muscle cells (SMC), endothelial cells, pericytes, perivascular adventitial cells, fibroblasts, monocytes differentiating into macrophages or fibrocytes, or fibrocytes originating from the bone marrow.

1.4 Distinctive Factors within Patients on Haemodialysis Contributing to the Development of NIH

1.4.1 Haemodynamics

Blood closest to the blood vessel endothelial wall flows slower than the blood at the centre of the vessel due to the viscosity of the blood. As a result, the blood moving across the endothelial surface results in a frictional dragging force, known as wall shear stress (WSS). There is a significant amount of evidence to suggest WSS is key in the development of NIH within the vasculature, a phenomenon Morbiducci *et al* described as the 'hemodynamic hypothesis' [66]. Indeed exposure of endothelial cells for 48 hours to oscillatory WSS results in important changes in gene expression, cytokine production and cytoskeleton organisation, including stimulating the production of Monocyte Chemoattractant Protein-1 (MCP-1) and IL-8 [67].

Development of NIH largely occurs in the venous segment of the AVF, with nearly half of all stenosis occurring in the venous anastomosis [68]. These regions directly correspond to zones of low and oscillatory WSS within the AVF [69]. From patient to patient, there are significant differences in the geometry of the AVFs. Using computational fluid dynamic modelling of the flow field in the AVFs of sixty patients undergoing upper limb AVF creation, Corbett *et al* found that even between patients with the same native AVF, there are significant differences in the AVF's arterial curvatures. These structural differences were found to be more pronounced in brachiocephalic fistulas in comparison to radiocephalic fistulas, with brachiocephalic fistulas being found to be less coplanar and have larger bifurcation angles. Corbett *et al* also found that the arterial curvatures heavily influenced the haemodynamics within the vessels, which may explain why brachiocephalic fistulas have higher non-maturation rates than radiocephalic fistulas [70]. Hence, the anatomical shape of the AVF influences the haemodynamic factors of the blood flow through the vessel, which in

turn influences the biochemical signals produced by endothelial cells, thus having a significant role in the development of NIH.

1.4.2 Vein rather than Artery

Whilst most studies investigating the development of NIH have focused on an arterial context, stenosis in AVFs usually occurs within the venous segment, which results in subtle yet important differences in NIH development. For example, it has been found that SMCs derived from veins express nearly a seven-fold greater density of PDGF receptor- β (PDGF-R β) than SMCs derived from arteries, and hence they have a significantly greater proliferative response to PDGF-BB than arterial SMCs [71].

Importantly, numerous studies have highlighted that pre-existing NIH present in veins of patients with late stage CKD prior to vascular access creation. Alpers *et al* carried out large scale histological analysis of the vein segments used for AVF creation from 554 patients. They found that 82% of the vein samples had large aggregates of collagen and proteoglycans within the media layer and most samples had some levels of pre-existing NIH formation [72]. Similarly, Wali *et al* noted that in contrast to the cephalic veins from young healthy organ donors ($n = 3$), all cephalic veins from patients with late stage CKD ($n = 20$) exhibited large degrees of generalised NIH and disruption or loss of internal elastic lamina [73]. This suggests that the vascular injury arising from complications of CKD, including uraemia and oxidative stress, results in pre-existing vascular abnormalities in patients on dialysis is likely to contribute to the development of NIH.

1.4.3 Oxidative Stress

Oxidative stress defined here as an imbalance between pro-oxidants with antioxidant defence mechanisms, which results in subsequent structural damage to biological molecules via oxidation. Oxidative stress is prevalent in patients on haemodialysis for numerous reasons. Firstly, conditions which normally accompany patients on dialysis

such as diabetes, old age and hypertension increase pro-oxidant activity. Secondly, patients on dialysis have a reduced antioxidant defence system due to having dietary recommendations to avoid fruits and vegetables, and an increase in pro-oxidants due a reduced glomerular filtration rate and chronic inflammation in the kidneys. Finally, the dialysis procedure itself is associated with a loss of antioxidants such as vitamin C, and an increase of pro-oxidants via inciting further inflammation [74].

The sources of oxidative stress can be endogenous or exogenous. Myeloperoxidase (MPO) is an enzyme that plays a key role in the immune response against pathogens and is an example of an endogenous source of oxidative stress. It is primarily found in the azurophilic granules of neutrophils and in the lysosomes of monocytes and macrophages to a lesser degree. When released into the extracellular space during cellular degranulation, MPO catalyses the production of hypochlorous acid (HOCl), a potent cytotoxic oxidant, from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) to carry out antimicrobial activity.

In the context of CKD, there is chronic inflammation and thus there is a frequent release of MPO into the vascular compartment. This extracellular MPO can remain as a soluble form in the circulation or bind to endothelial cells. Using rat common carotid arteries incubated with MPO and H_2O_2 , Yang *et al* demonstrated that MPO was able aggravate neointimal hyperplasia via its product, HOCl, eliciting chemical injury on endothelial cells [75]. More recently, Tiyerili *et al* fed apolipoprotein E-deficient mice a high cholesterol diet with MPO inhibitor in order to assess the effects of MPO on NIH formation. They found that higher dosages of MPO inhibitor decreased vascular oxidative stress, improved endothelial cell function and decreased NIH. Likewise, irradiated mice when repopulated with bone marrow derived cells from MPO knockout mice in comparison to wild type mice had reduced NIH formation [76]. Indeed, Stolic *et al* found that MPO was a statistically significant predictor of fistula failure [77].

Whilst undoubtedly oxidative stress can exacerbate the formation of NIH via damaging the endothelial layer, some have suggested that it can also be protective. Using a mouse model of AVF maturation, Sadaghianloo *et al* found that successful AVF creation corresponded with an early increase of reactive oxygen species and hypoxia-inducible factor 1-alpha (HIF-1 α) protein expression within the vessel. They theorised that exposure of this hypoxic injury potentially enhances early venous adaptation to the altered oxygenation of the arterial environment [78]. Therefore, whilst HIF-1 α may be necessary for preventing NIH during AVF maturation, its role may evolve in time, thus demonstrating that the effect of pro-oxidants will depend upon the context of the fistula itself.

1.4.4 Uraemia

Importantly, NIH in AVFs occurs within the context of a uremic milieu, which further contributes to increased oxidative stress and inflammation within the access circuit. Various solutes that are normally excreted by the kidneys are retained within the blood of patients undergoing dialysis, which is then associated with fluid, hormone, electrolyte and metabolic imbalances. As well as elevated levels of urea and creatinine, there are also elevated levels uremic solute molecules with biological toxicity, of which over 88 molecules have being identified thus far [79]. Most notable of these uremic toxins are protein-bound uremic toxins (PBUTs), which bind to large proteins such as albumin. Despite regular haemodialysis, PBUTs readily accumulate due to only the free fraction of PBUTs being able to diffuse through the dialysate. For instance, the PBUT indoxyl sulphate, which is 97.1% protein-bound, has been found to only be reduced by 31.8% via conventional haemodialysis [80].

Consequently, the uremic milieu in turn affects the circulating proteins and cells within patients undergoing haemodialysis. For instance, elevated levels of carbonyl compounds in uraemia act as the intermediates in the formation of advanced glycosylation end products (AGEs), with patients on haemodialysis being reported to

have over 6.75 higher levels of AGEs than healthy donors [81]. Interaction of AGEs with the receptor for advanced glycation end products (RAGE) results in an increase in pro-inflammatory cytokines and oxygen radicals through nuclear factor kappa-B (NFκB) gene induction. More recently, AGEs have also been shown to be able to cross link with collagen to alter vascular resistance in the vessel wall and thus further contribute to NIH development [82].

The effects various solutes have within the uremic milieu, however, can be controversial. For instance, Hsu *et al* found that the decrease in NO synthesis and increase in oxidative stress by human aortic endothelial cells in response to the addition of indoxyl sulphate can be attenuated by the addition of uric acid in a dose-dependent manner [83], a toxin often described as causing endothelial dysfunction by triggering inflammatory signalling pathways [84]. Indeed, Latif *et al* found that higher uric acid levels were associated with lower risk of all-cause and cardiovascular mortality in the haemodialysis population, which starkly contrasts the association of hyperuricemia with higher cardiovascular risk in the general population [85]. Hence, it is thought that some uremic toxins, such as ureic acid, may have some antioxidant capabilities in the context of an overstressed uremic environment and further study of them is warranted.

1.4.5 Changes in Leukocytes

The immune system can broadly be subdivided into the innate system, whereby there is an immediate non-specific immune response, and the adaptive system, whereby there is an antigen-specific response as a result of the action of professional antigen presenting cells priming adaptive cells. All immune effector cells, cells which actively respond to stimulus or stimuli to bring effects some change, appear to be affected in patients on haemodialysis. This can be highlighted by clinical observations such as the poor response to vaccine challenges and the higher rates of malignancy patients on

haemodialysis have. This indicates immune deficiency despite having a chronically activated immune system as a result of oxidative stress and uraemia [86].

Neutrophils, an innate type of immune cell, are the most abundant leukocyte, constituting to about 60-70% of leukocytes in the peripheral blood. Freshly isolated neutrophils, from patients on long-term haemodialysis, exhibit decreased phagocytosis rates and increased apoptosis in comparison to healthy controls [87]. This is in line with previous observations that neutrophils from healthy subjects incubated with uremic plasma have lower phagocytosis rates and higher apoptosis rates compared to neutrophils incubated with plasma from healthy volunteers [88]. In addition to this, contact between blood and haemodialysis membranes have been shown to increase the formation of platelet-neutrophil microaggregates, resulting in excess H_2O_2 production by neutrophils and coinciding with an increase in ROS production [89].

ROS and uraemia have also been attributed to a decrease in both the functionality and maturation in dendritic cells [90], and the overall numbers of circulating dendritic cells [91]. Given that dendritic cell activation leads to naïve T cell activation, it comes as no surprise that there is also an impaired T cell activation response in patients on dialysis [92]. Besides from this, patients on dialysis have been reported to have a lower thymic output, with Betjes *et al* reporting that patients on haemodialysis between 25-45 years old as having a significant loss of naïve T cells and an increase of memory T cells, T cells enriched to recall antigens that are similar to memory B cells, resembling that of 60-80 years old healthy controls [93].

Lymphocyte subset alterations seem to coincide with the progression of CKD. Lee *et al* describes that the percentage of CD16+ monocytes circulating in peripheral blood seems to increase as CKD progresses [94], though it is important to note that the overall number of monocytes in this study were not considered. The function of CD16+ monocytes are further detailed later in this thesis. Likewise, an increase in NK cells and

a decrease in B cells seems to correlate with further renal function impairment [95]. Despite the increase in Natural Killer (NK) cells in patients on dialysis, it has been reported that the NK cells from these patients have less expression of the activating receptor natural killer group 2 member D (NKG2D) than that of healthy donors [96]. NKG2D not only has a role in immune surveillance for malignancies and infection, but, more recently, the NKG2D/ligand interaction has also been described as a critical molecular link in propagating atherosclerosis [97].

There are a multitude of factors leading to functionality and phenotype leukocyte dysfunction, including haemodialysis, uraemia, oxidative stress and malnutrition. The overall consequences of leukocyte dysfunction undoubtedly have consequences in not just immune protection, but also the repair pathways triggered after damage to the endothelial layer of the fistula.

1.5 Biomarkers to Predict Restenosis following a Fistuloplasty

1.5.1 Patient Clinical Information Variables

A biomarker is defined here as a measurable characteristic, molecule or gene which can be used to indicate a biological process. There are few biomarker studies in the literature which focuses on predicting post-intervention patency in fistulas as there is more of a focus primarily is on predicting successful AVF maturation as well as AVF primary unassisted patency failure.

Of the few studies on post-intervention patency in fistulas there seems to be some consensus about certain patient demographic and clinical variables importance to patency outcome. Most recently, a retrospective analysis examining 124 consecutive patients who had successful interventions for dysfunctional AVFs found that thrombosis at the time of intervention, history of previous intervention, non-white ethnicity, lesion length, and patient age had a significant association with poor outcome following a fistuloplasty procedure, whilst older fistulas associated with good outcome [30]. This

was in consensus with a systematic review observing the patency of 965 fistulas after radiological intervention also found that lesion length and fistula age associated with outcome [98].

As important as these factors may be, there is little detailed explanation as to why they would influence AVF patency. For instance, whilst demographic variable such as age may be more palpable to explain, there have been no attempts to find a biological explanation as to why non-white ethnicity would influence AVF patency. This highlights the overall lack of real lack of detailed laboratory-based studies examining biomarkers predict outcome following a fistuloplasty procedure independent of clinical variables. Such studies would be more interesting in understanding the mechanisms of restenosis disease pathology as they could potentially be more relevant for developing new treatments.

1.5.2 Clinical Blood Test Biomarkers

The ideal laboratory biomarker would be able to predict the prognosis of patients with an easily obtainable simple blood test in a cost-effective manner. C-reactive protein (CRP) is synthesised by the liver in response to IL-6 and TNF- α secretion and its main physiological role is to bind to apoptotic cells and bacteria in order to activate the complement cascade or to mark opsonisation targets. Since inflammation is involved in NIH, traditionally people have looked at CRP as a potential measurable biomarker for AVF patency.

Despite CRP being a reliable measure of inflammation, there is some discordance as to how useful of marker it is for NIH. For instance, whilst Sirbu *et al* found that in 258 patients CRP level was an independent predictor of AVF primary patency loss [99], the “Choices for Healthy Outcomes in Caring for ESRD” study found no significant association with varying levels of CRP and AVF failure [100], nor did Bojakowski *et al* when carrying out multivariable analysis [101]. Indeed, as CRP becomes upregulated

by a multitude of inflammatory processes often as a secondary event to other biological molecules, it is likely to be too unspecific of a marker of NIH to be used as a biomarker on its own.

Likewise, the neutrophil-to-lymphocyte ratio (NLR) can be measured in a simple cost-effective blood test. The NLR is calculated by dividing the number of neutrophils in peripheral blood by the number of lymphocytes and a high NLR ratio is a marker of acute inflammation due to the numbers of neutrophils increasing during inflammatory responses. It is thought that the NLR may have some use in predicting AVF stenosis. For instance, Yilmaz *et al* found that patients with AVF stenosis (n = 64) had a higher mean NLR than patients without AVF stenosis (n = 44), and the severity of stenosis positively correlated with the NLR level [102]. More recently, Çildağ *et al* found that NLR correlates with restenosis after a fistuloplasty. The relatively small patient numbers and differential treatments the patients had, however, suggests that more detailed studies are required to further elucidate the value of the NLR as a biomarker in the setting of AVF restenosis [103]. Further limitations in NLR include the lack of verified reference values for what is to be a “normal” NLR for a patient on dialysis.

1.5.3 Biomarkers indicated from Coronary Angioplasty Studies

As more research on restenosis has been carried out in a cardiology context, much of the current knowledge in laboratory biomarkers predicting the occurrence of restenosis relies on studies focusing on outcome post percutaneous coronary angioplasty. As mentioned in section in 1.4, however, there are specific features in patients on haemodialysis which contrasts them to other patient groups thus bringing into question the applicability of the findings from coronary angioplasty studies.

Given that inflammation is one of the first steps to NIH development, it comes as no surprise that many studies have suggested that restenosis after coronary angioplasty is negatively associated with circulating levels of traditional inflammatory markers such as

CRP and IL-6 before or after the procedure [103-105]. More elegant studies have focused on the potential mediators of this inflammatory response. For instance, Zhou *et al* demonstrated that blockage of RAGE with its ligand interaction significantly suppressed NIH formation in both wild type and diabetic rats by modulating SMC properties after injury [107] and McNair *et al* highlighted the relevance of this to restenosis by associating the AGEs/RAGE ratio with outcome following a coronary angioplasty [108].

Coronary angioplasty studies have also indicated that platelets are key mediators of inflammation. For instance, Rong *et al* found that CD40 ligand (CD40L) increased NIH after carotid artery wire injury in atherosclerosis-prone mice. As approximately 95% of CD40L originates from activated platelets, the authors deduced this was a consequence of platelet-leukocyte aggregates. Interestingly, these were shown to mediate monocyte recruitment via regulation of MCP-1 [109]. Supporting this, Bann *et al* found that plasma levels of CD40L was increased by peripheral artery angioplasty, although there was no patient follow up to see whether this would have an impact on restenosis [110].

1.5.4 Monocytes as Potential Biomarkers for Restenosis

One of the few laboratory studies looking for biomarkers for restenosis following a fistuloplasty procedure found that MCP-1 is elevated up to two weeks post fistuloplasty, and an early increase in MCP-1 at two days post procedure was an independent predictor of restenosis [111]. Mori *et al* demonstrated that MCP-1 blockade prevented NIH formation after carotid artery angioplasty in hypercholesterolemic rabbits, thus directly showing its mechanistic importance in NIH formation [112]. MCP-1, also referred to as chemokine (C-C motif) ligand 2 (CCL2), is a chemokine that binds to chemokine (C-C motif) receptor 2 (CCR2) to induce to chemoattraction of mononuclear cells. As monocytes highly express CCR2, MCP-1 is well known to promote the recruitment of monocytes to sites of inflammation.

The importance of MCP-1 in restenosis highlights the importance of monocytes in NIH AVF pathology. Indeed, two animal models have already demonstrated that the depletion of monocytes with clodronate prevented NIH formation [113] [114]. Circulating monocytes can be subdivided into classical (CD14⁺⁺CD16⁻, resembling mouse lymphocyte antigen 6 complex (Ly6C)^{high} monocytes), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺, resembling mouse Ly6C^{low} monocytes). These subsets are phenotypically and functionally different. Classical monocytes are the most prevalent subset is considered pro-inflammatory, whilst CD16⁺ monocytes crawl on the endothelium, rendering them important for innate surveillance and repair of tissues.

Whilst there is a lack of literature on monocyte phenotype after an AVF fistuloplasty, increased intermediate monocyte subset levels have been associated with restenosis after peripheral artery percutaneous transluminal angioplasty [115]. *In vitro* co-culture studies have found that human non-classical monocytes induce endothelial cell proliferation in a VEGFA-dependent manner, and that Ly6C^{low} monocytes play a key role in endothelial regeneration after carotid artery injury [116]. There is a real need to investigate whether similar findings are evident following a fistuloplasty, especially considering how studies have described that there are more intermediate monocytes in patients on dialysis than in healthy controls, with intermediate monocytes also being found to independently be negatively associated with cardiovascular events in patients on dialysis [117].

1.6 Conclusions

With an ageing population, a rise in diabetes, hypertension and obesity, the number of patients requiring dialysis is set to continuously rise. Haemodialysis is the most common form of dialysis and for it to occur, the dialysis machine needs to have reliable access to the circulation. The preferred method of providing this access is by surgically making a connection between an artery and a vein and this connection is known as the arteriovenous fistula (AVF).

Vascular access failure caused by stenosis in the fistula is a common cause of morbidity in patients on haemodialysis. The fistuloplasty procedure is the established treatment for dysfunctional AVFs. Whilst it is effective, restenosis is a common problem with nearly half of patients requiring a repeat procedure within the first year of treatment. The Paclitaxel assisted balloon Angioplasty of Venous stenosis in haemodialysis access (PAVE) clinical trial is a double-blind, phase III, multi-centre randomised control trial with the aim of determining whether the use of Paclitaxel-coated balloons rather than standard high-pressure balloons during the fistuloplasty procedure could reduce the restenosis recurrence rate in patients on haemodialysis.

The stenotic lesions of the fistula are comprised of neointima hyperplasia (NIH). NIH refers to the proliferation and migration of vascular alpha smooth muscle actin expressing cells into the vessel's intima layer, which broadly follows the wound healing pathway. The lack of distinguishing cellular markers, the remarkable plasticity of cells and the impact of various models used to study NIH means that the origin of cells in NIH lesions, however, are often largely unknown. Due to this and the specific factors present in patients with end stage renal disease that are absent in other patient groups, the mechanism by which NIH develops in an AVF following a fistuloplasty procedure is largely unknown.

1.7 Hypothesis

The hypothesis of this thesis is that there is an increase in inflammatory mediators and phenotypic changes in circulating monocytes in response to the fistuloplasty procedure that can be observed 1-2 days post procedure and that these systemic factors can serve as biomarkers for AVF post intervention primary and or cumulative patency outcome.

1.8 Aims

The aims of the present study are summarised below:

1. To histologically further characterize the NIH lesions within AVFs.
2. To assess peripheral blood cells numbers for post fistuloplasty changes.
3. To assess the gene expression profile and phenotype of monocytes in peripheral blood for post fistuloplasty changes.
4. To identify the soluble factors in patient plasma that is upregulated or downregulated post fistuloplasty.
5. To correlate genes identified via microarray and soluble factors in patient plasma and cells with clinical outcome in order to suggest possible biomarkers which could indicate which patients may need a repeat fistuloplasty procedure earlier than others.

The following chapters detail the methods used and the results obtained when pursuing these aims.

Chapter 2 – Materials & Methods

2.1 Reagents used

All the reagents subsequently stated within this chapter are listed in Table 2-1. The antibodies and ELISA kits that were used are listed within the relevant methodology sections.

Table 2-1: A list of reagents used within this thesis detailing both the company and catalogue numbers.

Name	Company	Catalogue Number
1-step Fix/Lyse Solution (10X)	ThermoFisher	00-5333-54
Anti-mouse Ig.k/negative control compensation beads	Becton Dickinson	552843
Beckman Coulter Agencourt RNAClean XP Kit	Beckman Coulter	A63987
Bovine Serum Albumin (BSA)	Sigma	A7906-100G
CD14 positive selection microbeads	Miltenyi Biotech	130-050-201
Colour reagent A & B	R&D	DY999
Dibutylphthalate Polystyrene Xylene (DPX) mounting media	Sigma	06522-500ML
Dimethyl sulfoxide	Sigma	D2650
Dulbecco's Phosphate Buffered Saline	Sigma	D8537
EDTA	Fisher Scientific	BP2492-100
Elastic Stain Kit	Sigma	HT25A
Encore Biotin IL Module	NuGEN	4200-60
Eosin 1% Aqueous	ThermoFisher	12668616
Ficoll-Paque™	GE Healthcare	17144003
Fixable Viability Dye eFluor™ 450	ThermoFisher	65-0863-14
Human AB serum	Sigma	H4522-100ML
Hydrogen peroxide solution (30%)	Sigma	H1009-100ml

Name	Company	Catalogue Number
ImmPRESS® HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit	Vector Laboratories	MP-7402-15
ImmPRESS®-AP Anti-Rabbit IgG (alkaline phosphatase) Polymer Detection Kit	Vector Laboratories	MP-5401
Luna® Universal qPCR Master Mix	New England Biolabs	M3003S
Mayer's Haematoxylin	Sigma	MHS32-1L
MinElute Reaction Cleanup Kit	Qiagen	28204
Modified Verhoeff Van Gieson Elastic Stain Kit	Sigma	HT25A
Normal Horse Serum Blocking Solution	Vector Laboratories	S-2000-20
OPD Substrate	Sigma	P9187-5SET
Osteosoft®	Sigma	101728
Ovation Pico WTA System V2	NuGEN	3302-60
pH 5.2 phenol chloroform	Fisher Scientific	10295103
Proteinase K	Dako	S3020
Proteome Profiler Human XL Cytokine Array Kit	R&D	ARY022B
RevertAid™ First Strand cDNA synthesis kit	ThermoFisher	K1622
RNA 6000 Pico Chip Kit	Agilent	5067-1513
RNAlater	ThermoFisher	AM7020
RNeasy Mini kit	Qiagen	74104
RT-PCR Grade Water	ThermoFisher	AM9935
Sigmafast DAB tablet set	Sigma	D4293
Sigmafast Red TR tablet set	Sigma	F4648
Target Retrieval Solution Ready-to-Use	Dako	S1700
Trypan Blue	Sigma	T8154
TURBO™ DNase kit	ThermoFisher	AM1907
Xylene	Fisher Scientific	10784001

2.2 Summary of Patient Clinical Information

2.2.1 Patients and Healthy Donors used for PBMC and Plasma Isolation

In total, blood was processed from 121 different patients undergoing a fistuloplasty procedure from 13 different trusts across the UK, (Guy's & St Thomas, Royal Berkshire, Cardiff & Vale, Royal Devon & Exeter, Hull Royal Infirmary, East Kent Hospitals University, University Hospitals of Leicester, East & North Hertfordshire, Portsmouth Hospitals, Royal Free, Brighton and Sussex University Hospitals, Sheffield Teaching Hospitals NHS foundation trust). All patient blood samples were taken pre dialysis having had at least 10 hours between any previous dialysis sessions. Pre fistuloplasty blood samples were taken prior to the patient having a fistuloplasty procedure. For most patients this was performed on the day of the fistuloplasty procedure itself. Post fistuloplasty procedure blood was taken from 30 patients, 1-2 days post procedure, usually whilst the patient was attending a dialysis unit or following an overnight hospital stay.

It later was revealed that many of the blood samples were suboptimal to be used for various reasons. Reasons included: the patients did not go onto having a fistuloplasty procedure within 5 months of when their baseline sample was collected, the patient had a blood-borne virus infection, the sample was taken without adequate time between dialysis sessions, and the treated AVF had a post-intervention patency of less than 44 days due to the patient having to be censored. As a result, the data from only 75 patient fistuloplasty samples are shown within this thesis. Table 2-2 summarises which samples were used for which experiments.

The following demographic and clinical information was collected from the patients: their age at the time of their procedure, sex, ethnicity, whether the patient had a history of thrombosis or previous interventions in their fistula, the type of fistula being treated and the age of the fistula itself.

Appendix A – Patient Clinical Data Summary lists the demographic and clinical details from these patients and which samples were used for which subsequent experiment. Post-intervention primary patency and post-intervention cumulative patency information was collected from all the patients up until the end of the follow up date, which was the 4th of October 2019, or until post-intervention cumulative patency was reached. From the date the last patient was consented, this meant that there was a follow up period of at least 371 days. The AVF outcome data for these patients is also listed within the appendix.

Table 2-2: Table to show which samples from patients undergoing fistuloplasty procedures were used for which experiments.

Protein Screen* denotes that as these samples were processed overnight, these samples were only used for investigating proteins shown to not significantly change following overnight processing.

Patient Number	Pre vs. Post Fistuloplasty Protein Screen	Outcome Protein Screen	Pre vs. Post Fistuloplasty Clinical FBC & CRP	Outcome Clinical FBC & CRP	PBMC Subsets Flow Cytometry	Monocytes Subsets Flow Cytometry	Microarray	RT-PCR	Patient Number	Pre vs. Post Fistuloplasty Protein Screen	Outcome Protein Screen	Pre vs. Post Fistuloplasty Clinical FBC & CRP	Outcome Clinical FBC & CRP	PBMC Subsets Flow Cytometry	Monocytes Subsets Flow Cytometry	Microarray	RT-PCR
1	✓	✓	✓	✓	x	x	✓	✓	39	✓	✓	✓	✓	x	✓	x	✓
2	x	✓	✓	✓	x	x	x	✓	40	x	✓	✓	✓	x	x	x	✓
3	✓	✓	✓	✓	x	x	✓	✓	41	✓	✓	✓	✓	x	x	x	✓
4	✓	✓	✓	✓	x	x	x	✓	42	✓	✓	✓	✓	x	✓	x	✓
5	✓	✓	✓	✓	✓	✓	✓	✓	43	x	✓	✓	✓	x	x	x	✓
6	x	✓	✓	✓	x	x	x	✓	44	x	✓	✓	✓	x	x	x	✓
7	x	✓	✓	✓	x	x	x	✓	45	x	✓	✓	✓	x	x	x	x
8	✓	✓	✓	✓	✓	✓	✓	✓	46	x	✓	✓	✓	x	x	x	✓
9	✓	✓	✓	✓	x	x	✓	✓	47	x	✓	✓	✓	x	x	x	✓
10	✓	✓	✓	✓	✓	✓	✓	✓	48	x	✓	✓	✓	x	x	x	x
11	✓	✓	✓	✓	x	x	x	✓	49	x	✓	✓	✓	x	x	x	✓
12	✓	✓	✓	✓	x	x	✓	✓	50	x	✓*	x	✓	x	x	x	x
13	✓	✓	✓	✓	✓	✓	✓	✓	51	x	✓*	x	✓	x	x	x	x
14	✓	✓	✓	✓	✓	✓	✓	✓	52	x	✓*	x	✓	x	x	x	x
15	✓	✓	✓	✓	✓	✓	✓	✓	53	x	✓*	x	✓	x	x	x	x
16	x	✓	✓	✓	✓	✓	x	✓	54	x	✓*	x	✓	x	x	x	x
17	✓	✓	✓	✓	✓	✓	✓	✓	55	x	✓*	x	✓	x	x	x	x
18	✓	✓	✓	✓	✓	✓	✓	✓	56	x	✓*	x	✓	x	x	x	x
19	✓	✓	✓	✓	x	x	x	✓	57	x	✓*	x	✓	x	x	x	x
20	✓	✓	✓	✓	✓	✓	✓	✓	58	x	✓*	x	✓	x	x	x	x
21	✓	✓	✓	✓	✓	✓	✓	✓	59	x	✓*	x	✓	x	x	x	x
22	✓	✓	✓	✓	✓	✓	x	✓	60	x	✓*	x	✓	x	x	x	x
23	✓	✓	✓	✓	x	x	✓	✓	61	x	✓*	x	✓	x	x	x	x
24	✓	✓	✓	✓	x	x	x	x	62	x	✓*	x	✓	x	x	x	x
25	x	✓	✓	✓	x	x	x	✓	63	x	✓*	x	✓	x	x	x	x
26	✓	✓	✓	✓	x	x	x	✓	64	x	✓	x	✓	x	x	x	x
27	x	✓	✓	✓	x	x	x	✓	65	x	✓	x	✓	✓	x	x	x
28	x	✓	✓	✓	x	x	x	✓	66	x	✓	x	✓	x	x	x	x
29	✓	✓	✓	✓	✓	✓	x	✓	67	x	✓*	x	✓	x	x	x	x
30	x	✓	✓	✓	x	x	x	✓	68	x	✓*	x	✓	x	x	x	x
31	✓	✓	✓	✓	x	✓	x	✓	69	x	✓*	x	✓	x	x	x	x
32	✓	✓	✓	✓	x	✓	x	✓	70	x	✓	x	✓	x	x	x	✓
33	✓	✓	✓	✓	✓	✓	x	x	71	x	✓	x	✓	x	x	x	✓
34	x	✓	✓	✓	x	✓	x	✓	72	x	✓*	x	✓	x	x	x	x
35	x	✓	✓	✓	x	x	x	✓	73	x	✓*	x	✓	x	x	x	x
36	x	✓	✓	✓	x	✓	x	x	74	x	✓*	x	✓	x	x	x	x
37	✓	✓	✓	✓	x	✓	x	✓	75	x	✓*	x	✓	x	x	x	x
38	✓	✓	✓	✓	x	x	x	✓									

Blood was also taken from six patients undergoing a fistuloplasty for a time course experiment as further discussed in section 2.7.2. Clinical and follow up data was not collected from these patients. In addition, some patients had complications during their fistuloplasty procedure after their fistulogram which prevented a balloon from being dilated. Whenever possible, 1-2 day post procedure samples were collected from these fistulogram only patients to serve as controls for the administration of contrast.

Blood samples were also collected from healthy donors with no history of kidney disease. Table 2-3 details which healthy donor samples were used for which experiments. Overall healthy donors who donated samples were between the ages of 25 to 63 with a mean age of 38 and a standard deviation of 10 years, with 59% of donors being female.

Table 2-3: Table to show which healthy donor samples were used for which experiments and demographic information for each donor.

“PBMCs FC” refers to flow cytometry investigating PBMC subsets, whilst “Mono FC” refers to flow cytometry investigating monocyte subsets.

Healthy Donor	Age	Sex	Experiment
1	38	F	PBMC FC, Mono FC
2	32	F	PBMC FC, Mono FC
3	31	M	PBMC FC, Mono FC
4	49	M	PBMC FC, Mono FC
5	25	F	PBMC FC, Mono FC
6	35	M	PBMC FC, Mono FC
7	30	M	PBMC FC, Mono FC
8	50	F	PBMC FC, Mono FC
9	63	F	PBMC FC, Mono FC
10	28	M	PBMC FC, Mono FC, RT-PCR
11	30	F	PBMC FC, Mono FC, RT-PCR
12	39	F	PBMC FC, Mono FC, RT-PCR
13	34	M	PBMC FC, Mono FC, RT-PCR
14	31	M	PBMC FC, Mono FC, RT-PCR
15	55	M	PBMC FC, Mono FC, RT-PCR

16	30	F	PBMC FC, Mono FC, RT-PCR
17	40	F	PBMC FC, Mono FC, RT-PCR
18	55	F	PBMC FC, Mono FC, RT-PCR
19	27	F	PBMC FC, Mono FC, RT-PCR
20	56	M	PBMC FC, Mono FC, RT-PCR
21	37	F	RT-PCR
22	45	F	RT-PCR
23	32	M	RT-PCR
24	50	M	RT-PCR
25	27	F	RT-PCR
26	35	F	RT-PCR
27	31	M	RT-PCR
28	27	F	RT-PCR
29	42	F	RT-PCR

2.2.2 Donated Fistula Samples

Surgically removed stenotic fistulas were collected from four patients. All patients from which the AVF tissues were removed from were male and had functioning kidney transplants. Thus the AVFs were no longer in use and were excised for primarily cosmetic reasons, with the exception of AVF2 as this fistula also had a central stenosis resulting in the patient having arm and neck swelling. Two of the stenotic fistulas were basilic vein transposition AVFs and two were radio-cephalic fistulas. All fistula samples were known to have NIH and were not thrombosed.

2.3 Blood sample collection and processing

2.3.1 Blood Sample Collection

Whenever possible, blood for a full blood count (FBC) and C-reactive protein (CRP) test was sent to the clinical laboratory. These samples were collected at the same time as the research blood samples.

2.3.2 Plasma Processing

Blood for plasma collection was drawn into K2 ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged at 1000xg for 5 minutes at the lowest speed with no break on. After centrifugation, as much supernatant fluid as possible was carefully pipetted off the anti-coagulated blood layer. This was transferred into a 15ml conical tube and centrifuged at 1000xg for 5 minutes in order to separate the plasma from most of the platelets. The plasma layer was pipetted off and stored at -80°C in 1.5ml tubes.

2.3.3 Ficoll Density Gradient PBMC isolation

Most patients donated additional blood in EDTA tubes thus allowing for peripheral blood mononuclear cell (PBMC) isolation via Ficoll density gradient separation. A volume of sterile Dulbecco's phosphate buffered saline (PBS) equal to that of the volume of blood collected in EDTA vacutainer tubes were diluted 1:1 in a sterile 250ml pot. 15ml of Ficoll-Paque™ (GE healthcare) at room temperature was pipetted into the central hole of the 50ml SepMate™ (Stemcell technologies) tube insert. Keeping the SepMate tube vertical, 25-30ml of diluted blood sample was gently pipetted down the side of each SepMate tube. The tubes were centrifuged at 1200xg for 15 minutes at room temperature (20°C), normal acceleration with the brake on.

After centrifugation, 4 distinct layers could be visible within the SepMate tubes: a diluted plasma layer, a buffy coat layer containing PBMCs, a Ficoll layer and an erythrocyte and granulocyte layer at the bottom of the tube. The SepMate tubes were inverted for no longer than 2 seconds in order to pour the upper layer containing the diluted plasma layer and buffy coat layers into new 50ml conical tubes without the Ficoll, erythrocyte and granulocyte layers contaminating this. In order to remove contaminating Ficoll, these conical tubes were topped up with PBS so that the final volumes within each tube were 50ml before being centrifuged at 400xg for 10 minutes. The supernatant was then discarded carefully as the bottom cell pellet could have still been quite loose. The cell pellets were gently homogenized in each tube and

amalgamated into one 50ml conical tube. Tubes were topped up with sterile PBS so that the final volume in the tube was 50ml and cells were mixed by gently inverting the tube five times. Cells were pelleted by centrifuging the 50ml tubes at 300xg for 5 minutes in order to wash off contaminating platelets. The supernatant was carefully discarded, and the cell pellet was homogenized. The cell suspension was then transferred into one 15ml conical tube. PBS was added to the tube so that the final volume was 10ml and cells were mixed by gently inverting the tube 5 times before being prepared for counting.

Cell suspensions were combined with 0.4% Trypan Blue stain in a 96 well plate and mixed well by pipetting in order to achieve an accurate cell distribution. An Olympus BX51 microscope (Olympus Microscopy) set to phase contrast and a counting haemocytometer (improved Neubauer haemocytometer) was used for counting. A coverslip glass was placed over the haemocytometer and 15µl of cell dilution was passed through one chamber of the haemocytometer via capillary action. To work out the total number of cells within the cell suspension the following formula was used:

$$\frac{\text{No. of cells counted}}{\text{No. of chambers counted}} \times \text{dilution factor} \times 10,000 \times \text{cell suspension volume (ml)} = \text{Total cell count}$$

If more than 3×10^7 PBMCs were counted, then monocytes were purified from these PBMCs using a column based positive CD14⁺ selection kit (Miltenyi Biotec Inc) as detailed in section 2.3.4. Four volumes of PBMC cell suspensions containing 5×10^5 cells were placed into 1.5ml graduated conical tubes to be later stored in RNeasy as detailed in section 2.3.5.

The remaining cells were prepared for cryopreservation. Cells were pelleted via centrifugation at 500xg for 5 minutes and the supernatant was completely removed. 1ml of 4°C heat inactivated ‘off the clot’ male human AB serum was added per 10^7

cells. Slowly, drop by drop, an equal volume of 4°C human AB serum containing 20% dimethyl sulfoxide (DMSO) (Sigma) was added to the cell suspension. Aliquots of 5×10^6 to 10^7 PBMCs were placed into cryovials and then placed into a precooled StrataCooler cryopreservation module (Agilent). The StrataCooler was placed in a -80°C freezer for a minimum of 12 hours before the cryovials were transferred to liquid nitrogen for indefinite storage.

2.3.4 Monocyte Isolation

Isolated PBMCs were strained through a 40µm nylon mesh (Fisher Scientific) before being centrifuged at 300xg for 10 minutes in order to remove any clumps and enable optimal flow within the cell separation columns. The supernatant was carefully discarded, and the cell pellet was resuspended in 80µl of sterile MACs buffer (PBS, 0.5% bovine serum albumin (BSA), 2mM EDTA) per 10^7 PBMCs, and 20µl of positive selection CD14 microbeads (Miltenyi Biotec) per 10^7 cells. The cell suspension was left at 4°C for 20 minutes. After 20 minutes, the beads were washed off by adding 10ml of sterile MACs buffer to the cell suspension and then centrifuging the cells at 300xg for 10 minutes. The supernatant was discarded, and the cell suspension was resuspended in 2ml of MACs buffer.

A LS column (Miltenyi Biotec) was attached onto a MidiMacs separator magnet (Miltenyi Biotec) placed onto a MACs multistand (Miltenyi Biotec). 3ml of MACs buffer was flushed through the column, 1ml at a time, in order to prepare the column and to ensure that it is functional. Once the column stopped dripping MACs buffer, the cell suspension was flushed through the column 1ml at a time. 3ml of MACs buffer was then added 1ml at a time to the column again in order to limit contamination. The column was removed from the magnet and placed over a 15ml tube. The column was rinsed with 3ml of MACs buffer, 1ml at a time, in order to collect the magnetically labelled CD14+ cells. The column was then topped up with 3ml of MACs buffer and the provided plunger was used to flush through the rest of the CD14+ cells out of the

column. Based on the cell count, volumes of CD14⁺ cell suspensions containing 5×10^5 cells were placed into 1.5ml graduated conical tubes to be later stored in RNAlater as detailed in section 2.3.5.

The purity for CD14 expression of the bead isolated cells was assessed by applying cell-surface flow cytometric analysis with the use of antibodies listed in Table 2-8. Purity was good, with about 98% of PBMCs expressing CD14 following CD14 positive bead isolation as highlighted in Figure 2.1A. Closer analysis of monocyte subsets via the gating strategy described in Figure 2.7 (which gates out cells which are not monocytes), however, revealed that positive CD14 bead isolation completely depleted the non-classical monocytes (CD14⁺CD16⁺) subset, as shown in Figure 2.1B.

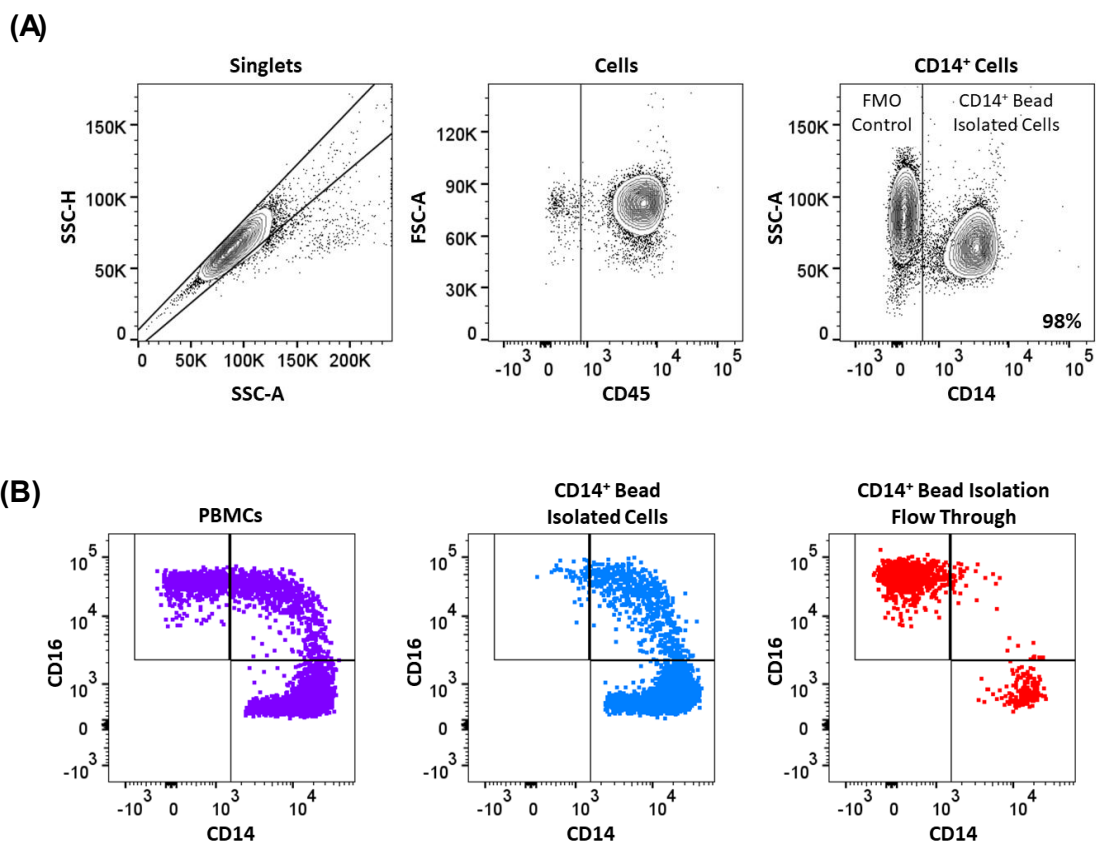


Figure 2.1: Separation of CD14⁺ cells from PBMCs using CD14 positive selection Microbeads.
(A) Flow cytometric analysis showing the purity of monocytes from CD14 positive bead isolation. CD14 positive bead isolated cells were initially gated for singlets, followed by CD45 to exclude debris. CD14 expression was compared against cells which were only stained for CD45 (FMO control) and revealed that 98% of cells were CD14 positive.
(B) Flow cytometric analysis examining monocyte subsets in PBMCs, CD14 positive bead isolated cells and the flow through of the bead isolation all from one donor. The gating strategy depicted in Figure 2.7 was used for all three plots. Analysis revealed the donor originally had visible amounts of classical, intermediate and non-classical monocytes (purple) but positive CD14 bead isolation depleted the non-classical monocytes (blue) as these were left in the dumped cells flow through (red).

2.3.5 Processing Cells to be Stored in RNAlater

Cell suspensions containing 5×10^5 cells in 1.5ml graduated conical tubes were centrifuged at 16,000xg for 5 minutes. The supernatant was then carefully aspirated before 200 μ l of RNAlater (ThermoFisher) was added to the pellet. Vigorous pipetting would allow the pellet to eventually resuspend in the RNAlater solution. This was left at room temperature for 2 minutes as a minimum before being transferred to 4°C for at least 18 hours. After this incubation time, samples were transferred to -80°C for indefinite storage.

2.4 Gene Expression Experiments

2.4.1 Extracting and Purifying RNA for Microarray & RT-PCR Applications

RNA was extracted from 16 pairs of pre and post fistuloplasty aliquots consisting of at least 5×10^5 CD14+ cells stored within 200 μ l of RNAlater using the RNeasy Mini kit (Qiagen) in accordance to the manufacturer's instructions.

900 μ l of PBS was added to the cells within RNAlater prior to being centrifuged at 10,000xg for 5 minutes. The supernatant was removed and 350 μ l of RLT buffer provided was added to the cells. The cells were vortexed for 15 seconds before being transferred to a QIAshredder silica membrane spin column and centrifuged for 4 minutes at 13,000xg in order to lyse the cells. In order to homogenise the lysate, an equal volume of 70% ethanol was added and vigorously pipetted up. The sample was then transferred to an RNeasy mini spin column and centrifuged for 30 seconds at 8,000xg. The flow through was discarded and 700 μ l of provided RW1 wash buffer, which removes non-specifically bound biomolecules such as fatty acids from the membrane, was added to the column. The column was then centrifuged at 8,000xg for 30 seconds and the flow-through was discarded. 500 μ l of RPE, a mild washing buffer which removes traces of salt from the column, was subsequently added to the column, which was then centrifuged at 8,000xg for 30 seconds. After discarding the flow-through, this wash step with RPE buffer was repeated. The column was then

centrifuged open at 13,000xg for 1 minute in order to dry the membrane of the column. Finally, the column was placed over a new collection tube. 22µl of RNase free water was added to the centre of the column's membrane, and the column was centrifuged at 8,000xg for 1 minute in order to elute the RNA. The eluted RNA was immediately stored at -80°C prior to microarray processing.

2.4.2 Purifying RNA for Microarray Applications

Genomic DNA contamination from the RNA was removed using the TURBO™ DNase kit (ThermoFisher). For each RNA sample, a 100µl mastermix containing 10µl of TURBO™ DNase, 10µl of 10X reaction buffer and RNase free water was centrifuged at 8,000xg for 10 seconds. The RNA samples containing the mastermix were then incubated at 37°C for 45 minutes. An equal volume of pH 5.2 phenol chloroform (Fisher Scientific) was then added to each sample. The samples were vortexed and left at room temperature for five minutes before being centrifuged at 10,000xg for 10 minutes. This treatment resulted into the samples dividing into two distinct layers, with the upper clearer layer containing the treated RNA. This layer was transferred into a Qiagen spin column available in the RNeasy Mini kit (Qiagen). After adding 120µl of 100% ethanol, the columns were centrifuged at 8,000xg for 15 seconds. The flow through was discarded and a subsequent three wash steps were carried out by centrifuging the columns with RPE buffer for 8,000xg for 15 seconds each time. Next, the column was left open in order to dry the membrane and then transferred to a new collection tube. 22µl of RNase free water was added to the centre of the column's membrane, and the column was centrifuged at 8,000xg for 1 minute in order to elute the purified RNA.

RNA integrity was assessed using Agilent's RNA 6000 Pico chip according to the manufacturer's instructions. Each Pico chip contains microfluidic channels that are used for the electrophoretic separation of the nucleic acid fragments based on their sizes. The gel matrix was prepared for this assay by vortexing 1µl of RNA dye with a 65µl gel aliquot and then centrifuging the mixture at 13,000xg for 10 minutes. 9µl of this

gel was added to the appropriate well of the chip and was plunged through the chip with a plunger within the chip priming station for 30 seconds. 9µl of gel and 5µl RNA marker was subsequently added to all the appropriate chip wells. Finally, 1µl of heat denatured RNA and RNA ladder was added to sample and ladder wells respectively. The chip was vortexed for a minute before being run on an Agilent 2100 Bioanalyzer for 20 minutes. The bioanalyser provided an RNA Integrity Number (RIN) value, a metric of total RNA quality ranging from 10 (highly intact RNA) to 1 (completely degraded RNA) for all the assayed samples. All samples had clearly visible 18S and 28S ribosomal peaks, and the disappearance of the additional peak between these ribosomal peaks confirmed the removal of the genomic DNA via the TURBO™ DNase kit (Figure 2.2).

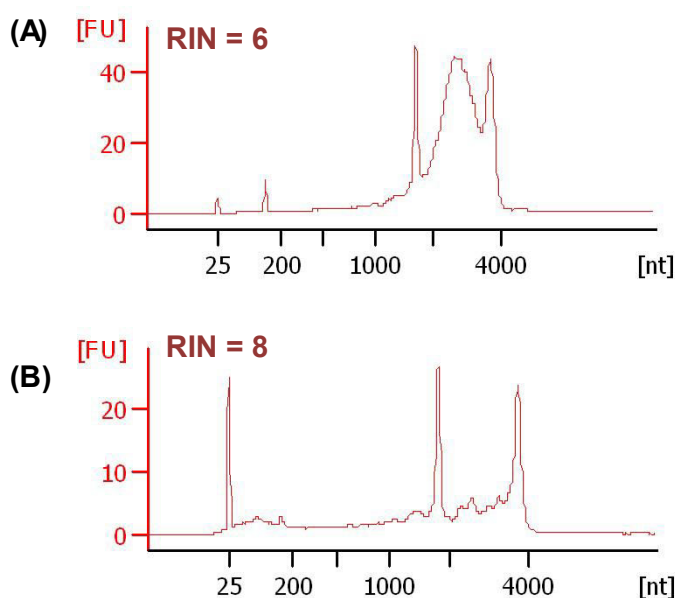


Figure 2.2: Representative figures to show quality analysis of RNA using the RNA 6000 Pico chip in the same RNA sample before and after DNA removal.

(A) Contaminating DNA is seen as an additional peak between the 18S and 28S ribosomal peak.

(B) Contaminating DNA is removed following DNase and chloroform treatment.

2.4.3 Synthesising and Amplifying cDNA for Microarray Applications

At least 4.6µg of RNA from each sample was converted into amplified single-stranded cDNA via the Ovation PicoSL WTA system Version 2 kit (NuGEN). 5µl of RNA sample and 2µl of First Strand Primer Mix was added to a 0.2ml PCR tube. Samples were thoroughly mixed before being heated to 65°C for 2 minutes to allow the primers to anneal to the RNA. Samples were cooled to 4°C before a 3µl First Strand Master Mix containing 2.5µl of First Strand Buffer Mix and 0.5µl of First Strand Enzyme Mix was added to each sample. Samples were thoroughly mixed before being heated to 25°C for 30 minutes, 42°C for 15 minutes, 70°C for 15 minutes, and then cooled to 4°C for first strand cDNA synthesis to occur. 10µl of Second Strand Master Mix was then made by combining 9.7µl of Second Strand Buffer Mix with 0.3µl of Second Strand Enzyme Mix. This was added to the samples before the samples were heated at 25°C for 10 minutes, 50°C for 30 minutes, 80°C for 20 minutes, and then cooled to 4°C for second strand cDNA synthesis to occur.

Solid-phase paramagnetic beads by Agencourt RNAClean XP Beads (Beckman Coulter) were then used to further purify the cDNA in accordance to the manufacturer's instructions. 32µl of brown bead suspension was added to each cDNA reaction mix and pipetted 10 times. The samples were incubated at room temperature for 10 minutes before being placed on the provided magnet for 5 minutes to separate the beads from the rest of the binding buffer. With the tubes still on the magnet, 45µl of clear binding buffer was then carefully removed from the bead solution. 200µl of freshly prepared 70% ethanol was then added to each of the samples and the samples were left in the magnet for a further minute. The ethanol was carefully removed, and this wash step was repeated a further two times. Beads were then air dried for 20 minutes to ensure that all residual ethanol was removed.

Following this, Single Primer Isothermal Amplification (SPIA) was carried out. This is a non-PCR based method that utilises a DNA chimeric primer to amplify the cDNA

appropriately for downstream microarray applications. All reagents for SPIA amplification were included in the Ovation PicoSL WTA system Version 2 kit (NuGEN). A 40µl SPIA Master mix was made by combining 20µl of SPIA buffer mix, 10µl of SPIA primer mix and 10µl of SPIA enzyme mix. This was added to the samples containing cDNA bound to the Agencourt RNAClean XP Beads. Samples were then heated to 47°C for 75 minutes, 95°C for 5 minutes, and then cooled to 4°C for SPIA to occur. The samples were then transferred to the magnet and allowed to stand for 5 minutes. This allowed the solution to form two distinct layers, the bead layer and the upper supernatant containing the eluted SPIA cDNA.

The eluted SPIA cDNA was further purified from the beads and previous enzymatic reactions using the MinElute Reaction Cleanup Kit (Qiagen). 300µl of the provided DNA stabilisation buffer ERC was added to and vortexed with each cDNA sample. The samples were then transferred to a spin column and washed by being centrifuged for 1 minute at 10,000xg with the flow through then being discarded. The remaining contaminating salts were then washed away by adding 500µl of the provided buffer PE and centrifuging the column for 1 minute at 10,000xg with the flow through then being discarded. This step was repeated before the column membrane was dried by centrifuging the column for 2 minutes at 10,000xg. The column tip was blotted on filter paper to remove residual wash buffer and then transferred into a collection tube. 20µl of room temperature nuclease free water was added to the centre of the column membrane and the column was centrifuged for 1 minute at 10,000xg in order to elute the purified cDNA.

2.4.4 Labelling cDNA for Microarray Applications

cDNA was assessed for quality via Agilent's RNA 6000 chip as previously described above and the profiles of all the cDNA samples were similar with most fragments being 150 nucleotides to 1500 nucleotides long, peaking around 200 nucleotides (Figure 2.3). At least 2µg of purified cDNA was biotinylated using the Encore Biotin IL module

(NuGEN). 5µl of uracil-DNA glycosylase (UNG) enzyme and buffer was added to each 25µl cDNA sample. Samples were thoroughly mixed before being heated at 50°C for 30 minutes and then being cooled to 4°C. This resulted in the creation of an abasic site in the cDNA strand. Following this 5µl of labelling buffer and labelling reagent was added to each cDNA sample. Samples were thoroughly mixed before being heated at 50°C for an hour and then being cooled to 4°C. This resulted in a biotin moiety chemically attaching to the previously created abasic site on each cDNA strand.

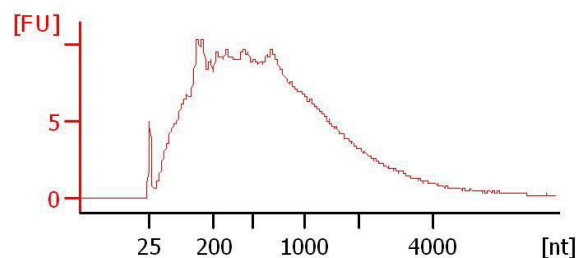


Figure 2.3: Representative figure to show quality analysis of cDNA using the RNA 6000 Nano chip. As expected, the most fragments of the cDNA were between 150 nucleotides to 1500 nucleotides long peaking at around 200 nucleotides.

Labelled cDNA was further purified with the MinElute Reaction Cleanup Kit (Qiagen) as previously described and was measured with a Nanodrop for cDNA concentration. 750ng of labelled cDNA was concentrated through the concentrator plus vacuum chamber (Eppendorf) by being heated at 45°C for 30 minutes and being resuspended in 5µL of DNase free water resulting in 5µl of 150ng/µL of cDNA ready for hybridization to the Illumina Whole Genome Expression BeadChip (Illumina).

2.4.5 cDNA Hybridization and Microarray Analysis

cDNA hybridization to the Illumina Whole Genome Expression BeadChip (Illumina) was carried out by Dr Paul Lavender (Respiratory Medicine & Asthma, King's College London, Guy's Hospital, UK). The chip was scanned using the Illumina iScan System and subjected to quantile normalization within the Genome Studio Suite v1.0 (Illumina). Expression data was analysed by Dr Paul Lavender using Partek Genomics Suite (Partek USA version 6.6) for the subsequently mentioned analysis of variance

(ANOVA), volcano plots and box and whisker plots detailing Vanin-1 gene mean expression. Ms Alessandra Bisquera (Population Health & Environmental Sciences, King's College London, Addison House, UK) carried out the Principal Components Analysis (PCA), and Multivariate distance matrix regression (MDMR) using the statistical programme R (v 3.3.3) using a statistical method as first described by McArdle and Anderson in 2001 [118].

2.4.6 Synthesising and Amplifying cDNA RT-PCR Applications

cDNA required for Reverse transcription polymerase chain reaction (RT-PCR) applications was transcribed using 100ng/ml of RNA per sample using the RevertAid™ First Strand cDNA synthesis kit (ThermoFisher). The RNA was incubated with cDNA mastermix containing 1µl of random hexamer primer, 4µl of 5X reaction buffer, 1µl of RiboLock RNase inhibitor, 2µl of 10mM dNTP mix, 1µl 200IU RevertAID H minus M-MuLVRT and the appropriate volume of nuclease free water to bring the final volume in each PCR tube to be 20µl.

The contents of each tube were mixed by pipetting up and down gently ten times. Tubes were incubated at 25°C for 5 minutes, and then 42°C for an hour before the reaction was terminated by heating the tubes to 70°C for 5 minutes. cDNA samples were stored at -20°C for no longer than four weeks before being used for downstream RT-PCR processes.

2.4.7 Optimisation of RT-PCR

RT-PCR was carried out to assess if the gene expression of calreticulin (CALR), Calcium-binding protein (S100)A4, S100A9, vannin-1 (VNN1) and vannin-2 (VNN2) would correlate with patient outcome as alluded to by previous microarray findings. Hydrolysis probes (TaqMan) covalently labelled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at its 5' end and a dark quencher dye at its 3' end was used for investigating these genes. A 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein

(VIC) labelled 18s rRNA probe was used as an endogenous control for the FAM labelled target genes. The probes used for qPCR are listed in Table 2-4:

Table 2-4: The probes used for RT-PCR.

Target	Taq Probe set ID	Probe label	Catalogue Number
CALR	Hs00189032_m1	FAM	4453320
S100A4	Hs00243202_m1	FAM	4453320
S100A9	Hs00610058_m1	FAM	4453320
VNN1	Hs01546812_m1	FAM	4448892
VNN2	Hs01100378_g1	FAM	4351372
Eukaryotic 18S rRNA Endogenous Control	-----	VIC™/MGB probe	4319413E

The general principle of RT-PCR is that the 5'-3' exonuclease activity of Taq polymerase cleaves the quencher dye from the fluorescent dye during hybridization to the complementary specific target sequence, which results in a fluorescent signal. As this fluorescence would directly correspond with gene amplification the fluorescence signal allows for the quantitative measurement of the accumulation of the product during the exponential stages of the PCR.

2.5µl of the 250ng/mL of cDNA was dispensed in triplicates on a 384 well plate followed by a Master Mix containing 5µl of Luna® Universal qPCR Master Mix (New England Biolabs), fluorescent dye labelled target gene primer and PCR grade water (ThermoFisher) with a final volume of 10µl. The plate was sealed with an adhesive PCR plate seal before being centrifuged at 200xg for 5 minutes to remove bubbles. PCR reactions were run on ViiA 7 RT-pPCR system (ThermoFisher Scientific) with the cycle following parameters: 1 minute at 95°C for one cycle, 45 cycles of 15 seconds at 95°C then 30 seconds of 60°C. Cycle threshold (Ct) values, the number of cycles required for the fluorescent signal to exceed the background level, were obtained.

These values were normalised against the endogenous reference gene control by subtracting the Ct of 18S rRNA with that of the gene of interest to obtain the Δ Ct value.

The RT-PCR assays carried out required some optimisation in order to run reliably. Initially as the endogenous control and the genes of interest were labelled with VIC and FAM respectively, multiplexing these probes was attempted. 2.5 μ l of cDNA, 5 μ l Luna® Universal qPCR Master Mix, 0.25 μ l of 18S probe, 0.25 μ l FAM probe and 2 μ l of PCR grade water was used, but this yielded improper amplification curves with a very low standard curve efficiency as shown in Figure 2.4A. In order to rule out the possibility that this was occurring due to cDNA quality, the same protocol was applied to freshly made healthy donor cDNA, however the same poor amplification pattern appeared again (Figure 2.4B). To evaluate if this was due to the concentration of probe within the master mix, different concentrations of 18S probe was applied, yet this made little to no impact as highlighted in Figure 2.4C. Finally, to evaluate if this was specifically due to incompatibility with the 18S probe and the selected FAM labelled probes, FAM labelled dectin-1 probe was multiplexed with 18S in both freshly made and patient cDNA. Multiplexing Dectin-1 with 18S revealed good amplification curves for both cDNA samples (Figure 2.4D), highlighting that the probes for the genes of interest were simply suboptimal when multiplexing with 18S. Thus, without multiplexing, 2.5 μ l of cDNA, 5 μ l of Luna® Universal qPCR Master Mix, 0.25 μ l of individual fluorescent dye labelled target gene primer and 2.25 μ l PCR grade water yielded optimal amplification plots (Figure 2.4E). A sample without cDNA was run as a control for cDNA contamination within the PCR reagents each time and revealed minimal contamination.

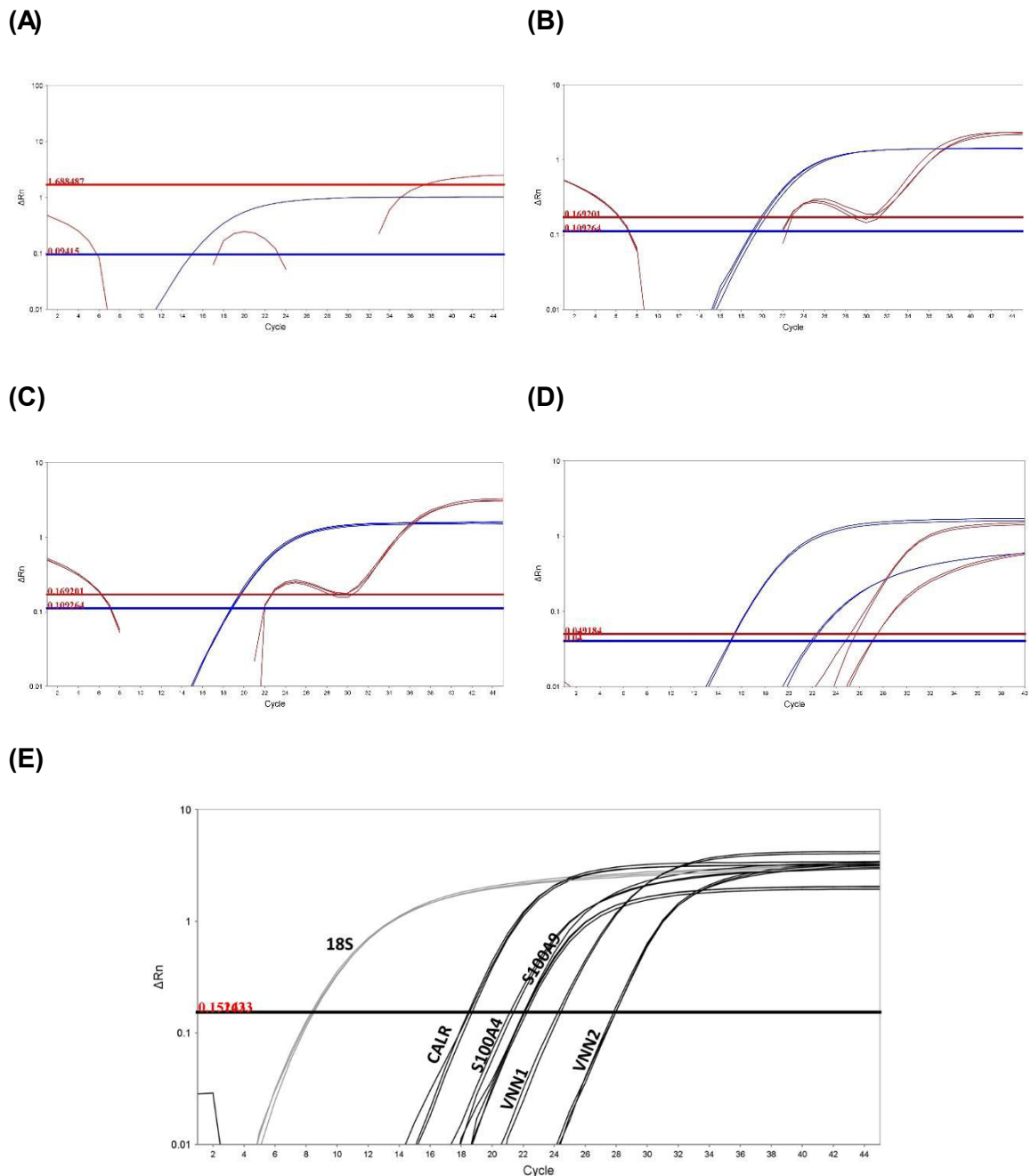


Figure 2.4: RT-PCR amplification plots depicting PCR optimisation.

(A) Amplification plot of multiplexing CALR with 18S using stored fistuloplasty patient cDNA. Blue line indicates 18S amplification curve, red line indicates CALR amplification curve.

(B) Amplification plot of multiplexing CALR with 18S using freshly made healthy donor cDNA. Blue line indicates 18S amplification curve, red line indicates CALR amplification curve.

(C) Amplification plot of multiplexing CALR with a lower concentration of 18S using freshly made healthy donor cDNA. Blue line indicates 18S amplification curve, red line indicates CALR amplification curve.

(D) Amplification plot of multiplexing Dectin-1 with 18S using freshly made healthy donor cDNA and stored fistuloplasty patient cDNA. Blue line indicates 18S amplification curve, red line indicates CALR amplification curve.

(E) Representative amplification plot revealing peaks of all probes used without multiplexing from a single stored fistuloplasty patient cDNA sample.

2.5 Protein Screen Experiments

2.5.1 Proteome Profiler

The proteome profiler human XL cytokine array kit (R&D), a membrane-based antibody array which enables the detection of 105 proteins via chemiluminescence, was used on four pairs of pre and post fistuloplasty derived plasma samples in order to indicate as to whether there were any changes in the levels of plasma cytokines and chemokines after the fistuloplasty procedure.

The kit was used according to the manufacturer's instructions. Using forceps, each nitrocellulose membrane containing 105 pairs of capture antibodies and control antibodies was carefully placed facing upwards into separate wells of a multi-dish. 2ml of blocking reagent Array Buffer 6 was subsequently added to each membrane. Whilst the membranes were incubated for one hour on a rocking platform shaker, the patient plasma samples were prepared by diluting 200µl of plasma in 1.3ml of Array Buffer 6. After an hour, Array Buffer 6 was aspirated from the wells of the multi-dish and the diluted plasma samples were pipetted onto the membranes. A lid was placed on the multi-dish and it was incubated overnight at 4°C on a rocking platform.

On the second day, each membrane was placed into a new multi-dish with 15ml of provided wash buffer and left on a rocking platform for 10 minutes. This was aspirated, and the washing step was repeated a further two times. In the meantime, Array Buffer 4/6 was prepared by adding 4ml of Array Buffer 4 to 8ml of Array Buffer 6. Subsequently, 30µl of detection antibody cocktail was diluted in 1.5ml of Array Buffer 4/6, thus preparing the detection antibody cocktail. After draining the wash buffer from the membranes, the membranes were incubated for one hour on a rocking platform shaker with the detection antibody cocktail.

The membranes were then washed a further three times with wash buffer as previously described. In the meantime, Streptavidin-HRP was prepared for each membrane by

diluting 1µl of Streptavidin-HRP in 1,999µl of Array Buffer 6. The membranes were then incubated with 2ml of the diluted Streptavidin-HRP for 30 minutes on a rocking platform shaker. Each membrane was then washed a further three times as previously described.

After blotting off excess wash from the membranes, each membrane was placed on a plastic sheet protector with the identification number facing upwards. A chemiluminescent detection reagent was prepared by pipetting 500µl of Chemi Reagent 1 to 500µl of Chemi Reagent 2. Each membrane was incubated with 1ml of detection reagent for one minute ensuring that the reagent mix spread evenly to all corners of each membrane. The membranes were covered with a plastic sheet protector and any air bubbles were gently smoothed out. The membranes were visualised under the Image quant LAS4000 mini (GE Healthcare Life Sciences) with exposure for at least 5 seconds and the exposure type set at precision. Image Studio lite version 5.2 (Licor) was used to quantify the positive signals on the developed images.

2.5.2 ELISAs

The ELISA kits that were used in this project and the respective plasma dilutions are listed in Table 2-5. All ELISAs were R&D Duoset ELISAs and were carried out in a 96 well flat bottom microplates (Nunc), were carried out according to the manufacturer's instructions and followed the same general protocol.

Table 2-5: Table showing the ELISA kits used.

Target	Dilution of Plasma	Substrate Used	Supplier	Catalogue Number
MPO	1:150	OPD	R&D	DY3174
M-CSF	1:4	OPD	R&D	DY216
Adiponectin	1:8000	OPD	R&D	DY1065
CRP	1:25000	OPD	R&D	DY1707
PDGF-BB	1:50	OPD	R&D	DY220
TfR	1:2000	1:1 Colour Reagent A & Colour Reagent B	R&D	DY2474
Calprotectin	1:2000	1:1 Colour Reagent A & Colour Reagent B	R&D	DY8226
VEGF	1:2	1:1 Colour Reagent A & Colour Reagent B	R&D	DY293B

In all instances, the reconstituted capture antibody was initially diluted to the working concentration in PBS. 100µl of working concentration capture antibody was pipetted into each well of the plate using a multichannel pipette. The plate was sealed and incubated overnight in the dark. The following day, each well was aspirated and 200µl of wash buffer (0.05% Tween 20 (Sigma) in PBS) was added to each well. The wash buffer was then aspirated off and the washing step repeated a further two times before the plate was blotted dry against clean paper towels in order to remove any unbound capture antibody.

The following day, to reduce the residual binding capacity of the wells, 300µl of 1% BSA diluted in PBS was added to each well of the plate. The plate was covered and incubated at room temperature for a minimum of an hour. After this incubation period, the plates were washed three times as previously described. ELISA standards and plasma samples were then diluted in 1% BSA. 100µl of the respective standards and samples were added to each well of the plate in duplicates before the plate was covered and incubated for two hours. Following the incubation period, each well was aspirated and washed three times. The detection antibody was diluted in 1% BSA and

100µl was added into each well. The plate was covered and incubated for a further two hours before the plate was washed as previously described.

100µl per well of *o*-Phenylenediamine dihydrochloride (OPD) (Sigma) or a 1:1 of colour Reagent A & Colour Reagent B (R&D) was used as the substrate for the ELISAs. Dependent on target protein concentration within approximately 30 minutes of the plate being left in the dark, OPD resulted in the solution within the wells turning from colourless to a shade of yellow, whilst 1:1 of colour Reagent A & Colour Reagent B resulted in the solution within the wells turning from colourless to a shade of blue. The reaction was stopped by the addition of 50µl 1M H₂SO₄ per well which turned wells yellow. The plate was then analysed using a SpectraMax Plus384 Microplate Reader (Molecular Devices) at wavelength 492nm. The mean averages for all standards and samples were taken and the optical density for the average zero standard was subtracted from all samples. A standard curve was generated using Prism (Graphpad), and all standard curves had an R² value of at least 0.98. The target protein concentrations were extrapolated from the standard curves and multiplied by the dilution factor used to give a final concentration.

2.5.3 Luminex Assays

Luminex assays are solid phase sandwich immunoassay which allows one to look for the concentrations of many proteins within the same sample. Magnetic or polystyrene microspheres are coated with a capture antibody for a protein of interest and are internally dyed with a corresponding red to infrared fluorophore a set intensity denoted as a 'bead region.' Therefore, multiple analyte-specific beads can be multiplexed so long as they do not have overlapping bead regions.

All Luminex assays carried out were MagPlex assays and were carried out in 96 well Luminex Bio-Plex Pro™ Flat Bottom Plates (Bio-Rad) according to the manufacturer's

instructions. All the provided reagents in vials were centrifuged 30 seconds at 1000xg followed by being vortexed for five seconds prior to their cap being removed.

Initially 10ml of the provided wash buffer was brought to room temperature and diluted in 240ml of deionised water. Plasma samples were diluted 1:1 with calibrator diluent, all except for angiogenin and PF4, which were diluted 1:200. Standards were diluted with calibrator diluent in a serial dilution of 1:3, or 1:4 for the TNF- α and IL-6 Luminex kit. The microparticle cocktail was prepared by mixing 500 μ l of microparticle cocktail with 5ml of diluent RD2-1 in a brown mixing bottle. 50 μ l of the diluted microparticle cocktail, standards and samples were then pipetted into each well of the plate. The plate was then securely covered with a foil plate sealer and incubated for 2 hours, or 3 hours for TNF- α and IL-6 Luminex kit, on a horizontal plate shaker set at 800 $\pm$ 50 RPM.

After 2-3 hours, the plate was placed onto a magnet for 2 minutes. 100 μ l of diluted wash buffer was placed into each well of the plate before the plate was tipped whilst the magnet was attached. This step was repeated a further two times. The biotin antibody cocktail was then prepared by adding 500 μ l of biotin antibody cocktail to 5ml of diluent RD2-1. 50 μ l of this diluted biotin antibody cocktail was then to each well. The plate was securely covered before being incubated for one hour on a horizontal plate shaker set at 800 $\pm$ 50 RPM.

During the incubation period, streptavidin-PE was prepared by adding 220 μ l of streptavidin-PE to 5.35ml of diluted wash buffer. After the incubation period, the plate was placed onto the magnet for two minutes before being washed three times as previously described. 50 μ l of diluted streptavidin-PE was subsequently added to each well. The plate was covered and incubated for 30 minutes on a horizontal plate shaker set at 800 $\pm$ 50 RPM before being washed as previously described.

On the final step, 100µl of wash buffer was added to each well and the plate was incubated for two minutes on a horizontal plate shaker set at 800±50 RPM. The plate was read within 30 minutes on FlexMap 3D (Luminex) with the analysis set to quantitative and the appropriate doublet discrimination values entered.

2.6 Human AVF Tissue Experiments

2.6.1 Tissue Sample Collection and Processing

Excised AVF tissue was collected in PBS and transported to the laboratory for processing within 1 hour of removal. Known areas of NIH were marked with blue tattoo ink (Figure 2.5A). Samples were longitudinally cut open allowing for regions containing excess fat and aneurysm to be optimally visualised and discarded (Figure 2.5B). The remaining segments were cut into 1cm cubes (Figure 2.5C) and placed into 10% formalin overnight. The AVF samples were then placed into a mild decalcifier-solution, Osteosoft® (Sigma) until they reached a rubber-like consistency exhibiting weak resistance, which was usually reached within 18 hours.

In addition to excised AVF tissue, surplus saphenous vein tissue was collected from patients undergoing coronary artery bypass graft (CABG) operations at St. Thomas hospital. All veins were transported in PBS to the central laboratory for processing within 2 hours of harvest. Each vein was placed into a sterile 100mm x 15mm petri dish filled with PBS where it was cleaned of excess adipose tissue using a sterile scalpel blade. The ends of each vein were discarded by removing at least 2mm of tissue to account for potential trauma during harvesting. Varicose veins and sections of veins containing branching regions and valves were also excluded.

Samples were placed into an automated tissue processor in order to be prepared for wax embedding. AVF tissue was embedded upright so that the adventitia, media and intima layer would be evident in each microscope slide.

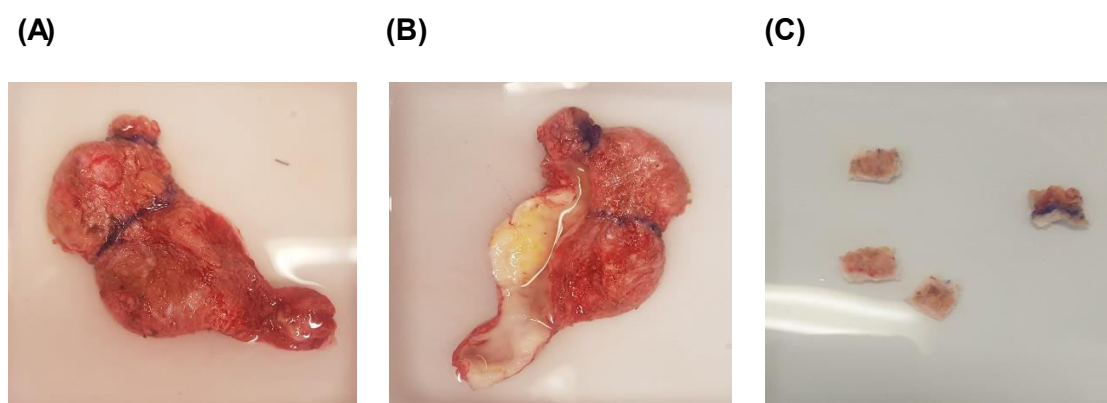


Figure 2.5: Processing excised AVF tissue.

(A) An excised AVF tissue with known regions of NIH marked with blue tattoo ink.

(B) An excised AVF tissue cut open to reveal a yellow region of fat.

(C) 1cm explants of AVF tissue ready for tissue processing.

Paraffin embedded sections were cut at 1 μ m thickness using a Leica RM2235 microtome and were baked onto the slides at 60°C for a minimum of one hour before staining. Polysine coated slides (ThermoScientific) were used for immunohistochemistry procedures.

2.6.2 Haematoxylin and Eosin Staining

Haematoxylin and Eosin (H&E) staining is widely used to visualise the general distribution of cells within a tissue's structure. Nuclei are stained blue via the action of haematoxylin whilst cytoplasm and extracellular matrix components are counterstained shades of pink via the action of eosin. Slides were placed in xylene (Fisher Scientific) for ten minutes in order to dissolve the wax. Tissues were rehydrated through being submerged in 1:1 xylene: 100% ethanol, 100% ethanol, 90% ethanol, 70% ethanol for five minutes each. They were subsequently washed in deionised water for five minutes. The slides were then treated with Mayer's Haematoxylin (Sigma) for 30 minutes and washed with running tap water for 5 minutes. Slides were placed into Eosin 1% aqueous (ThermoFisher) for 5 minutes. They were dehydrated through being placed in 100% ethanol, 1:1 xylene: 100% ethanol and then xylene. Coverslips were mounted on sections using Dibutylphthalate Polystyrene Xylene (DPX) mounting media (Sigma).

2.6.3 Modified Elastin Staining

In order to accurately quantify the degree of NIH, a reliable protocol for histologically distinguishing each layer of the vessel had to be established. The protocol established was using a Modified Verhoeff Van Gieson Elastic Stain Kit (Sigma) according to the manufacturer's instructions with minor modifications. Slides were deparaffinised in xylene, followed by rehydration in decreasing concentrations of ethanol to deionised water as previously described in 2.6.2. Slides were overstained in working elastic stain solution (55.5% alcoholic haematoxylin, 8.3% ferric chloride solution, 22.2% weigert's iodine solution, 14% deionised water) for at least 30 minutes before being rinsed in deionised water. Elastic fibres were then differentiated by being placed in ferric chloride solution, washed in running tap water for 2 minutes and then placed in 95% ethanol for a further 2 minutes. Importantly, it was essential to check each section under the microscope to observe the differentiation of the internal elastic lamina from the background staining as the internal elastic lamina has notoriously weak elastin staining. Finally, slides were counterstained in Van Gieson solution which stained collagen fibres red and other components yellow before being placed in 100% ethanol for 1 minute to wash off the excess Van Gieson solution. Slides were placed into xylene and then permanently mounted using DPX.

2.6.4 Immunohistochemistry

Tissues were placed in xylene and rehydrated as previously described in 2.6.2 before being prepared for antigen retrieval. The antibodies used for immunohistochemistry and the required antigen retrieval technique needed for each antibody is listed in Table 2-6.

Table 2-6: Immunohistochemistry antibodies and the corresponding antigen retrieval technique carried out

Marker	Clone	Format	Supplier	Catalogue Number	Antigen Retrieval Method
aSMA	1A4	Mouse Monoclonal	DAKO	MO851	Heat induced
SMMHC	SMMS-1	Mouse Monoclonal	DAKO	M3558	Heat induced
Vimentin	V9	Mouse Monoclonal	DAKO	M0814	Heat induced
Desmin	D33	Mouse Monoclonal	DAKO	M0616	Heat induced
vWF	F8/86	Mouse Monoclonal	DAKO	M0725	Heat induced
CD34	QBEnd 10	Mouse Monoclonal	DAKO	A0398	Heat induced
CD68	KP1	Mouse Monoclonal	DAKO	M7165	Heat induced
TNF-α	28401	Mouse Monoclonal	R&D	MAB610	Enzymatic

The formalin-fixed tissues all required an antigen retrieval step for optimal staining. Without antigen retrieval, primary antibodies were prevented from being able to bind to the corresponding antigen due to the fixation process causing methylene bridges to form over the antigen binding sites. Antigen retrieval refers to any technique that breaks these methylene bridges thus exposing the tissue's antigenic sites to primary antibodies.

For heat induced antigen retrieval, slides were placed into 50ml falcon tubes filled with 35ml of pre-warmed Target Retrieval Solution Ready-to-use (Dako). They were placed in a water bath at 80°C for 20 minutes and then the tubes were removed from the water bath and allowed to slowly cool at room temperature for a further 20 minutes. For enzymatic antigen retrieval, a drop of Proteinase K (Dako) was dropped onto the slides for 10 minutes. Slides were subsequently washed in deionised water within a glass trough containing a magnetic stirrer for 3 minutes. All subsequent washes utilised a magnetic stirrer.

Slides were blocked with normal horse serum (vector laboratories) for 20 minutes before being washed in deionised water for 3 minutes. 30% peroxidase block (Sigma) was diluted to 3% in deionised water before being added to the slides for at least 5 minutes. Following this, slides were washed 3 times with PBS for 3 minutes each before 200µl of the appropriate primary antibody diluted 1:200 in PBS was added on per tissue section. Slides were incubated in a condenser for one hour before being washed twice in PBS for 3 minutes each.

Following this, the appropriate secondary antibody was placed on for 20 minutes with the slides protected from the dark. For monoclonal antibodies this was utilised by adding a drop of manufacturer readymade ImmPRESS HRP Anti-Mouse IgG (Vector laboratories). Slides were then washed three times with PBS for 3 minutes each time before the appropriate substrate was placed on. For monoclonal antibodies this was with a Sigmafast 3'3-Diaminobenzidine (DAB) tablet set (sigma) dissolved in 5ml of deionised water. Slides were observed under the microscope for colour change before being washed in deionised water three times for 3 minutes each time. Following this, slides were placed in Mayer's Haematoxylin (Sigma) for 40 minutes and then were washed in deionised water for 3 minutes. Samples treated with the DAB substrate were rehydrated and coverslips were mounted using DPX.

Cells within fistula tissue regions were counted manually within three independent representative 400X field magnification views with blinding against which tissue was being examined.

2.7 FACs Experiments

2.7.1 PBMC subsets and Monocyte Subsets FACs

Fluorochrome conjugated antibody stain solutions of 100µl per sample were prepared in Eppendorf tubes using the panel of antibodies listed in Table 2-7 for investigating

PBMC subsets and in Table 2-8 for investigating monocyte subsets from cryopreserved PBMC samples.

Table 2-7: Fluorochrome conjugated antibodies used for FACs analysis of PBMC subsets from cryopreserved PBMCs.

Marker	Fluorochrome	Clone	Amount used in 100µl	Supplier	Catalogue Number
CD14	APC	M5E2	2µl	Becton Dickinson	555399
CD19	FITC	HIB19	12µl	Becton Dickinson	555412
CD3	PerCP	OKT3	1µl	Biolegend	317335
CD8	BV510	SK1	2µl	Biolegend	344731
γδ	PE	B1	3µl	Biolegend	331209
NKp46	Alexa Fluor 700	9E2	2µl	Biolegend	331931

Table 2-8: Fluorochrome conjugated antibodies used for FACs analysis of monocyte subsets from cryopreserved PBMCs.

Marker	Fluorochrome	Clone	Amount used in 100µl	Supplier	Catalogue Number
CD14	APC	M5E2	2µl	Becton Dickinson	555399
CCR2	FITC	K036C	2µl	Biolegend	357215
CD3	PerCP	OKT3	1µl	Biolegend	317335
CD19	PerCP-C5.5	SJ25C1	2µl	Biolegend	363015
γδ TCR	PerCP-C5.5	GL3	1µl	Biolegend	118117
NKp46	PerCP-C5.5	9E2	2µl	Biolegend	331919
CD66b	PerCP-C5.5	G10F5	2µl	Biolegend	305108
CD16	BV510	3G8	2µl	Becton Dickinson	563829
HLA-DR	PE	L243	2µl	Biolegend	307606
CD11b	Alexa Fluor 700	ICRF44	2µl	Biolegend	301355

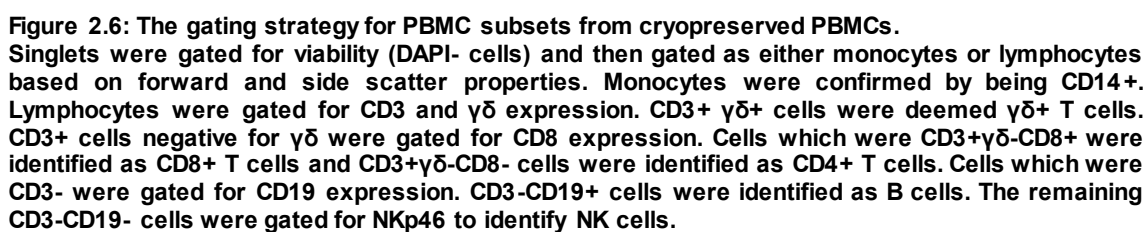
Cyrovials containing 5×10^6 - 10^7 PBMCs were placed into the waterbath set to 37°C and defrosted for 1 minute. Drop by drop, add the cells were added to 12ml of Roswell Park Memorial Institute (RPMI) 1640 Medium before being spun at 400xg for 5 minutes. The supernatant was discarded, and cells were resuspended in 15ml of PBS before being spun at 400xg for 5 minutes. Cells were resuspended in 1ml of PBS and transferred into FACs tubes. 1µl of Fixable Viability Dye eFluor™ 450 was added to the 1ml cell suspensions within the FACs tubes for live dead staining. The tubes were then incubated for 15 minutes at room temperature in the dark. The tubes were topped up with 1ml of FACs buffer and centrifuged at 400xg for 5 minutes.

Cells were resuspended in 200µl of FACs buffer and plated onto round well 96 well plates (Nunc). The plate was spun at 300xg for 5 minutes and the supernatant was removed by tipping the plate. Fluorochrome conjugated antibody stain solutions of 100µl were added to each of the wells and the plate was incubated for 30 minutes at room temperature in the dark. The wells were topped up with 100µl of FACs buffer before being spun at 200xg for 10 minutes. The supernatant was discarded, and cells were suspended in 100µl of FACs buffer. Cell suspensions were transferred to FACs tubes and topped up with FACs buffer so that their final volume was 500µl. Compensation controls were carried out using anti-mouse Ig.k/negative control compensation beads (Becton Dickinson) and was carried out simultaneously with PBMC staining. One drop of positive and negative beads was placed into FACs tubes and was stained with 100µl of single stain antibody mix and was incubated for 30 minutes at room temperature in the dark. The tubes were topped up with 2ml of FACs buffer before being spun at 200xg for 10 minutes. The supernatants were discarded and FACs tubes and topped up with FACs buffer so that their final volume was 500µl.

FACs tubes were protected from the light and left at 4°C before being acquired on the LSRT Fortessa™ and FACSDIVA software (Becton Dickinson) within 3 hours acquired at

a low speed with at least 10,000 events live cell events being recorded for each condition.

The gating strategy for identifying different PBMC subsets is depicted in Figure 2.6. Cells were initially gated for singlets based on side scatter area (SSC-A) and side scatter height (SSC-H). Dead cells were separated from live cells based on Viability Dye eFluor™ 450 staining detected on the Pacific Blue channel. Generous gates were drawn on monocyte cells and lymphocyte cells based on these subpopulation's characteristic forward and side scatter properties. Potential monocytes were gated for CD14 expression and the percentage of CD14⁺ cells were used for the monocyte percentages. Cells within the lymphocyte gate were gated for CD3 and $\gamma\delta$ expression. CD3⁺ $\gamma\delta$ ⁺ cells were deemed as $\gamma\delta$ ⁺ T cells. CD3⁺ $\gamma\delta$ ⁻ cells were further gated for CD8 expression of which cells which were CD3⁺CD8⁺ were CD8⁺ T cells, whilst cells which were CD3⁺CD8⁻ were assumed to be CD4⁺ T cells. The cells which were CD3⁻ $\gamma\delta$ ⁻ cells were further gated for CD19 expression. CD3⁻CD19⁺ cells were noted as B cells, whilst the remaining cells were gated for NKp46. The cells which were CD3⁻CD19⁻NKp46⁺ were regarded as NK cells.



96

to 2% enabling the optimal presentation to differentiate between the different monocyte subset populations

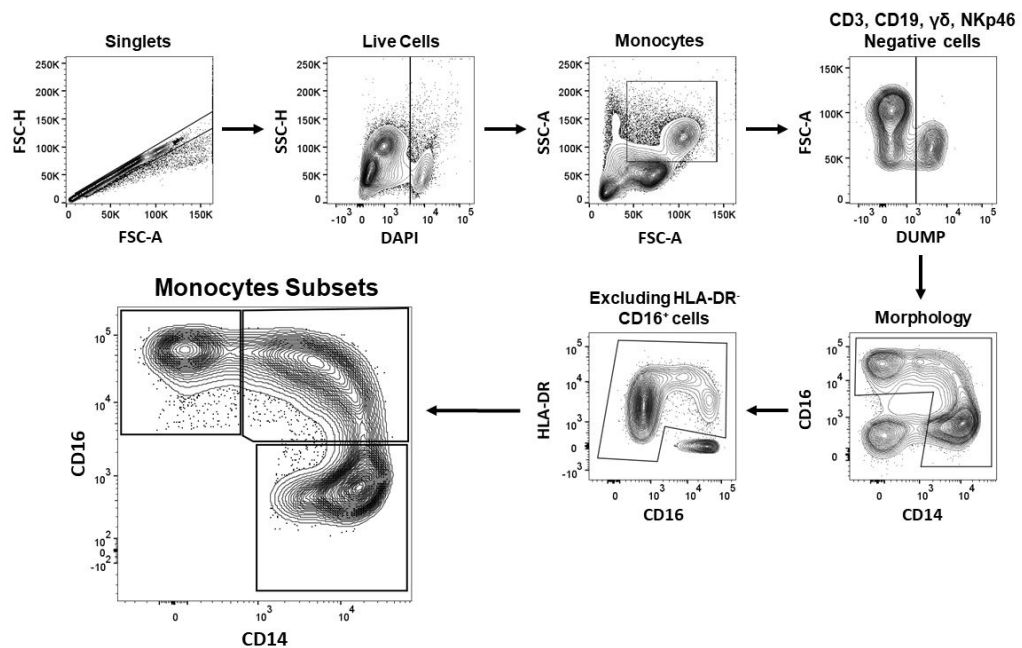


Figure 2.7: The gating strategy for monocyte subsets from cryopreserved PBMCs. Singlets were gated for viability (DAPI- cells) and then gated as potential monocytes by drawing a generous gate based on forward and side scatter properties. CD3+, CD19+, γδ+ and NKp46+ cells were dumped. The remaining cells were then gated based on monocyte's characteristic morphology when gated for CD14 and CD16. Contaminating cells were further removed by gating HLA-DR with CD16. Monocyte subsets were then gated based on CD14 and CD16 expression.

2.7.2 Whole blood FACs for Monocyte Subsets & Neutrophils

In order to further investigate whether any changes in monocyte subsets was immediate and to be able to get absolute cell numbers whole blood flow cytometry examining was carried out using counting beads. 4ml EDTA blood samples were taken from patients during the following time points:

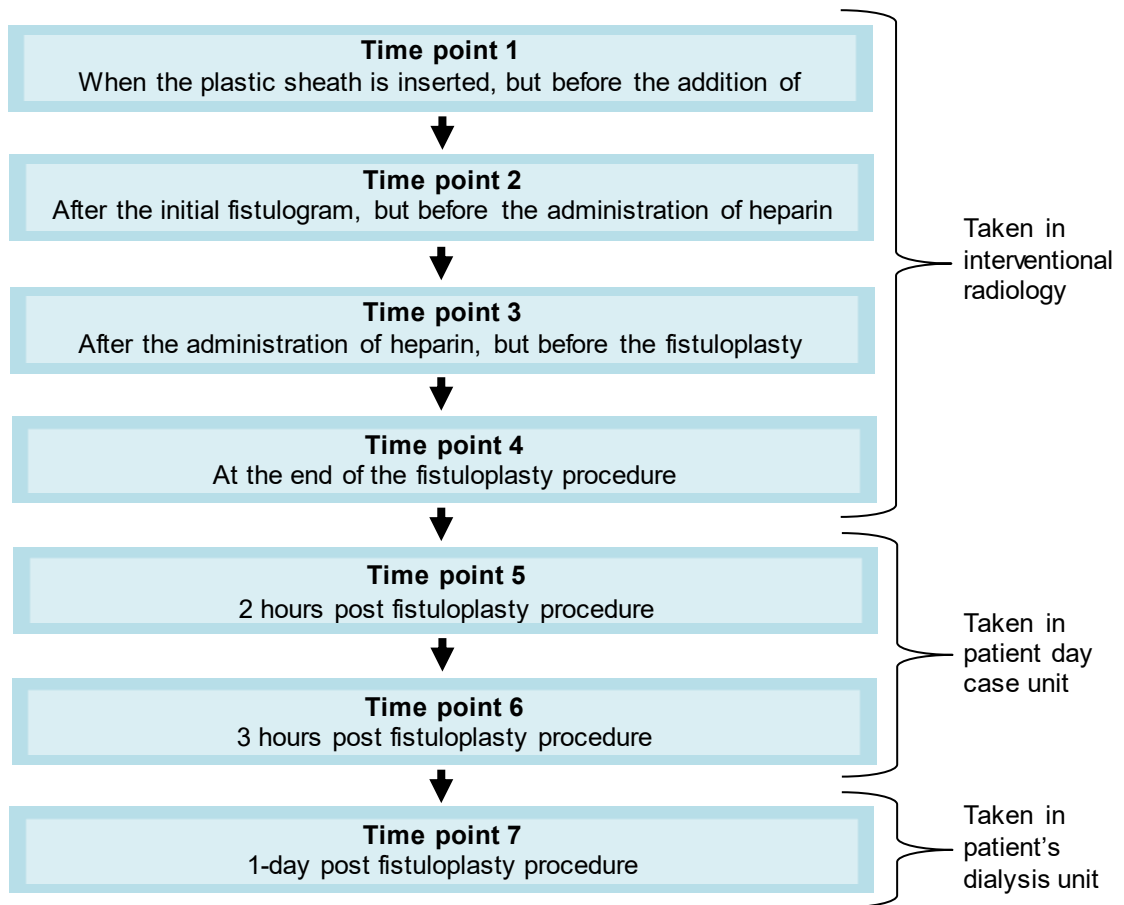


Figure 2.8: Time points for collecting blood from patients undergoing a fistuloplasty
4ml of EDTA blood was collected from patients at each of the following time points.

Exactly 100µl of whole blood was pipetted carefully through the metal retainer of the Absolute Counting Tubes (Becton Dickinson). Fluorochrome conjugated antibody stain solutions of 100µl per sample were prepared in Eppendorf tubes using the antibodies listed in Table 2-9 and FACs buffer (0.5% BSA, 5mM EDTA 1XPBS). 100µl of these antibody solutions was then pipetted to the 100µl of blood within the Absolute Counting Tubes.

Table 2-9: Fluorochrome conjugated antibodies used for FACs analysis of whole blood monocyte subsets and neutrophils.

Marker	Fluorochrome	Clone	Amount used in 100µl	Supplier	Catalogue Number
CD14	Alexa Fluor 647	M5E2	2µl	Biolegend	367128
CD16	BV510	3G8	2µl	Biolegend	302048
CD66b	Alexa Fluor 700	G10F5	2µl	Biolegend	305103
NKp46	FITC	9E2	2µl	Biolegend	331921
CD11b	FITC	ICRF44	4µl	Biolegend	301356
CD3	FITC	UCHT1	4µl	Biolegend	300440
CD19	FITC	H1B19	4µl	Becton Dickinson	555412
HLA-DR	BUV395	G46-6	4µl	Becton Dickinson	564040
CCR2	BV421	48607	4µl	Becton Dickinson	564067
CD45	BV786	HI30	5µl	Becton Dickinson	563716

FACs tubes were vortexed briefly before being incubated at room temperature for 20 minutes protected from the light. 900µl of working concentration 1-step fix/lyse solution (ThermoFisher) was then added to each tube. Tubes were vortexed for 20 seconds before being incubated for 15 minutes at room temperature in the dark. Tubes were vortexed once more before being stored at 4°C in the dark before being run on an LSRFortessa™ with FACsDIVA software (Becton Dickinson). Compensation controls were carried out simultaneously with samples with compensation beads as described in the previous section. Whilst acquiring samples on the Fortessa, a P1 stopping gate was drawn around the counting bead population and a threshold was set on BV786 to 500 to reduce signal noise from non-cellular events. At least 5000 bead events were collected from each fully stained tube at the low acquiring speed, with the same number of bead events collected from samples from the same donor.

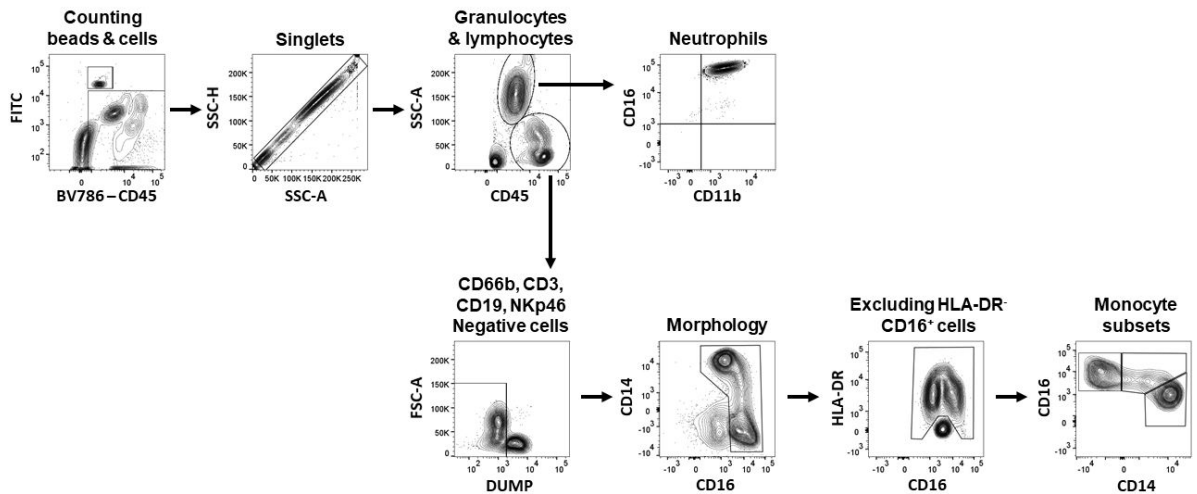


Figure 2.9: The whole blood gating strategy for neutrophils and monocyte subsets.

Counting beads were identified based on their high FITC intensity whilst cell events were identified based on CD45 expression. Cells were gated for doublet exclusion and then separated based on side scatter morphology with CD45. Large cells were gated as CD11b+CD16+ were deemed to be neutrophils. Lymphocytes were dumped for CD66b+, CD3+, CD19+, NKp46+ cells. The remaining cells were gated based on monocyte's characteristic shape when gated for CD14 and CD16. Contaminating cells were further removed from the remaining monocyte population by gating out HLA-DR low CD16 high expressing cells. Monocytes were gated for their subsets based on CD14 and CD16 expression.

The gating strategy used is depicted in Figure 2.9. Initially counting beads and cellular events were gated for based on the beads having a high FITC intensity and cellular events being CD45+. Next, doublet cell exclusion was carried out based on a side scatter area SSC-A and side scatter height SSC-H. Singlet monocyte and granulocyte populations were generously gated for based on side scatter properties when gated for CD45 expression. The cell count events for neutrophils were collected from the CD11b+ CD16+ cells within the granulocyte drawn gate as these markers removed the few contaminating eosinophils and basophils within the neutrophil population. Cells which were gated as potential monocytes based on their CD45 and side scatter properties were subsequently dumped for CD66b+, CD3+, CD19+, NKp46+ expression. The remaining cells were gated based on characteristic “ γ ” morphology when gating for CD16 and CD14 expression. A CD16+HLA-DR- NK cell population was then removed from the remaining monocytes by gating for CD16 and HLA-DR expression. The pure monocytes remaining were gated for CD14 vs. CD16 to reveal

the different subset populations. The absolute number of cell populations was calculated using the following formula:

$$\text{Cells per } \mu\text{l of blood} = \frac{\left(\frac{\text{No. FACS cell events counted}}{\text{No. FACS bead events counted}} \right) \times \left(\frac{\text{Supplier provided no. of beads per tube}}{\text{Test volume (1100 } \mu\text{l)}} \right)}{\text{Amount the blood diluted was by (11)}}$$

2.8 Statistics

The specific statistical tests used within each experiment are stated within the relevant results sections. Microarray expression data was analysed using Genomics Suite (Partek Inc. version 6.6) by Dr Paul Lavender and Ms Alessandra Bisquera using the statistical programme R (version 3.3.3). Cox regression analysis and Kaplan-Meier analysis was carried out in IBM SPSS statistics (version 26). Further details to the multivariate cox regression statistical model are in section 2.8.1. All other statistics was carried out using GraphPad Prism (GraphPad software version 8.1) with p values of less than 0.05 being considered as statistically significant.

2.8.1 Multivariate Cox Regression

Multivariate analysis was carried out in order to seek for patterns and relationships between several variables simultaneously and in case associations were being made undetectable due to a larger influence of a clinical variable on our data set. For all multivariate analysis, a manually carried out Forward (Step-Up) Selection was used due to the large number of variables and high possibility of co-linearity. The below describes the step method:

Step 0: Initially a univariate cox regression analysis is carried out whereby each of the x variables (be it a clinical variable such as the log age of fistula, or something measured within the chapter, such as plasma levels of IL-6) one by one are fitted against patency outcome. The variable with the lowest p value is selected and denoted as var1.

Step 1: A multivariate cox regression analysis is carried out whereby each of the remaining (x - var1) variables are combined with var1 and is one by one are fitted against patency outcome.

The variable with the lowest p value in combination with var1 is selected and denoted as var2 (so long as the p value is less than the STOP value).

Step 2: A multivariate cox regression analysis is carried out whereby each of the remaining (χ - var1 and var2) variables are combined with var1 and var2 and are one by one are fitted against patency outcome. The variable with the lowest p value in combination with var1 and var2 is selected and denoted as var3 (so long as the p value is less than the STOP value).

Step 3: A multivariate cox regression analysis is carried out whereby each of the remaining (χ - var1, var2 and var3) variables are combined with var1, var2 and var3 and are one by one are fitted against patency outcome. The variable with the lowest p value in combination with var1, var2 and var3 is selected and denoted as var4 (so long as the p value is less than the STOP value).

The steps are repeated until there are no more variables with a p value of less than the STOP value and thus no more variables can be selected for the next step.

When a variable enters this model, it cannot be deleted during the step phase. The STOP p value was set either to 0.1 if there was significance in the univariate analysis or 0.15 if there was no significance in the univariate analysis. Whilst higher than a traditional p value of 0.05, it is important to note that multivariate analysis by definition is exploratory analysis and hence a STOP p value of 0.05 – 0.2 is common in the literature.

Co-linearity, (which is when the correlation between variables is high enough to say that the variation in one variable explains the variation in the other) is problematic in regression models because when predictor variables in the same regression model are correlated, they cannot independently predict the value of the outcome. Hence, co-linearity reduces the statistical significance of regression models. As a result of this, following the initial selection of predictor variables found in the multivariate cox regression, a correlation matrix is carried out to show the correlations between the variables of interest. If two predictor variables correlate with one another, the variable that was first selected in the multivariate cox regression analysis is kept and the other variable is discarded.

In addition, whilst multivariate analysis can show if a nominal variable (like ethnicity, a variable which has two or more categories but does not have an intrinsic order such as how patient age would) associates with outcome, it would not be able to show which

ethnicity did better specifically. Hence, Kaplan-Meier curves were drawn for the all the nominal variables studied (gender, ethnicity and type of native fistula). These curves are in Appendix B – Kaplan-Meier Curves for Patient Clinical Nominal Data.

2.9 Research Ethics

Tissue samples, healthy donor and fistuloplasty time course blood samples were collected under “the Dialysis and Vascular Access Study” ethics, approved by South Central Berkshire B Research Ethics Committee (REC reference: 15/SC/0764). All other blood samples were collected using the ethics for the PAVE clinical trial, approved by London-Chelsea Research Ethics Committee (REC reference: 15/LO/0638).

Chapter 3 – Results

3.1 Investigating the Histopathology of Venous Stenosis in Dysfunctional AVFs

3.1.1 Introduction

An arteriovenous fistula (AVF) is created by connecting an artery and a vein together through anastomosis. During a successful maturation process, both the arterial and the venous segments of the AVF undergo vascular outward remodelling. The muscular media layer of the venous segment thickens, and the lumen of both the artery and the vein dilates. Neointima hyperplasia (NIH) in an AVF post fistuloplasty very rarely occurs outside of this venous segment.

Both arteries and veins are comprised of three distinct layers: the intima, the media and the adventitia. The intima is the inner most layer of the blood vessel and under homeostasis is a single layer of continuous endothelial cells supported by a subendothelial layer of connective tissue and supportive cells. The media is the middle layer of the blood vessel and is often separated from the intima by the internal elastic lamina, which is prominent in arteries, but very thin in large veins or absent in small veins. The internal elastic lamina is a cross-linked polymer of tropoelastin which is largely secreted by smooth muscle cells (SMC)s and fibroblasts. It helps provide elasticity and hemodynamic stress resilience to the vessel. It is also thought to be an important regulator in outward remodelling in AVFs during maturation [119]. The media is comprised of SMCs, elastic and connective tissues arranged circularly around the vessel. In arteries this layer is thick, whilst in veins this layer has only a few layers of smooth muscle cells dependent on the size of the vessel. In AVFs, however, due to the arterialisatation process during maturation, the media layer of the vein resembles that of an artery. The adventitia is the outermost layer of the vessel. It is often separated from the media via the external elastic lamina, but this is again very thin in large veins and absent in small veins. The adventitia is comprised almost entirely of loose irregular

connective fibres such as collagen and elastic fibres. There are a few cells present in the adventitia and they are namely fibroblasts which are smooth muscle actin (SMA) positive. Blood micro vessels known as vasa vasorum (literally meaning vessels of vessels in latin) penetrate the adventitia episodically with the main function of providing sustenance for the blood vessel.

When NIH occurs, histologically there is an increase in the size of the intimal layer as a consequence of an increase of SMA expressing cells within the layer. If the extent of NIH is great, eventually the luminal diameter of the vessel cannot be compensated for by outward re-modelling and a separate process of luminal narrowing occurs. The result of this is a decrease in luminal space, which may result in a reduction in blood flow and to the development of thrombosis. Descriptions of NIH were noted well over 100 years ago, with Nobel prize winning Alexis Carrel describing bi-terminally transplanted vein grafts as becoming “enormously thickened” after 15 days [120].

There are many studies suggesting that patients with chronic kidney disease (CKD) are pre-disposed to developing NIH in their AVF due to the complications of CKD including uraemia and oxidative stress. Alpers *et al* carried out histology on vein segments collected during AVF creation used for anastomosis enrolled in the Hemodialysis Fistula Maturation Study, which was aimed at identifying predictors of fistula maturation failure. They found that 88% of the 365 veins studied already had some degree of NIH, with the 20% of luminal area loss because of overgrowth of the intima in nearly 60% of cases [72]. This overall highlighted the contribution of baseline effects of CKD in developing pre-existing vascular abnormalities in patients on dialysis.

Both AVFs which develop stenosis post fistuloplasty or fail to mature have NIH as their main pathology. Roy-Chaudhury *et al* histologically examined the stenotic venous segments from four patients with AVFs that failed to mature adequately, with early AVF failure in the paper being defined as an AVF that could not be used for dialysis or failed

within the first three months of use. Tissues were examined for SMA, vimentin and desmin expression in order to distinguish contractile smooth muscle cells from myofibroblasts, with myofibroblasts being defined as vimentin positive and desmin negative in the paper. Roy-Chaudhury *et al* define the NIH of these AVFs as primary being composed of myofibroblasts in an eccentric orientation, with very few contractile smooth muscle cells [53].

Whilst the composition of the NIH in AVFs that failed to adequately mature may look like this, it is uncertain whether the NIH composition of an AVF post fistuloplasty would be highly similar or very distinct. It has been shown that pre-existing or postoperative NIH does not increase the risk AVF maturation failure [121] [122]. Using a pig model of AVF maturation failure, Chan *et al* describes how the majority of the cells within the stenotic lesions of the AVF at 7 days is comprised of active myofibroblasts, and suggests that these cells migrated into the intima layer from the adventitia [123]. Duque *et al* hypothesises operative trauma to the adventitia resulting in early adventitial activation is a major contributing factor to AVF maturation failure, which is less likely to be important in later patency failures [122]. Hence, whilst NIH may be common in AVFs with primary failure and secondary failure, the pathophysiology resulting in these lesions is likely to differ greatly.

The NIH lesion which starts to develop in an AVF post fistuloplasty in humans is poorly defined histologically as treated functional AVFs are rarely excised. Recently a group developed a clinically relevant model of angioplasty in mice with an AVF to be able to investigate histologically the short term effects of a fistuloplasty procedure [124]. To replicate CKD, mice underwent a partial nephrectomy. 28 days subsequent to this they had an AVF created from the right external jugular vein to the left carotid artery. Two weeks subsequent to this, the mice either had an angioplasty or a sham procedure. Mice were sacrificed two weeks after this procedure for histological evaluation. The group found that those mice that had the angioplasty rather than the sham procedure

had significantly less NIH implying that the procedure had treated the NIH that had started to form following the AVF formation. Angioplasty treated animals also had significantly less monocyte or macrophage infiltration (defined by CD68 expression), a disrupted internal elastic lamina and a decrease in cell density which they noted implied acute cell death and vascular remodelling following an angioplasty procedure. Whilst interesting, the study is of limited use in understanding the late NIH changes that would have occurred in the vessel as the follow up period post fistuloplasty was too short. There are also clear dissimilarities between murine and human AVF haemodynamics which could have altered the NIH disease process.

Overall, little is known about alterations in structural compartments of the stenosis in an AVF post fistuloplasty and its potential pathophysiologic significance. For this reason there is a real need to further investigate the histopathology of venous stenosis in dysfunctional human AVFs.

3.1.2 Aims

The aim of this chapter was to histologically assess excised AVF tissue. Surgically removed stenotic fistulas were collected from four patients who had well established NIH. Haematoxylin and eosin (H&E) and modified elastin staining was carried out as well as immunohistochemistry for smooth muscle actin (SMA), von Willebrand Factor (vWF), smooth muscle myosin heavy chain (SMMHC), vimentin, desmin, CD34 and CD68.

3.1.3 The Extent of Fibrosis within the Lesion Varies from Patient to Patient

Four AVF excised tissues were obtained for histological investigation. AVF1 and AVF2 were basilic vein transposition fistulas whilst AVF3 and AVF4 were radio-cephalic fistulas.

Histologically, NIH was defined as the region between the endothelial layer and the internal elastic lamina, which rather than being a single cell layer thick as seen in normal tissue (Figure 3.1) would be grossly enlarged. Initially a modified elastin stain was carried out on the AVF tissues in order to visualise the NIH to further investigate (Figure 3.2). AVF1-4 all had large and highly visible degrees of NIH. Interestingly, the size of the NIH between AVF1-2 was very similar, likewise to the size of the NIH between AVF3-4. As tissue sections were cut in serial sections, all subsequent stains were cross referenced against the modified elastin stained slides.

Fibrosis, histologically defined as an accumulation of extracellular matrix proteins and a decrease in cellularity, is the prevalent event in late stage NIH growth. Hence, next, H&E staining was carried out in order to determine the levels of fibrosis within the NIH region (Figure 3.3). The intima of AVF1 had many cells with rounded nuclei. In contrast, the intima of AVF2-4 had few cells and those cells largely had elongated nuclei. Thus, even in the end stage of NIH development in the AVF, the extent of fibrosis within the lesion can greatly vary from patient to patient.

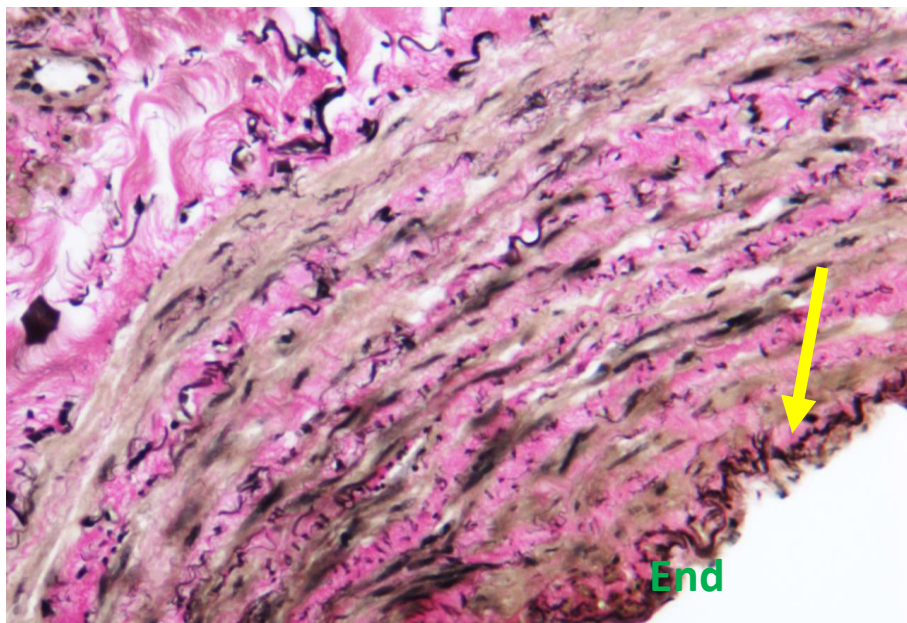


Figure 3.1: Modified elastin staining of a saphenous vein (X400 magnification). Elastic fibres are shown in navy. Collagen fibres are red and other components as yellow. 'End' indicates the endothelial layer. Yellow arrow indicates the internal elastic lamina.

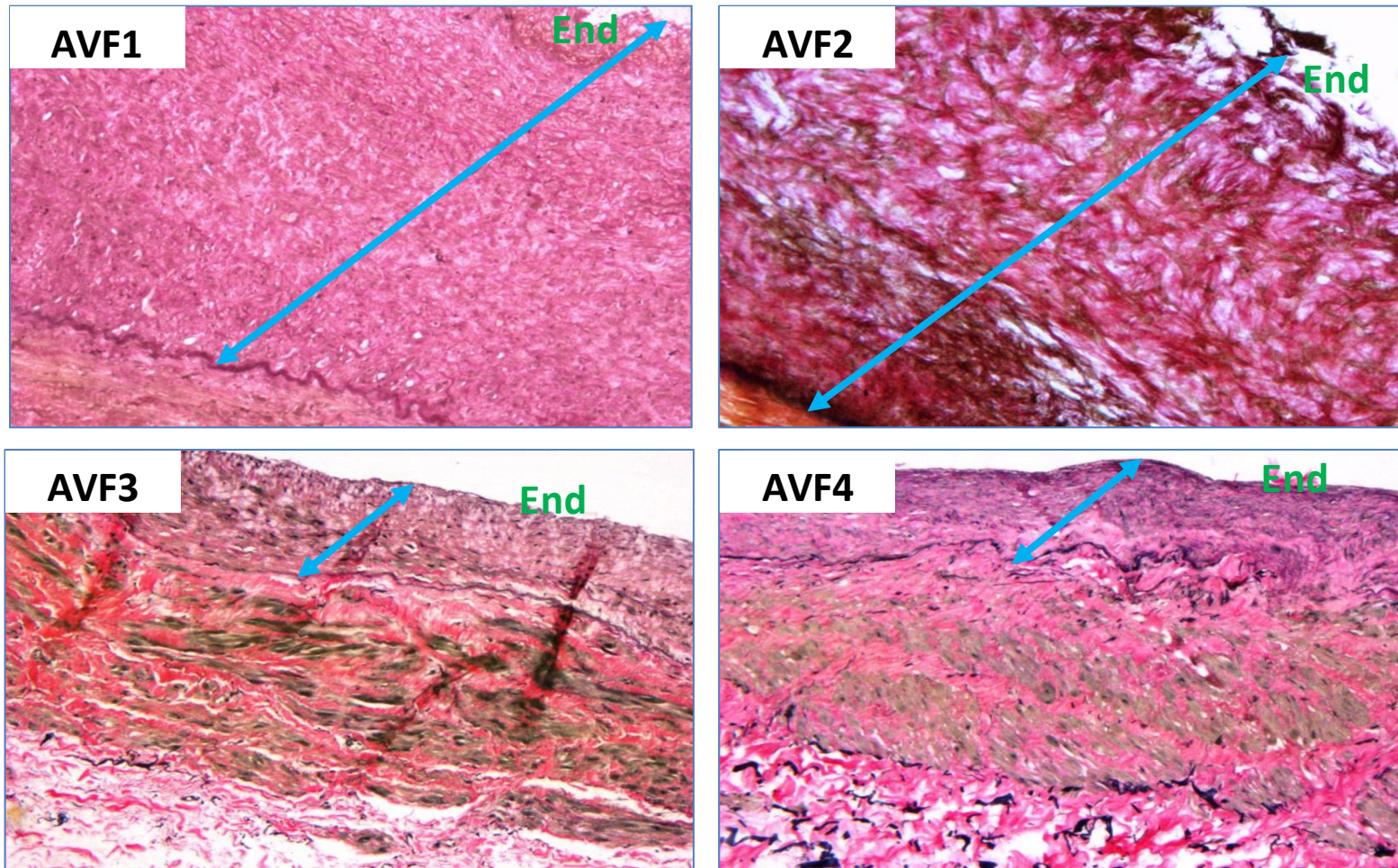


Figure 3.2: Modified elastin staining of four AVF tissue's intima sections (X100 magnification). Elastic fibres are shown in navy. Collagen fibres are red and other components as yellow. 'End' indicates the endothelial layer. Blue arrows indicate the NIH region. Note the variations in degree of NIH between the four AVF tissues.

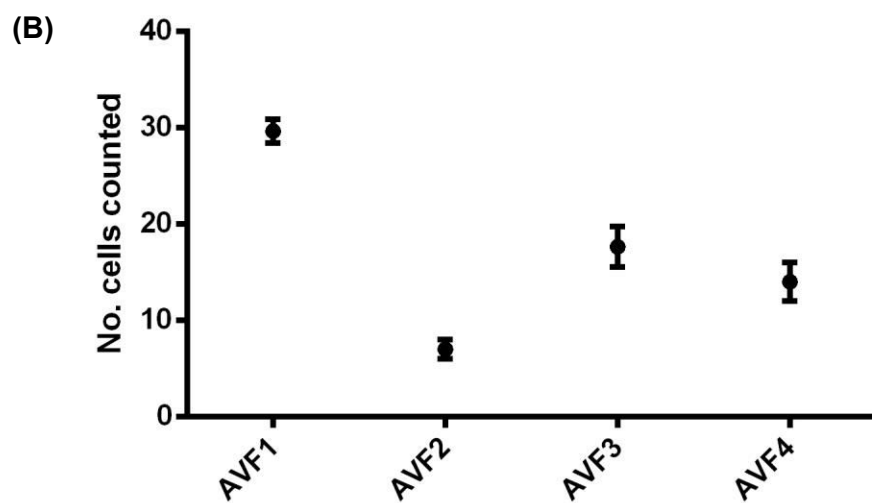
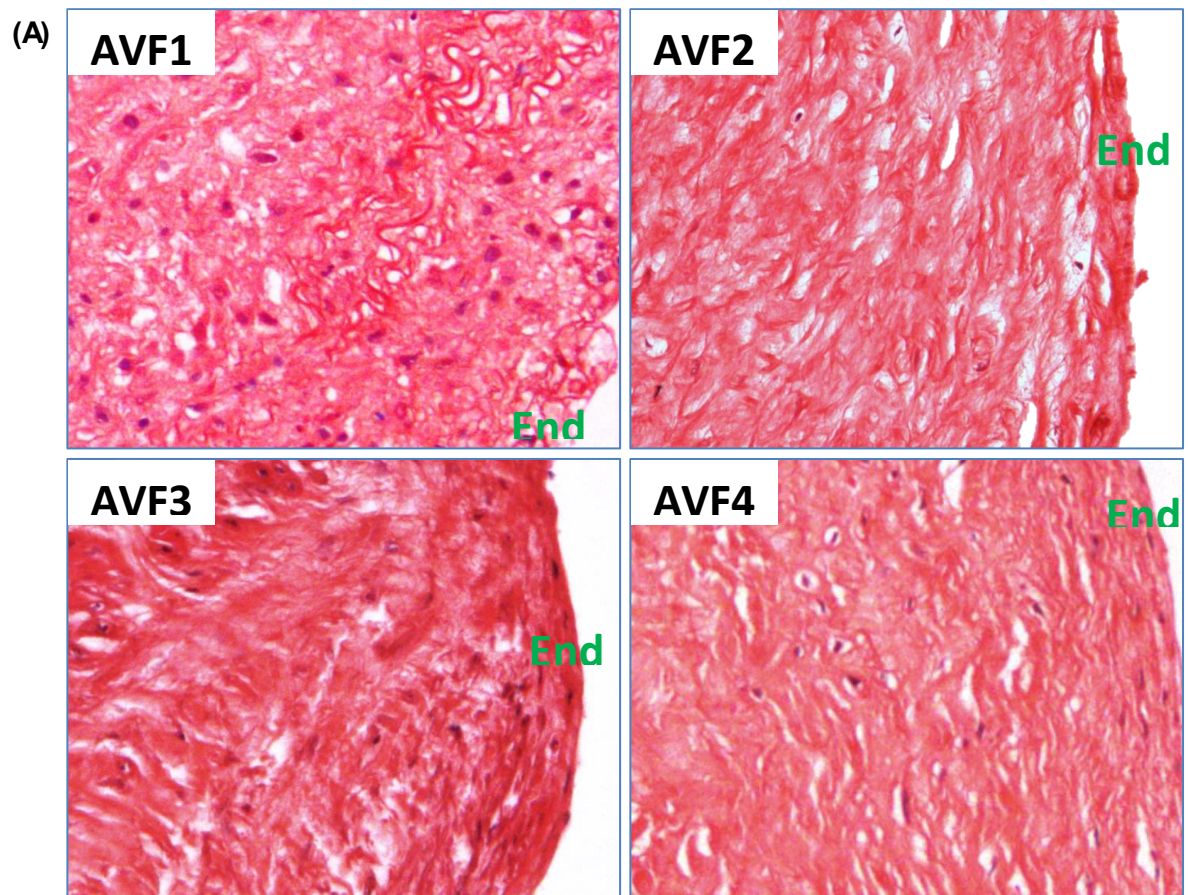


Figure 3.3: The degree of fibrosis within the intima section varies across four AVF tissues.

(A) Haematoxylin and Eosin (H&E) staining of four AVF tissues intima section (X400 magnification). Nuclei are stained blue via the action of haematoxylin whilst cytoplasm and extracellular matrix components are counterstained shades of pink via the action of eosin. 'End' indicates the endothelial layer. Note the variations in cellularity in the intima between the four AVF tissues.

(B) The quantification of nucleated cells within four H&E AVF tissues intima in an X400 view field. Cells within three independent representative field views of X400 were counted. Data are mean ± SEM.

3.1.4 The Primary Composition of the Late Stage NIH Lesion within AVF Tissue are Myofibroblasts

Next I aimed to characterise the primary cell composition of the late stage NIH within our AVF tissue. Each time immunohistochemistry was carried out, a control was carried out in which no primary antibody was used, but all other steps, including antigen retrieval and the secondary antibody placement, was used. For each antibody, staining was present in at least one segment of the AVF tissue, showing that the staining procedure had worked. In addition to this, long saphenous venous tissue that had little NIH was used as control for the primary antibody for CD34, CD68, SMA, SMMHC, vimentin and vWF as shown in Figure 3.4.

The staining patterns for vWF, SMA, SMMHC, desmin and vimentin in the adventitia, media and intima was consistent for all four AVF tissues and so only representative images are shown for these stains (Figure 3.5 and Figure 3.6). In all instances, little evidence of non-specific staining was found. As expected, staining for vWF revealed that endothelial cells expressing vWF lined the intima of the AVF and the vasa vasorum in adventitia, whilst there was no evidence for vWF staining in the media. SMA and vimentin staining is present throughout the vessel, though the degree of staining is less so in the adventitia and intima due to there being less cellularity in these regions in comparison to the media. In contrast, desmin stained around the vasa vasorum in adventitia and in the media, but the only few cells within the lesion were positive for desmin. Similarly, SMMHC stained around the vasa vasorum in adventitia, but very few cells within both the media and the lesion were positive for SMMHC.

The staining for the fistula tissue were similar to the staining patterns of the healthy saphenous vein tissue, with the exception of CD34, CD68, SMMHC and vimentin. The immunohistochemistry for CD34 and CD68 staining was not consistent between the four AVF tissues as discussed in section 3.1.5. In the saphenous vein, there was staining for SMMHC within the intima and not for vimentin, contrasting the AVF tissue's

intima layer. The findings here imply that the neointima of stenosed AVFs are primary composed of myofibroblasts based on the majority of the cells expressing SMA and vimentin, but not desmin and SMMHC, whilst myofibroblasts are rarely present in healthy vein tissue intima layer. Those cells which did express desmin and SMMHC are likely to be contractile smooth muscle cells and are unlikely to be the main contributor to NIH development.

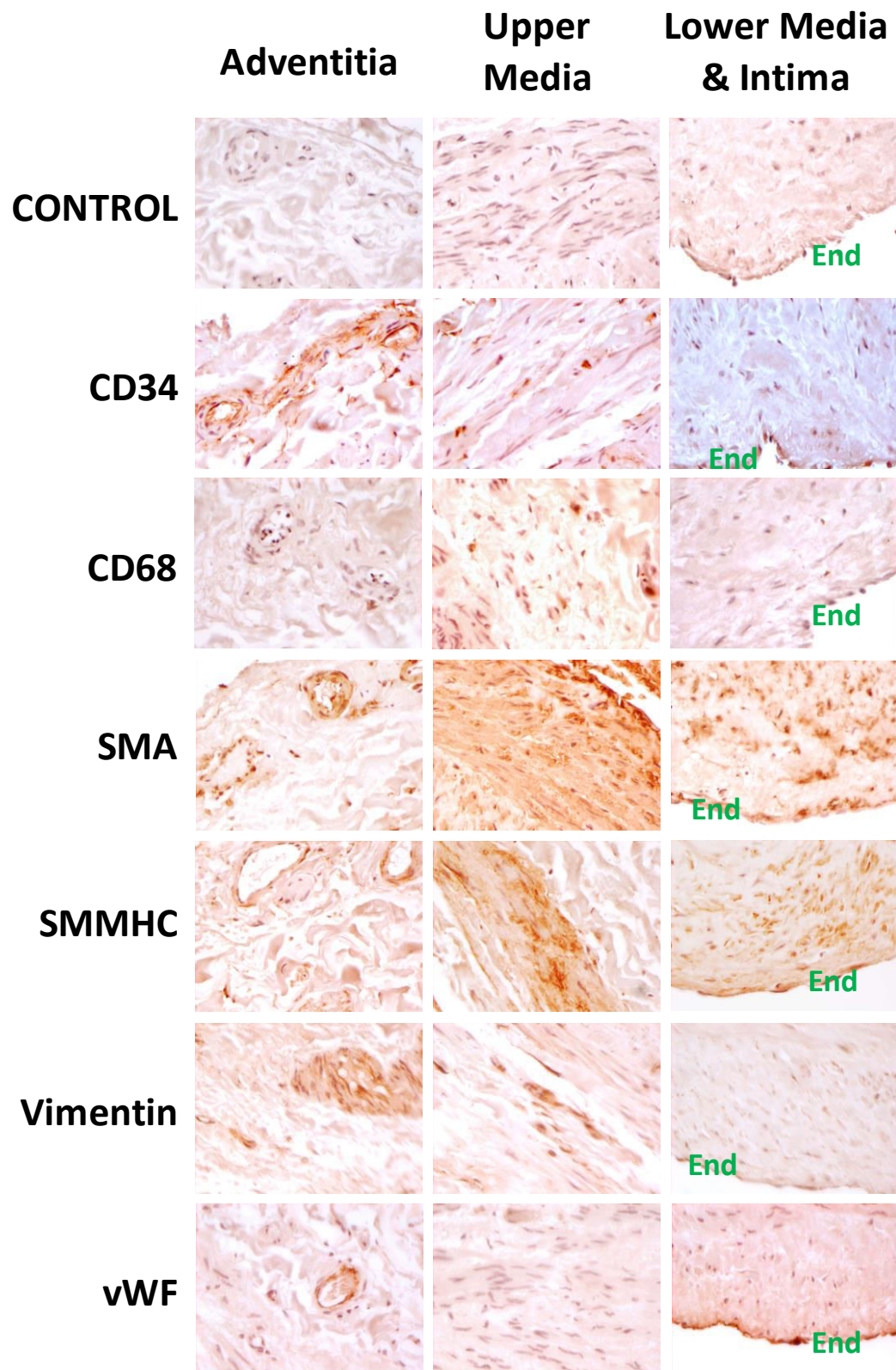


Figure 3.4: Immunohistochemistry characterising the adventitia, upper media and lower media with intima layer in a healthy saphenous vein tissue (X400 magnification). Images showing representative immunohistochemistry staining for the control (staining carried out without a primary antibody), CD34, CD68, Alpha smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC), vimentin and von Willebrand factor (VWF). The adventitia, media and intima layer sections for each stain are derived from the same saphenous vein tissue. 'End' indicates the endothelial layer.

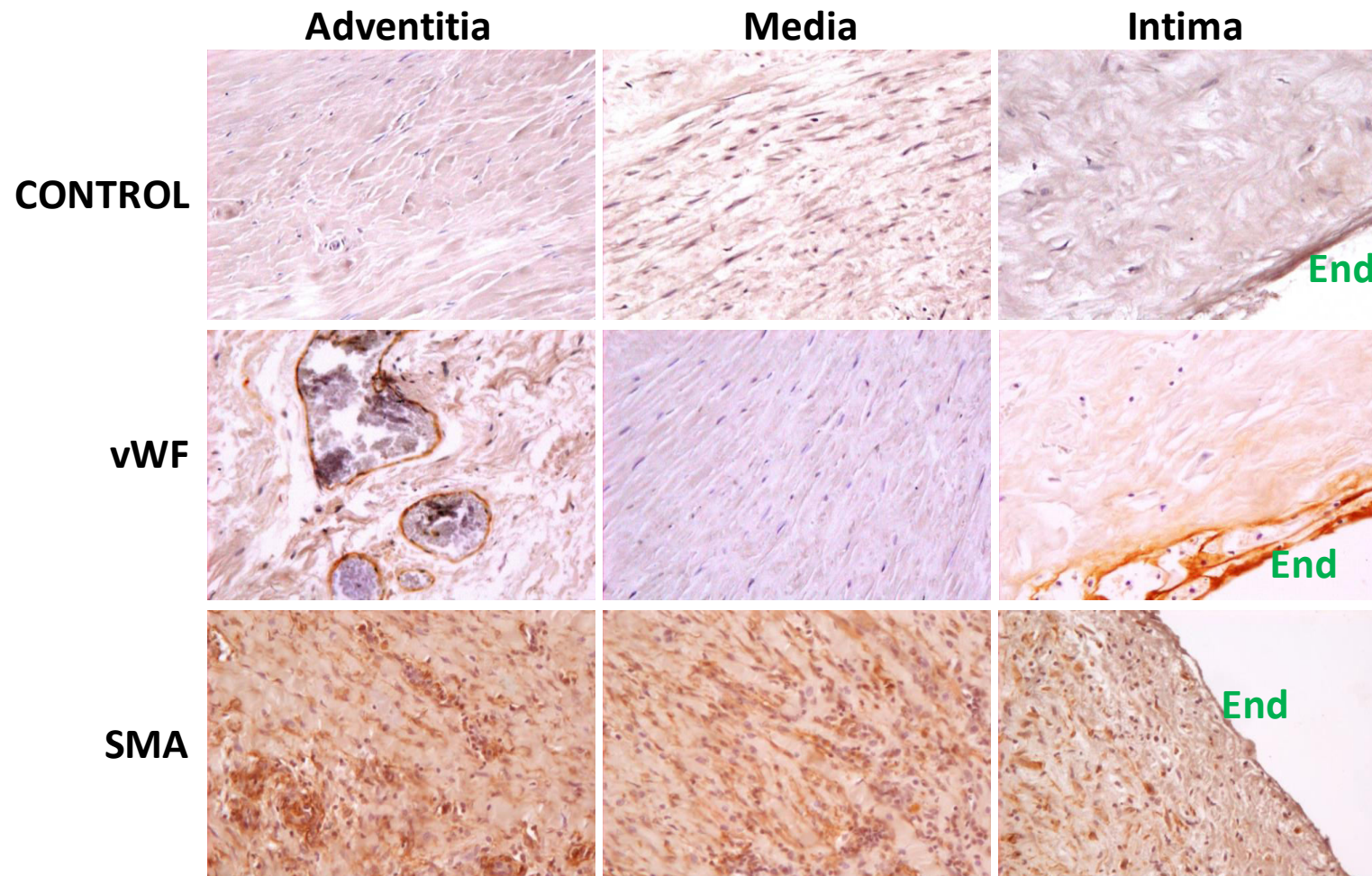


Figure 3.5: Immunohistochemistry characterising the adventitia, media and intima layer in representative AVF tissue (X200 magnification). Images showing representative immunohistochemistry staining for the control (staining carried out without a primary antibody), von Willebrand factor (VWF) and alpha smooth muscle actin (SMA). The adventitia, media and intima layer sections for each stain are derived from the same fistula tissue. 'End' indicates the endothelial layer.

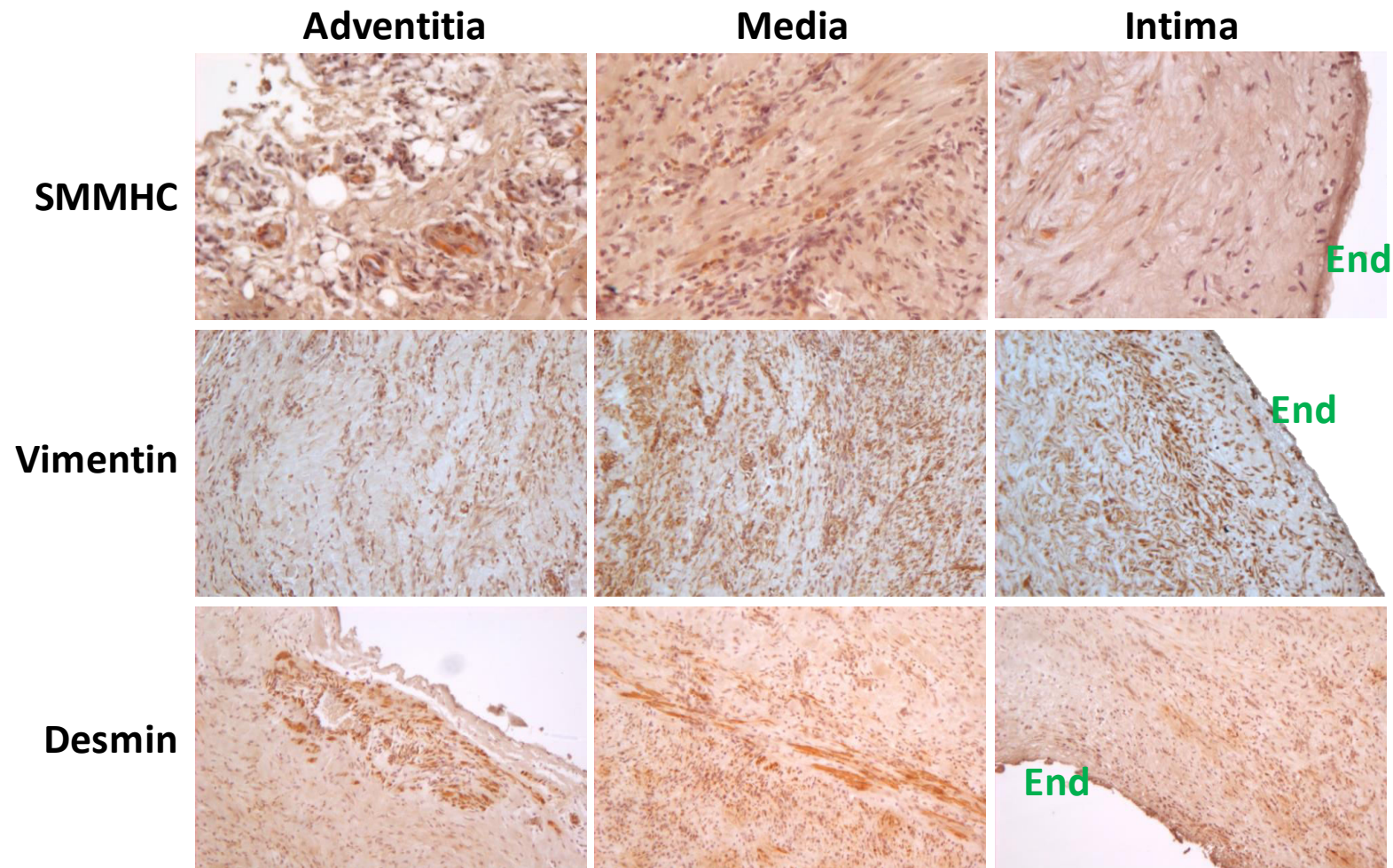


Figure 3.6: Immunohistochemistry characterising the adventitia, media and intima layer in representative AVF tissue (X200 magnification). Images showing representative immunohistochemistry staining for smooth muscle myosin heavy chain (SMMHC), vimentin and desmin. The adventitia, media and intima layer sections for each stain are derived from the same fistula tissue. 'End' indicates the endothelial layer.

3.1.5 The Origin of the Myofibroblasts may vary from Patient to Patient

Unlike the staining for vWF, SMA, SMMHC, vimentin and desmin as discussed in the previous section, the immunohistochemistry for CD34 and CD68 staining was not consistent between the four AVF tissues.

CD34 is a marker for cells of hematopoietic stem cell origin, including for vascular endothelial progenitor cells. CD34 staining followed the same pattern in AVF2-4, but was markedly different in AVF1 (Figure 3.7). In AVF1 there were a few spindle shaped CD34+ cells within the intima, and many found in clusters within the media. In contrast for AVF2-4 (with AVF2 being used for representative images), small CD34+ cells were only found around or proximal to the endothelial layer of the vessel or vasa vasorum in the adventitia. This CD34 staining in the adventitia was the same in AVF1.

CD68 is commonly used to identify monocytes and macrophages and this staining was also carried out for the AVF tissue (Figure 3.8). All AVF tissues had some small cells stained with CD68 within the adventitia approximately near the vasa vasorum. For AVF3 and AVF4, there was no CD68 staining in the intima and media layers in contrast to AVF1 and AVF2. AVF1 had few CD68+ cells within the intima. Those cells were large and small implying a mixed population of monocytes and macrophages. The media was full of CD68+ cells, all of which were small and rounded implying they were monocytes rather than macrophages. This contrasted AVF2, which had large granular CD68+ cells in the intima close to the endothelial layer and a few macrophages within the media.

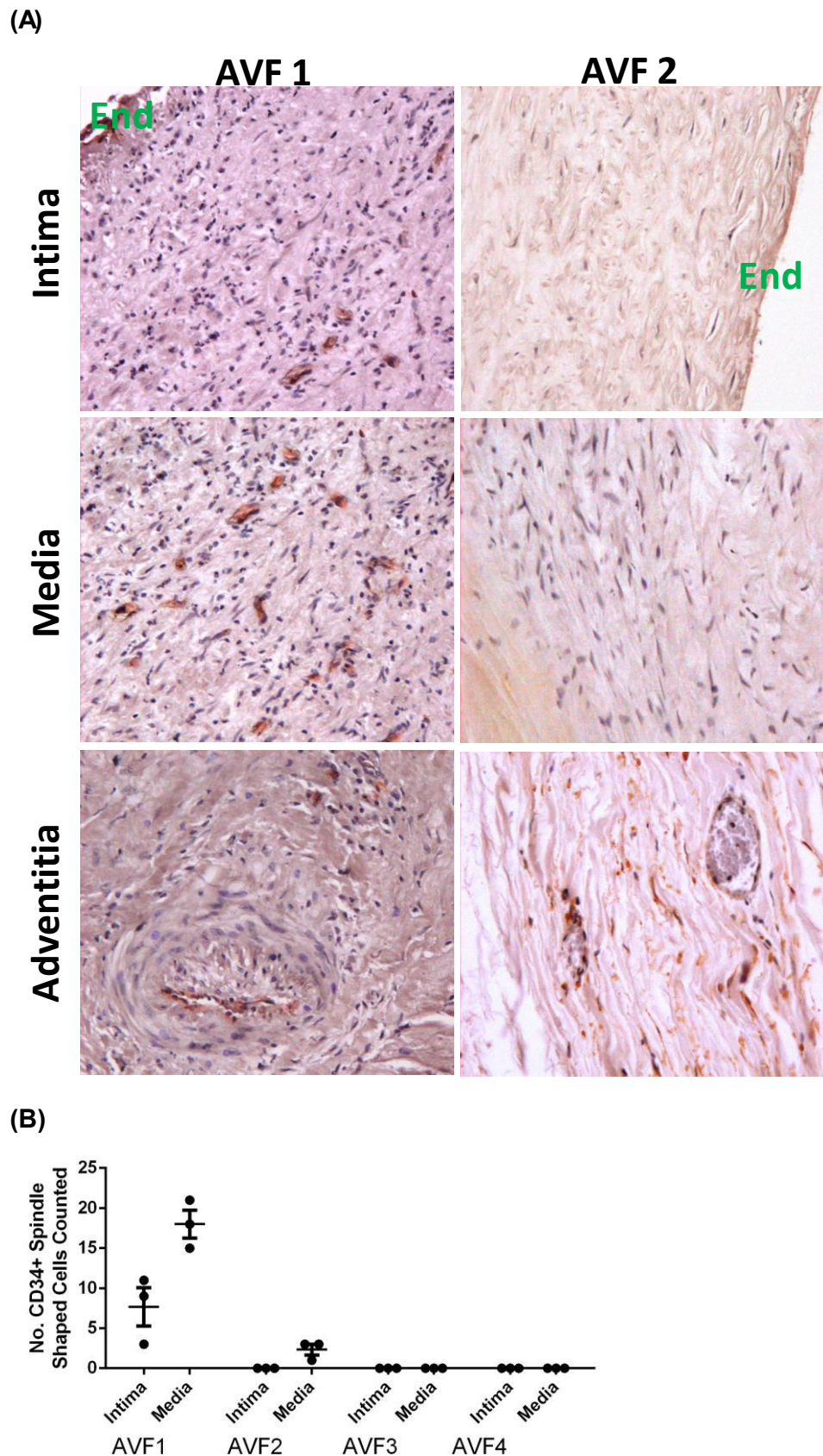
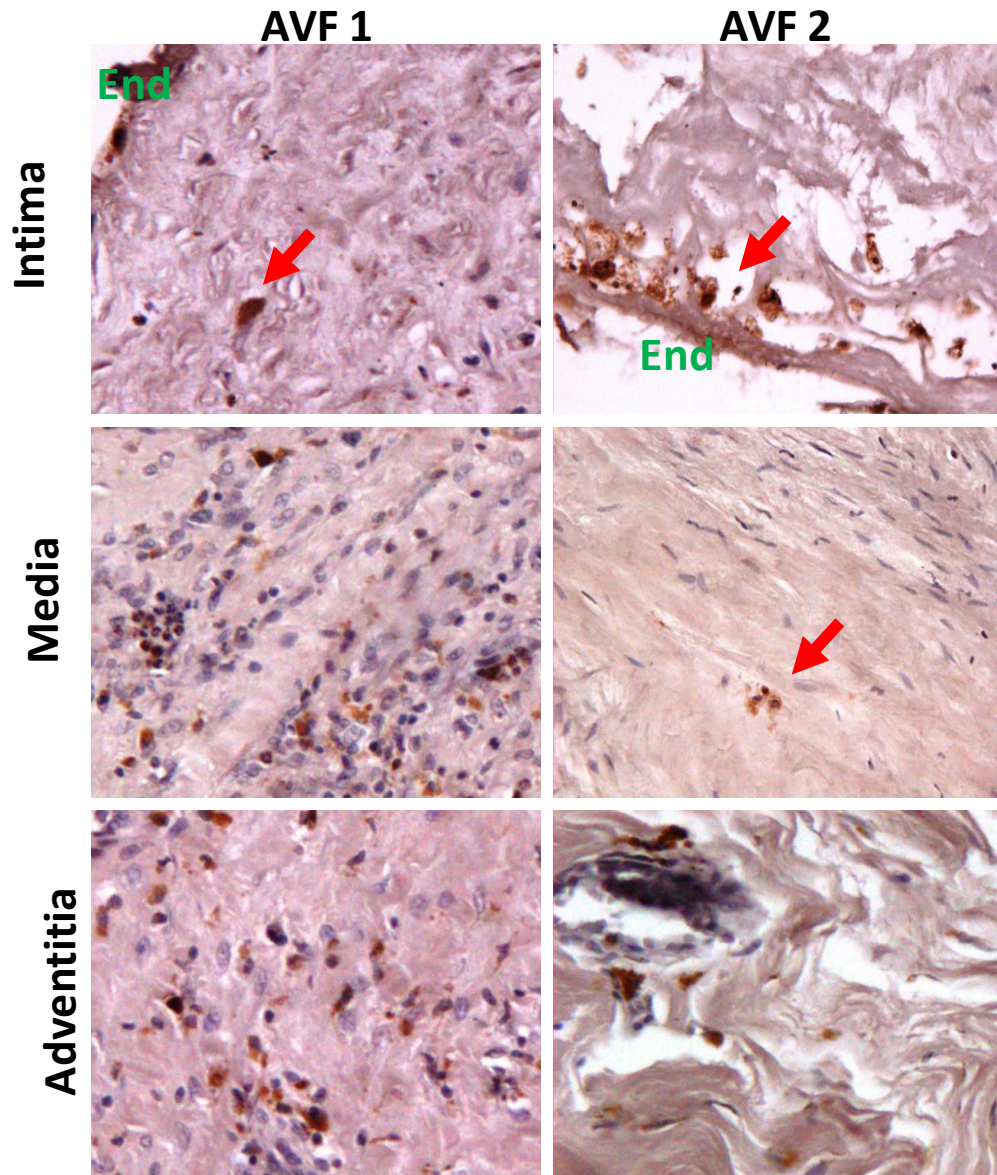


Figure 3.7: Immunohistochemistry of CD34 within AVF tissue.

(A) CD34 staining in the adventitia, media and intima layer in two representative AVF tissues (X200 magnification). 'End' indicates the endothelial layer.

(B) The quantification of CD34 spindle shaped cells within the intima and media layer within an X400 view field. Cells within three independent representative field views of X400 were counted. Data are mean $\pm$ SEM.

(A)



(B)

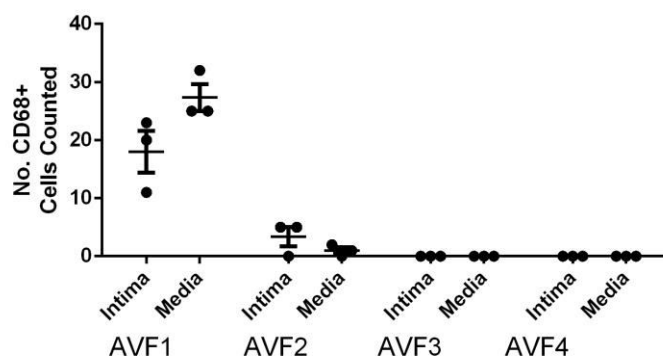


Figure 3.8: Immunohistochemistry of CD68 within AVF tissue.

(A) CD68 staining in the adventitia, media and intima layer in two representative AVF tissues (X400 magnification). 'End' indicates the endothelial layer.

(B) The quantification of CD68 cell infiltrates within the intima and media layer within an X400 view field. Cells within three independent representative field views of X400 were counted. Data are mean $\pm$ SEM.

3.1.6 Discussion

H&E, modified elastin staining and immunohistochemistry for vWF, SMA, SMMHC, vimentin and desmin was carried out on four AVF excised vessels with the aim of characterising the late stage NIH that occurs in AVFs. All four patients may at some point had a fistuloplasty, but it is important to note that the NIH characterised here may not have specifically been in the segment that had the fistuloplasty procedure and that these fistulas had been abandoned for some time prior to being excised. Thus the NIH observed within the tissue would not have necessarily have been as a result of balloon injury. In addition all veins, in particular AVF1 and AVF2, had some degree of aneurysmal enlargement as an additional pathology in the veins. Whilst only areas of known NIH were used for histology, it is unknown as to whether the development of an aneurysm would have affected the composition of the NIH as well. With a limited number of specimens it is hard to draw conclusions, but it should be emphasised these vessels were difficult to obtain and thus they simply serve as exploratory tools for making further hypotheses.

AVF1 and AVF2 were basilic vein transposition fistulas whilst AVF3 and AVF4 were radio-cephalic fistulas. Cumulative patency slightly varies between basilic vein transposition fistulas and radio-cephalic fistulas in favour of basilic vein transposition fistulas [125]. It would have been interesting therefore to see if AVF1 and AVF2 histologically looked very different to AVF3 and AVF4 yet had similarities between them. The degree of NIH in AVF1 and AVF2 were similar likewise to the degree of NIH in AVF3 and AVF4 (Figure 3.2). H&E staining revealed that AVF1 was densely packed with many cells with rounded nuclei. The intima of AVF2-4 in contrast had visibly fewer cells and those cells largely had elongated nuclei (Figure 3.3). It is possible that the NIH lesion of AVF2-4 were older than that of AVF1, as generally during the last stage of NIH development there is the extracellular matrix remodelling phase, during which there is reduced cellularity and an increase fibrosis within the enlarged media layer.

Indeed Farb *et al* reported a 45% reduction in SMCs between 3 and 9 months in NIH lesions within in stent coronary arteries [49].

Roy-Chaudhury *et al* described that most cells within the venous NIH of fistulas with early failure as being myofibroblasts with a few contractile SMCs. They stained for SMA, vimentin and desmin. There is overlap between the staining for these markers between myofibroblasts and contractile SMCs, but the consensus is that myofibroblasts do not express desmin and contractile SMCs stain for vimentin weaker than myofibroblasts [53]. In accordance to this, we also stained AVF1-4 for SMA, vimentin and desmin, and also added SMMHC as an additional marker for contractile SMCs (Figure 3.5 and Figure 3.6). Similarly to Roy-Chaudhury *et al*'s findings, I found that the intima was comprised of SMA and vimentin, but had very few SMMHC and desmin positive cells and hence the intima of an AVF which got NIH much later on is also comprised of myofibroblasts. As discussed in section 1.3.2, many cells can develop into myofibroblasts. It is still interesting to see the cells in the AVF NIH adopt a vimentin expressing phenotype.

vWF was used as a marker for endothelial cells, and all AVF tissue was found to have a conserved endothelial layer showing minimal damage during processing (Figure 3.5). The development of an intact endothelial layer is one of the later stages of NIH development, again reflecting that the NIH lesions in the AVF were very much at the final stages rather than at the early stages of development. vWF also stained the endothelial cells lining the vasa vasorum in the adventitia, and no vWF staining was present in the media. Alpers *et al* had found microvessels were present in 25 out of 48 of the neointimas examined in segments of the vein used for anastomosis creation. These microvessels were identified via CD31 staining marking endothelial cells surrounding the microvessel lumen. From this they concluded neoangiogenesis was occurring in the intima and, like how tumour angiogenesis promotes the growth of cancers, neoangiogenesis enables NIH growth [72]. Perhaps if more AVF tissues had been

obtained, some neoangiogenesis may have been observed. The lack of neoangiogenesis observed in the intima of the sampled AVF tissues is nonetheless interesting as it has been suggested that the importance of neoangiogenesis is dependent on the stage of lesion development: a critical “trigger” in the early phase, but receding in later phase lesions [126], further highlighting that the NIH observed in AVF1-4 were indeed mature lesions.

It is becoming increasingly apparent that adult blood vessels are not terminally differentiated tissues, but dynamic tissues containing stem and progenitor cell populations. The AVF tissue is no different in this respect. In all AVF tissues CD34 cells lined the endothelial layer of both the vessel and the vasa vasorum in the adventitia. CD34 expressing cells were also found in the adventitia in close proximity to the vasa vasorum. These CD34 expressing cells are known to be endothelial progenitor cells, multipotent pericytes and also highly proliferative self-renewing adventitial stem cell antigen-1–positive (Sca-1+) cells and are thought to be important in overall vessel homeostasis.

Similarly to the CD34 cells, CD68 expressing cells were found in all the AVF tissues adventitia in close proximity to the vasa vasorum (Figure 3.8). CD68 is used to identify monocytes and macrophages and the presence of these cells have classically been thought as a marker of inflammation. The continual presence of CD68+ cells in the adventitia of blood vessels, however, is well documented. It is apparent that these long-term residents are also important for vessel homeostasis. Indeed the source of CD68+ cells in the adventitia may also be from the adventitia itself. Psaltis *et al* found that the Sca-1+ cells localized in the adventitia, particularly expressed by cells lining the vasa vasorum, in mature murine aorta act as a local vascular source for CD68+ cells found within the adventitia [127]. In 309 studied saphenous veins, CD68+ cells were never found in the intima unless they were also expressed in the adventitia or media layers

[128]. This implies that dysfunction of these local CD68⁺ cells may result in the migration of CD68⁺ cells to the NIH lesion.

Alpers *et al* [72] rarely observed many CD68⁺ cells in the neointimas examined in segments of the vein used for anastomosis creation, with approximately half of the studied veins (n = 48) having CD68⁺ cells, and a mean of 7.7 cells per involved neointima, which were rarely in a cluster [72]. Interestingly, AVF1 and AVF2 had CD68⁺ staining in both the intima and the media implying that CD68⁺ may be more important in later stage NIH development in AVFs. The CD68⁺ cells found in clusters in AVF2 media and intima were large and granular in appearance thus suggesting that they were macrophages rather than monocytes. In the intima they were present in the endothelial layer, suggesting they had just recently infiltrated from the blood stream.

In contrast to this, AVF1 had small rounded CD68⁺ cells in the media and intima, implying they were monocytes rather than macrophages. The paradigm that once monocytes enter tissue, they develop into macrophages or dendritic cells established as early as the 1960s has been challenged by more recent observations that some extravasated monocytes retain much of their monocyte phenotype [129]. Circulating monocytes can be subdivided into classical (CD14⁺⁺CD16⁻, resembling mouse lymphocyte antigen 6 complex (Ly6C)^{high} monocytes), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺, resembling mouse Ly6C^{low} monocytes). Whilst it cannot be verified which subcategory the monocytes were without CD16 staining, previous studies can allow us to theorise.

In the mouse, Auffray *et al* found that since Ly6C^{low} monocytes interact closer to the endothelium layer compared to Ly6C^{high} monocytes, their infiltration dynamics into inflamed tissues are quicker in response to aseptic wound healing or *Listeria* monocytophenes [130]. These monocytes, however, differentiated into macrophages, hence they are unlikely to be the initial source of the monocytes in the intima and

media of AVF1, and are more likely to be the source of the macrophages in AVF2. It is important to note that classical monocytes can differentiate into non-classical monocytes. Chousterman *et al* found that in a mouse model of sterile hepatic injury classical monocytes were recruited early and persisted for at least 48 hours before converting into non classical monocytes, and this phenotypic conversion was essential for optimal repair [131]. The consensus seems to be that the time of entry of CD68+ cells into the intima, be it monocytes or macrophages, dictates its function, with the presence of CD68+ cells at a later stage corresponding to wound repair anti-inflammatory functions.

AVF1 also had CD34 expressing cells within the media and intima layer in close proximity to the media layer. The large spindle morphology with oval nuclei of these CD34 expressing cells makes it tempting to conclude that these cells are fibrocytes. Whether fibrocytes are also the source of the myofibroblasts within the NIH of this lesion remains unknown and is impossible to determine with histology alone. Indeed, the differentiation of fibrocytes into myofibroblasts involves a progressive loss in the markers commonly used to differentiate them from other cell types. For instance, Mori *et al*, using a skin wound healing model on irradiated female mice receiving a male whole bone marrow transplant, found that that over 80% of the myofibroblasts that originated from fibrocytes lost expression of CD34 at day 7 after injury in skin [132]; hence it is easy to underestimate the contribution fibrocytes have to a NIH lesion. Monocytes are also known to be a source of fibrocytes [64] and given the corresponding abundance of monocytes within AVF1, it is possible that these cells could have been the sources of the fibrocytes, which in turn could have been the source of myofibroblasts in AVF1. Whilst these are simply speculations based on a few observations, the observations made thus far indicate that further investigation with more samples could bring about very interesting findings.

3.2 Investigating the changes in Plasma Proteins following a Fistuloplasty Procedure

3.2.1 Introduction

Vascular injury after a fistuloplasty procedure results in persistent inflammation and adverse vascular remodelling, overall bringing about the development of neointima hyperplasia (NIH) as detailed in section 1.3.1. Several proteins are involved in mediating all stages of the pathogenesis of NIH, including cytokines, chemokines, damage-associated molecular pattern molecules, matrix metalloproteinases and growth factors.

Cytokines are small signalling proteins, about 25kDa, released from cells in response to stimuli, which result in an induction of a response by binding to specific receptors. Dependent on the secreted cytokine and its transportation within the blood, they can act in an autocrine manner, affecting the cell which released them, a paracrine manner, affecting the neighbouring cell, or in an endocrine manner, affect the behaviour of a distant cell. Cytokines can be grouped into families based on the structures of the receptors they interact with, but given how members of the same family can have very different functions, it is perhaps more useful to group cytokines based on the actions they have. The formation of an inflammatory milieu is a key stage of NIH. Many cytokines can be grouped as being 'pro-inflammatory' and 'anti-inflammatory' and during the acute phase of NIH it is expected that balance between these cytokines would tip towards inflammatory. These cytokines orchestrate or amplify the inflammatory response and initiate a cascade of mediators directly responsible for the various events associated with inflammation. For instance, CRP is released as a consequence of IL-6 production. In the context of wound healing, anti-inflammatory cytokines dampen the release of pro-inflammatory cytokines, or limit the actions of pro-inflammatory cytokines by, for instance, induce the production of IL-1 receptor agonist (IL-1ra) or tumour necrosis factor receptor superfamily (TNFRSF) such as CD30, which

blocks the actions of IL-1 and TNF- α respectively [133]. Figure 3.9 groups some cytokines in pro-inflammatory and anti-inflammatory groups.

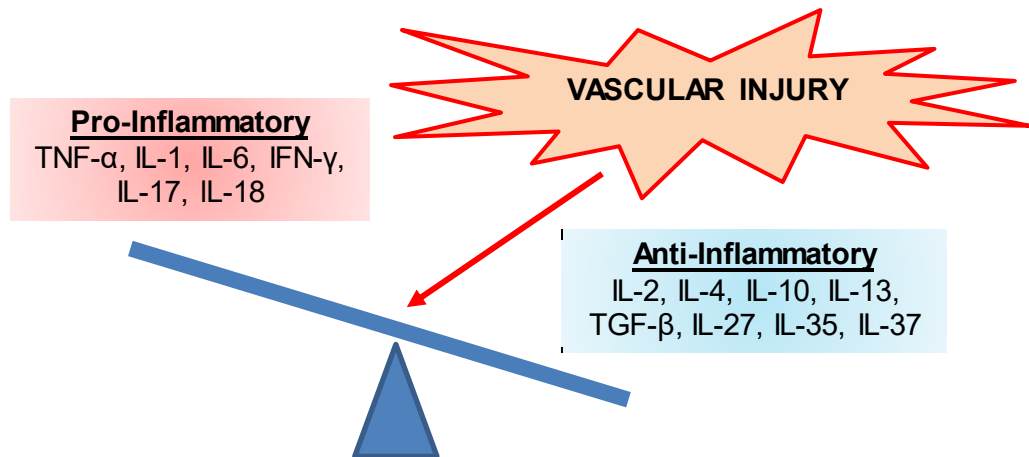


Figure 3.9: Vascular injury tips the balance of pro-inflammatory and anti-inflammatory cytokines towards pro-inflammatory.

In the context of wound healing, TNF- α , IL-1, IL-6, IFN- γ , IL-17, IL-18 are examples conventional of pro-inflammatory cytokines whilst IL-2, IL-4, IL-10, IL-13, TGF- β , IL-27, IL-35, IL-37 are conventional examples of anti-inflammatory cytokines.

Many cytokines can also induce changes in cell adhesiveness and cell cytoskeleton structure resulting in the cell migrating towards a protein signal gradient. These cytokines are commonly referred to as chemokines. Chemokines are grouped according to their amino acid structure. There are two main subfamilies of chemokines: CC chemokines, which have two cysteine residues adjacent to one another, and CXC chemokines, which have the cysteine residue separated by a single amino acid, except for CX3CR1 which has three separating amino acids.

Chemokines are instrumental in attracting leukocytes during the granulation phase during NIH development. For instance, monocyte chemoattractant protein-1 (MCP-1, other known as CCL2) released by endothelial and fibroblasts interacting with the CCR2 receptor of monocytes results in monocyte chemotaxis to the site of injury and further MCP-1 production by monocytes. Chemokines are also instrumental in shaping the composition of the NIH lesion itself. For instance, an initial burst of apoptosis of injured SMCs is known to trigger the release of the chemokine stromal cell-derived factor (SDF, other known as CXCL12). The interaction of SDF with CXCR4 on bone marrow progenitor cells (i.e. fibrocytes) is thought to be crucial in the recruitment of

bone marrow progenitor cells to the lesion and thus the subsequent composition of cells [134].

It is also important to note that the fistuloplasty procedure results in the injury of the vascular walls and thus the release of damage-associated molecular patterns (DAMPs), endogenous molecules that can perpetuate the non-infectious inflammatory response. Many S100 proteins, which are small calcium-binding cytosolic proteins, are thought to be DAMPs. These S100 proteins can bind to the RAGE (receptor for advanced glycation end products), which leads to nuclear factor kappa B (NF- κ B) activation, a transcription factor important for several genes involved in inflammation. S100B is an example of this. Under normal physiological conditions it is mainly localized in astrocytes, but the observations that S100B levels increase after vascular injury from the adventitia layer to the intima layer in a time-dependent manner led researchers to study its link with NIH development. Indeed, it has been proposed that the interaction of S100B with RAGE is an inducer of SDF-1 α and thereby important for NIH arising from bone marrow progenitor cells [135].

Moreover, S100A8 and S100A9 (also known as calgranulins A and B, or myeloid-related proteins (MRP) 8 and 14 respectively) together can exist a heterodimer known as calprotectin. Calprotectin comprises as much as 60% of the soluble protein content of the cytosol of neutrophils and is also expressed on the membrane of monocytes. Neutrophil activation or endothelial adhesion of monocytes can result in the release of calprotectin [136]. Given the importance of these cells, it comes as no surprise that calprotectin has been associated neutrophil and monocyte dependent vascular inflammation and blocking it attenuates the severity of vascular injury responses *in vivo* [137].

Neutrophils and macrophages recruited to the NIH lesion can secrete proteases which degrade specific components of the extracellular matrix such as the internal elastic

lamina. These proteases are known as matrix metalloproteinases (MMPs) and are named according to the gene in which encodes for them. Structural remodelling of the extracellular matrix allows space to be carved for new cells to proliferate and as a result it comes as no surprise that many MMPs are associated with NIH formation. An example of this is matrix metalloproteinase-8 (MMP-8, also known as collagenase-2), which has been shown to promote the differentiation of adventitial progenitor cells into smooth muscle cells (SMCs) [138].

MMPs also results in the release of a variety of growth factors that had previously been tethered in an inactive form to the proteoglycans of the extracellular matrix. A growth factor regulates defined phases of the cell cycle, often resulting in increased cell division. It can also stimulate cell growth, differentiation and survival. Many cytokines are also growth factors and the terms are at times used interchangeably. For instance, IL-2 is a growth factor for B cells. Perhaps the most well studied examples of this are the colony stimulation factors: Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). These glycoprotein growth factors bind to receptors on the surfaces of haemopoietic stem cells to induce differentiation.

During the development of NIH following a fistuloplasty procedure, injury to the endothelial layer from the mechanical action of the balloon results in platelets aggregating to the damaged surface. Aggregated platelets release many growth factors from their alpha granule platelets such as platelet derived growth factors (PDGF), vascular derived growth factor (VEGF) well known to act as potent mitogens for endothelial and SMCs. More recently exploratory studies have been linking proteins with NIH development which they would have otherwise overlooked. An example of this is brain derived neurotrophic factor (BDNF), identified from whole-genome microarray analysis. BDNF was found to increase proliferation of pulmonary arterial SMCs *in vitro*

and has found to be upregulated in the arteries of patients suffering from idiopathic pulmonary arterial hypertension, which is characterised by NIH [133].

It is tempting to associate the increased cell proliferation by growth factors with an increase in NIH development when this is not necessarily the case. Indeed, more recent studies are finding that growth factors targeting endothelial cell proliferation specifically may be NIH limiting. An example of this is hepatocyte growth factor (HGF), which was originally identified to stimulate the growth of hepatocytes, but more recently has been demonstrated to be a mitogen for endothelial cells without also inducing the replication of SMCs. Studies using *in vivo* gene transfer of HGF in rabbit abdominal aortae found that HGF gene transfer inhibits the early formation of NIH following ultrasound-guided balloon injury, most likely by enhancing re-endothelialisation and thus limiting the amount of SMC proliferation [139]. This can also be illustrated by the balance between growth factors angiopoietin-1 and angiopoietin-2, which are both vascular-specific growth factors that compete for the Tie2 receptor expressed on endothelial and certain hematopoietic cells. Angiopoietin-2 production by early recruited myofibrocytes directly stimulates proliferation and enhanced survival of neointimal cells *in vivo*, whilst angiopoietin-1, mainly produced by pericytes, seen maintain vascular quiescence [140]. Disruption of the balance in favour of angiopoietin-2 production results in NIH, which can be counteracted by the addition of angiopoietin-1 [141].

To investigate plasma proteins, a protein screening panel was designed based on the existing literature and the results from a preliminary experiment.

3.2.2 Aims

Initially the aim of this section was to explore the changes in soluble proteins following a fistuloplasty procedure using paired pre and 1-2-day post fistuloplasty plasma samples using a protein panel of 52 proteins. Proteins found to have significantly

changed 1-2 days post fistuloplasty were aimed to be explored in greater detail using a time course experiment. Finally, the aim was to explore whether the plasma proteins could be associated with patient outcome.

3.2.3 Summary of the Patient Samples used

In total, blood samples from 75 patients undergoing a fistuloplasty procedure were used within this thesis. Pre fistuloplasty plasma from all of these patients were used within this results chapter in section 3.2.8 in order to investigate if there is an association between pre fistuloplasty plasma proteins with post-intervention primary patency and post-intervention cumulative patency outcome. Table 2-2 lists which patient samples were used for which experiment whilst Appendix A – Patient Clinical Data Summary lists the clinical data for each patient.

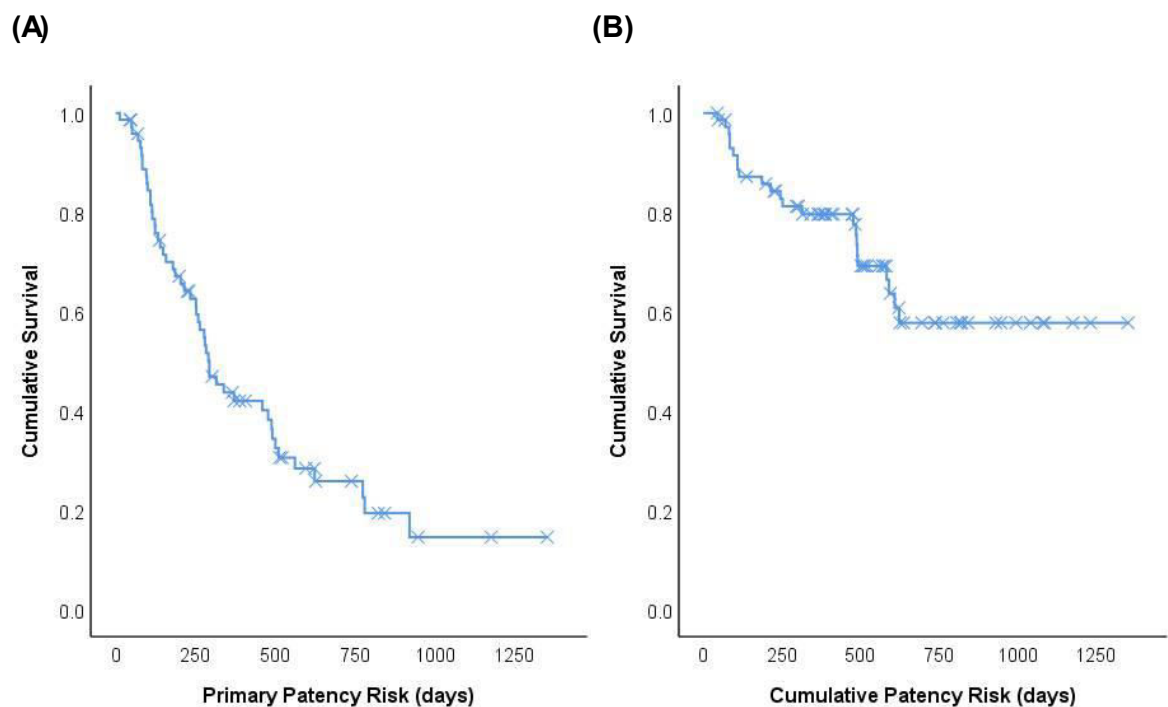
Table 3-1 summarises the clinical data of all 75 patients. The influence of nominal patient clinical variables (gender, ethnicity and type of native fistula) on post-intervention primary patency and post-intervention cumulative patency can be seen in Appendix B – Kaplan-Meier Curves for Patient Clinical Nominal Data.

Table 3-1: A Summary of the clinical data for all 75 patients undergoing a fistuloplasty procedure who donated blood samples.

Gender	34 female, 41 male.
Mean Patient Age (years $\pm$ SD) [Range]	61($\pm$ 13) [20 – 88]
Ethnicity	10/75 asian, 28/75 black, 36/75 white, 1/75 other.
Fistula Type	18/75 basilica vein transposition, 40/75 brachio-cephalic, 19/75 radio-cephalic fistula.
Mean Fistula Age (log years $\pm$ SD) [Range]	2.38 ($\pm$ 1.89) [0.08 – 12.75]
History of Thrombosis	2/75 had a history of thrombosis.
History of Previous Interventions	22/75 had a history of previous interventions within their fistula.

24 out of 75 (32%) of patients never lost post-intervention primary patency. The main reason for a loss of primary patency in patents was because they had a radiological procedure (24 patients, 32%), followed by thrombosis (10 patients, 13%), AVF abandonment (10 patients, 13%) and a surgical procedure (7 patients, 9%).

In contrast, 51 out of 75 patients (68%) never lost post-intervention cumulative patency. For 27 out of 51 of these patients it was because they reached the end of follow up. Figure 3.10 shows Kaplan-Meier analysis for AVF's post-intervention primary patency and post-intervention cumulative patency risk.



Number of patients at risk:
0 days: 75, 250 days: 40, 500 days: 17,
750 days: 8, 1000 days: 2, 1250 days: 1

Number of patients at risk:
0 days: 75, 250 days: 54, 500 days: 33,
750 days: 14, 1000 days: 6, 1250 days: 1

Figure 3.10: Kaplan-Meier analysis for AVF's post-intervention primary patency and post-intervention cumulative patency risk.

(A) Kaplan-Meier analysis for post-intervention cumulative patency.

(B) Kaplan-Meier analysis for post-intervention primary patency.

Steps represent survival function. X marks patient censoring.

3.2.4 There are a Few Proteins which Consistently Change Post Fistuloplasty

A proteome profiler human XL cytokine array kit (R&D), a membrane-based antibody array which enables the detection of 105 proteins via chemiluminescence, was used on four pairs of pre and post fistuloplasty derived plasma samples in order to help select proteins for a protein panel to apply to a larger cohort using ELISAs and Luminex assays. Some proteins could be visualised as having changed post fistuloplasty as seen in Figure 3.11.

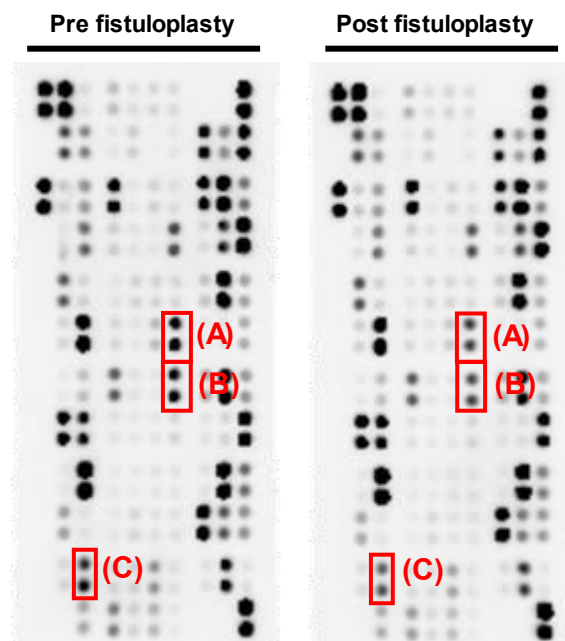


Figure 3.11: Representative figure of a pair of proteome profiler membranes using pre and post fistuloplasty plasma samples from one patient. (A) Duplicate proteins marked in red boxes represent brain derived neurotrophic factor (BDNF), (B) Dipeptidyl peptidase-4 (DDP4) and (C) PF4 have visibly decreased in this patient after the fistuloplasty procedure.

Figure 3.12 summarises the data generated from the four pairs of proteome profiler membranes. Flt-ligand, G-CSF, GM-CSF and IL-1 α protein levels were found to have increased post fistuloplasty, whilst MPO and TfR levels were found to have decreased post fistuloplasty and thus these proteins were included on the protein panel. The final protein panel decided is shown in Table 3-2.

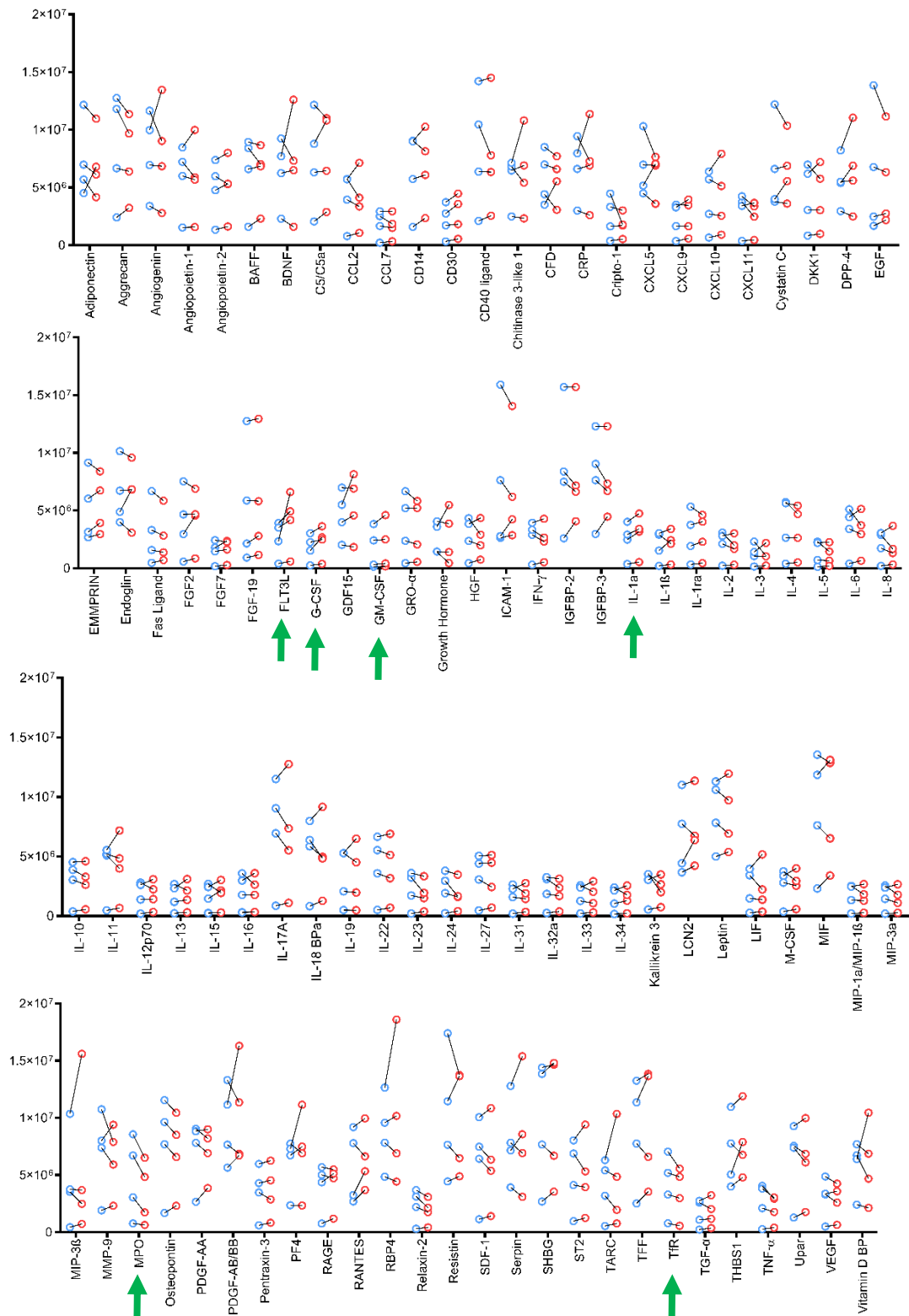


Figure 3.12: Proteins levels in pre and post fistuloplasty samples as detected by a proteome profiler.

105 different proteins were measured in four pairs of fistuloplasty plasma samples. Data from the lowest possible exposure time detectable for both pre fistuloplasty and post fistuloplasty samples was used for each data point. The Y axis indicates the mean pixel density. Blue represents pre fistuloplasty samples. Red indicates post fistuloplasty samples. Green arrows indicate the proteins which were found to consistently change within 4 out of 4 patients. Flt-ligand, G-CSF, GM-CSF and IL-1 α protein levels consistently increased post fistuloplasty, whilst MPO and Tfr levels consistently decreased post fistuloplasty.

Table 3-2: Table showing all the proteins investigated either by ELISAs or Luminex techniques. 1 = Indicated by proteome profiler

Protein Panel			
Adiponectin	G-CSF ¹	IL-18	PDGF-AA
Angiogenin	GM-CSF ¹	IFN- γ	PDGF-BB
Angiopoietin-1	HGF	MCP-1	PF4
Angiopoietin-2	IL-1 α ¹	M-CSF	RAGE
BDNF	IL-1 β	MIF	RANTES
Calprotectin	IL-1r a	MIG	Resistin
CD30	IL-2R α	MIP-3 α	SCGF
CD40 Ligand	IL-2	MIP-3 β	SDF-1 α
CRP	IL-4	MPO ¹	S100B
CTACK	IL-6	MMP-1	S100A9
FIt-3 Ligand ¹	IL-10	MMP-8	TNF- α
Eotaxin	IL-16	MMP-12	TfR ¹
Endothelin-1	IL-17A	MMP-13	VEGF

3.2.5 IL-6, TNF- α and MPO levels Change Post Fistuloplasty

To explore the protein changes after a fistuloplasty procedure, a protein screen consisting of a panel of 52 proteins were applied to patient samples pre and 1-2 days post fistuloplasty via a combination of ELISAs and Luminex assays. As only 40 samples would fit on a typical single ELISA or Luminex plate when run in duplicates, initially this panel was applied to 20 pairs of pre and post fistuloplasty samples. This revealed that many of the proteins were undetectable within the existing protein panel and therefore they were excluded from further analysis on the remaining 10 pairs of patient plasma samples. This applied to endothelin-1, G-CSF, GM-CSF, IFN- γ , IL-10,

1L-1 β , IL-1 α , IL-2, IL-4, resistin and SDF-1 α . Table 3-3 lists these proteins and the number of patients that they were detected in for both the pre and post fistuloplasty samples.

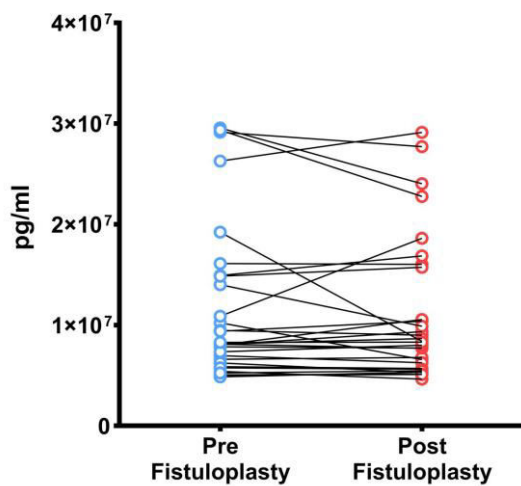
Table 3-3: List of the proteins which failed to be reliably detected in 20 pairs of pre and 1-2 days post patient plasma samples.

Protein	Number of Pre Fistuloplasty Samples Protein was Detected in	Number of Post Fistuloplasty Samples Protein was Detected in	Detection limit of the assay (pg/ml)	Reason for not being detected
Endothelin-1	0/20	0/20	11.6 - 2,810	Below standard curve
G-CSF	0/20	0/20	24 - 5,840	Below standard curve
GM-CSF	0/20	0/20	12.2 - 2,970	Below standard curve
IFN-γ	1/20	0/20	58.5 - 14,209	Below standard curve
IL-10	0/20	0/20	4.8 - 1,162	Below standard curve
IL-1β	8/20	8/20	19.5 - 4,744	Below standard curve
IL-1α	8/20	7/20	5.2 - 1,270	Below standard curve
IL-2	4/20	5/20	29.6 - 7,200	Below standard curve
IL-4	11/20	8/20	14.6 - 3,550	Below standard curve
Resistin	5/20	8/20	36.5 - 8,880	Above standard curve
SDF-1α	11/20	11/20	151 - 36,720	Below standard curve

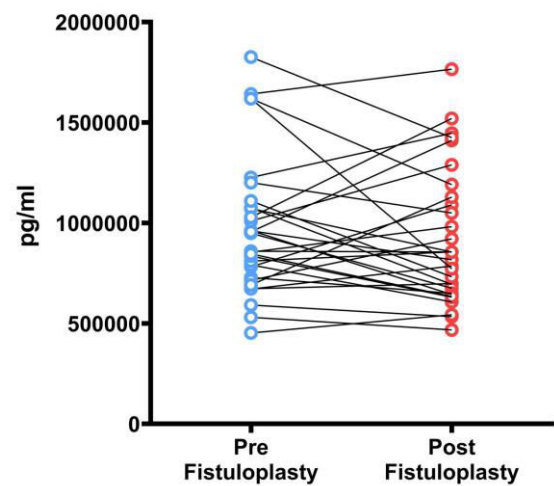
Figure 3.13 and Figure 3.14 shows the levels of the remaining 41 detectable plasma proteins in the patient plasma samples before and after their fistuloplasty procedure. Of all of the assayed proteins, only the levels of IL-6, TNF- α and MPO significantly changed post fistuloplasty. IL-6 levels significantly increased post fistuloplasty ($p = 0.0003$), increasing in 22/30 patients and remaining undetectable in 2/30 patients (Figure 3.14A). This increase in IL-6 was statistically significant even when adjusting for a multiple comparisons effect testing using the Bonferroni correction method (with

0.00096 being the corrected threshold for p value significance using this method). Without adjusting for multiple comparisons, TNF- α also significantly increased post fistuloplasty ($p = 0.0064$), increasing in 24/30 patients (Figure 3.14B). In contrast, MPO significantly decreased post fistuloplasty ($p = 0.0397$), decreasing in 19/30 patients (Figure 3.14C). CD30 and Stem Cell Growth Factor (SCGF) were trending towards significance ($p = 0.076$ and $p = 0.0809$ respectively), with CD30 decreasing in 17/30 patients and SCGF increasing in 20/30 patients, but the importance of these proteins should not be overstated.

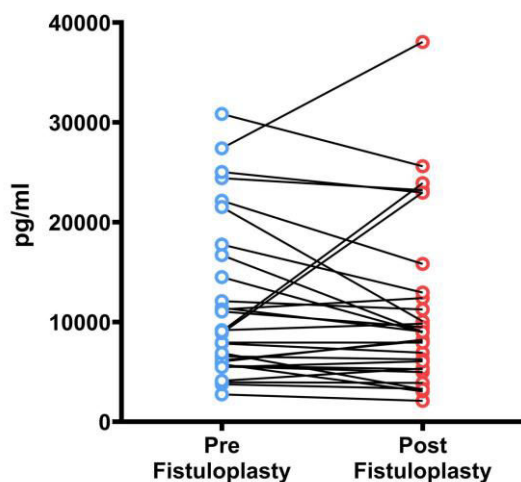
(A) Adiponectin



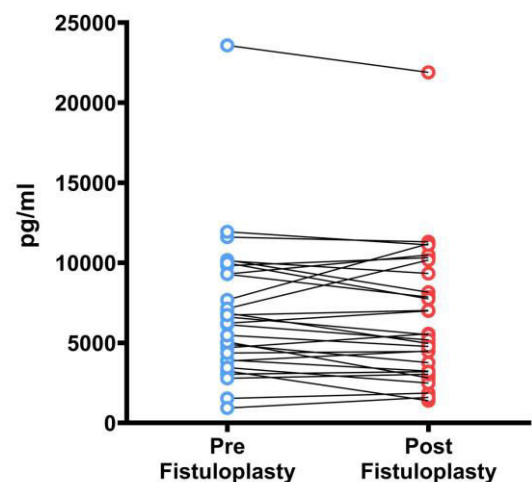
(B) Angiogenin



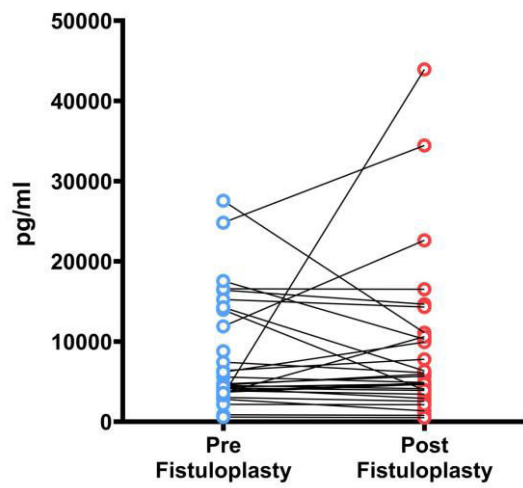
(C) Angiopoietin-1



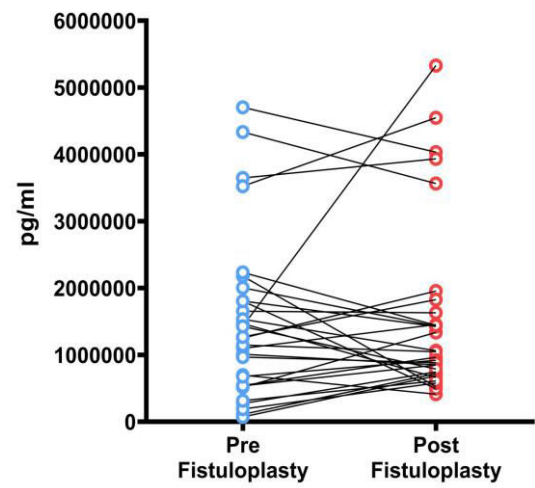
(D) Angiopoietin-2



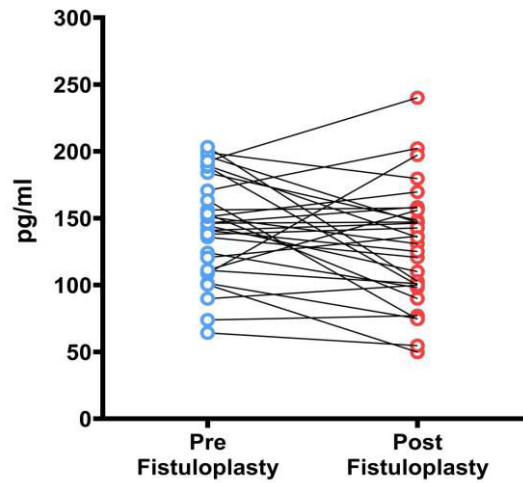
(E) BDNF



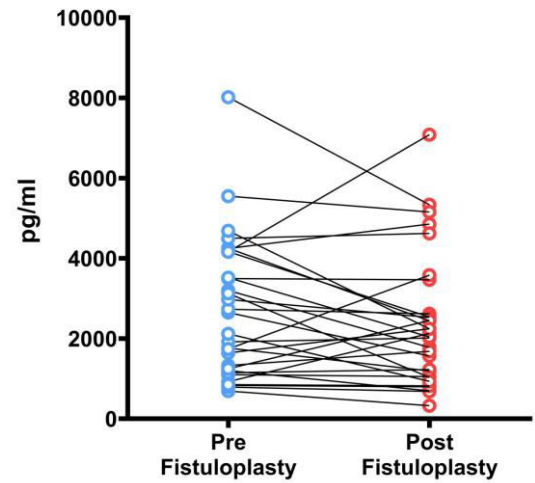
(F) Calprotectin



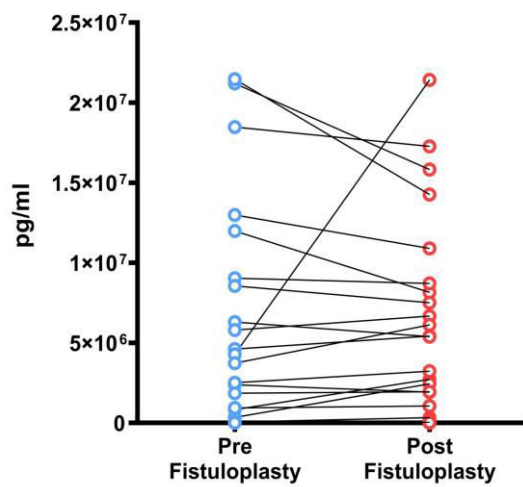
(G) CD30



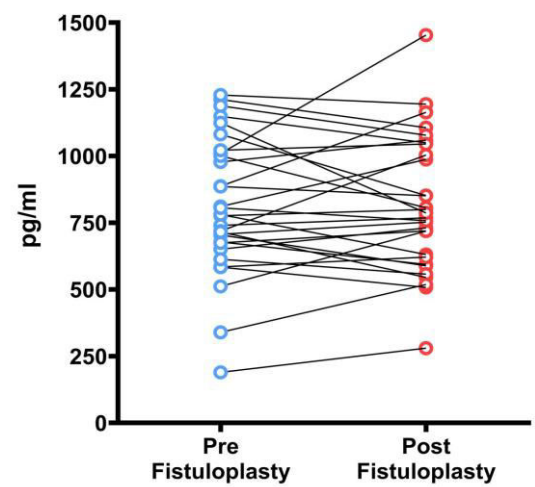
(H) CD40 Ligand



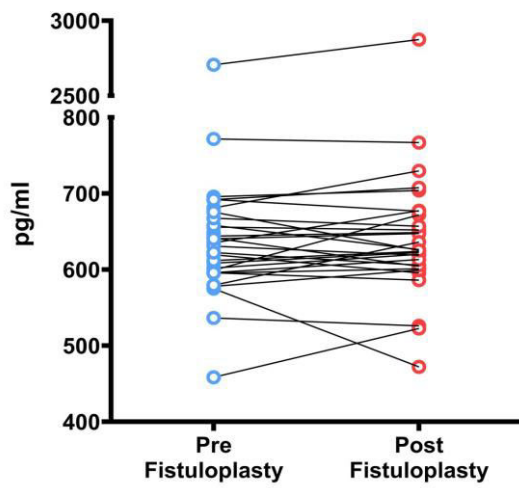
(I) CRP



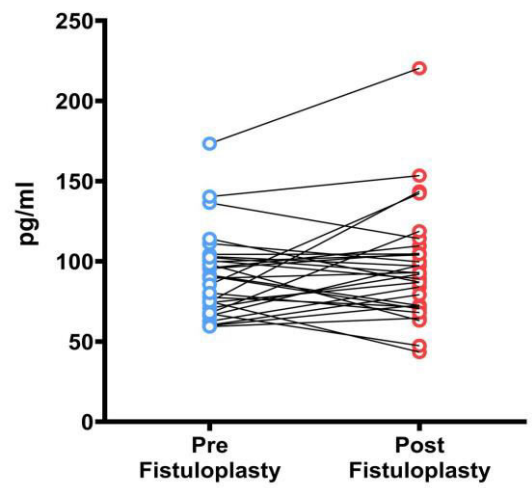
(J) CTACK



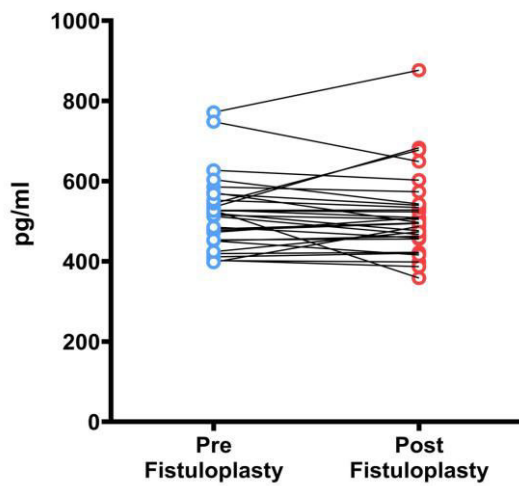
(K) Eotaxin



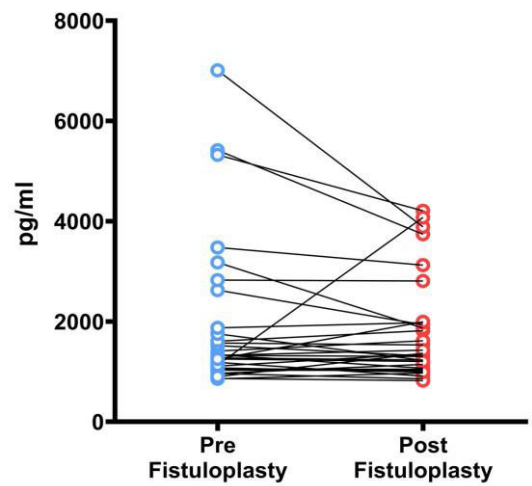
(L) Flt-3 Ligand



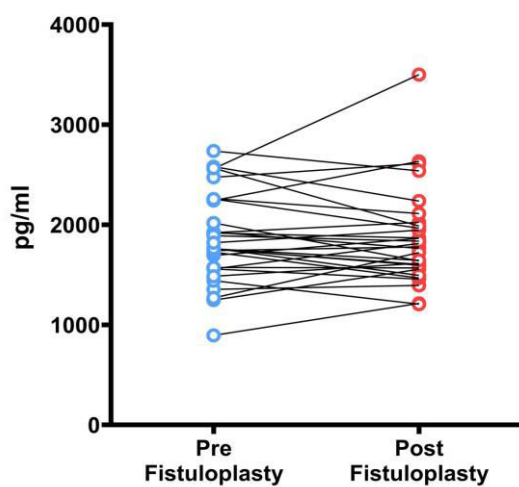
(M) HGF



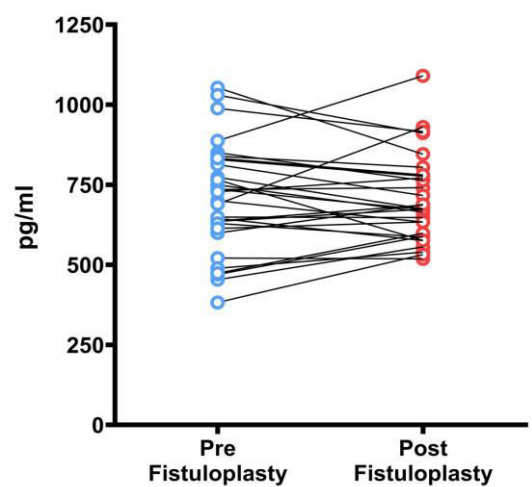
(N) IL-1ra



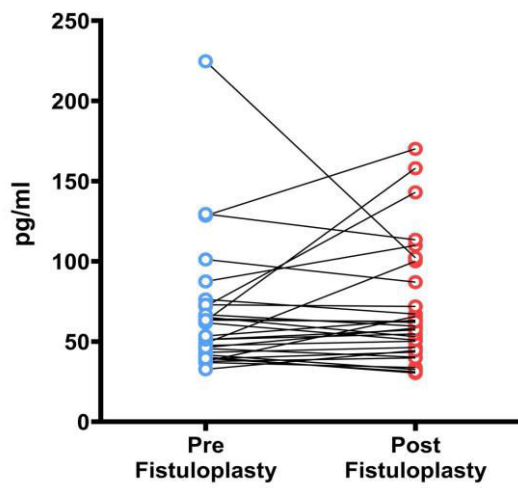
(O) IL-2R α



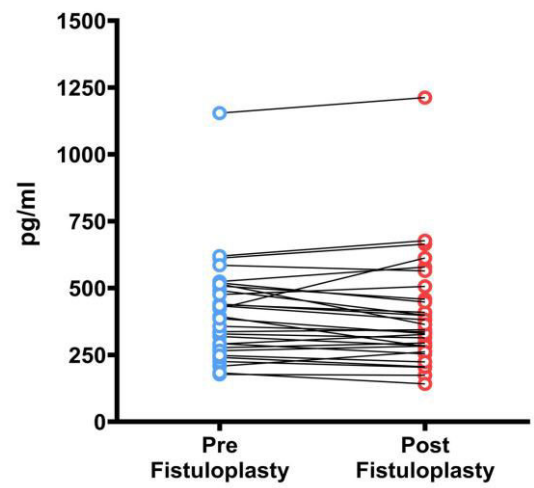
(P) IL-16



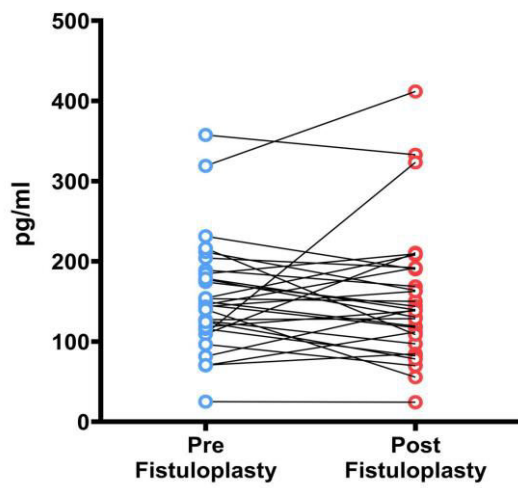
(Q) IL-17A



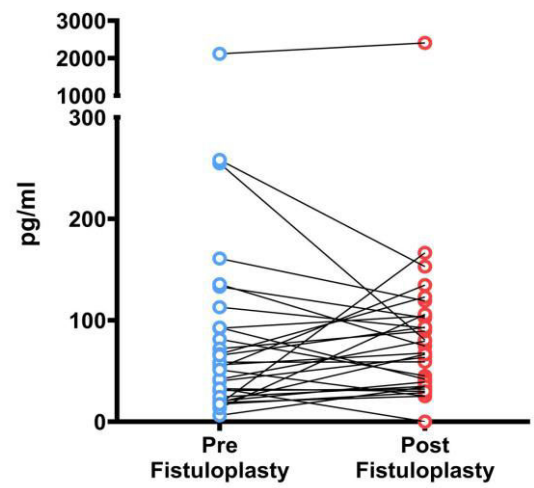
(R) IL-18



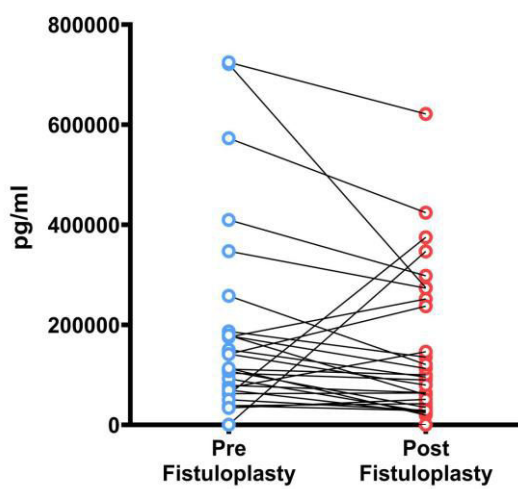
(S) MCP-1



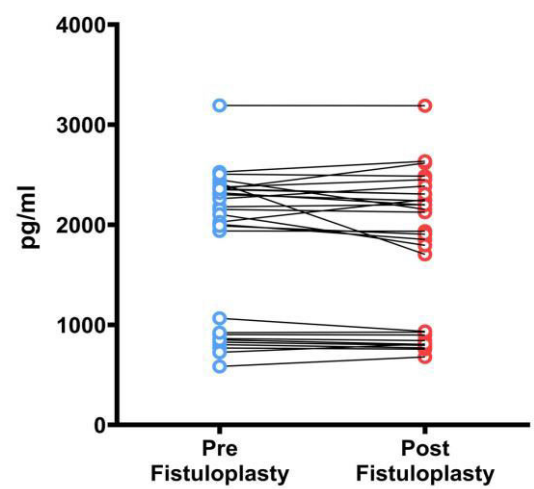
(T) M-CSF



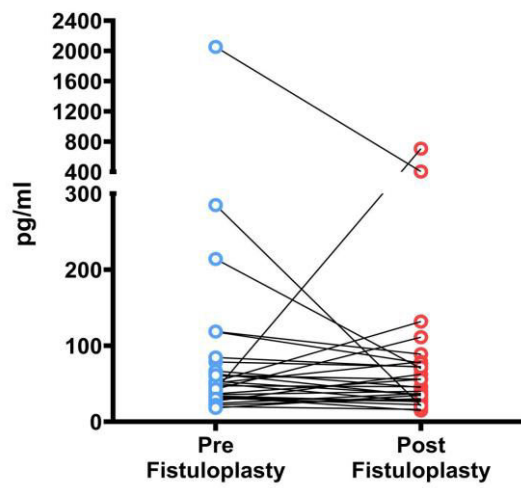
(U) MIF



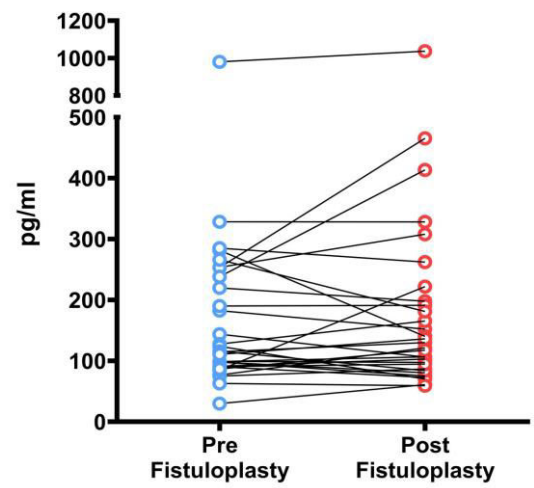
(V) MIG



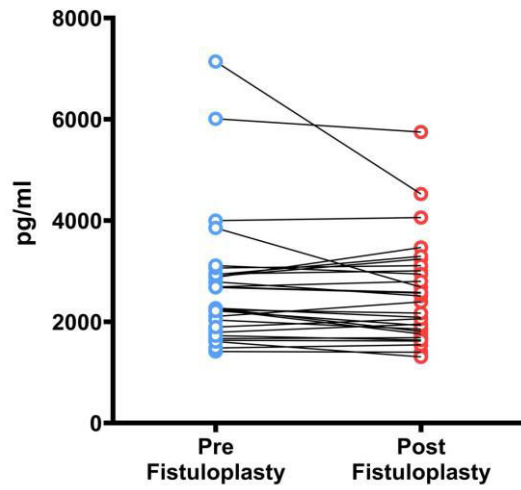
(W) MIP-3 α



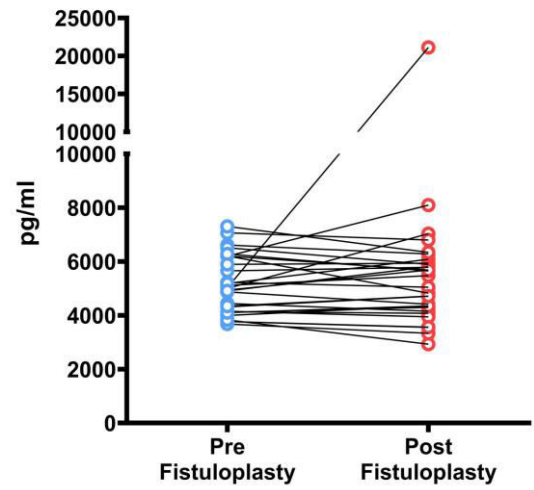
(X) MIP-3 β



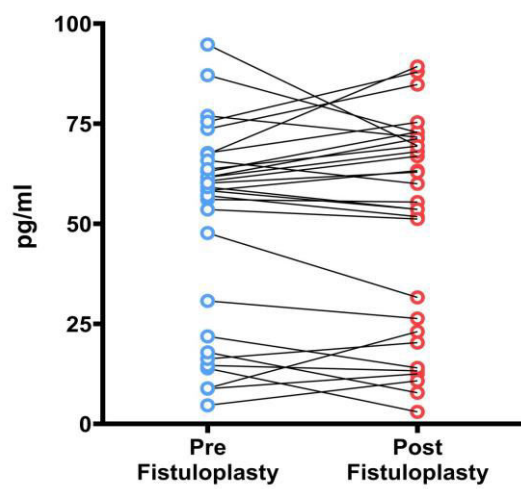
(Y) MMP-1



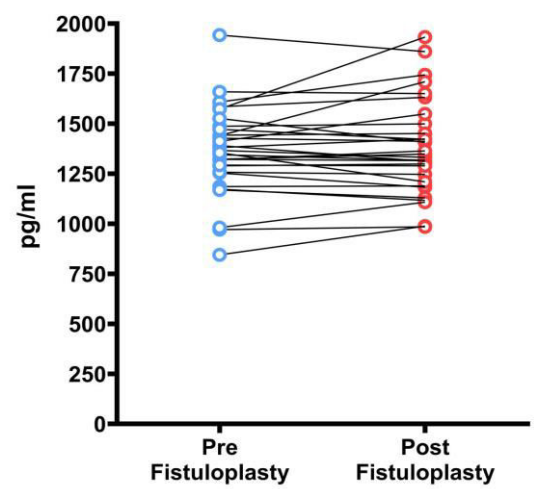
(Z) MMP-8



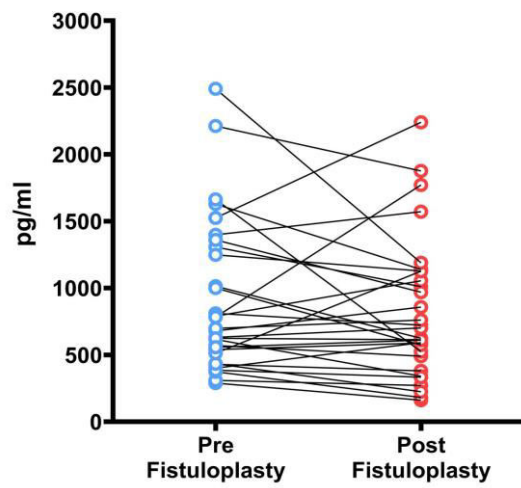
(AA) MMP-12



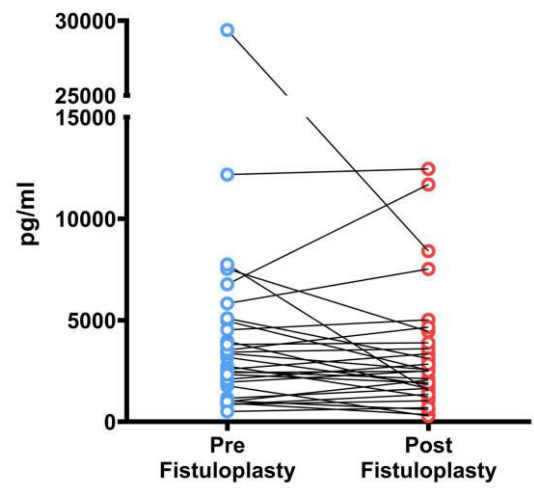
(AB) MMP-13



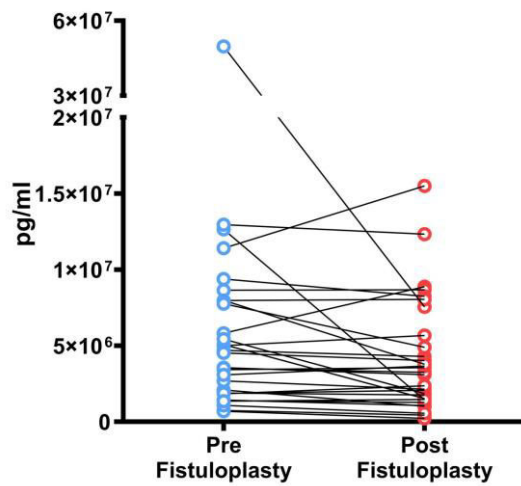
(AC) PDGF-AA



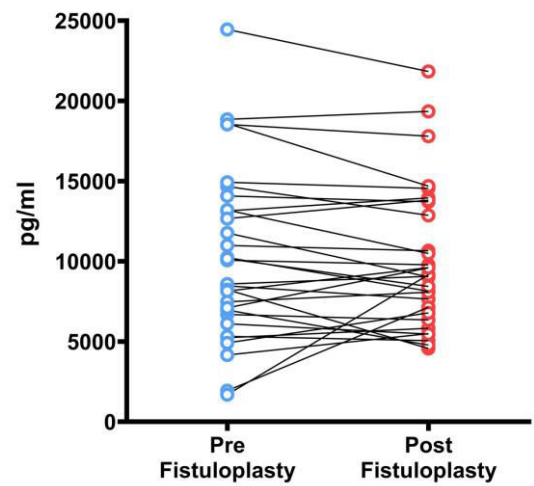
(AD) PDGF-BB



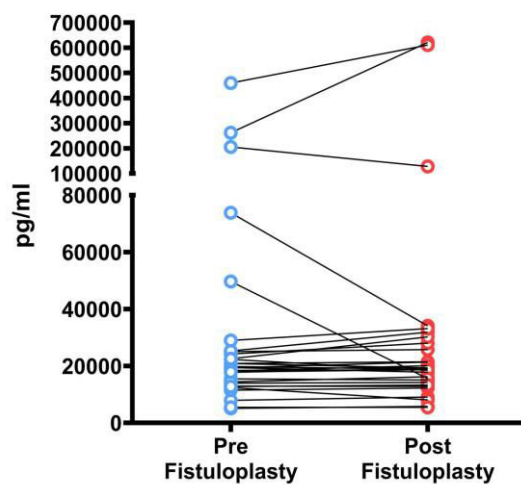
(AE) PF4



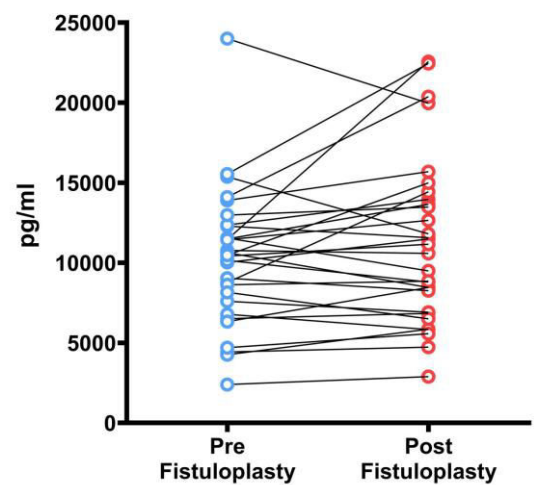
(AF) RAGE



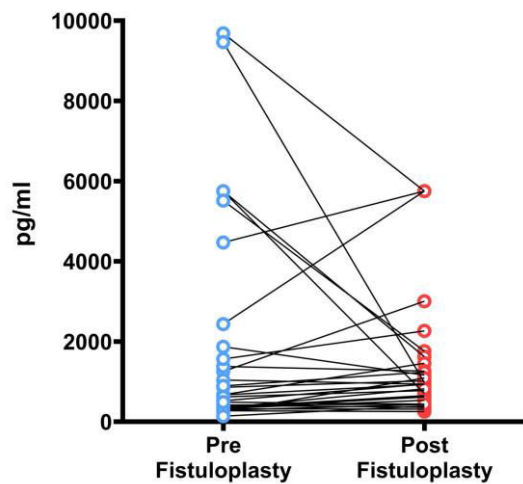
(AG) RANTES



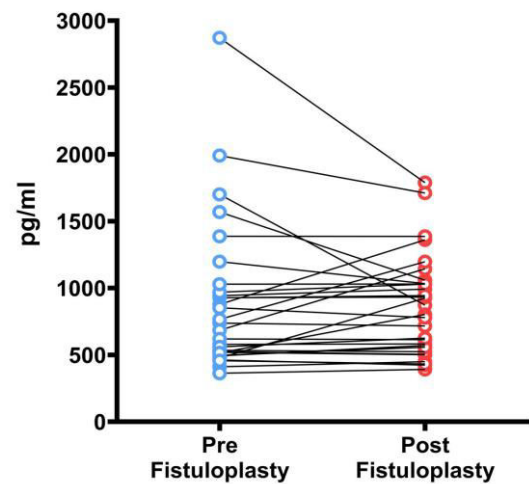
(AH) SCGF



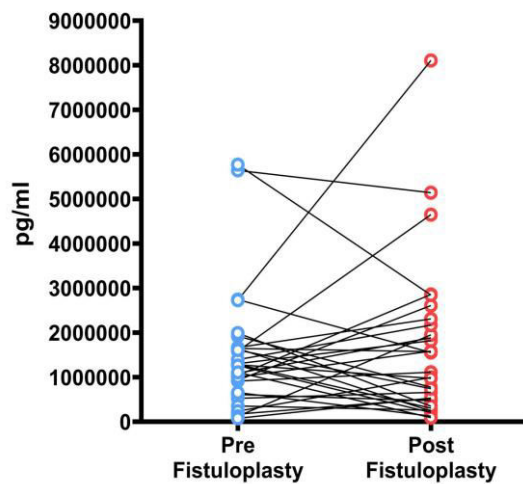
(AI) S100A9



(AJ) S100B



(AK) TfR



(AL) VEGF

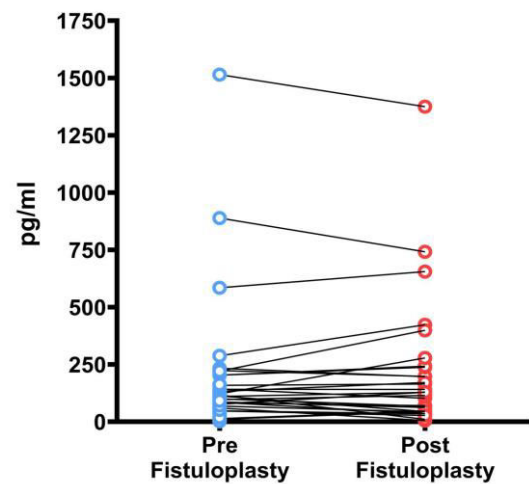


Figure 3.13: Protein levels in paired patient plasma samples before and 1-2 days after their fistuloplasty.

n = 30 paired samples. Data was analysed using a paired *t* test.

(A) Adiponectin: $p = 0.3875$

(B) Angiogenin: $p = 0.3989$

(C) Angiopoietin-1: $p = 0.7084$

(D) Angiopoietin-2: $p = 0.2816$

(E) BDNF: $p = 0.5929$

(F) Calprotectin: $p = 0.6701$

(G) CD30: $p = 0.0761$

(H) CD40 Ligand: $p = 0.1725$

(I) CRP: $p = 0.8470$

(J) CTACK: $p = 0.8506$

(K) Eotaxin: $p = 0.3378$

(L) Flt-3 ligand: $p = 0.2775$

(M) HGF: $p = 0.8460$

(N) IL-1ra: $p = 0.3731$

(O) IL-2R α : $p = 0.9561$

(P) IL-16: $p = 0.8605$

(Q) IL-17A: $p = 0.4961$

(R) IL-18: $p = 0.3396$

(S) MCP-1: $p = 0.9186$

(T) M-CSF: $p = 0.4381$

(U) MIF: $p = 0.2946$

(V) MIG: $p = 0.2120$

(W) MIP-3 α : $p = 0.4625$

(X) MIP-3 β : $p = 0.3381$

(Y) MMP-1: $p = 0.1524$

(Z) MMP-8: $p = 0.3117$

(AA) MMP-12: $p = 0.9034$

(AB) MMP-13: $p = 0.2911$

(AC) PDGF-AA: $p = 0.2929$

(AD) PDGF-BB: $p = 0.1938$

(AE) PF4: $p = 0.1475$

(AF) RAGE: $p = 0.7826$

(AG) RANTES: $p = 0.3503$

(AH) S100A9: $p = 0.2542$

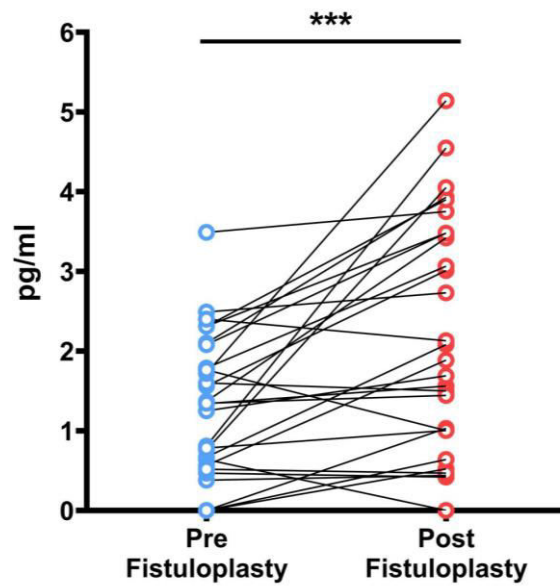
(AI) S100B: $p = 0.7580$

(AJ) SCGF: $p = 0.0809$

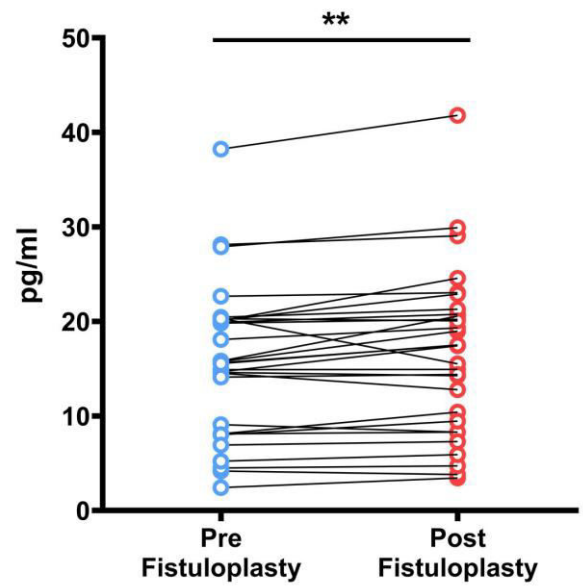
(AK) TfR: $p = 0.5545$

(AL) VEGF: $p = 0.8639$

(A) IL-6



(B) TNF- α



(C) MPO

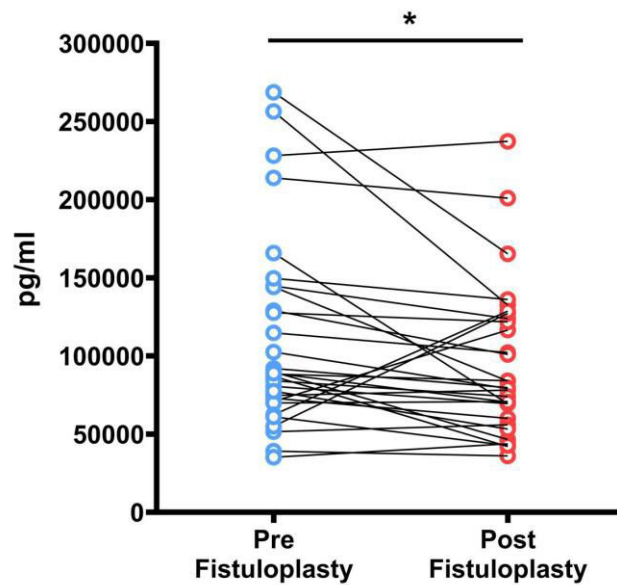


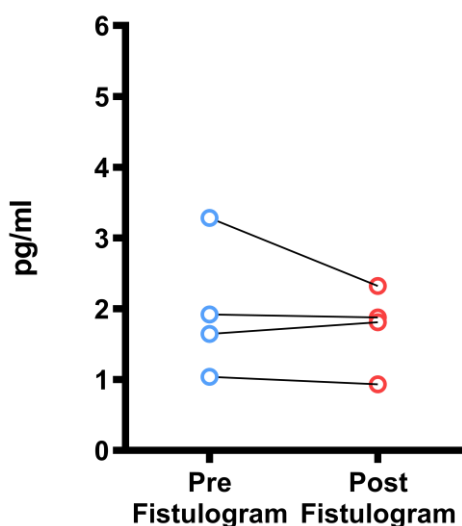
Figure 3.14: IL-6, TNF- α and MPO protein levels significantly changed in paired patient plasma samples.

(A) IL-6 ($p = 0.0003$), (B) TNF- α , ($p = 0.0064$) and (C) MPO ($p = 0.0397$) levels in paired patient plasma samples before and 1-2 days after their fistuloplasty. $n = 30$ paired samples. Data was analysed using a paired t test.

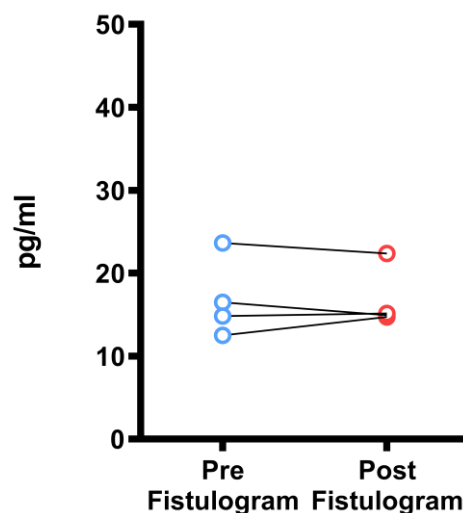
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The significant change in the levels of IL-6, TNF- α and MPO 1-2 days post fistuloplasty were unlikely to be due to the administration of contrast as there was no observable trend in the four patients who simply had a fistulogram procedure (Figure 3.15).

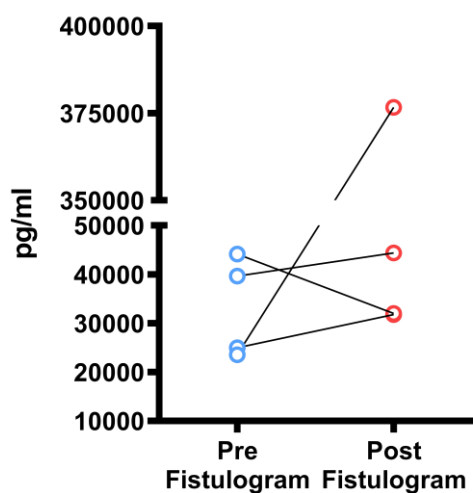
(A) IL-6



(B) TNF- α



(C) MPO



(D) CRP

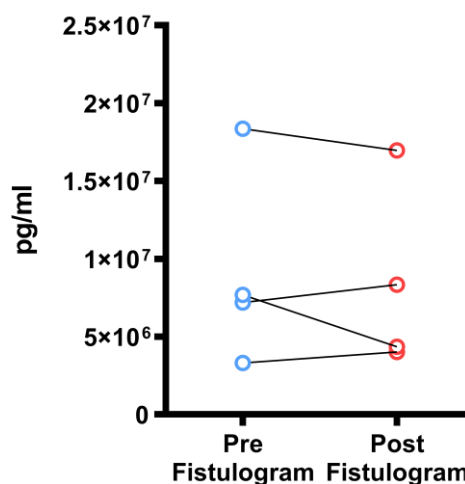


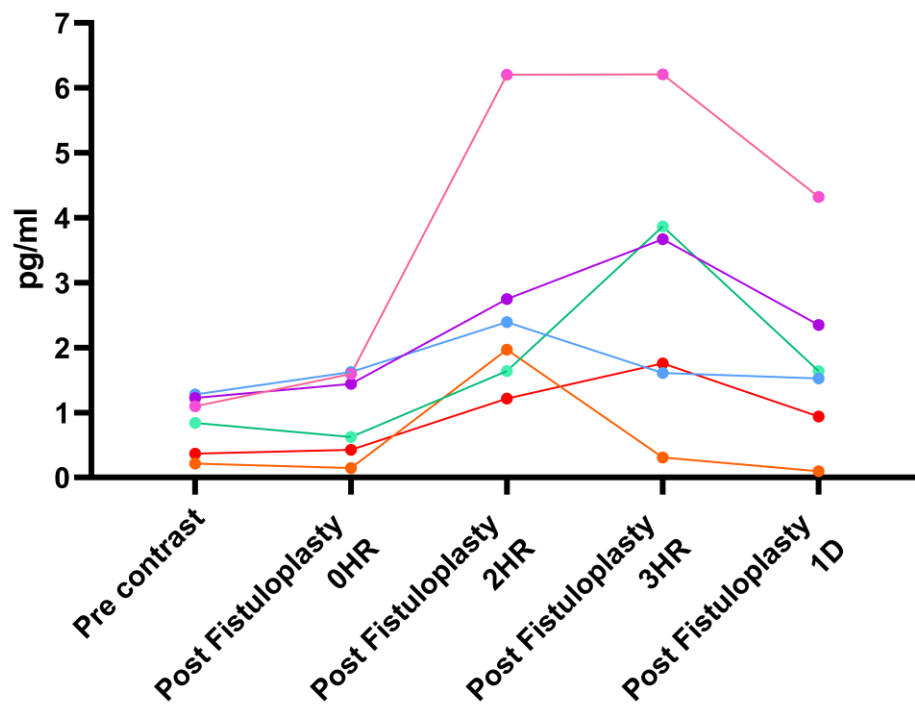
Figure 3.15: IL-6, TNF- α , MPO and CRP pre and 1-2 days post fistulogram plasma protein levels (A) IL-6, (B) TNF- α , (C) MPO (D) CRP levels in paired patient plasma samples before and 1-2 days after their fistulogram. (n = 4 paired samples). Data was analysed using a paired t test.

3.2.6 There are Transient Changes in Protein Levels Post Fistuloplasty

Changes in the protein levels of IL-6, TNF- α and MPO post fistuloplasty were further investigated using a time course experiment. CRP was also further investigated due to its historic importance as a biomarker. All time points were compared to baseline (pre contrast) levels. The administration of contrast and heparin did not have any immediate changes on the levels of IL-6 (Figure 3.16B), TNF- α (Figure 3.16D) MPO (Figure 3.16F) and CRP (Figure 3.16H). IL-6 and TNF- α both peaked between at 2-3 hours rather than 1-day post fistuloplasty. IL-6 peaked at 2 hours for 2/6 patients and at 3 hours for 4/6 patients. TNF- α also peaked at 2 hours for 2/6 patients and at 3 hours for 4/6 patients, but these were different patients. Interestingly MPO went up in 5/6 patients at 3 hours post fistuloplasty. CRP significantly decreased immediately post fistuloplasty ($p = 0.0486$) and then was close to significantly increasing ($p = 0.0502$) (Figure 3.16).

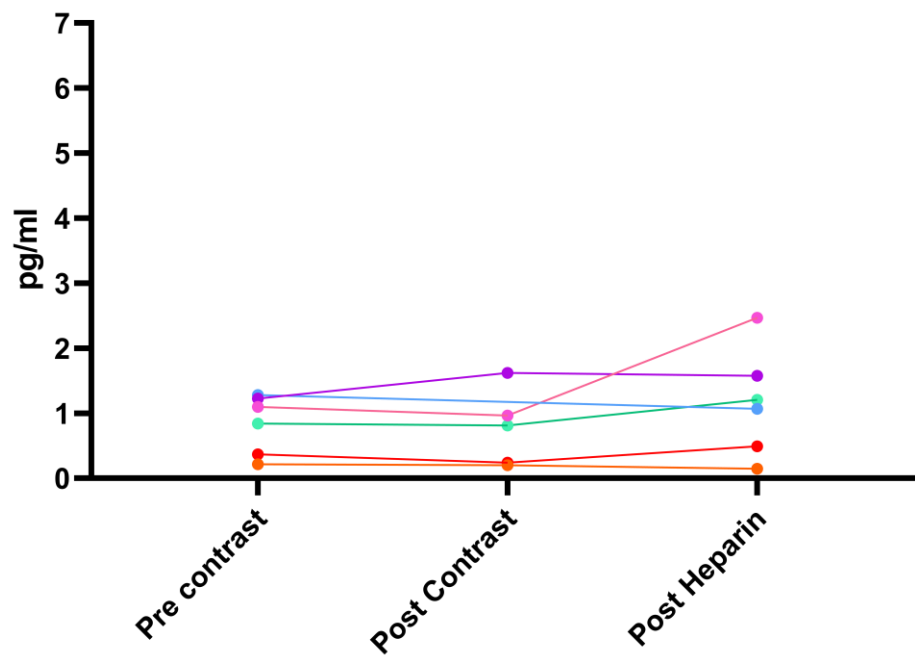
(A)

IL-6



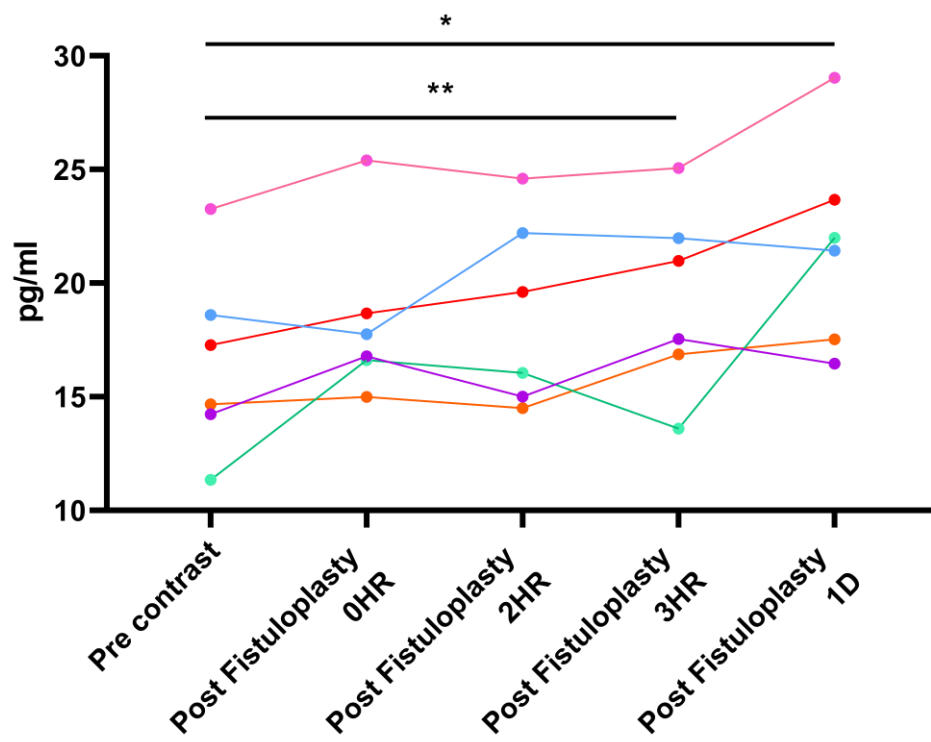
(B)

IL-6



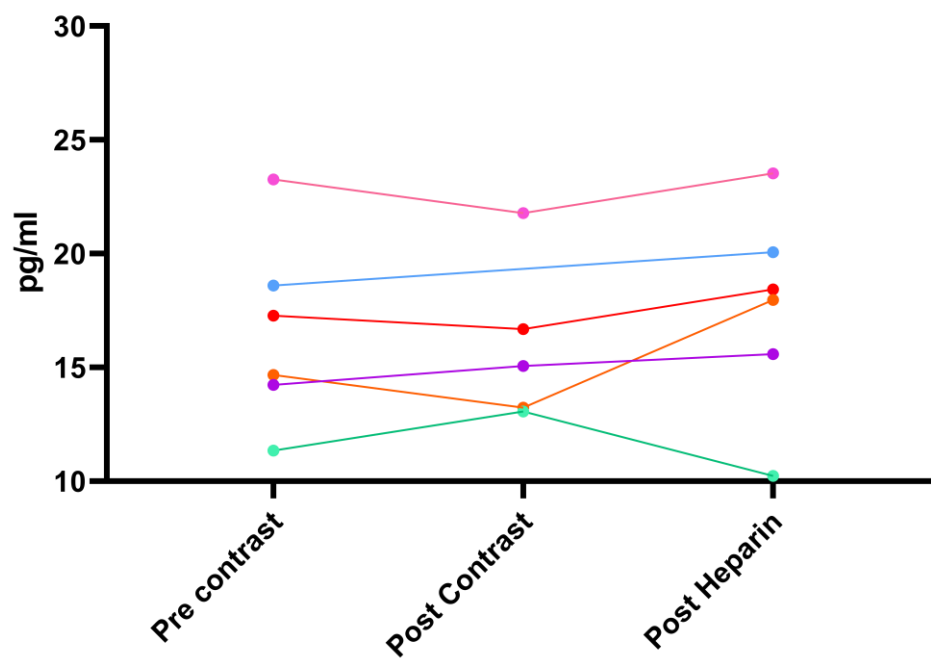
(C)

TNF- α



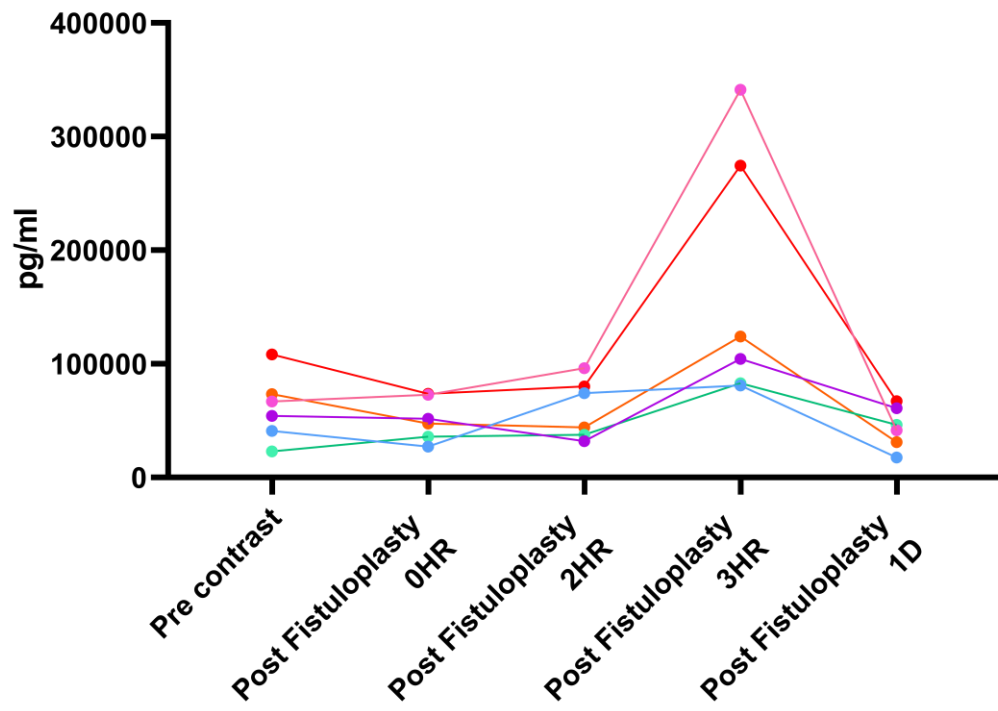
(D)

TNF- α



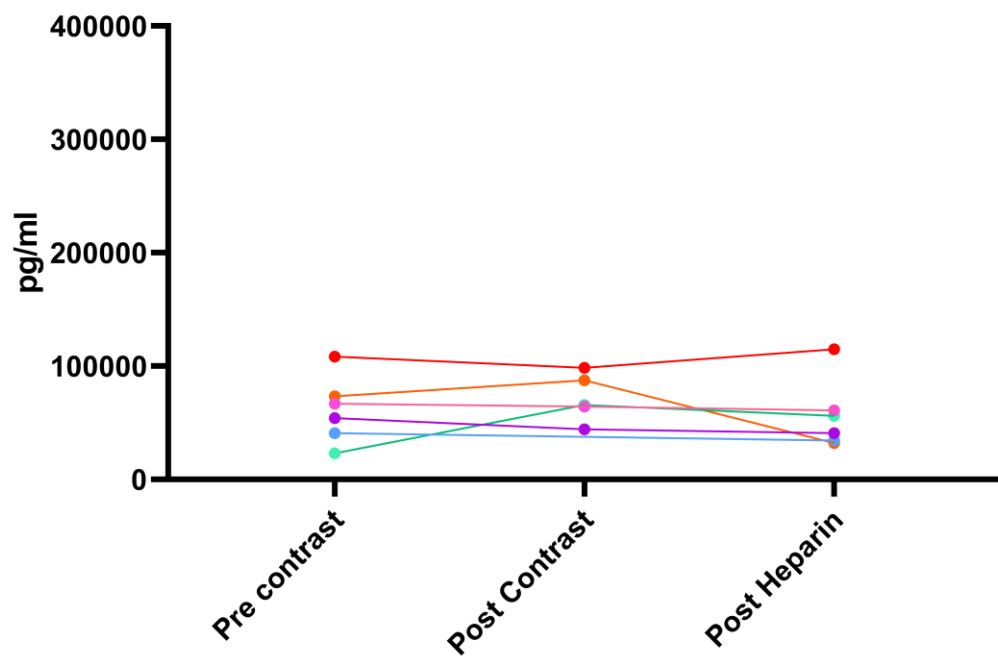
(E)

MPO



(F)

MPO



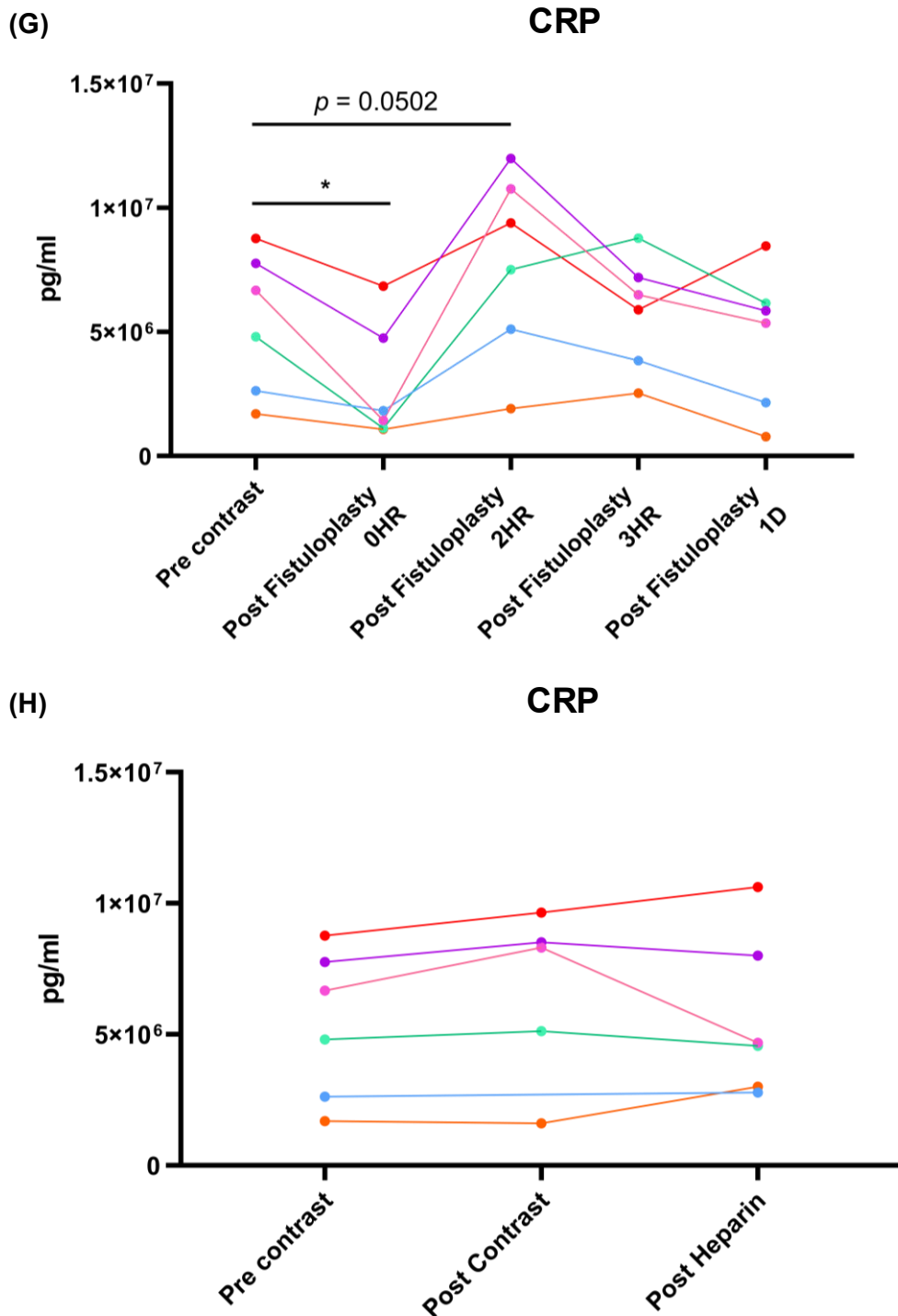


Figure 3.16: Plasma protein levels of IL-6, TNF- α , MPO and CRP at through the course of the fistuloplasty procedure.

(n = 6 patients) RM-one-way ANOVA, Giesser-Greenhouse correction with the mean of each data set compared against the mean of the pre-contrast (baseline) sample. * $p < 0.05$, ** $p < 0.01$.

(A) IL-6 levels pre contrast, 0 hours post fistuloplasty, 2 hours post fistuloplasty, 3 hours post fistuloplasty and 1-day post fistuloplasty.

(B) IL-6 levels pre contrast, post contrast and post heparin.

(C) TNF- α levels pre contrast, 0 hours post fistuloplasty, 2 hours post fistuloplasty, 3 hours post fistuloplasty and 1-day post fistuloplasty.

(D) TNF- α levels pre contrasts, post contrast and post heparin.

(E) MPO levels pre contrast, 0 hours post fistuloplasty, 2 hours post fistuloplasty, 3 hours post fistuloplasty and 1-day post fistuloplasty.

(F) MPO levels pre contrast, post contrast and post heparin.

(G) CRP levels pre contrast, 0 hours post fistuloplasty, 2 hours post fistuloplasty, 3 hours post fistuloplasty and 1-day post fistuloplasty.

(H) CRP levels pre contrast, post contrast and post heparin.

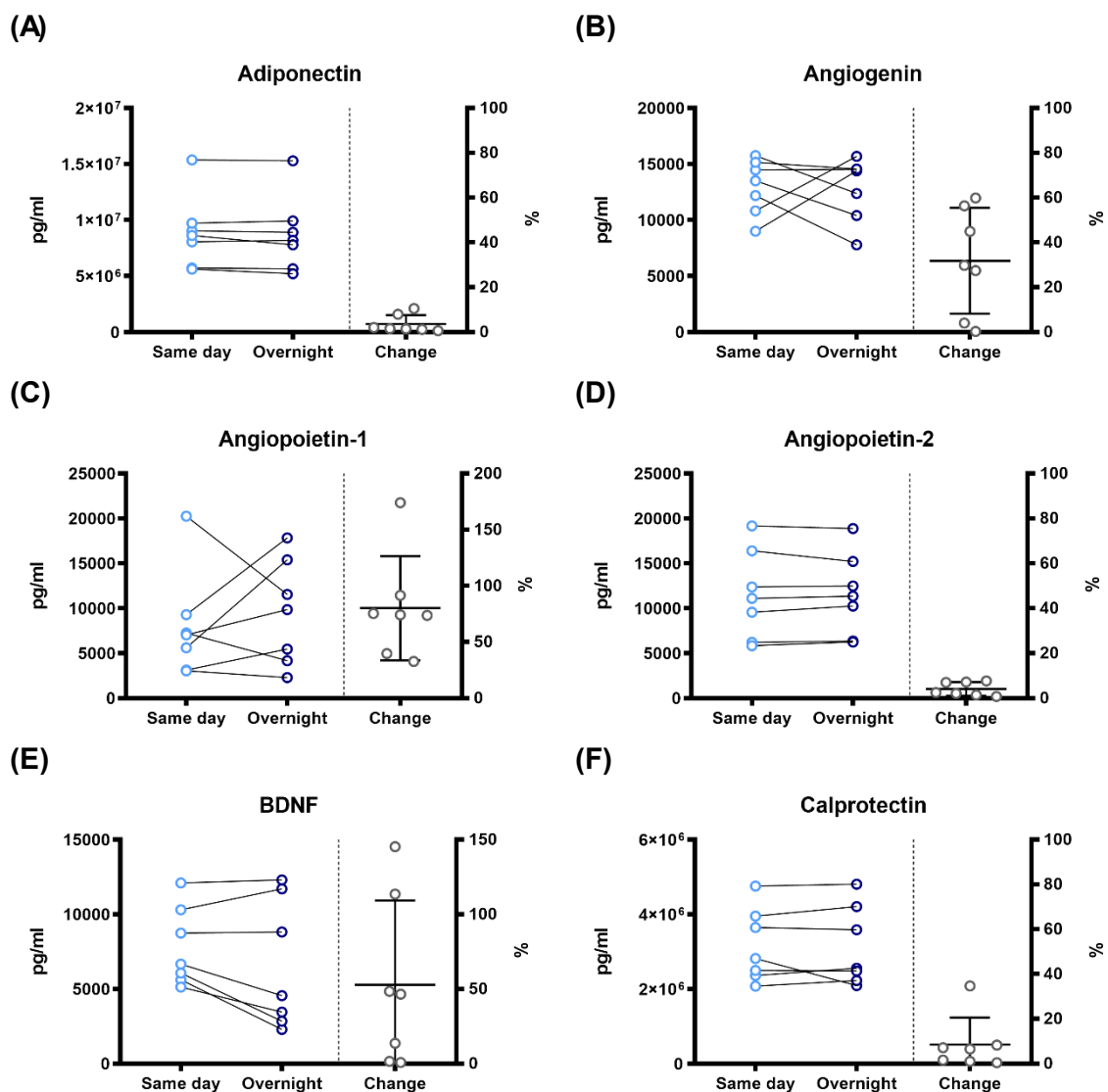
3.2.7 Many Proteins are Sensitive to Processing Time

In order to investigate the association of plasma proteins with post-intervention primary and cumulative patency, ELISA and Luminex assays were run on all 75 patients' pre fistuloplasty plasma samples. For cost and logistical reasons, some blood samples from external sites (for instance, samples from the Hull Royal Infirmary) had to be processed the following day after being taken from the patient. Whilst ELISA and Luminex assays were carried out measuring plasma protein levels on all patient samples regardless of the processing time, it was important to establish which proteins would be sensitive to an overnight processing in order to know which overnight protein sample measurements to exclude from statistical analysis. In order to investigate this, a pair of 5ml EDTA blood samples was collected pre-dialysis from patients who dialysed at Guy's Hospital locally, with one tube being processed straight away and the other tube being left overnight to be processed the following day. Same day plasma samples were always assayed on the same plate as their overnight paired sample. The results from these experiments are shown in Figure 3.17.

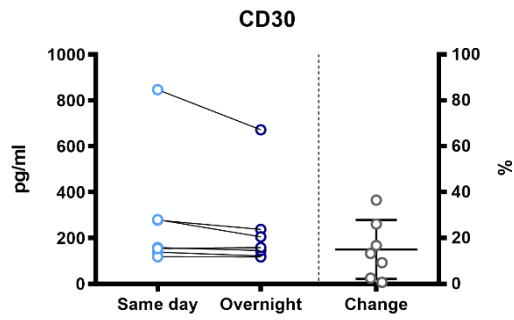
Adiponectin, angiogenin-2, calprotectin, CD30, CRP, Eotaxin, HGF, IL-2R α , IL-6, M-CSF, Macrophage Migration Inhibitory Factor (MIF), Monokine induced by Gamma (MIG), macrophage inflammatory protein (MIP)-3 β , RAGE, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES), S100B, SCGF, Tfr, TNF- α , VEGF had a mean change of less than 26% with no significance towards increasing or decreasing when processed the following day. In contrast, levels of cutaneous T-cell-attracting chemokine (CTACK), fms-like tyrosine kinase-3 (FLT-3) ligand, MMP-1, PF4 and PDGF-AA significantly decreased when processed the next day, whilst levels of IL-16, IL-18, MMP-8, and S100A9 significantly increased when processed the next day. Angiogenin, angiopoietin-1, BDNF, CD40 ligand, IL-1ra, MIP-3 α and PDGF-BB had a mean change of more than 26%, but with no visible trend towards increasing or decreasing when processed the following day. Despite the assays for IL-17A, MMP-12 and MMP-13 working for the pre vs 1-2 days post

fistuloplasty comparisons, for technical reasons, the assays for these proteins stopped working following these experiments and a comparison could not be made between same day and overnight plasma samples for IL-17A, MMP-12 and MMP-13.

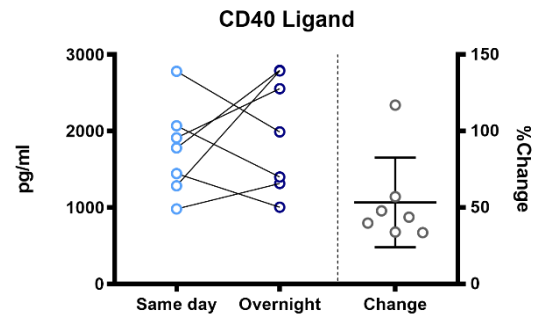
Hence, based on this, data from fistuloplasty plasma patient samples that were processed the following day for proteins which changed by more than 26% and or had significance towards increasing or decreasing when processed the following day, IL-17A, MMP-12 and MMP-13 were retrospectively removed.



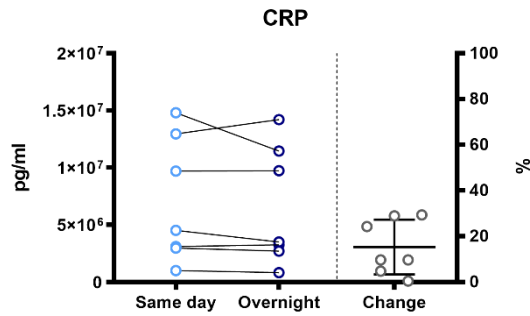
(G)



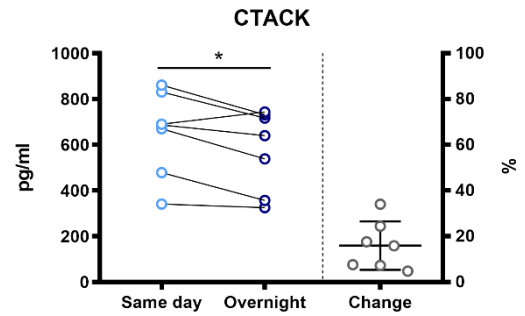
(H)



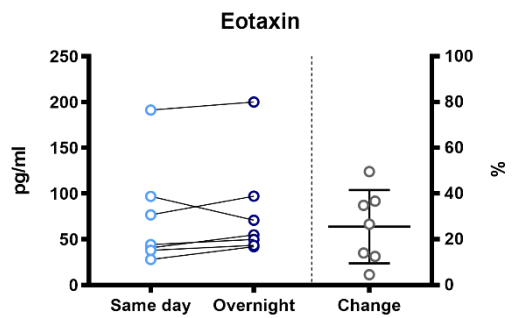
(I)



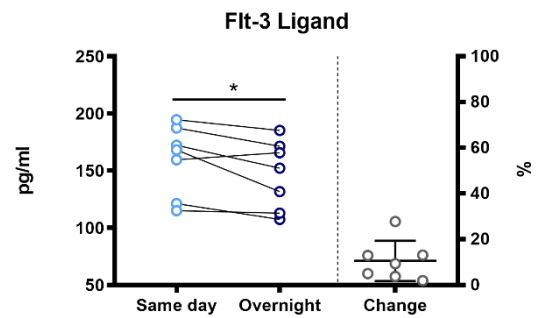
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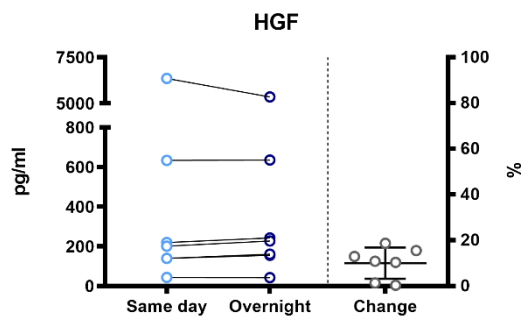
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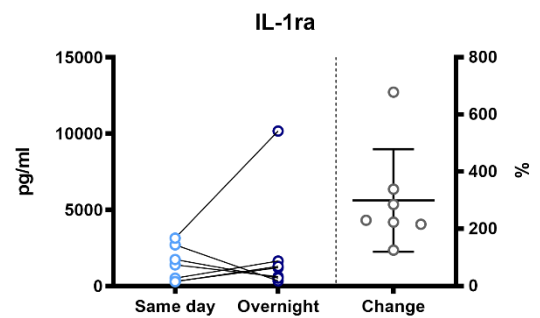
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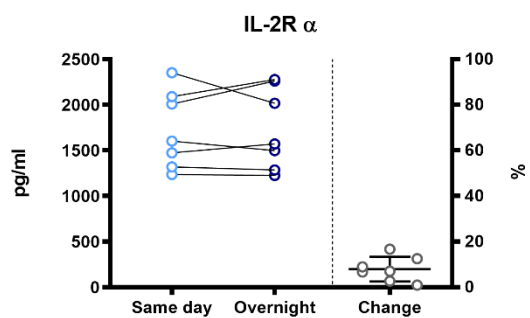
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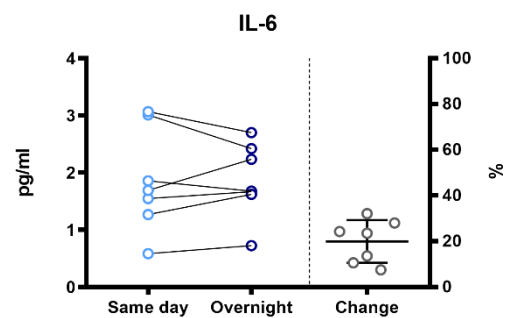
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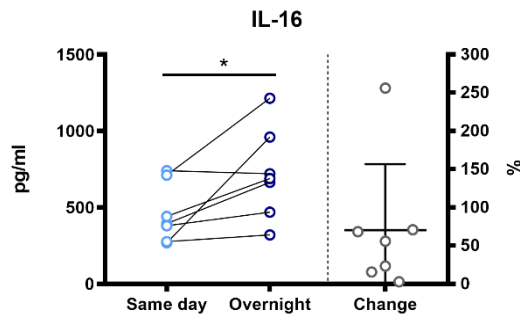
(O)



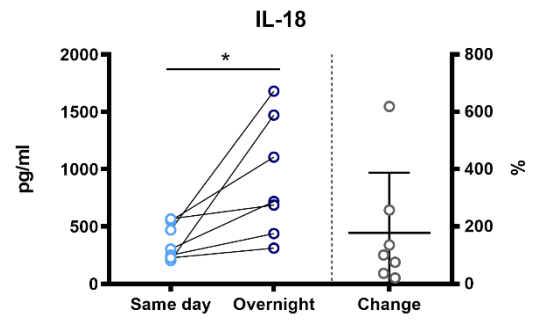
(P)



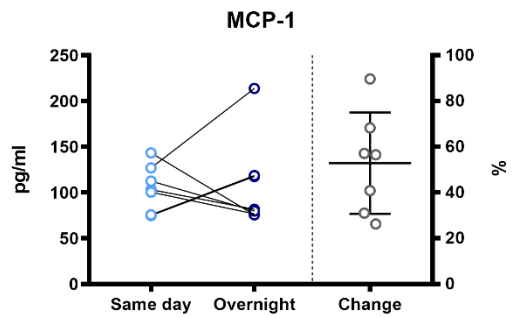
(Q)



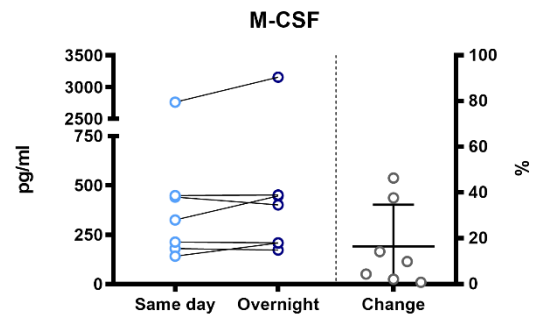
(R)



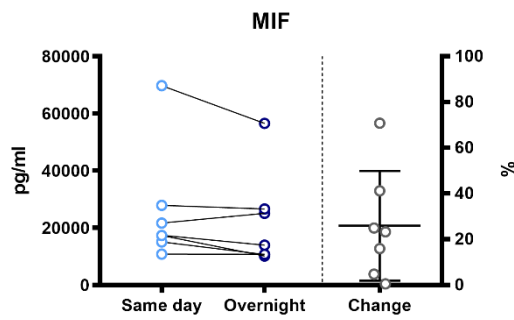
(S)



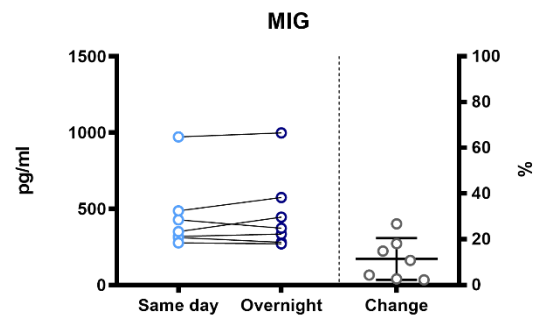
(T)



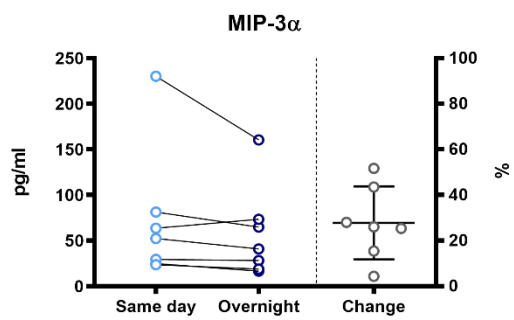
(U)



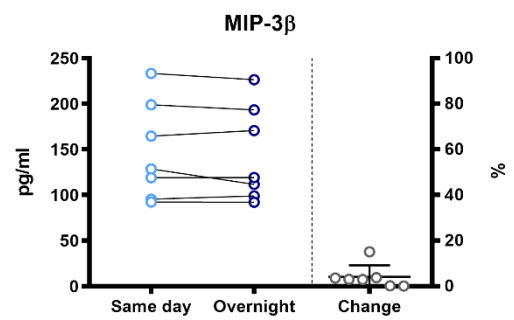
(V)



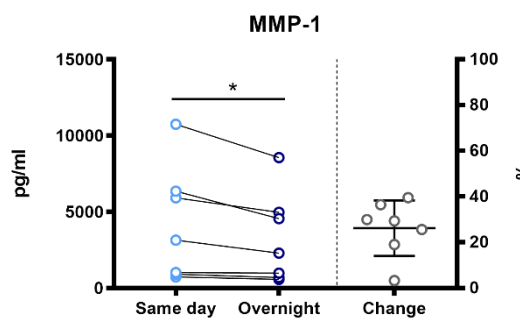
(W)



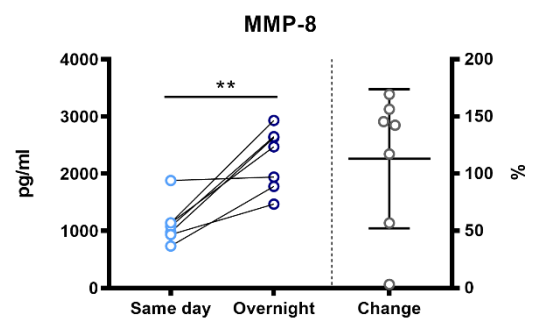
(X)



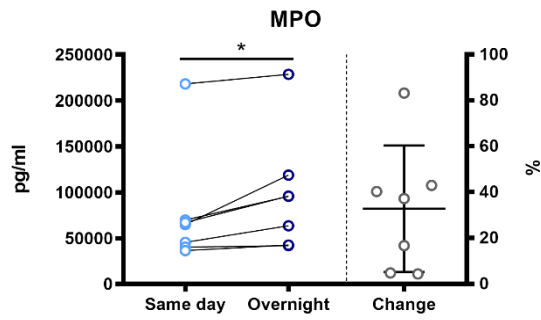
(Y)



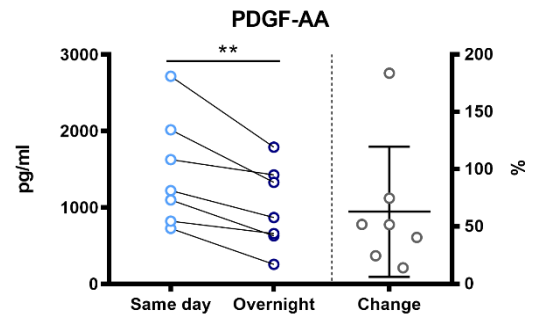
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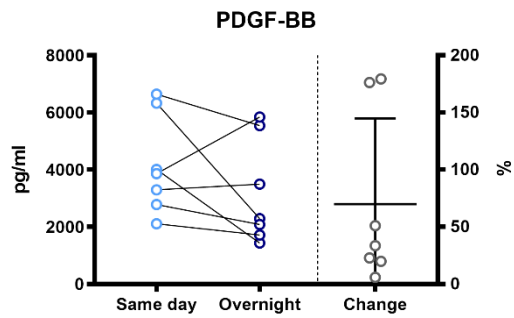
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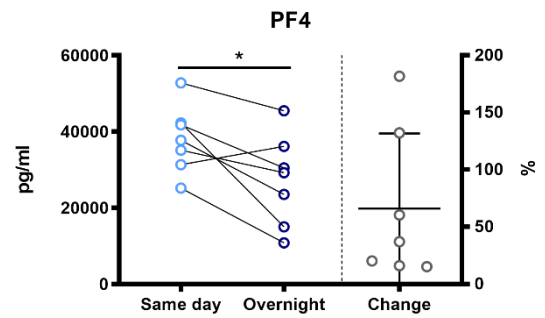
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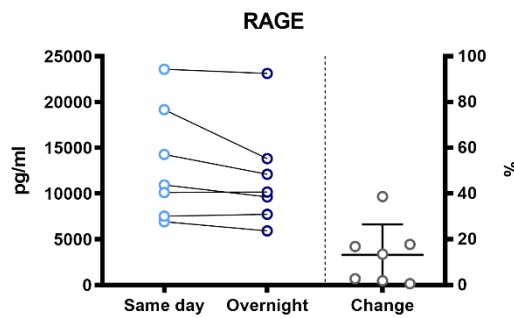
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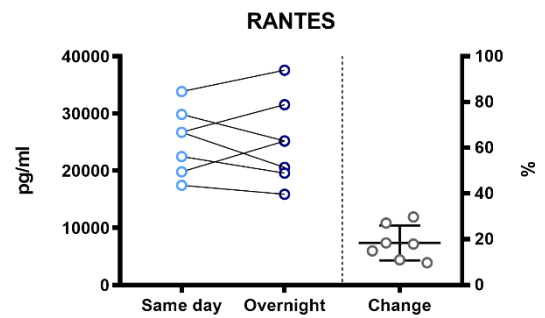
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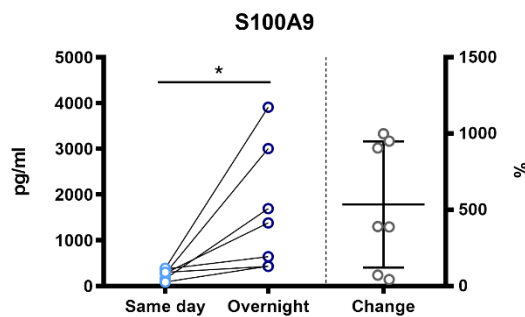
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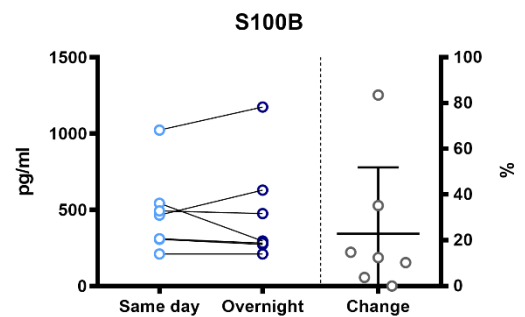
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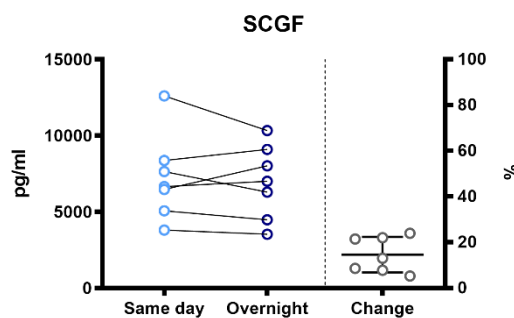
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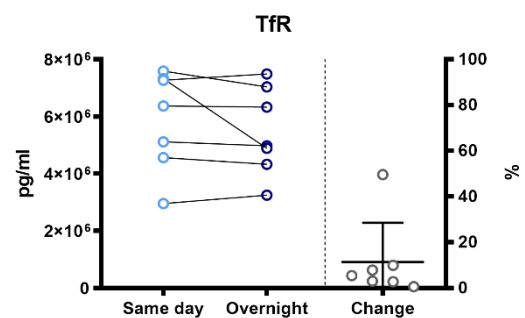
(AH)



(AI)



(AJ)



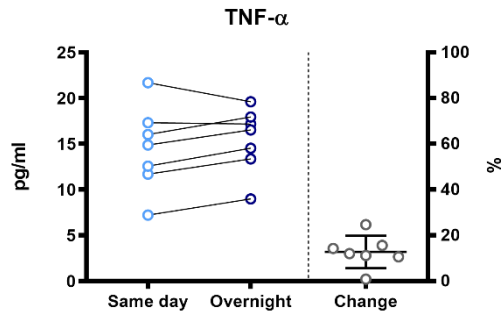
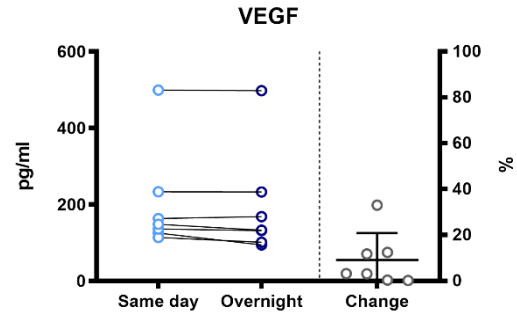
(AK)**(AL)**

Figure 3.17: Changes in plasma protein levels when processed the next day rather than the same day.

Plasma protein levels of samples processed the same day samples (light blue) or processed the following day (dark blue). (n = 7). Data was analysed using a paired *t* test, **p*<0.05, ***p*<0.01. Percentage change data was worked out by subtracting the larger number from the smaller number and then multiplying by 100. The data on the graph for percentage change is expressed as a mean $\pm$ standard deviation.

- (A) Adiponectin: 3.62% mean percentage change.
- (B) Angiogenin: 31.83% mean percentage change.
- (C) Angiopoietin-1: 80.19% mean percentage change.
- (D) Angiopoietin-2: 4.13% mean percentage change.
- (E) BDNF: 52.84% mean percentage change.
- (F) Calprotectin: 8.54% mean percentage change.
- (G) CD30: 15.04% mean percentage change.
- (H) CD40 Ligand: 53.27% mean percentage change.
- (I) CRP: 15.32% mean percentage change.
- (J) CTACK: 15.95% mean percentage change.
- (K) Eotaxin: 25.55% mean percentage change.
- (L) Flt-3 ligand: 10.59% mean percentage change.
- (M) HGF: 10.02% mean percentage change.
- (N) IL-1ra: 299.8% mean percentage change.
- (O) IL-2R α : 7.93% mean percentage change.
- (P) IL-6: 19.97% mean percentage change.
- (Q) IL-16: 70.44% mean percentage change.
- (R) IL-18: 171.19% mean percentage change.
- (S) MCP-1: 52.85% mean percentage change.
- (T) M-CSF: 16.47% mean percentage change.
- (U) MIF: 25.93% mean percentage change.
- (V) MIG: 11.42% mean percentage change.
- (W) MIP-3 α : 27.79% mean percentage change.
- (X) MIP-3 β : 4.12% mean percentage change.
- (Y) MMP-1: 26.19% mean percentage change.
- (Z) MMP-8: 113.05% mean percentage change.
- (AA) MPO: 37.79% mean percentage change.
- (AB) PDGF-AA: 63.04% mean percentage change.
- (AC) PDGF-BB: 69.88% mean percentage change.
- (AD) PF4: 66.14% mean percentage change.
- (AE) RAGE: 13.17% mean percentage change.
- (AF) RANTES: 18.44% mean percentage change.
- (AG) S100A9: 535.71% mean percentage change.
- (AH) S100B: 22.86% mean percentage change.
- (AI) SCGF: 14.60% mean percentage change.
- (AJ) TfR: 11.33% mean percentage change.
- (AK) TNF- α : 12.78% mean percentage change.
- (AL) VEGF: 9.16% mean percentage change.

3.2.8 Some Plasma Proteins are likely to be Associated with Post-intervention Primary and Cumulative patency

I then sought to establish if the assayed proteins would associate with post fistuloplasty outcome. Initially univariate cox regression tests were carried out examining for associations with post-intervention primary patency and post-intervention cumulative patency. Pre fistuloplasty plasma protein levels, post fistuloplasty plasma protein levels and the change in plasma protein levels (calculated by subtracting post fistuloplasty protein levels with pre fistuloplasty protein levels) were investigated.

Table 3-4 is a summary of the univariate analysis findings. Only the levels of pre fistuloplasty MPO associated with post-intervention primary patency and no other proteins, including post level proteins and change in level proteins, associated with post-intervention primary patency outcome. The pre fistuloplasty levels of MIF ($p = 0.024$), PF4 ($p = 0.013$), S100A9 ($p = 0.004$) and TNF- α ($p = 0.038$) significantly associated with post-intervention cumulative patency outcome, and MMP12 and MMP13 were close to significance ($p = 0.052$ and $p = 0.054$ respectively). It is interesting that there was a similar pattern of proteins for post fistuloplasty plasma proteins and change in plasma proteins that associated with cumulative patency outcome. For post fistuloplasty plasma proteins, MMP12 ($p = 0.039$), MMP13 ($p = 0.016$) and S100A9 ($p = 0.005$) significantly associated with post-intervention cumulative patency, as well as protein PDGF-BB ($p = 0.005$). In addition to pre fistuloplasty levels, the change in PF4 ($p = 0.033$) and S100A9 ($p = 0.036$) also associated with post-intervention cumulative patency, as did MPO ($p = 0.021$).

Table 3-4: Univariate cox regression analysis showing the association of assayed plasma proteins with patient outcome.

Table showing the *p* values of the associations between the levels of each pre fistuloplasty protein, post fistuloplasty protein and change in protein level (calculated by subtracting post fistuloplasty with the pre fistuloplasty plasma protein levels) with either post-intervention primary patency or post-intervention cumulative patency outcome. Blue cells highlight protein associations found to be significant or very close to significance.

n = 75 or 54 patients for pre fistuloplasty plasma proteins (dependent on whether overnight samples were included as discussed in section 3.2.7), *n* = 30 post fistuloplasty and change in plasma proteins patient samples.

Plasma protein time point	Pre Fistuloplasty		Post Fistuloplasty		Change (Post minus Pre)	
Post-intervention Outcome	Primary Patency	Cumulative Patency	Primary Patency	Cumulative Patency	Primary Patency	Cumulative Patency
Adiponectin	0.216	0.736	0.223	0.122	0.833	0.622
Angiogenin	0.248	0.424	0.678	0.658	0.819	0.651
Angiopoietin-1	0.427	0.220	0.455	0.844	0.645	0.452
Angiopoietin-2	0.464	0.244	0.938	0.068	0.934	0.994
BDNF	0.503	0.147	0.615	0.248	0.677	0.891
Calprotectin	0.633	0.999	0.485	0.484	0.631	0.260
CD30	0.714	0.620	0.455	0.194	0.463	0.221
CD40 Ligand	0.790	0.234	0.375	0.374	0.963	0.966
CRP	0.809	0.692	0.484	0.128	0.930	0.523
CTACK	0.948	0.591	0.949	0.740	0.287	0.512
Eotaxin	1.000	0.422	0.311	0.070	0.719	0.660
Flt3 Ligand	0.313	0.305	0.732	0.663	0.648	0.132
HGF	0.257	0.804	0.542	0.187	0.935	0.945
IL-16	0.560	0.937	0.640	0.163	0.439	0.660
IL-17A	0.888	0.668	0.721	0.417	0.900	0.983
IL-18	0.950	0.581	0.442	0.424	0.945	0.608
IL-1ra	0.474	0.945	0.467	0.867	0.766	0.563
IL-2R α	0.447	0.629	0.443	0.115	0.424	0.547
IL-6	0.097	0.958	0.226	0.412	0.158	0.761
MCP-1	0.353	0.496	0.571	0.888	0.989	0.821
M-CSF	0.367	0.298	0.710	0.709	0.289	0.784
MIF	0.401	0.024	0.806	0.390	0.576	0.410
MIG	0.765	0.309	0.747	0.166	0.268	0.178
MIP-3 α	0.529	0.199	0.245	0.753	0.165	0.106
MIP-3 β	0.849	0.675	0.966	0.441	0.219	0.265
MMP1	0.904	0.791	0.942	0.845	0.634	0.199
MMP12	0.743	0.052	0.614	0.039	0.624	0.246
MMP13	0.900	0.054	0.625	0.016	0.147	0.172
MMP8	0.426	0.840	0.712	0.254	0.752	0.417
MPO	0.003	0.081	0.306	0.608	0.108	0.021
PDGF-AA	0.973	0.342	0.422	0.795	0.920	0.776
PDGF-BB	0.926	0.149	0.226	0.005	0.202	0.173
PF4	0.160	0.013	0.641	0.144	0.132	0.033
RAGE	0.583	0.267	0.116	0.205	0.965	0.811
RANTES	0.212	0.200	0.317	0.175	0.438	0.471
S100A9	0.265	0.004	0.156	0.005	0.436	0.036
S100B	0.230	0.552	0.755	0.821	0.507	0.397
SCGF	0.678	0.396	0.794	0.170	0.988	0.759
TfR	0.717	0.839	0.341	0.213	0.320	0.651
TNF- α	0.166	0.038	0.325	0.383	0.937	0.149
VEGF	0.816	0.785	0.393	0.978	0.820	0.682

I then sought to carry out multivariate analysis in order to seek for patterns and relationships between several variables simultaneously. I included patient clinical variables (gender, ethnicity, patient age, type of native fistula, log age of fistula, history of previous interventions and previous thrombosis) in case protein associations were being made undetectable due to a larger influence of a clinical variable on the data set. The multivariate analysis carried out manually was a forward analysis as explained in detail in the methods chapter, section 2.8. The exploratory 'stop' p value was set to a limited 0.1 due to the high amount of significances found within the univariate analysis. Multivariate analysis was not carried out for post fistuloplasty proteins and change in plasma proteins to find associations with post-intervention primary patency as there were no factors within the univariate analysis for these that had a p value of less than 0.1.

Therefore, a multivariate analysis was carried out for the following:

- Pre fistuloplasty proteins with associations for post-intervention primary patency (Table 3-5),
- Pre fistuloplasty proteins with associations for post-intervention cumulative patency (Table 3-7),
- Post fistuloplasty proteins with associations with post-intervention cumulative patency (Table 3-9),
- Change in plasma proteins with associations with post-intervention cumulative patency (Table 3-11).

Table 3-5: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and pre fistuloplasty protein levels with post-intervention primary patency.

The 'start' step denotes *p* values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was MPO as it had the lowest *p* value. 'Step 1' shows the *p* values of the first multivariate cox regression analysis carried out in which each variable (both clinical and protein levels) was combined with var1. The protein found to have the lowest *p* value here denoted as variable 2 (var2), and in this case is PF4. 'Step 2' shows the *p* value of the second multivariate cox regression carried out in which each protein was combined with var1 (MPO) and var2 (PF4) to find the protein with the next lowest *p* value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a *p* value of less than 0.10. Light blue cells highlight the protein found to have the lowest *p* value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest *p* value. *n* = 75 or 54 patients for pre fistuloplasty plasma proteins (dependent on whether overnight samples were included as discussed in section 3.2.7).

	0	Step 1	Step 2	STOP
Gender	0.188	0.381	0.433	0.661
Ethnicity	0.022	0.277	0.215	0.274
Patient Age	0.639	0.726	0.681	0.264
Type of Native Fistula	0.423	0.501	0.437	0.848
Log Age of Fistula	0.143	0.408	0.283	0.489
Previous Interventions	0.602	0.389	0.383	0.667
Previous Thrombosis	0.852	0.996	0.981	0.411
Adiponectin	0.503	0.374	0.442	0.773
Angiogenin	0.216	0.059	0.080	0.250
Angiopoietin-1	0.948	0.509	0.916	0.999
Angiopoietin-2	0.809	0.512	0.502	0.478
BDNF	1.000	0.820	0.675	0.947
Calprotectin	0.464	0.217	0.129	0.105
CD30	0.248	0.743	0.647	0.594
CD40 Ligand	0.790	0.717	0.698	0.733
CRP	0.714	0.341	0.293	0.617
CTACK	0.427	0.701	0.852	0.408
Eotaxin	0.633	0.784	0.588	0.455
Flt3 Ligand	0.313	0.366	0.314	0.583
HGF	0.257	0.316	0.164	0.658
IL-16	0.560	0.430	0.192	0.543
IL-17A	0.888	0.575	0.819	0.793
IL-18	0.950	0.926	0.915	0.959
IL-1ra	0.474	0.261	0.214	0.287
IL-2R α	0.447	0.517	0.642	0.720
IL-6	0.097	0.370	0.408	0.692
MCP-1	0.353	0.075	0.055	0.115
M-CSF	0.367	0.805	0.795	0.796
MIF	0.401	0.761	0.468	0.721
MIG	0.765	0.131	0.106	0.608
MIP-3 α	0.529	0.740	0.727	0.110
MIP-3 β	0.849	0.474	0.504	0.813
MMP1	0.904	0.493	0.552	0.887
MMP12	0.743	0.452	0.345	0.528
MMP13	0.900	0.371	0.338	0.695
MMP8	0.426	0.177	0.057	0.365
MPO	0.003	X	X	X
PDGF-AA	0.973	0.490	0.635	0.835
PDGF-BB	0.926	0.717	0.285	0.408
PF4	0.160	0.057	X	X
RAGE	0.583	0.861	0.817	0.696
RANTES	0.212	0.436	0.423	0.564
S100A9	0.265	0.705	0.718	0.376
S100B	0.230	0.256	0.179	0.360
SCGF	0.678	0.075	0.046	X
TfR	0.717	0.254	0.172	0.580
TNF- α	0.166	0.938	0.950	0.624
VEGF	0.816	0.566	0.569	0.780

Following each multivariate analysis, a matrix regression of coefficients table was then carried out in order to assess for co-linearity between variables identified.

- Table 3-6 for pre fistuloplasty proteins associated with post-intervention primary patency,
- Table 3-8 for pre fistuloplasty proteins associated with post-intervention cumulative patency,
- Table 3-10 for post fistuloplasty proteins associated with post-intervention cumulative patency,
- Table 3-12 for change in fistuloplasty proteins associated with post-intervention cumulative patency.

Table 3-6: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-5.

	MPO	PF4	SCGF
MPO			
PF4	0.617		
SCGF	0.191	0.635	

Table 3-7: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and pre fistuloplasty protein levels with post-intervention cumulative patency.

The 'start' step denotes p values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was S100A9 as it had the lowest p value. 'Step 1' shows the p values of the first multivariate cox regression analysis carried out in which each variable (both clinical and protein levels) was combined with var1. The protein found to have the lowest p value here denoted as variable 2 (var2), and in this case is PF4. 'Step 2' shows the p value of the second multivariate cox regression carried out in which each protein was combined with var1 (S100A9) and var2 (PF4) to find the protein with the next lowest p value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a p value of less than 0.10. Light blue cells highlight the protein found to have the lowest p value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest p value. $n = 30$ patients.

	0	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Step 10	Step 11	Step 12	Step 13	STOP
Gender	0.343	0.207	0.244	0.143	0.323	0.047	X	X	X	X	X	X	X	X	X
Ethnicity	0.564	0.257	0.159	0.116	0.494	0.239	0.273	0.233	0.181	0.205	0.183	0.223	0.080	0.085	X
Patient Age	0.025	0.021	0.018	0.021	0.022	X	X	X	X	X	X	X	X	X	X
Type of Native Fistula	0.235	0.027	0.015	0.005	X	X	X	X	X	X	X	X	X	X	X
Log Age of Fistula	0.898	0.719	0.442	0.730	0.609	0.906	0.778	0.550	0.759	0.651	0.938	0.979	0.994	0.646	0.255
Previous Interventions	0.076	0.111	0.798	0.811	0.326	0.543	0.984	0.820	0.731	0.555	0.179	0.203	0.293	0.232	0.209
Previous Thrombosis	0.083	0.820	0.108	0.071	0.882	0.589	0.812	0.855	0.168	0.611	0.080	0.374	0.652	0.550	0.256
Adiponectin	0.736	0.519	0.594	0.690	0.581	0.993	0.567	0.756	0.678	0.867	0.930	0.927	0.344	0.949	0.717
Angiogenin	0.424	0.697	0.858	0.892	0.750	0.280	0.261	0.388	0.271	0.986	0.845	0.827	0.965	0.927	0.959
Angiopoietin-1	0.220	0.562	0.499	0.536	0.418	0.508	0.317	0.769	0.938	0.996	0.986	0.499	0.826	0.677	0.383
Angiopoietin-2	0.244	0.091	0.077	0.085	0.137	0.332	0.198	0.238	0.699	0.957	0.487	0.097	0.068	X	X
BDNF	0.147	0.287	0.922	0.807	0.813	0.754	0.656	0.362	0.440	0.266	0.337	0.475	0.893	0.789	0.586
Calprotectin	0.999	0.919	0.615	0.717	0.413	0.461	0.248	0.458	0.292	0.313	0.513	0.798	0.671	0.688	0.785
CD30	0.620	0.961	0.822	0.997	0.659	0.760	0.343	0.898	0.852	0.940	0.651	0.681	0.366	0.619	0.603
CD40 Ligand	0.234	0.577	0.527	0.657	0.756	0.700	0.313	0.607	0.893	0.752	0.855	0.436	0.633	0.569	0.268
CRP	0.692	0.913	0.775	0.833	0.978	0.323	0.452	0.589	0.274	0.146	X	X	X	X	X
CTACK	0.591	0.332	0.241	0.195	0.373	0.100	0.296	0.252	0.164	0.326	0.521	0.613	0.579	0.825	0.740
Eotaxin	0.422	0.100	0.145	0.092	0.130	0.478	0.260	0.130	X	X	X	X	X	X	X
Flt3 Ligand	0.305	0.970	0.870	0.857	0.563	0.822	0.478	0.854	0.693	0.868	1.000	0.954	0.652	0.680	0.737
HGF	0.804	0.911	0.550	0.607	0.681	0.268	0.327	0.440	0.097	X	X	X	X	X	X
IL-16	0.937	0.621	0.229	0.290	0.338	0.181	0.320	0.695	0.174	0.763	0.875	0.711	0.861	0.625	0.526
IL-17A	0.668	0.103	0.910	0.842	0.975	0.543	0.555	0.608	0.601	0.490	0.846	0.955	0.979	0.535	0.618
IL-18	0.581	0.357	0.206	0.209	0.197	0.088	0.152	0.132	0.118	0.210	0.547	0.817	0.618	0.396	0.697
IL-1ra	0.945	0.839	0.730	0.878	0.943	0.916	0.883	0.965	0.727	0.960	0.956	0.960	0.898	0.990	0.824
IL-2R α	0.629	0.329	0.415	0.624	0.615	0.595	0.391	0.641	0.510	0.818	0.584	0.303	0.746	0.621	0.837
IL-6	0.958	0.307	0.254	0.282	0.250	0.522	0.733	0.428	0.550	0.348	0.104	0.095	0.134	0.260	0.363
MCP-1	0.496	0.592	0.717	0.846	0.982	0.911	0.516	0.723	0.696	0.991	0.838	0.880	0.701	0.994	0.370

	0	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Step 10	Step 11	Step 12	Step 13	STOP
M-CSF	0.298	0.023	0.009	X	X	X	X	X	X	X	X	X	X	X	X
MIF	0.024	0.896	0.565	0.578	0.954	0.996	0.593	0.611	0.813	0.608	0.641	0.835	0.355	0.381	0.358
MIG	0.309	0.996	0.911	0.799	0.876	0.387	0.686	0.737	0.220	0.580	0.923	0.075	X	X	X
MIP-3 α	0.199	0.623	0.613	0.618	0.732	0.597	0.781	0.847	0.833	0.593	0.096	0.433	0.847	0.628	0.267
MIP-3 β	0.675	0.475	0.412	0.293	0.199	0.598	0.928	0.895	0.675	0.670	0.838	0.699	0.683	0.791	0.988
MMP1	0.791	0.773	0.934	0.749	0.648	0.742	0.756	0.816	0.658	0.784	0.545	0.773	0.612	0.773	0.787
MMP12	0.052	0.230	0.391	0.378	0.146	0.698	0.988	0.461	0.406	0.458	0.619	0.230	0.372	0.683	0.744
MMP13	0.054	0.982	0.825	0.846	0.622	0.241	0.260	0.336	0.224	0.175	0.377	0.249	0.174	0.103	0.101
MMP8	0.840	0.880	0.400	0.498	0.403	0.272	0.409	0.927	0.443	0.733	0.917	0.379	0.553	0.929	0.458
MPO	0.081	0.189	0.119	0.513	0.991	0.766	0.397	0.472	0.453	0.764	0.924	0.778	0.881	0.754	0.911
PDGF-AA	0.342	0.802	0.961	0.919	0.810	0.890	0.425	0.689	0.966	0.651	0.344	0.810	0.326	0.516	0.763
PDGF-BB	0.149	0.351	0.682	0.548	0.956	0.840	0.806	0.661	0.660	0.576	0.235	0.128	0.078	0.163	0.160
PF4	0.013	0.005	X	X	X	X	X	X	X	X	X	X	X	X	X
RAGE	0.267	0.111	0.088	0.084	0.093	0.208	0.318	0.201	0.282	0.350	0.834	0.892	0.809	0.956	0.962
RANTES	0.200	0.097	0.082	0.082	0.068	0.209	0.276	0.334	0.985	0.214	0.141	0.136	0.120	0.100	0.105
S100A9	0.004	X	X	X	X	X	X	X	X	X	X	X	X	X	X
S100B	0.552	0.685	0.448	0.516	0.478	0.309	0.079	X	X	X	X	X	X	X	X
SCGF	0.396	0.356	0.485	0.718	0.391	0.886	0.824	0.584	0.166	0.402	0.079	X	X	X	X
TfR	0.839	0.461	0.304	0.168	0.377	0.350	0.573	0.560	0.782	0.281	0.315	0.123	0.069	0.097	0.261
TNF- α	0.038	0.143	0.071	0.069	0.136	0.527	0.516	0.691	0.782	0.395	0.251	0.387	0.168	0.256	0.510
VEGF	0.785	0.521	0.559	0.262	0.231	0.304	0.216	0.428	0.327	0.277	0.211	0.120	0.302	0.336	0.257

Table 3-8: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-7.
Variables highlighted yellow are *p<0.05, orange **p<0.01, red *p<0.001.**

	S100A9	PF4	MCSF	Fistula Type	Patient Age	Gender	S100B	Eotaxin	HGF	CRP	SCGF	MIG	Angio-poitin2	Eth-nicity
S100A9														
PF4	0.719													
MCSF	0.494	0.613												
Fistula Type	0.393	0.654	0.251											
Patient Age	0.408	0.577	0.918	0.194										
Gender	0.998	0.306	0.495	0.325	0.167									
S100B	0.012	0.143	0.145	0.606	0.769	0.457								
Eotaxin	0.439	0.354	0.348	0.611	0.038	0.612	0.378							
HGF	0.706	0.191	0.702	0.853	0.036	0.220	0.539	< 0.001						
CRP	0.052	0.811	0.932	0.421	0.037	0.384	0.490	0.180	0.614					
SCGF	0.027	0.635	0.523	0.840	0.107	0.987	0.103	0.453	0.579	0.020				
MIG	0.004	0.919	0.927	0.416	0.083	0.468	0.339	0.005	0.149	< 0.001	< 0.001			
Angiopoietin2	0.925	0.499	0.486	0.963	0.151	0.037	0.214	0.626	0.296	0.026	0.936	0.173		
Ethnicity	0.412	0.777	0.162	0.009	0.164	0.772	0.516	0.753	0.183	0.496	0.274	0.455	0.639	

Table 3-9: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and post fistuloplasty protein levels with post-intervention cumulative patency.

The 'start' step denotes *p* values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was PDGF-BB and S100A9 as they had the lowest *p* values. 'Step 1' shows the *p* values of the first multivariate cox regression analysis carried out in which each variable (both clinical and protein levels) was combined with var1. The protein found to have the lowest *p* value here denoted as variable 2 (var2), and in this case is patient age. 'Step 2' shows the *p* value of the second multivariate cox regression carried out in which each protein was combined with var1 (PDGF-BB and S100A9) and var2 (patient age) to find the protein with the next lowest *p* value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a *p* value of less than 0.10. Light blue cells highlight the protein found to have the lowest *p* value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest *p* value. *n* = 30 patients.

	0	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	STOP
Gender	0.726	0.848	0.462	0.332	0.321	0.977	0.182	0.257	0.119	0.269	0.398
Ethnicity	0.988	0.765	0.841	0.587	0.567	0.715	0.992	0.072	0.578	0.882	0.597
Patient Age	0.032	0.018	X	X	X	X	X	X	X	X	X
Type of Native Fistula	0.144	0.222	0.335	0.464	0.507	0.416	0.454	0.072	0.167	0.652	0.735
Log Age of Fistula	0.418	0.976	0.600	0.852	0.421	0.187	0.697	0.099	0.760	0.875	0.686
Previous Interventions	0.762	0.415	0.674	0.835	0.550	0.591	0.373	0.239	0.184	0.328	0.797
Previous Thrombosis	0.179	0.180	0.688	0.759	0.704	0.464	0.939	0.220	0.105	0.079	0.673
Adiponectin	0.122	0.154	0.090	0.106	0.094	0.219	0.232	0.301	0.345	0.429	0.602
Angiogenin	0.658	0.601	0.604	0.848	1.000	0.557	0.566	0.299	0.706	0.613	0.546
Angiopoietin-1	0.844	0.443	0.758	0.171	0.269	0.193	0.730	0.340	0.089	0.657	0.660
Angiopoietin-2	0.068	0.170	0.673	0.711	0.950	0.032	0.333	0.667	0.696	0.312	0.988
BDNF	0.248	0.511	0.039	X	X	X	X	X	X	X	X
Calprotectin	0.484	0.538	0.959	0.377	0.432	0.486	0.021	X	X	X	X
CD30	0.194	0.289	0.245	0.118	0.205	0.389	0.038	0.127	0.207	0.279	0.483
CD40 Ligand	0.374	0.900	0.934	0.599	0.883	0.713	0.396	0.211	0.066	0.212	0.799
CRP	0.128	0.333	0.997	0.697	0.610	0.520	0.162	0.875	0.123	0.238	0.450
CTACK	0.74	0.664	0.877	0.770	0.505	0.517	0.403	0.258	0.648	0.587	0.675
Eotaxin	0.07	0.202	0.814	0.965	0.995	0.130	0.382	0.541	0.847	0.219	0.389
Flt3 Ligand	0.663	0.598	0.558	0.907	0.937	0.149	0.040	0.247	0.093	0.550	0.378
HGF	0.187	0.633	0.866	0.462	0.462	0.602	0.266	0.878	0.881	0.218	0.455
IL-16	0.163	0.956	0.853	0.888	0.900	0.472	0.891	0.269	0.983	0.176	0.431
IL-17A	0.417	0.771	0.778	0.856	0.821	0.045	0.084	0.144	0.432	0.354	0.639
IL-18	0.424	0.673	0.533	0.611	0.489	0.862	0.819	0.078	0.682	0.255	0.871
IL-1ra	0.867	0.431	0.776	0.358	0.481	0.576	0.487	0.166	0.694	0.122	0.282
IL-2R α	0.115	0.261	0.449	0.154	0.228	0.600	0.277	0.126	0.836	0.734	0.621
IL-6	0.412	0.929	0.937	0.608	0.419	0.182	0.256	0.144	0.401	0.477	0.519
MCP-1	0.888	0.664	0.657	0.301	0.356	0.151	0.214	0.132	0.178	0.078	X
M-CSF	0.709	0.677	0.490	0.325	0.039	X	X	X	X	X	X
MIF	0.39	0.099	0.095	0.043	X	X	X	X	X	X	X
MIG	0.166	0.223	0.846	0.324	0.045	0.090	0.094	0.234	0.051	X	X
MIP-3α	0.753	0.811	0.554	0.772	0.340	0.406	0.776	0.035	X	X	X
MIP-3β	0.441	0.259	0.798	0.775	0.942	0.383	0.723	0.075	0.168	0.735	0.799
MMP1	0.845	0.848	0.703	0.250	0.501	0.060	0.071	0.098	0.152	0.272	0.314
MMP12	0.039	0.225	0.705	0.271	0.574	0.746	0.287	0.043	0.239	0.939	0.821
MMP13	0.016	0.449	0.972	0.880	0.189	0.285	0.922	0.372	0.632	0.724	0.662
MMP8	0.254	0.659	0.971	0.166	0.284	0.012	X	X	X	X	X
MPO	0.608	0.919	0.855	0.844	0.462	0.827	0.251	0.504	0.108	0.342	0.473
PDGF-AA	0.795	0.322	0.410	0.046	0.039	X	X	X	X	X	X
PDGF-BB	0.005	X	X	X	X	X	X	X	X	X	X
PF4	0.144	0.885	0.153	0.708	0.558	0.604	0.195	0.813	0.963	0.250	0.331
RAGE	0.205	0.398	0.750	0.910	0.498	0.296	0.053	0.599	0.419	0.940	0.771
RANTES	0.175	0.022	0.088	0.085	0.064	0.253	0.504	0.167	0.134	0.415	0.876
S100A9	0.005	X	X	X	X	X	X	X	X	X	X
S100B	0.821	0.230	0.459	0.153	0.234	0.386	0.236	0.220	0.829	0.179	0.390
SCGF	0.17	0.549	0.357	0.670	0.618	0.880	0.344	0.333	0.142	0.701	0.945
TfR	0.213	0.441	0.565	0.856	0.716	0.897	0.327	0.202	0.219	0.370	0.603
TNF-α	0.383	0.547	0.822	0.356	0.611	0.735	0.189	0.149	0.094	0.139	0.257
VEGF	0.978	0.302	0.332	0.500	0.443	0.104	0.130	0.072	0.220	0.233	0.873

Table 3-10: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-9. Variables highlighted yellow are *p<0.05, orange **p<0.01, red *p<0.001.**

	PDGFBB	S100A9	Patient Age	BDNF	MIF	MCSF	PDGFAA	MMP8	Cal-protectin	MIP3a	MIG	MCP1
PDGFBB												
S100A9	0.007											
Patient Age	0.714	0.282										
BDNF	0.082	0.903	0.701									
MIF	0.001	0.009	0.675	0.080								
MCSF	0.585	0.967	0.864	0.616	0.668							
PDGFAA	0.008	0.255	0.885	0.001	0.013	0.307						
MMP8	0.077	0.008	0.908	0.019	0.587	0.565	0.084					
Calprotectin	0.375	0.715	0.181	0.199	0.518	0.810	0.255	0.652				
MIP3a	0.854	0.849	0.299	0.474	0.690	0.980	0.465	0.617	0.271			
MIG	0.371	0.129	0.130	0.042	0.248	0.495	0.042	0.181	0.706	0.025		
MCP1	0.513	0.814	0.836	0.483	0.516	0.650	0.251	0.721	0.135	0.910	0.725	

Table 3-11: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and change in fistuloplasty protein levels with post-intervention cumulative patency.

The 'start' step denotes p values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was MPO as it had the lowest p values. 'Step 1' shows the p values of the first multivariate cox regression analysis carried out in which each variable (both clinical and protein levels) was combined with var1. The protein found to have the lowest p value here denoted as variable 2 (var2), and in this case is PF4. 'Step 2' shows the p value of the second multivariate cox regression carried out in which each protein was combined with var1 (MPO) and var2 (PF4) to find the protein with the next lowest p value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a p value of less than 0.10. Light blue cells highlight the protein found to have the lowest p value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest p value. $n = 30$ patients.

	0	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	STOP
Gender	0.726	0.528	0.612	0.982	0.777	0.663	0.215	0.959
Ethnicity	0.988	0.806	0.8	0.752	0.480	0.853	0.117	0.499
Patient Age	0.032	0.045	0.051	0.075	0.103	0.003	X	X
Type of Native Fistula	0.144	0.106	0.11	0.322	0.178	0.198	0.867	0.104
Log Age of Fistula	0.418	0.753	0.872	0.651	0.702	0.503	0.151	0.335
Previous Interventions	0.762	0.299	0.263	0.21	0.392	0.139	0.561	0.698
Previous Thrombosis	0.179	0.137	0.112	0.183	0.983	0.515	0.777	0.217
Adiponectin	0.622	0.558	0.479	0.389	0.679	0.314	0.512	0.366
Angiogenin	0.651	0.985	0.656	0.333	0.470	0.505	0.7	0.735
Angiopoietin1	0.452	0.763	0.913	0.219	0.298	0.005	0.402	0.513
Angiopoietin2	0.994	0.878	0.641	0.461	0.506	0.662	0.741	0.647
BDNF	0.891	0.379	0.397	0.897	0.838	0.134	0.437	0.473
Calprotectin	0.26	0.145	0.115	0.351	0.716	0.84	0.118	0.103
CD30	0.221	0.393	0.577	0.698	0.601	0.227	0.173	0.147
CD40Ligand	0.966	0.875	0.651	0.269	0.312	0.037	0.895	0.724
CRP	0.523	0.523	0.354	0.746	0.422	0.386	0.086	0.141
CTACK	0.512	0.633	0.527	0.912	0.735	0.53	0.309	0.213
Eotaxin	0.66	0.472	0.437	0.946	0.229	0.481	0.734	0.566
Flt3Ligand	0.132	0.756	0.508	0.981	0.475	0.29	0.639	0.465
HGF	0.945	0.74	0.732	0.27	0.721	0.33	0.244	0.183
IL16	0.66	0.759	0.607	0.946	0.324	0.74	0.467	0.329
IL17A	0.983	0.557	0.532	0.831	0.921	0.443	0.731	0.311
IL18	0.608	0.502	0.45	0.231	0.431	0.362	0.608	0.767
IL1Ralpha	0.563	0.812	0.683	0.98	0.731	0.45	0.608	0.616
IL2Ralpha	0.547	0.842	0.944	0.953	0.678	0.665	0.498	0.453
IL6	0.761	0.646	0.476	0.762	0.949	0.163	0.855	0.529
MCP1	0.821	0.747	0.76	0.389	0.273	0.397	0.343	0.159
MCSF	0.784	0.236	0.226	0.318	0.488	0.613	0.173	0.129
MIF	0.41	0.732	0.839	0.749	0.974	0.765	0.617	0.917
MIG	0.178	0.75	0.804	0.701	0.361	0.442	0.845	0.991
MIP3a	0.106	0.079	0.066	0.114	0.624	0.239	0.681	0.609
MIP3b	0.265	0.242	0.255	0.195	0.138	0.448	0.35	0.6
MMP1	0.199	0.223	0.154	0.283	0.071	X	X	X
MMP12	0.246	0.27	0.037	X	X	X	X	X
MMP13	0.172	0.651	0.706	0.982	0.358	0.999	0.423	0.651
MMP8	0.417	0.998	0.951	0.766	0.508	0.011	0.166	0.218
MPO	0.021	X	X	X	X	X	X	X
PDGFAA	0.776	0.397	0.302	0.87	0.908	0.011	0.855	0.315
PDGFBB	0.173	0.281	0.169	0.374	0.265	0.448	0.7	0.603
PF4	0.033	0.022	X	X	X	X	X	X
RAGE	0.811	0.963	0.879	0.283	0.225	0.863	0.065	X
RANTES	0.471	0.471	0.494	0.585	0.630	0.782	0.906	0.957
S100A9	0.036	0.159	0.104	0.022	X	X	X	X
S100B	0.397	0.983	0.925	0.842	0.214	0.622	0.202	0.109
SCGF	0.759	0.828	0.695	0.403	0.984	0.494	0.243	0.302
tfR	0.651	0.526	0.509	0.586	0.535	0.332	0.852	0.303
TNFa	0.149	0.161	0.072	0.068	0.642	0.594	0.496	0.937
VEGF	0.682	0.555	0.242	0.643	0.749	0.578	0.74	0.249

Table 3-12: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-11.
Variables highlighted yellow are *p<0.05, orange **p<0.01.

	MPO	PF4	MMP12	S100A9	MMP1	Patient Age	RAGE
MPO							
PF4	0.981						
MMP12	0.844	0.005					
S100A9	0.094	0.768	0.152				
MMP1	0.934	0.681	0.182	0.988			
Patient Age	0.405	0.484	0.998	0.749	0.240		
RAGE	0.821	0.695	0.049	0.382	0.791	0.780	

The preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and pre fistuloplasty proteins included MPO, PF4 and SCGF with a final *p* value of 0.001 (Table 3-13). The preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and pre fistuloplasty proteins included S100A9, PF4, SCGF, patient age, gender and ethnicity with a final *p* value of less than 0.001 (Table 3-14). The preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and the change in proteins post fistuloplasty included S100A9, patient age, M-CSF, calprotectin, MIP-3 α and MCP-1 with a final *p* value of 0.025 (Table 3-15). The preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and the change in proteins post fistuloplasty included MPO, PF4, S100A9, MMP1 and patient age with a final *p* value of less than 0.001 (Table 3-16). Asides from age, gender and ethnicity, none of the identified proteins had a hazard ratio with a magnitude deemed clinically important. Despite this, the persistent appearance of S100A9, PF4 and MPO in both univariate and multivariate models, different patency outcomes and different protein measurements does make it likely that S100A9, PF4 and MPO are important for post-intervention AVF patency and the lack of hazard ratios is more due to a having inadequate numbers of patient samples.

Table 3-13: Table summarising a preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and pre fistuloplasty plasma proteins.

Omnibus Tests of Model Coefficients				
Significance		0.001	-2 Log Likelihood	240.303
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
MPO	< 0.001	1.000	1.000	1.000
PF4	0.022	1.000	1.000	1.000
SCGF	0.046	1.000	1.000	1.000

Table 3-14: Table summarising a preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and pre fistuloplasty plasma proteins.

Omnibus Tests of Model Coefficients				
Significance		< 0.001	-2 Log Likelihood	103.025
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
S100A9	0.001	1.000	1.000	1.001
PF4	0.015	1.000	1.000	1.000
M-CSF	0.001	1.001	1.000	1.002
Patient Age	0.002	1.084	1.030	1.141
Gender	0.020	3.434	1.219	9.673
Ethnicity	0.031	2.355	1.084	5.119

Table 3-15: Table summarising a preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and post fistuloplasty plasma proteins.

Omnibus Tests of Model Coefficients				
Significance		0.025	-2 Log Likelihood	61.188
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
S100A9	0.010	1.000	1.000	1.001
Patient Age	0.060	1.043	1.043	1.089
M-CSF	0.612	0.994	0.994	1.016
Calprotectin	0.612	1.000	1.000	1.000
MIP-3 α	0.790	0.999	0.999	1.005
MCP-1	0.975	1.000	1.000	1.008

Table 3-16: Table summarising a preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and the change in plasma proteins post fistuloplasty.

Intestoplasty.

Omnibus Tests of Model Coefficients				
Significance	< 0.001		-2 Log Likelihood	37.115
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
MPO	0.504	1.000	1.000	1.000
PF4	0.004	1.000	1.000	1.000
S100A9	0.009	0.999	0.999	1.000
MMP1	0.008	1.007	1.002	1.012
Patient Age	0.009	1.230	1.052	1.437

3.2.9 Some Patient Demographic and Clinical Variables are Associated with Post-intervention Primary and Cumulative patency

Given that none of the proteins assessed had a hazard ratio of a significant magnitude, additional statistical modelling was performed to assess the relative impact of standard patient demographic and clinical variables, such as gender and log age of fistula. It was found that non-white ethnicity significantly associated with post-intervention primary patency ($p = 0.013$, hazard ratio of 1.533) (Table 3-17) and patient age significantly associated with post-intervention cumulative patency ($p = 0.025$, hazard ratio of 1.038) (Table 3-18). Multivariate analysis was also carried out and did not find any other associations (not shown).

Table 3-17: Univariate cox regression analysis showing associations of patient demographic and clinical variables with post-intervention primary patency outcome. Blue cells highlight associations found to be significant. n = 75 patients.

	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Gender	0.188	1.453	0.833	2.535
Ethnicity	0.022	1.533	1.065	2.208
Patient Age	0.639	1.005	0.984	1.026
Type of Native Fistula	0.423	1.170	0.796	1.720
Log Age of Fistula	0.143	0.617	0.323	1.177
Previous Interventions	0.602	0.848	0.456	1.576
Previous Thrombosis	0.852	1.145	0.276	4.750

Table 3-18: Univariate cox regression analysis showing associations of patient demographic and clinical variables with post-intervention cumulative patency outcome. Blue cells highlight associations found to be significant. n = 75 patients.

	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Gender	0.343	1.487	0.655	3.373
Ethnicity	0.564	1.177	0.677	2.046
Patient Age	0.025	1.038	1.005	1.072
Type of Native Fistula	0.235	1.429	0.793	2.574
Log Age of Fistula	0.898	0.935	0.336	2.603
Previous Interventions	0.076	2.112	0.925	4.822
Previous Thrombosis	0.083	3.751	0.843	16.690

3.2.10 Discussion

To explore the protein changes after a fistuloplasty procedure, a protein screen consisting of a panel of 52 proteins was applied to patient samples pre and post fistuloplasty via a combination of ELISAs and Luminex assays. Initially this was applied to 20 pairs of samples and it was found that 11 of the selected proteins (endothelin-1, G-CSF, GM-CSF, IFN- γ , IL-10, IL-1 β , IL-1 α , IL-2, IL-4, resistin, SDF-1 α) were undetectable. With the exception of resistin, this was because the proteins were below the lower detection limits of the kits used and hence the detection limits of the assays for these proteins were a limitation of this study.

Another limitation of this study is the possibility that some of the proteins degraded overtime. For instance, de Jager *et al* found that within four years, several plasma cytokines degraded to 75% of baseline values [142]. Given that initially I was only looking for post fistuloplasty changes and that the post sample was taken only a few days after the baseline sample at most, this was not initially a large concern as I was only interested in the change post fistuloplasty rather than the amount of protein. When associating proteins with post-intervention outcome, however, the amount of protein was important and thus this is a limitation that must be considered. Secondly freeze thawing plasma is known to result in a degradation of proteins. Whilst pre and post samples were always assayed together with the same number of freeze thaw cycles and great care was taken not to freeze thaw patient plasma samples many times, it is conceded that each patient sample underwent at least one freeze thaw cycle which may have affected some results. Indeed freeze thaw cycles may affect some proteins more than others.

Of the remaining 41 proteins assayed in 30 pairs of patient samples, only IL-6, TNF- α and MPO were found to have significantly changed 1-2 days post fistuloplasty. These proteins were also measured in patients who only had a fistulogram rather than a fistuloplasty procedure. Whilst only four patients met this category, it is unlikely the

changes in IL-6, TNF- α and MPO were due to the administration of contrast or heparin as there was no observable trends 1-2 days post fistulogram in these four patients. These samples served as important controls because radiological contrast media and heparin is known to bind to a large number of plasma proteins, including PF4 [143]. Plasma levels of IL-6, TNF- α , MPO and CRP were further studied using a time point experiment which included samples immediately taken after contrast and heparin administration. The levels of these plasma proteins remained consistent so it is unlikely that contrast or heparin had an immediate effect.

IL-6 was also found to significantly increase 1-2 days post fistuloplasty, peaking between 2-3 hours. Similarly, TNF- α significantly increased 1-2 days post fistuloplasty, peaking at 3 hours post. Both cytokines are key pro-inflammatory cytokines and their increase is in keeping with a typical inflammatory response to balloon treatment [144] [145]. When comparing pre and 1-2 day post fistuloplasty plasma samples, MPO was found to significantly decrease post fistuloplasty. Interestingly, the detailed time course experiment found that MPO levels spiked at 3 hours post fistuloplasty. Whilst this was not statistically significant with the few samples I had, it is worth further exploring this trend. Aminian *et al* also reported a similar phenomenon with an increase of serum MPO in patients with stable angina undergoing coronary stenting one post procedure, followed by a subsequent decrease in serum MPO below patient baseline levels six hours post procedure [146]. Similarly, Matijevic *et al*, who carried out the Atherosclerosis Risk in Communities Carotid Magnetic Resonance Imaging Study, found that monocyte MPO levels were significantly lower in patients with peripheral arterial disease (PAD), which is caused by progressive atherosclerosis that occludes the arterial lumen [147]. Both authors give similar theories to reason MPO decreased post procedure, namely that circulating MPO decreases due to it binding to the site of injury, with an initial increase being linked to an acute increase in neutrophil and monocyte inflammatory activity. Indeed, newly released positively charged MPO directly interacts with the heparin-sulfate proteoglycans associated with the

endothelium layer via electrostatic forces [148]. Therefore, it could be that post fistuloplasty there was an acute increase in neutrophil and monocyte activity, hence the increase of MPO at 3 hours, followed by a decrease in cell activation and the circulating MPO binding to the injured endothelium.

Circulating CRP within the plasma was not shown to significantly change 1-2 days post fistuloplasty, but because of its clinical significance as a potential biomarker, it was further investigated using with a time course experiment and CRP was found to significantly increase 0 hours post fistuloplasty with a trend towards increasing at 2 hours ($p = 0.502$) before starting to decrease again. The fluctuations in CRP post fistuloplasty perhaps highlights why there are discrepancies in the literature about how useful it is as a biomarker for AVF patency. Perhaps if CRP had measured in all patients 2 hours post fistuloplasty it would have better associated with patient outcome. Indeed for many of the proteins I measured there was no significant change 1-2 days post fistuloplasty, but that does not mean a change would not have occurred 2 or 3 hours post fistuloplasty. Also quite notably, many proteins assayed were very sensitive to overnight processing time. Therefore a further limitation of this study was that plasma samples processed within 1 hour in contrast to plasma samples processed within 4 hours would not also have notable protein levels fluctuations.

PF4 (CXCL4) was found to significantly associate with post-intervention patency: In the univariate analyses, pre fistuloplasty PF4 levels and the change in PF4 plasma levels associated with cumulative patency. In the multivariate analysis pre fistuloplasty PF4 levels associated with primary patency and pre fistuloplasty and change in PF4 protein levels associated with cumulative patency. PF4 is the most abundant platelet chemokine and is released in high concentrations from the alpha granules of platelets upon platelet stimulation. PF4 has directly been shown to interact with SMCs by stimulating them to have an inflammatory synthetic phenotype and has been directly linked to excoriating NIH formation hence the rationale for antiplatelet therapy in

patients at risk for atherosclerosis [149] [150]. Besides from its interactions with SMCs, activated platelets have been shown to bind to monocytes, to form platelet–leukocyte aggregates during which they deposit PF4 on the monocyte surface, which results in increased monocyte adhesion to the inflamed endothelium [151]. Monocytes cultured with PF4 show increased survival and differentiation into macrophages [152].

MPO was found to significantly associate with post-intervention patency: In the univariate analyses, pre fistuloplasty MPO levels associated with primary patency and change in MPO levels associated with cumulative patency. In the multivariate analysis, pre fistuloplasty MPO levels again associated with primary patency and change in MPO levels again associated with cumulative patency. The potential importance of MPO as a biomarker for AVF patency has briefly been explored before. Stolic *et al* found that serum MPO was a statistically significant predictor of thrombosis of AVFs [77]. Wildgruber *et al* found that MPO expression on intermediate monocytes measured at 3, 6 and 12 months associated with an increased restenosis risk in patients following a peripheral percutaneous transluminal angioplasty [115]. MPO has directly been linked to NIH formation through two studies. Yang *et al* demonstrated that HOCl, the product of MPO, was able to elicit chemical injury on endothelial cells resulting increased apoptosis. HOCl had dose and time dependent effects on NIH formation [75]. Tiyerili *et al* demonstrated that inhibiting MPO in apolipoprotein E-deficient mice on a high cholesterol diet had less NIH formation than mice treated without the inhibitor. Likewise, irradiated mice when repopulated with bone marrow derived cells from MPO knockout mice in comparison to wild type mice had reduced NIH formation [76].

S100A9 was found to significantly associate with post-intervention patency: In the univariate analyses, pre fistuloplasty, post fistuloplasty and change in MPO levels associated with cumulative patency. In the multivariate analysis, pre fistuloplasty, post fistuloplasty and change in MPO levels again associated with cumulative patency. The

gene expression of S100A9 was also identified by the microarray in section 3.4, and the relative gene expression of S100A9 was found to significantly be higher in patient PBMCs in comparison to healthy donor PBMCs, which supports the existing literature [153] [154]. S100A9 overexpression has been linked to NIH in AVFs before, with Martinez *et al* finding that S100A9 expression was upregulated in pre-access veins that developed NIH and thus failed to mature [155]. Croce *et al* found that mice that lack calprotectin after femoral artery wire injury had significant reductions in monocytes and macrophages accumulation, cellular proliferation, and NIH formation when compared to wild-type mice [137]. The primary source of systemic S100A9 is thought to be through neutrophils followed by monocytes [156]. A prerequisite for S100A9 secretion by neutrophils and monocytes is that they are activated and contact an inflamed endothelium or extracellular matrix proteins. Therefore an excess of S100A9 in the plasma can indicate an increase in inflammatory cells coming in contact with the endothelial layer. Moreover, it has also been shown that S100A9 contact with the endothelial layer further activates it, with endothelial cells significantly altering genes linked to permeability, platelet aggregation and adhesion after being cultured with calprotectin, thus further recruiting neutrophils and monocytes to the endothelial layer [157].

Whilst PF4, MPO and S100A9 had significant associations with post-intervention outcomes, none of these proteins had hazard ratio with a magnitude deemed important. In contrast, it was found that non-white ethnicity significantly associated with post-intervention primary patency ($p = 0.013$, hazard ratio of 1.533) and patient age significantly associated with post-intervention cumulative patency ($p = 0.025$, hazard ratio of 1.038). It therefore seems likely that patient clinical and demographic factors are more important predictors of fistula patency than the plasma factors. Despite this, it is worth further investigating the proteins found here in a larger cohort as they represent possible future treatment targets.

3.3 Investigating the changes in Leukocytes following a Fistuloplasty Procedure

3.3.1 Introduction

The immune system is a multi-cellular organism's defence against the invasion of pathogens, tumour cells and toxins that may cause harm to the host. In jawed vertebrates, Gnathostomata, there are two major subsystems of the immune system which are elegantly interconnected to one another: the innate and the adaptive immune systems.

The innate immune system is the first line of defence mounted. The response is immediate, but not antigen specific and results in no immunological memory against antigens. Several mechanical and biological barriers, such as the skin and lysozymes in saliva, protect the host from pathogens. Following a barrier breach, pathogens can encounter innate immune cells which can initiate an immunological response. These specialised cells include neutrophils, natural killer (NK) cells and monocytes. The cellular innate response is usually identified by germline encoded pattern recognition receptors, which detect pathogen-associated molecular patterns (PAMPs), associated with pathogens or damage-associated molecular patterns (DAMPs), which is associated with host cell damage or death. Antigen presentation, in addition to activation of pattern recognition receptors on innate cells and the surrounding cytokine milieu generated has a direct effect on downstream adaptive immune responses.

The adaptive immune system is broadly made up of T cells and B cells. These cells can rearrange cell surface receptors to generate almost an unlimited diversity in the ability to recognise epitopes. B cells secrete antibodies and present antigens. T cells can be subdivided into CD4⁺ helper, cytotoxic CD8⁺ and $\gamma\delta$ ⁺ T cells. CD4⁺ T cells are key cells of the adaptive immune response, and their main role is to regulate immunological responses through the release of cytokines or through cell to cell

interactions. Dendritic cell and CD4⁺ T cell interactions results in the activation of CD8⁺ T cells which can induce cell apoptosis through cell-cell interactions and can release potent cytotoxins. In contrast, $\gamma\delta$ ⁺ T cells are often placed between the innate and adaptive immune system in that they can provide rapid immunological responses yet can also develop a memory phenotype- a key aspect of adaptive immunity.

One of the major obstacles of the immune system is to be able to mount an effective immune response whilst limiting excessive inflammation and tissue damage. In patients with CKD there are clear alterations in the immune system resulting in defects. Patients have poor response to vaccine challenges and higher rates of malignancy [86]. There is also an increased risk of morbidity and mortality of 50% and 20% respectively from cardiovascular disease and infections in patients with CKD. As discussed in section 1.4.5, there are several alterations in key leukocyte populations leading to a defective immune system, namely from complications arising from uraemia and excess oxidative stress.

A recent study found that a higher monocyte count was associated with increased risk of death overall in patients with CKD [158]. Indeed, monocytes have long been considered an important cell type in the orchestration of inflammatory responses, particularly for vascular injury. In rabbits, Mori *et al* had previously shown that monocytes infiltrate the neointima after an artery angioplasty and that MCP-1 blockade prevented NIH [112]. Moreover, in at least two different animal models it has been shown that depletion of monocytes via clodronate prevented the formation of NIH [113] thus further highlighting the importance of monocytes in NIH formation.

Monocytes comprise about 2% to 10% of all leukocytes in adults and 4% of leukocytes in mouse blood. They have diverse effector functions demonstrating their incredible plasticity. Monocytes can differentiate into macrophages, which are large professional antigen presenting phagocytes described as capable of releasing either 'inflammatory'

or ‘repair’ cytokines depending on whether they had M1 or M2 differentiation respectively. They can also differentiate into myeloid lineage dendritic cells in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 when cultured *in vitro* [159]. More recently monocytes have been described as having the capacity to differentiate into fibrocytes, cells which express the progenitor marker CD34, the haematopoietic marker CD45 and stromal markers such as collagen type 1 [64].

There are three major subsets of monocytes identified according to their differential expression of their pattern recognition receptor CD14 and Fc receptor CD16: “classical” CD14⁺⁺CD16⁻ monocytes, “intermediate” for CD14⁺⁺CD16⁺ monocytes, and “non-classical” for CD14⁺CD16⁺ monocytes. This nomenclature was standardised in 2010 by Ziegler-Heitbrock *et al* following discussions with the International Union of Immunological Societies and the World Health Organization [160]. In mice there are Ly6C^{high} monocytes, which are akin to human classical monocytes, and Ly6C^{low} monocytes, which are broadly like CD16⁺ human monocytes [16]. Classical monocytes constitute about 80-95% of the monocyte population, whilst intermediate and non-classical constitute to 2-8% and 2–11% respectively [161].

The exact role for each monocyte subset is debatable as different responses have been observed depending on the context of their activation. Broadly speaking, classical monocytes are often described as “inflammatory monocytes” due to their ability to migrate to inflammatory sites and their importance in microbial defence. Indeed, only classical monocytes can differentiate into monocyte derived dendritic cells [161]. In culture, CD16 expressing monocytes have been shown to be able to differentiate into both M1 and M2 macrophages [161], but there is some doubt as to whether *in vivo* they significantly contribute to the pool of macrophages. Indeed, in Nr4a1 knockout mice, which have normal numbers of myeloid progenitor cells and Ly6C^{high} monocytes, but no Ly6C^{low} monocytes, it has been shown that there were normal numbers of macrophages in all of the mouse tissues with the exception of the thymus [162].

Non-classical monocytes are often referred to as “resident monocytes.” They are smaller, longer surviving and have poor phagocytotic and reactive oxygen species (ROS) production abilities. They are thought to be important in the innate local surveillance of the vasculature as they patrol the endothelium layer clearing debris, scanning for an inflammatory stimulus and repairing the endothelium [163]. The function of intermediate monocytes is less defined with some studies suggesting their function is more related to the classical subset and others suggesting they are more related to the non-classical subset. They are the most effective at antigen presentation and ROS production [164].

Whole genome transcriptome analysis studies indicate that all monocyte subsets originate from a common myeloid progenitor cell. CD16 expressing monocytes are also suggested to be at the more advanced stage of differentiation in comparison to classical monocytes due to having more of a dendritic cell and macrophage cell like transcription program [165]. Human *in vivo* deuterium labelling studies have shown that intermediate and non-classical monocytes emerge sequentially from the pool of classical monocytes with a model of sequential transition [166]. Therefore, it could also be that one of the main roles of intermediate monocytes is to give rise to non-classical monocytes. Given the distinct functional attributes in the monocyte subsets, it comes as no surprise that they differentially express many markers. Indeed, between CD16+ and CD16- monocytes, transcriptional profiling reveals 2,759 genes differentially expressed between these two groups [165]. CX₃CR₁ expression is lowest in classical monocytes and increases with CD16 expression. In contrast CCR2 and CD11b are highest in classical monocytes and decreases with CD16 expression. Intermediate monocytes express the highest level of HLA-DR [164].

Previous findings have found that patients with CKD have a higher percentage of intermediate monocytes compared to healthy subjects in the blood circulation, with

authors demonstrated this is part attributed to the effect of uremic sulphates [167] [168]. There is mounting evidence to support the notion that CD16+ monocytes may have an adverse role in these patients. Indeed, intermediate monocytes have been associated with increased cardiovascular events in patients [169] as well as with the progression of anaemia in CKD [170].

CD16+ monocytes are thought to be particularly important in the development of NIH. Indeed, monocyte subset distributions have been found to differentially be affected by angioplasty of lower limb arteries in patients with and without restenosis with increased frequencies of intermediate monocytes being associated with restenosis [115]. *In vitro* co-culture studies have found that human non-classical monocytes induce endothelial cell proliferation in a VEGFA-dependent manner, and that Ly6C^{low} monocytes play a key role in endothelial regeneration after carotid artery injury [116]. Given the importance of monocyte subsets in the health of patients with CKD and in an angioplasty context, investigating monocyte subsets following a fistuloplasty procedure seems important.

3.3.2 Aims

The initial aim of this chapter was to determine if there was a change to the overall numbers of leukocyte populations post fistuloplasty by comparing pre and 1-2 days post fistuloplasty PBMC samples, and by looking at patient clinical FBC data. I then focused solely on investigating whether there were any changes to monocytes subset population (classical, intermediate and non-classical) and whether these population changes had any associations with outcome.

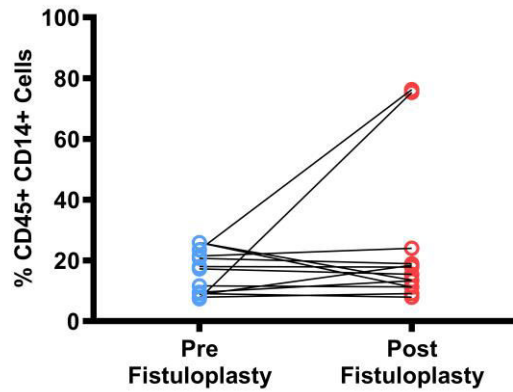
3.3.3 There are No Changes to the Overall Numbers of Circulating Leukocyte Populations Post Fistuloplasty

First I sought to establish whether there are any significant changes in the numbers of leukocyte subsets 1-2 days following a fistuloplasty procedure. Flow cytometry was carried out on 13 pairs of pre and post fistuloplasty cryopreserved PBMCs from patients investigating the percentages of monocytes (based on being CD45+CD14+), $\gamma\delta$ + T cells (based on being CD45+CD3+ $\gamma\delta$ +), CD8+ T cells (based on being CD45+CD3+CD8+), CD4+ T cells (based on being CD45+CD3+CD8-), B cells (based on being CD45+CD3-CD19+) and NK cells (based on being CD45+CD3-CD19-NKp46+). The analysis on the 13 pairs of patient PBMC samples revealed that there were no significant changes in the percentages of PBMC subsets post fistuloplasty (Figure 3.18) and so further patient samples were not included.

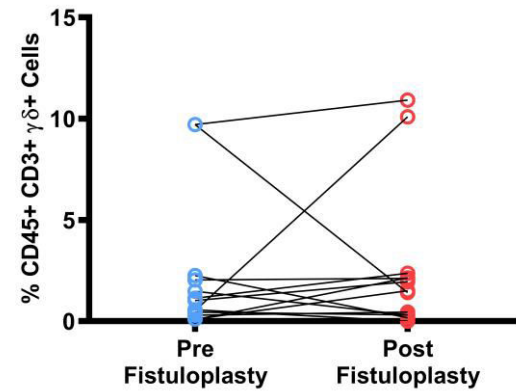
Clinical full blood count data from paired patient samples before and 1-2 days after their fistuloplasty procedure were also examined in 49 patients who had this clinical data available (Figure 3.19). This enabled us to look at the overall full white blood cell count, platelets, neutrophils and haemoglobin. In a similar manner to the flow cytometry data, the full blood count data also showed that there were no significant changes in the number of circulating cells, but interestingly, there was a significant decrease in patient haemoglobin post fistuloplasty procedure ($p = 0.0008$).

The percentages of PBMC subsets in pre fistuloplasty patient samples were also compared to that of 8 cryopreserved healthy donor PBMCs. There was no significant difference in the percentages of monocytes, $\gamma\delta$ + T cells, CD8+ T cells, B cells and NK cells between pre fistuloplasty patients and healthy donors (data not shown), however, the percentage of CD4+ T cells were found to be significantly lower in patients than in healthy donors ($p = 0.026$) (Figure 3.20).

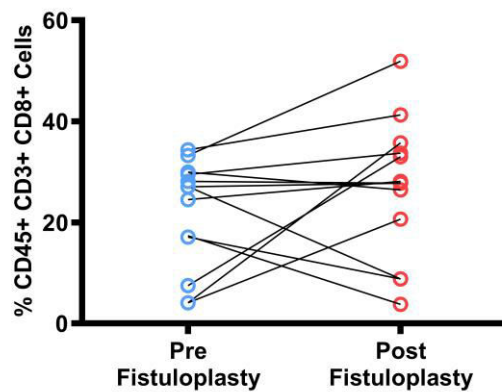
(A) Monocytes



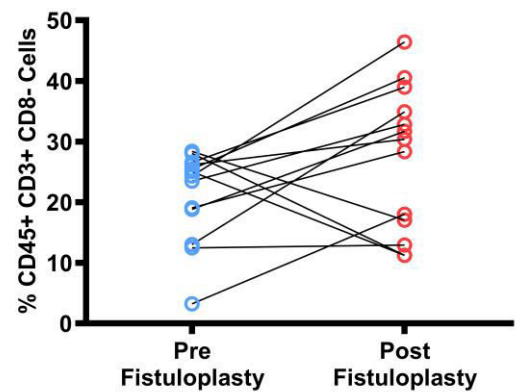
(B) $\gamma\delta$ + T cells



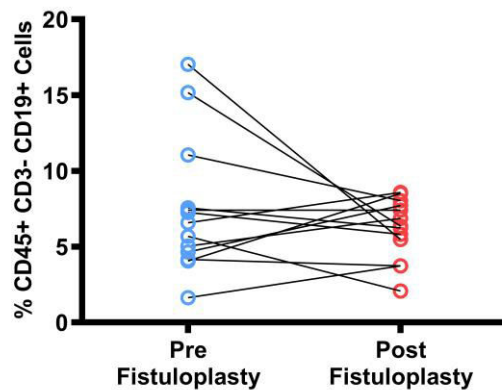
(C) CD8+ T cells



(D) CD4+ T cells



(E) B cells



(F) NK cells

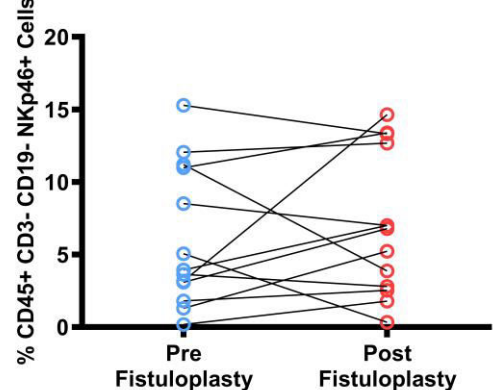
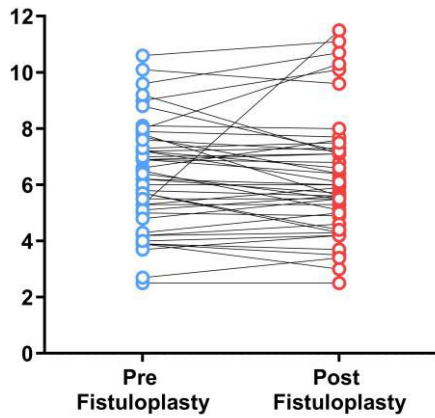


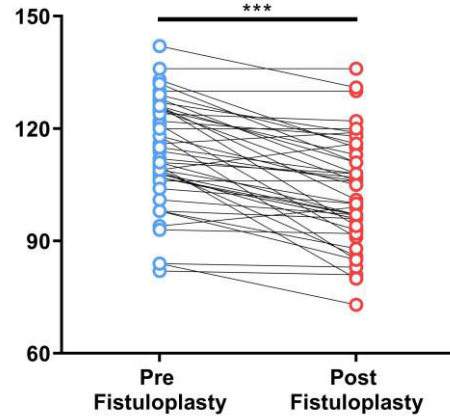
Figure 3.18: Percentages of PBMC subsets in paired patient PBMC samples before and 1-2 days after their fistuloplasty procedure.

The percentages of (A) monocytes, (B) $\gamma\delta$ + T cells, (C) CD8+ T cells, (D) CD4+ T cells, (E) B cells and (F) NK cells pre and 1-2 days post fistuloplasty. Data was analysed using a paired t test. (n = 13 paired pre and post fistuloplasty samples).

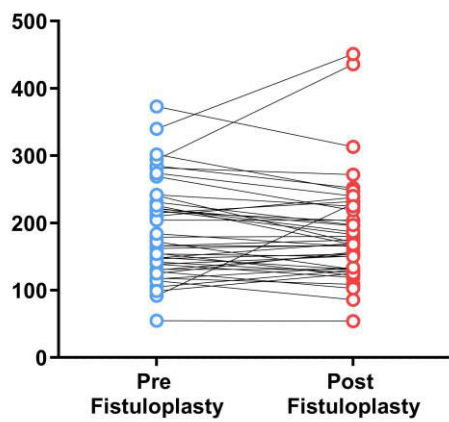
(A) WBC



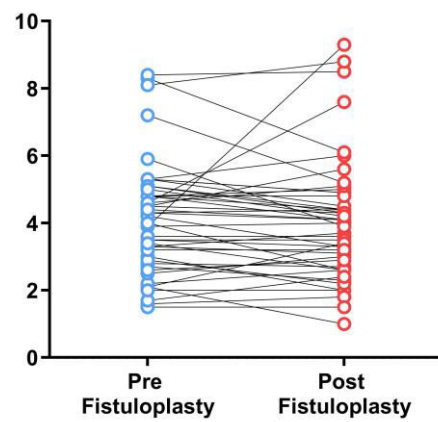
(B) Haemoglobin



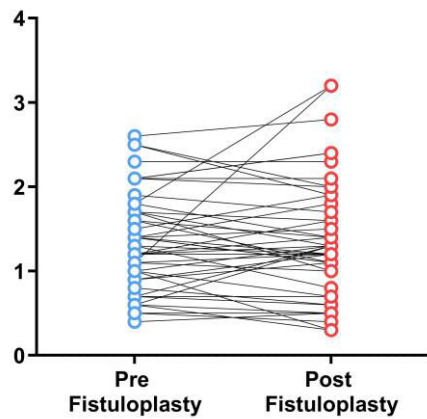
(C) Platelets



(D) Neutrophils



(E) Lymphocytes



(F) Monocytes

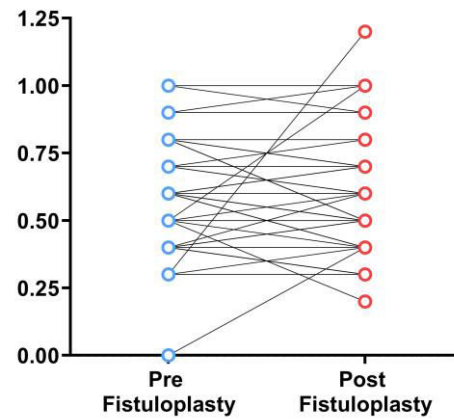


Figure 3.19: Clinical full blood count data from paired patient samples before and 1-2 days after their fistuloplasty procedure.

The numbers of (A) white blood cells, (B) platelets, (C) neutrophils, (D) lymphocytes and (E) monocytes (F) pre and 1-2 days post fistuloplasty expressed in $\times 10^9/l$. Data was analysed using a paired t test. (n = 49 paired pre and post fistuloplasty samples). ***p<0.001.

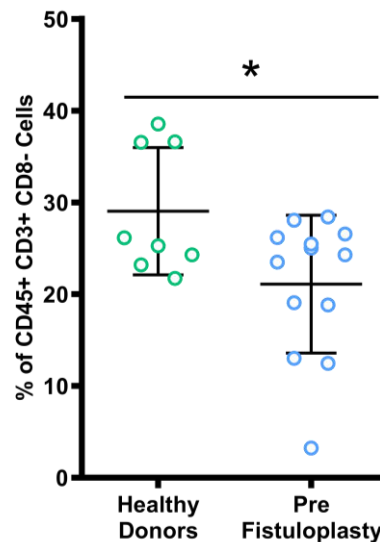


Figure 3.20: CD4+ T cells are significantly lower in patients when compared to healthy controls. The percentage of CD4+ T cells in healthy (green) and pre fistuloplasty (blue) patient samples. Results are expressed as mean $\pm$ SD. (n = 8 healthy donors and 13 patient samples). Significant differences were determined an unpaired t test. * $p < 0.05$.

3.3.4 Blood Counts and Patency Outcome

I then sought to establish if any variables in the full blood count data (WBC, haemoglobin, platelets, neutrophils, lymphocytes and monocytes) would associate with post-intervention patency outcome. Initially a univariate cox regression tests were carried out examining for associations with post-intervention primary patency and post-intervention cumulative patency. Pre fistuloplasty full blood count data and post fistuloplasty full blood count data were investigated for associations.

Table 3-19 is a summary of the univariate analysis findings for primary patency and Table 3-20 is a summary of summary of the univariate analysis findings for cumulative patency. A hazard ratio of less than one would indicate an association with better outcome, whilst a hazard ratio of more than one would indicate an association with worse outcome. For both post-intervention primary patency and post-intervention cumulative patency univariate findings, haemoglobin was significant. For primary patency, higher post fistuloplasty levels of haemoglobin significantly associated with better outcome ($p = 0.030$, hazard ratio of 0.967), whilst for cumulative patency, higher

levels of pre fistuloplasty haemoglobin significantly associated with better outcome ($p = 0.006$, hazard ratio of 0.961).

Table 3-19: Univariate cox regression exploring for associations with full blood count blood data with post-intervention primary patency outcome. n = 75 pre fistuloplasty patients, n = 49 post fistuloplasty patients. Green highlights significance.

Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Pre WBC	0.652	1.038	0.883	1.221
Pre Platelets	0.387	1.002	0.997	1.007
Pre Haemoglobin	0.121	0.985	0.965	1.004
Pre Neutrophils	0.771	1.026	0.864	1.217
Pre Monocytes	0.983	1.002	0.813	1.236
Pre Lymphocytes	0.543	1.162	0.717	1.882
Pre NLR	0.359	0.962	0.887	1.045
Pre NMR	0.432	0.977	0.923	1.035
Post WBC	0.788	0.976	0.820	1.162
Post Platelets	0.731	0.731	0.992	1.006
Post Haemoglobin	0.030	0.967	0.939	0.997
Post Neutrophils	0.818	0.818	0.812	1.178
Post Monocytes	0.334	0.334	0.094	2.230
Post Lymphocytes	0.844	0.844	0.656	1.674
Post NLR	0.792	0.990	0.922	1.064
Post NMR	0.388	1.053	0.937	1.183

Table 3-20: Univariate cox regression exploring for associations with full blood count blood data with post-intervention cumulative patency outcome. n = 75 pre fistuloplasty patients, n = 49 post fistuloplasty patients. Green highlights significance.

Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Pre WBC	0.715	1.043	0.833	1.306
Pre Platelets	0.801	0.999	0.993	1.006
Pre Haemoglobin	0.006	0.961	0.934	0.989
Pre Neutrophils	0.898	1.016	0.798	1.293
Pre Monocytes	0.550	0.801	0.387	1.658
Pre Lymphocytes	0.889	0.947	0.436	2.053
Pre NLR	0.531	0.958	0.839	1.095
Pre NMR	0.819	0.991	0.913	1.075
Post WBC	0.822	0.972	0.758	1.246
Post Platelets	0.522	1.003	0.994	1.012
Post Haemoglobin	0.116	0.971	0.935	1.007
Post Neutrophils	0.692	0.948	0.727	1.236
Post Monocytes	0.903	0.880	0.112	6.889
Post Lymphocytes	0.771	1.102	0.572	2.123
Post NLR	0.292	0.918	0.784	1.076
Post NMR	0.993	1.001	0.857	1.169

I then sought to carry out multivariate analysis in order to seek for patterns and relationships between several variables simultaneously. I included patient clinical variables (gender, ethnicity, patient age, type of native fistula, log age of fistula, history of previous interventions and previous thrombosis) in case WBC, haemoglobin, platelets, neutrophils, lymphocytes and monocytes associations were being made undetectable due to a larger influence of a clinical variable on the data set. The multivariate analysis carried out manually was a forward analysis as further explained in the methods chapter, Statistics. The exploratory 'stop' p value was set to a limited 0.10 due to the significance found with haemoglobin in the univariate analysis.

Table 3-21 shows the multivariate analysis carried out on the full blood count data and clinical variables for post-intervention primary patency outcome, whilst Table 3-32 shows the multivariate analysis carried out on the full blood count data and clinical variables for post-intervention cumulative patency outcome. In both analyses, only haemoglobin remained the only full blood count data associating with post-intervention outcome. For primary patency, ethnicity (in which being non-white associated worse with outcome), patient age (in which older patients had a worse outcome), log age of fistula (in which older fistulas had a better outcome) and gender (in which females did worse), in addition to post fistuloplasty haemoglobin levels, associated with primary patency outcome. For cumulative patency, patient age (in which older patients had a worse outcome), in addition to pre fistuloplasty haemoglobin levels, associated with cumulative patency outcome.

Table 3-21: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and variables measured within the FBC with post-intervention primary patency.

The 'start' step denotes *p* values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was ethnicity as it had the lowest *p* value. 'Step 1' shows the *p* values of the first multivariate cox regression analysis carried out in which each variable was combined with var1. The variable found to have the lowest *p* value here denoted as variable 2 (var2), and in this case is patient age. 'Step 2' shows the *p* value of the second multivariate cox regression carried out in which each variable was combined with var1 (ethnicity) and var2 (patient age) to find the variable with the next lowest *p* value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a *p* value of less than 0.10. Light blue cells highlight the protein found to have the lowest *p* value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest *p* value. *n* = 75 pre fistuloplasty patients, *n* = 49 post fistuloplasty patients.

	0	Step 1	Step 2	Step 3	Step 4	STOP
Gender	0.188	0.240	0.097	0.293	0.079	X
Ethnicity	0.022	X	X	X	X	X
Patient Age	0.639	0.036	X	X	X	X
Type of Fistula	0.423	0.785	0.887	0.761	0.751	0.441
Log age of Fistula	0.143	0.125	0.095	0.039	X	X
Previous Interventions	0.602	0.123	0.339	0.637	0.554	0.991
Previous Thrombosis	0.852	0.109	0.609	0.940	0.761	0.559
Pre WBC	0.652	0.830	0.231	0.255	0.252	0.478
Pre Platelets	0.387	0.989	0.374	0.555	0.604	0.397
Pre Haemoglobin	0.121	0.091	0.102	0.587	0.348	0.767
Pre Neutrophils	0.771	0.812	0.424	0.287	0.247	0.446
Pre Monocytes	0.983	0.684	0.976	0.533	0.703	0.808
Pre Lymphocytes	0.543	0.726	0.918	0.587	0.594	0.947
Pre NLR	0.359	0.266	0.607	0.890	0.996	0.992
Pre NMR	0.432	0.400	0.639	0.689	0.487	0.428
Post WBC	0.788	0.830	0.946	0.995	0.687	0.615
Post Platelets	0.731	0.414	0.718	0.779	0.446	0.719
Post Haemoglobin	0.030	0.850	0.031	X	X	X
Post Neutrophils	0.818	0.788	0.968	0.946	0.729	0.813
Post Monocytes	0.334	0.600	0.263	0.249	0.144	0.258
Post Lymphocytes	0.844	0.866	0.860	0.738	0.843	0.663
Post NLR	0.792	0.335	0.783	0.641	0.539	0.712
Post NMR	0.388	0.842	0.347	0.292	0.183	0.266

Table 3-22: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and variables measured within the FBC with post-intervention cumulative patency.

The 'start' step denotes p values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was pre fistuloplasty levels of haemoglobin as it had the lowest p value. 'Step 1' shows the p values of the first multivariate cox regression analysis carried out in which each variable was combined with var1. The variable found to have the lowest p value here denoted as variable 2 (var2), and in this case is patient age. 'Step 2' shows the p value of the second multivariate cox regression carried out in which each variable was combined with var1 (pre fistuloplasty levels of haemoglobin) and var2 (patient age) to find the variable with the next lowest p value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a p value of less than 0.10. Light blue cells highlight the protein found to have the lowest p value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest p value. n = 75 pre fistuloplasty patients, n = 49 post fistuloplasty patients.

	0	Step 1	STOP
Gender	0.343	0.529	0.207
Ethnicity	0.564	0.642	0.151
Patient Age	0.025	0.036	X
Type of Fistula	0.235	0.226	0.395
Log age of Fistula	0.898	0.772	0.579
Previous Interventions	0.076	0.123	0.149
Previous Thrombosis	0.083	0.109	0.191
Pre WBC	0.715	0.830	0.807
Pre Platelets	0.801	0.989	0.713
Pre Haemoglobin	0.006	X	X
Pre Neutrophils	0.898	0.812	0.430
Pre Monocytes	0.550	0.684	0.706
Pre Lymphocytes	0.889	0.726	0.449
Pre NLR	0.531	0.266	0.191
Pre NMR	0.819	0.400	0.419
Post WBC	0.822	0.830	0.457
Post Platelets	0.522	0.414	0.612
Post Haemoglobin	0.116	0.850	0.854
Post Neutrophils	0.692	0.788	0.296
Post Monocytes	0.903	0.600	0.636
Post Lymphocytes	0.771	0.866	0.568
Post NLR	0.292	0.355	0.133
Post NMR	0.993	0.842	0.664

A matrix of regression coefficients table was then carried out in order to assess for co-linearity between the variables identified in the multivariate analysis. Table 3-23 shows the correlation matrix for the variables identified for primary patency and Table 3-24 shows the correlation matrix for the variables identified for cumulative patency. In both instances, no correlations were found.

Table 3-23: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-19.

	Ethnicity	Patient Age	Post Haemoglobin	Log Age of Fistula	Gender
Ethnicity					
Patient Age	0.163				
Post Haemoglobin	0.242	0.780			
Log Age of Fistula	0.418	0.416	0.120		
Gender	0.772	0.167	0.800	0.512	

Table 3-24: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-20.

	Pre Haemoglobin	Patient Age
Pre Haemoglobin		
Patient Age	0.581	

The preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and full blood count data included ethnicity, patient age, post haemoglobin levels, log age of the fistula and gender with a final *p* value of 0.014 (Table 3-25). The preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and full blood count data included pre fistuloplasty haemoglobin levels and patient age with a final *p* value of 0.002 (Table 3-26).

Table 3-25: Table summarising a preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and FBC variables.

Omnibus Tests of Model Coefficients				
Significance	0.014	-2 Log Likelihood		186.049
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Ethnicity	0.174	1.474	0.843	2.577
Patient Age	0.054	1.030	0.999	1.062
Post Haemoglobin	0.026	0.970	0.944	0.996
Log age of Fistula	0.012	0.345	0.150	0.793
Gender	0.079	2.081	0.918	4.719

Table 3-26: Table summarising a preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and the patient clinical variables and FBC variables.

Variables.

Omnibus Tests of Model Coefficients				
Significance	0.002	-2 Log Likelihood		154.263
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Pre Haemoglobin	0.011	0.963	0.935	0.991
Patient Age	0.036	1.035	1.002	1.069

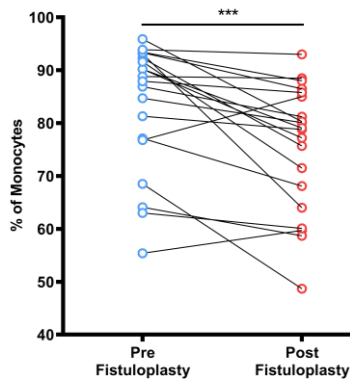
3.3.5 Monocytes shift to a CD16 expressing Phenotype Post Fistuloplasty

The proportion of monocyte subsets were analysed in 20 pairs of pre and 1-2 days post fistuloplasty patient PBMC samples, which was all of the paired PBMC samples I had at the time. There was a significant shift in the monocyte populations towards a CD16 expressing phenotype 1-2 days post fistuloplasty as the percentage of classical monocytes significantly decreased ($p = 0.0009$) (Figure 3.21A), whilst the percentage of intermediate (Figure 3.21B) and non-classical monocytes (Figure 3.21C) significantly increased ($p = 0.0015$ and $p = 0.0019$ respectively). Figure 3.21D shows a representative flow cytometry plot of this shift. These changes were unlikely to be due to the administration of contrast as in the three patients who had a fistulogram, yet no

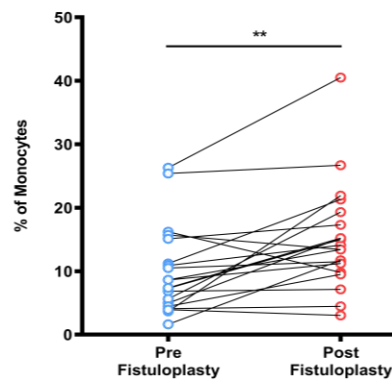
fistuloplasty, no observable trend in a shift of monocyte population was visible (Figure 3.22).

The MFI of markers CCR2, CD11b and HLA-DR were also examined in within the patient populations as shown in Figure 3.23. As expected, HLA-DR was most highly expressed in intermediate monocytes, whilst CCR2 and CD11b were most highly expressed in classical monocytes, with the least expression in non-classical monocytes. CCR2 expression significantly decreased in intermediate and non-classical monocytes ($p = 0.0254$ and $p = 0.0046$ respectively), but not in classical monocytes. Likewise, HLA-DR expression significantly decreased in intermediate and non-classical monocytes, but not in classical monocytes ($p = 0.0119$ and $p = 0.0220$ respectively). In contrast, CD11b only significantly decreased in non-classical monocytes ($p = 0.0444$).

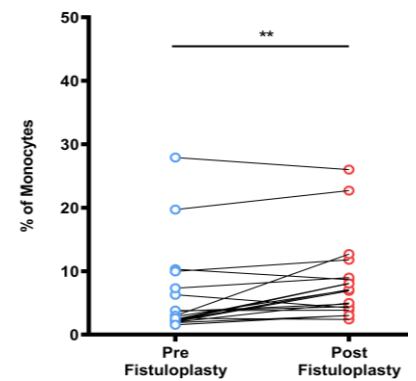
(A) Classical Monocytes



(B) Intermediate Monocytes



(C) Non-Classical Monocytes



(D) Representative flow cytometry plot

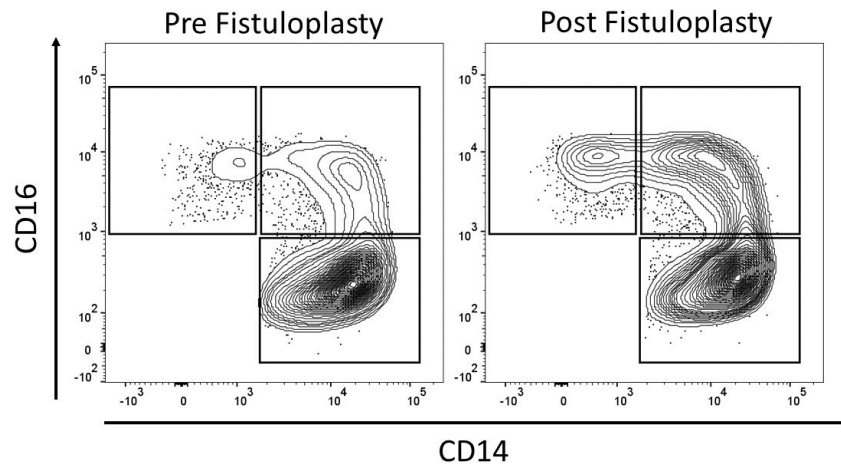
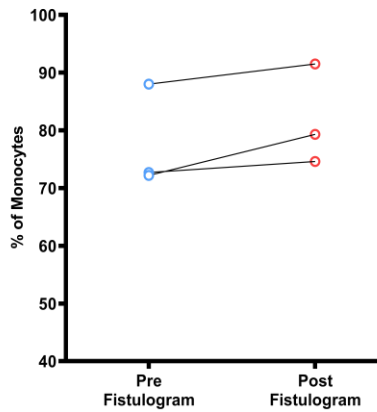
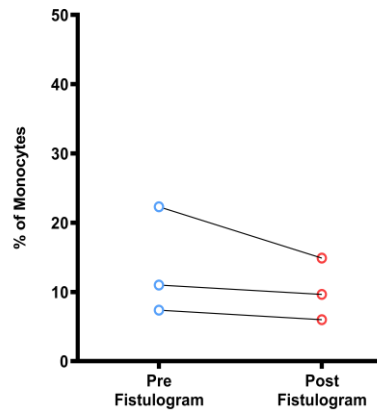


Figure 3.21: Percentages of monocyte subsets in paired patient PBMC samples before and 1-2 days after their fistuloplasty procedure. The percentages of (A) classical, (B) intermediate and (C) non-classical monocytes pre and 1-2 days post fistuloplasty. Data was analysed using a paired *t* test. (n = 20 paired pre and post fistuloplasty samples). **p<0.01, ***p<0.001. (D) A representative flow cytometry plot of monocyte subsets pre and 2 days post fistuloplasty from a patient.

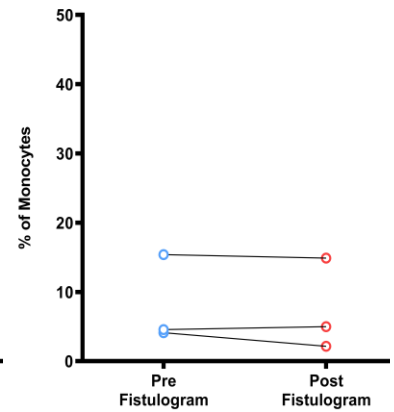
(A) Classical Monocytes



(B) Intermediate Monocytes



(C) Non-Classical Monocytes



(D) Representative flow cytometry plot

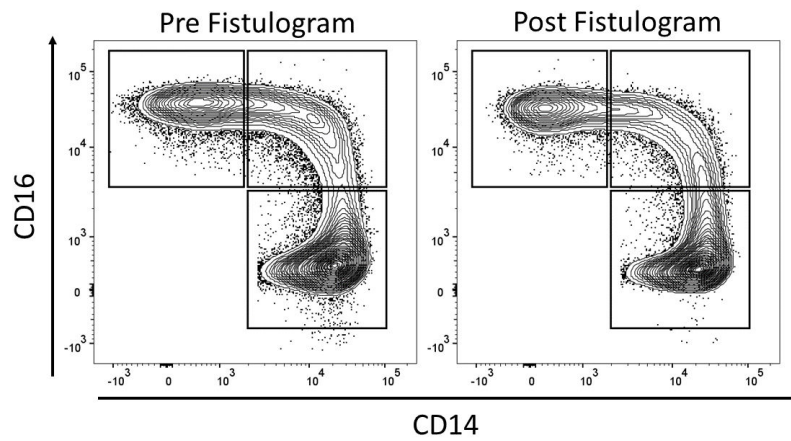
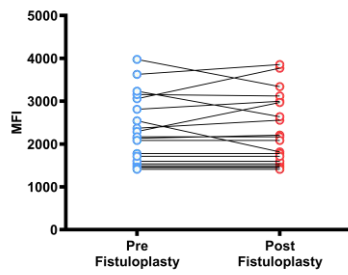
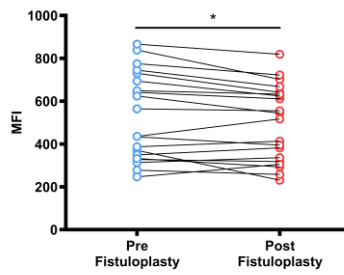


Figure 3.22: Percentages of monocyte subsets in paired patient PBMC samples before and 1-2 days after their fistulogram procedure.
The percentages of (A) classical, (B) intermediate and (C) non-classical monocytes pre and 1-2 days post fistuloplasty. (n = 3 paired pre and post fistulogram samples).
(D) A representative flow cytometry plot of monocyte subsets pre and 2 days post fistulogram from a patient.

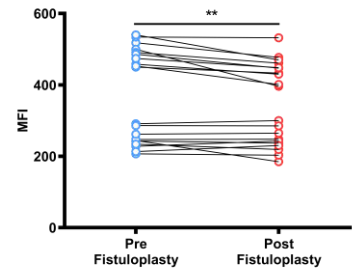
(A) Classical Monocytes - CCR2



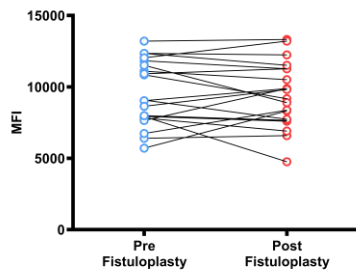
(B) Intermediate Monocytes - CCR2



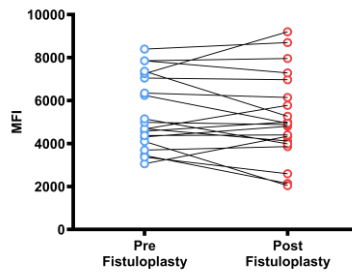
(C) Non-Classical Monocytes - CCR2



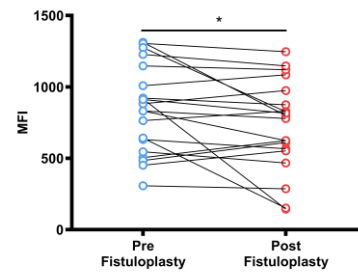
(D) Classical Monocytes - CD11b



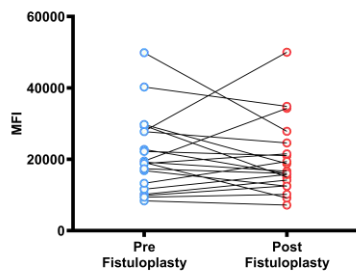
(E) Intermediate Monocytes - CD11b



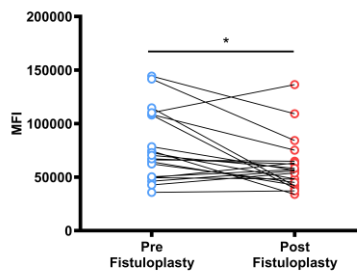
(F) Non-Classical Monocytes - CD11b



(G) Classical Monocytes - HLA-DR



(H) Intermediate Monocytes - HLA-DR



(I) Non-Classical Monocytes - HLA-DR

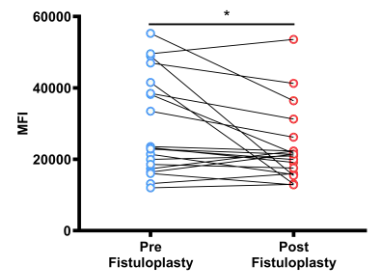
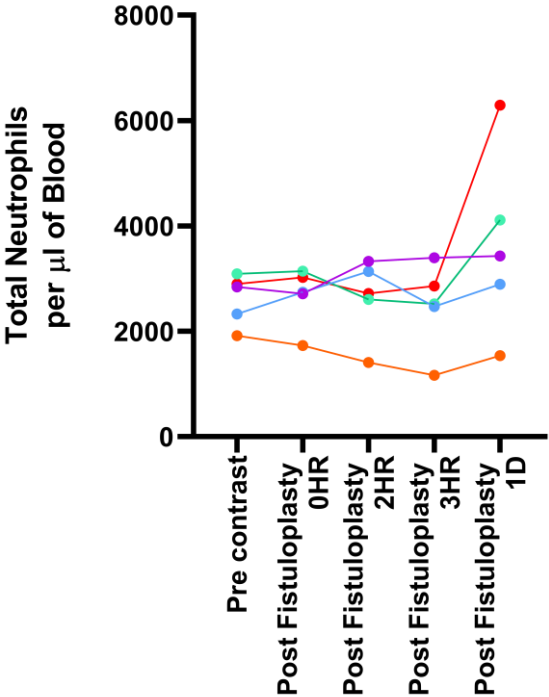


Figure 3.23 Monocyte subsets MFIs of CCR2, CD11b and HLA-DR in paired patient PBMC samples before and 1-2 days after their fistuloplasty.
n = 20 paired pre and post fistuloplasty samples, n = 3 paired pre and post fistulogram, samples.
Data was analysed using a paired t test. **p<0.01, *p<0.001.**

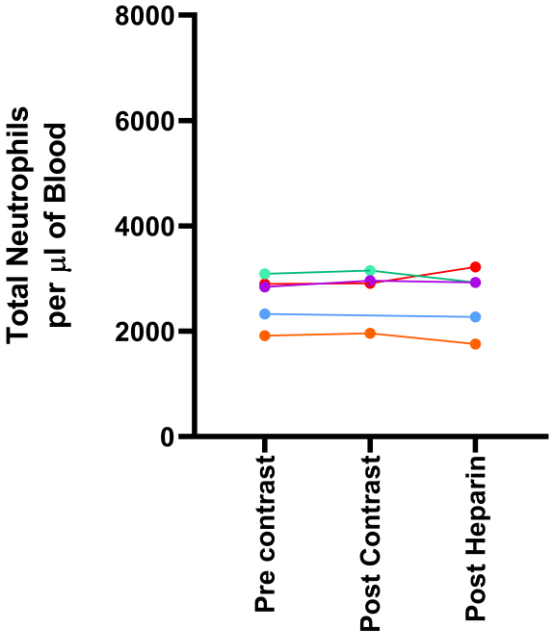
The increase in the percentage of CD16 expressing monocytes observed 1-2 days post fistuloplasty within the patient PBMC samples could have been due to either a decrease in the overall number of classical monocytes or because of a population increase of CD16 expressing monocytes post fistuloplasty. In order to determine this, another flow cytometry experiment was carried out using absolute counting beads on whole blood which would enable the exact quantification of the number of cells within each monocyte sub population. Another question the previous experiment on cryopreserved PBMCs raised was whether this observed shift in monocyte populations occurred immediately post fistuloplasty and so the experiment was carried out as a time course. Blood taken from patients before the administration of contrast served as a baseline sample. Blood taken post contrast and heparin administration served as controls for the immediate effect of these procedures. Blood was then taken immediately, 2 hours, 3 hours, and 1-day post fistuloplasty. Another advantage of using whole blood for flow cytometry was that it allowed us to also quantify patient neutrophils. Figure 3.24 shows the results from this experiment which was carried out on five patients.

There were no significant changes in the neutrophil and classical monocytes absolute numbers (Figure 3.24A-D). The absolute number of non-classical monocytes only significantly increased 1 day post fistuloplasty ($p = 0.0190$) (Figure 3.24G) showing that the shift in monocyte subsets was both not due to the reduction in CD14 monocytes nor was it an immediate effect. Interestingly, the intermediate monocytes significantly decreased 3 hours post fistuloplasty ($p = 0.0065$) before recovering or increasing 1-day post fistuloplasty (Figure 3.24E). Figure 3.25 shows a representative flow cytometry plot illustrating the change in the monocyte subsets from baseline levels to 3 hours and 1 day post fistuloplasty. The administration of contrast or heparin had no immediate effect on neutrophils or monocyte subsets as shown in Figure 3.24B, D, F and H.

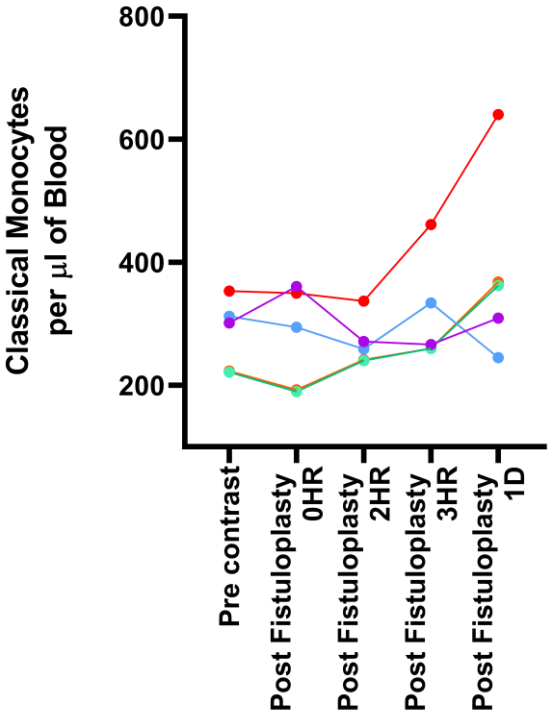
(A) Neutrophils



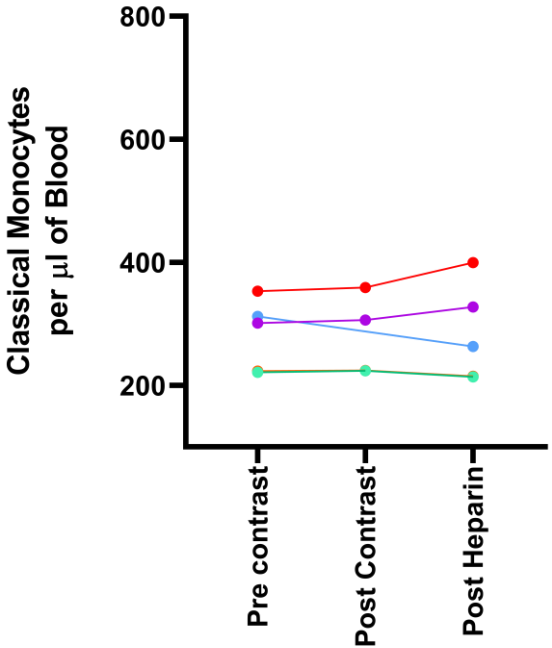
(B) Neutrophils



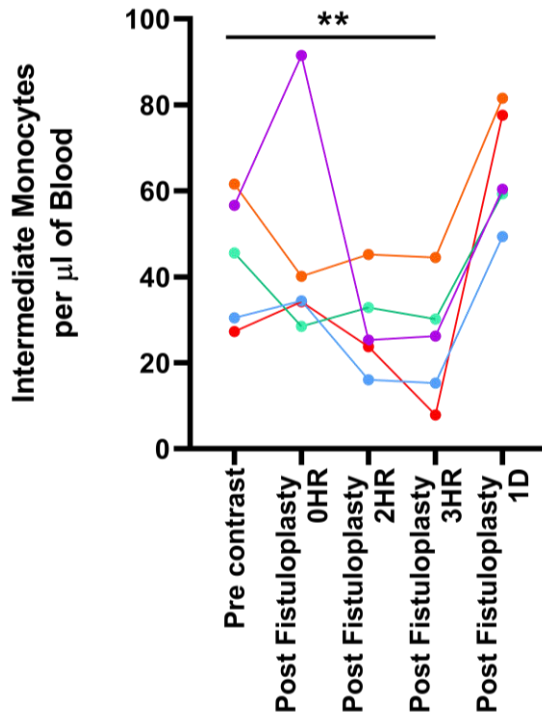
(C) Classical Monocytes



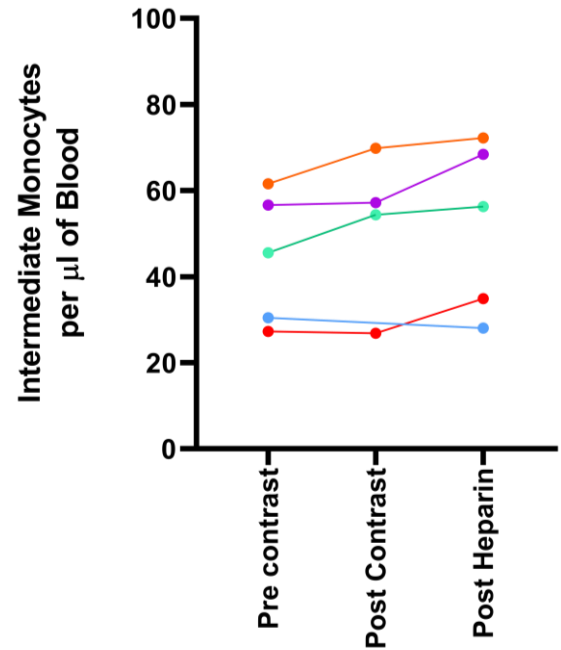
(D) Classical Monocytes



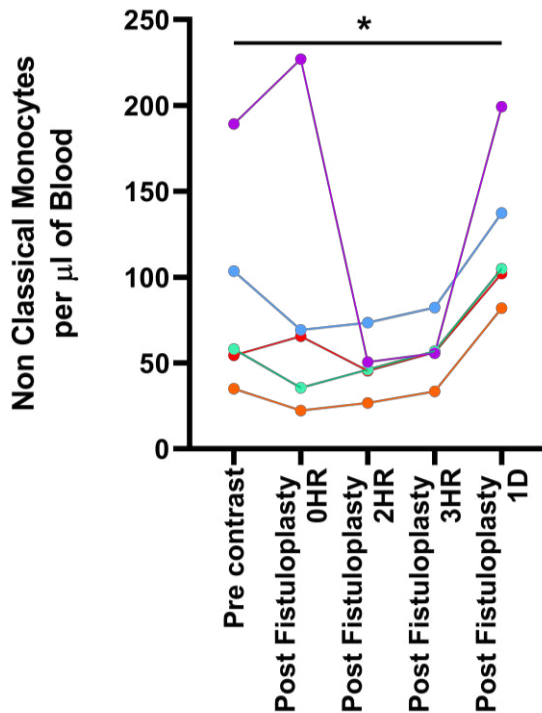
(E) Intermediate Monocytes



(F) Intermediate Monocytes



(G) Non-Classical Monocytes



(H) Non-Classical Monocytes

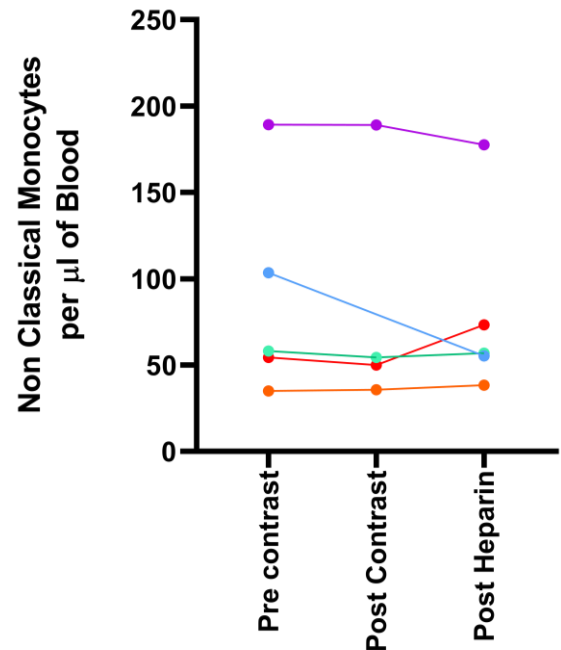


Figure 3.24: Absolute numbers of neutrophils and monocyte subsets in whole blood from multiple time points before, during, and after a fistuloplasty procedure. Graphs showing the absolute numbers of (A) neutrophils, (C) classical, (E) intermediate and (G) non-classical monocytes prior to the administration of contrast, immediately, 2 hours, 3 hours and 1-day post fistuloplasty in five donors. Graphs showing the absolute numbers of (A) neutrophils, (C) classical, (E) intermediate and (G) non-classical monocytes prior to the administration of contrast, after the administration of contrast and after heparin administration the same in five donors. RM-one-way ANOVA, Giesser-Greenhouse correction with the mean of each data set compared against the mean of the pre-contrast (baseline) sample. * $p < 0.05$, ** $p < 0.01$.

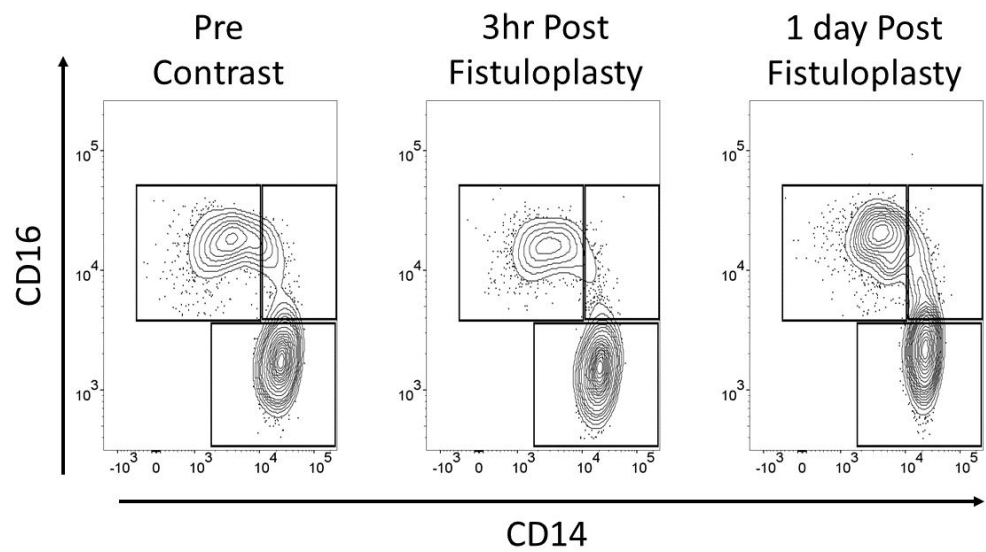


Figure 3.25: Representative flow cytometry plot of monocyte subsets pre contrast, 3 hours post contrast and 1 day post fistuloplasty. Intermediate monocytes visibly seem to decrease at 3 hours post fistuloplasty before recovering 1 day post fistuloplasty, whilst non-classical monocytes seem to be visibly increasing 1 day post fistuloplasty.

3.3.6 Monocyte Phenotype is Associated with Outcome

The shift of monocytes to a CD16 expressing phenotype was investigated for potential associations with post-intervention primary patency and post-intervention cumulative patency patient outcome. Despite the small samples size of 20 patients, both pre fistuloplasty and post fistuloplasty non-classical monocytes significantly negatively associated with post-intervention primary patency (hazard ratio 1.081, $p = 0.046$ and hazard ratio 1.087, $p = 0.053$ respectively) (Table 3-27). Multivariate analysis was not carried out for this data for two reasons: (1) there are large degrees of co-linearity between monocyte subset percentage variables (2) because in the 20 patients in this analysis, none of the patient clinical variables had significantly associated with outcome in the univariate analysis.

Table 3-27: Cox regression of the association of monocyte subsets on post-intervention primary patency and cumulative patency outcome. n = 20 pre fistuloplasty patients, n = 20 post fistuloplasty patients. Green highlights significance.

Monocyte %	Patency Outcome	P value of model	Hazard Ratio	95% CI
Pre fistuloplasty classical	Post-intervention primary patency	0.207	0.934	9.034 - 1.015
Post fistuloplasty classical	Post-intervention primary patency	0.501	0.986	0.946 - 1.028
Pre fistuloplasty intermediate	Post-intervention primary patency	0.592	1.018	0.953 - 1.087
Post fistuloplasty intermediate	Post-intervention primary patency	0.847	0.994	0.932 - 1.059
Pre fistuloplasty non-classical	Post-intervention primary patency	0.046	1.081	1.001 - 1.167
Post fistuloplasty non-classical	Post-intervention primary patency	0.053	1.087	0.999 - 1.183
Pre fistuloplasty classical	Post-intervention cumulative patency	0.696	0.987	0.927 - 1.052
Post fistuloplasty classical	Post-intervention cumulative patency	0.284	1.045	0.964 - 1.131
Pre fistuloplasty intermediate	Post-intervention cumulative patency	0.891	0.993	0.892 - 1.105
Post fistuloplasty intermediate	Post-intervention cumulative patency	0.094	0.877	0.752 - 1.023
Pre fistuloplasty non-classical	Post-intervention cumulative patency	0.258	1.062	0.957 - 1.179
Post fistuloplasty non-classical	Post-intervention cumulative patency	0.780	1.019	0.891 - 1.167

3.3.7 Discussion

There was no significant change in the overall white blood cell count, neutrophils, platelets, lymphocytes, monocytes, T cells, B cells or NK cells 1-2 days post fistuloplasty. Despite this, I did find that CD4⁺ T cells are significantly higher in pre fistuloplasty patients when compared to healthy controls. A limitation of comparing healthy donors with the patients on dialysis was the age discrepancy between the healthy controls and patient samples and the lack of CD4 antibody used in the flow cytometry panel. However, given the use of a CD8 and $\gamma\delta$ antibody, the vast majority of the CD3 cells gated as negative for these would most likely be CD4⁺ T cells. It should be stressed that the finding that CD4⁺ T cells are less in patients on dialysis is not surprising. Indeed Betjes *et al* found that patients on dialysis have less CD4⁺ T cells due to having a lower thymic output and CD4⁺ T cells with a higher susceptibility to apoptosis [93].

Interestingly, Okba *et al* found that whilst CD4⁺ T cells decrease with dialysis, there is an unusual subset of CD4⁺ T cells which lacks the CD28 co-stimulatory receptor that increases with dialysis. This subset is significantly more pro-inflammatory and increases with atherosclerosis [171]. Whilst CD4⁺ T cells did not increase post fistuloplasty, it would be interesting to further investigate whether this particular inflammatory CD4⁺CD28⁻ T cell subset did. Recent advances in technology suggest that there are also heterogeneous populations of mature neutrophils in various physiological and pathological conditions. Neutrophils are conventionally isolated during in the red blood fraction during Ficoll-Paque isolation. Recently, however, neutrophils have been found in the PBMC fraction. These neutrophils have been referred to as 'low density neutrophils' (LDNs), whilst conventional neutrophils are referred to as 'normal density neutrophils' (NDNs) [172]. NDNs, which include neutrophils with an exhausted phenotype or pro-inflammatory phenotype, have been shown to increase in patients with various inflammatory autoimmune diseases such as systemic lupus erythematosus [173]. Currently there is no literature as to whether these

types of neutrophils are prevalent in patients on dialysis, but given how 'immune exhaustion' is a feature of dialysis, it is quite possible that NDNs would be prevalent. Whether these NDNs would decrease or increase would also be interesting to investigate. Likewise, there are many more subsets of NK cells and B cells of which the ratios may have shifted post fistuloplasty.

Haemoglobin levels did significantly decrease post fistuloplasty. It is uncertain whether this finding is clinically significant, as the drop in haemoglobin could simply be a short term consequence of the fistuloplasty procedure itself. It is unlikely to be due to taking research blood samples as not enough blood was taken to significantly impact haemoglobin levels. More importantly, higher haemoglobin levels associated with better post-intervention primary patency (post haemoglobin levels, $p = 0.030$, hazard ratio of 0.967) and cumulative patency outcome (pre haemoglobin levels, $p = 0.006$, hazard ratio of 0.961). This both compliments and contrasts some of the existing literature. For instance, Wen *et al* carried out a retrospective survival analysis of 100 patient's mature AVFs for primary unassisted patency failure. They found that patients with haemoglobin of less than 80g/L had more frequent AVF dysfunction than those patients with haemoglobin between 80-100g/L, but haemoglobin above 100g/L did not seem to benefit AVF survival in their study [174]. Of note, the patients within this study both pre and post fistuloplasty for ethical reasons all had a haemoglobin of above 80g/L (with the exception of one patient, who had a haemoglobin of 76g/L of which a much reduced blood volume was taken from), with most patients having a haemoglobin of above 100g/L. Thus the finding that haemoglobin levels of above 100g/L associated with better outcome here contrasts the findings of Wen *et al*. Whether therefore high haemoglobin is something to be strived for patients who are to undergo a fistuloplasty, however, is controversial, as highlighted by a large meta-analysis' findings that higher levels of haemoglobin is associated with strokes, vascular access thrombosis and serious cardiovascular event in patients with CKD [175].

Haemoglobin levels were not found to correlate with the levels of CRP, or the plasma proteins found to associate with post-intervention primary or cumulative patency listed in tables 3-14 – 3-17. To answer whether haemoglobin is a general marker for health or whether it directly relates to NIH formation warrants further direct investigation. It is, however, known that low haemoglobin could result in anaemic hypoxia, in which there is a reduction in the oxygen carrying ability of the blood and thus oxidative stress. Oxidative stress has been shown to stimulate NIH formation in various studies, including in an angioplasty model in mice where oxidative stress was shown to increase SMC apoptosis and leads to the local accumulation of inflammatory cytokines [176].

Monocytes were then further investigated by looking at their subsets and the expression of CCR2, CD11b and HLA-DR within these subsets. It was found that 1-2 days post fistuloplasty, monocytes shift to a CD16 expressing phenotype. This shift in monocyte subsets did not happen immediately. Indeed, at 3 hours post fistuloplasty, intermediate monocytes significantly decreased prior to increasing. It is not likely that these changes occurred as a result of contrast or heparin administration as in the three patients who only had a fistulogram, no observable trend could be seen, nor was there any observable trend in the monocyte subsets in the time course experiment immediately post contrast or post heparin. The observation that monocytes do not switch to a CD16 expressing phenotype post balloon treatment is not new, with Maga *et al* finding that there was no shift to CD16 expressing monocytes immediately (0hr) post percutaneous transluminal angioplasty [177]. This however is the first time a shift in monocyte subsets post fistuloplasty has been reported.

The observation of the shift in monocytes post fistuloplasty leads to the question of what causes this, which the studies carried out here unfortunately cannot provide a direct answer to. *In vivo* deuterium labelling studies have previously shown that human

monocytes mature in the bone marrow and are released into circulation as classical monocytes, before progressively giving rise to intermediate followed by non classical monocytes. Therefore the increase in CD16 expressing monocytes would have corresponded to signals to classical monocytes to upregulate CD16 expression [166]. M-CSF blockade has been shown in rheumatoid arthritis patients to result in decreased levels of CD16 expressing monocytes and so M-CSF is likely to be important in monocyte subset conversion [178].

CD16⁺ monocytes had significantly less CCR2 and CD11b expression post fistuloplasty, but this was not observed in classical monocytes. The significance of the decrease in these markers needs to be further explored as whilst statistically significant, the relative change in MFI was little. CD11b regulates monocyte transmigration and adhesion and CCR2 plays an important role in the transmigration and extravasation of monocytes during inflammation. Carlin *et al* found that CCR2 knockout mice had the same numbers and of Ly6C^{low} monocytes in the bloodstream and crawling on the endothelium as control mice [179]. Carlin *et al* argues that “the choice between extravasation and intravascular retention may also correspond to distinct properties of different leucocyte cell types.” Thus given that CD16 monocytes are specialised towards surveying the endothelium for damage and there is damage to the endothelium layer post fistuloplasty, a reduction of CCR2 and CD11b expression on CD16 expressing monocytes can indicate monocytes shifting their “choice” towards remaining on the endothelial layer for facilitating repair rather than vessel extravasation ability.

Moreover, HLA-DR was significantly lower in CD16 expressing monocytes post fistuloplasty, but not in classical monocytes. HLA-DR is the human MHC class II cell surface receptor and plays a central role in the adaptive immune response as they are required for the antigen presentation to CD4⁺ T cells. Therefore lower HLA-DR results in less antigen presentation capacity of monocytes. A reduction in HLA-DR on

monocytes is known to happen after inflammatory trauma such as sepsis [180]. Kim *et al* found that HLA-DR is regulated differently on CD16⁺ and CD16⁻ monocytes and HLA-DR levels would decrease later in CD16⁺ monocytes than in classical monocytes [181]. Likewise to the CCR2 and CD11b data, the down regulation of HLA-DR could indicate a modulation of CD16 expressing monocytes to focus on reparative functions rather than antigen presentation.

Both pre and post fistuloplasty monocyte count did not associate with post-intervention primary or cumulative patency. This finding contrasts with Wildgruber *et al* observations that patients with higher baseline monocyte counts had an increased risk for developing restenosis following peripheral percutaneous transluminal angioplasty [115] and Schillinger *et al* findings that baseline monocyte counts were associated with restenosis after femoropopliteal percutaneous transluminal angioplasty [182]. The shift in monocyte subsets to a CD16 expressing phenotype, however, was found to be important in restenosis post fistuloplasty as even in the small patient cohort of 20, CD16 expressing monocytes associated with post-intervention primary patency outcome using a univariate cox regression (pre fistuloplasty non classical monocytes $p = 0.046$, hazard ratio of 1.081, and post fistuloplasty non classical monocytes $p = 0.054$, hazard ratio of 1.087). Likewise, Wildgruber *et al* found that increased CD16 expressing monocytes have been associated with restenosis after peripheral percutaneous transluminal angioplasty [115]. This study, however, examined monocytes at baseline, 3, 6, and 12 months thus implying that CD16 expressing monocytes may play a longer term role in NIH development rather than simply during the degranulation phase.

It is evident that there is a link between CD16 monocytes and NIH post fistuloplasty, but whether CD16 monocytes directly contribute to NIH is yet to be explored. Getzin *et al* found that Ly6C^{low} monocytes helped repair the damaged endothelial monolayer repair and hence arterial regeneration subsequent to carotid artery injury [183].

Therefore the association with higher levels of CD16 monocytes and higher restenosis risk may simply be a consequence of higher endothelial damage resulting in more CD16 cell recruitment for repair rather than CD16 monocytes directly causing restenosis. Equally, CD16 monocytes could facilitate an over reparative response which is fitting in line with NIH being an exaggerated wound healing response. CD16 monocytes release TNF- α more than classical monocytes and in response to TNF- α , endothelial cells upregulate integrin expression, thus allowing further capture of neutrophils and increasing the inflammatory response [184].

3.4 Investigating the changes in Monocytes Gene Expression following a Fistuloplasty Procedure

3.4.1 Introduction

Monocytes originate from a committed myeloid precursor cell in the bone marrow and constitute to about 5-10% of all leukocytes in healthy adults. They are key cells of mononuclear-phagocyte system and can give rise to tissue macrophages and dendritic cells. In PBMCs, monocytes are classically identified by their expression of the pattern recognition receptor CD14 surface antigen. This makes it possible to isolate monocytes in a mixed PBMC population using immunomagnetic bead separation targeting CD14 expression. As discussed in section 3.3.1, monocytes are thought to be particularly important in the development of neointima hyperplasia (NIH) and hence monocytes were investigated for potential gene expression changes following a fistuloplasty procedure.

The term "gene expression" is used to describe the transcription of the information contained within heritable DNA into messenger RNA (mRNA), the molecules that are translated into the proteins that perform most of the critical functions of cells. A microarray can be used to quantify gene expression in thousands of genes at once by utilising the ability of a given mRNA molecule to hybridize specifically to the DNA template from which it originated. If a gene is overexpressed post fistuloplasty, then more post fistuloplasty cDNA, as compared to pre fistuloplasty cDNA, will hybridize to the spot representing that expressed gene. This allows the microarray technique to identify genes of potential importance in monocytes and thus generate hypotheses that can further be confirmed with RT-PCR on larger patient cohorts.

3.4.2 Aims

The aim of this chapter was to assess the gene expression profile of CD14 positive bead isolated cells for post fistuloplasty changes using a microarray assay. The

patients used for this microarray analysis were then categorised as either having a 'good' or a 'bad' post-intervention primary patency outcome and pre fistuloplasty samples were assessed for gene associations with this outcome. Identified genes of potential interest were subsequently investigated in a larger cohort with RT-PCR and associated with post-intervention primary patency and post-intervention cumulative patency with and without adjustment for patient clinical variables. The relative gene expression of these genes within pre fistuloplasty PBMCs were also compared to that of healthy donors.

3.4.3 Summary of the Patient Samples used within this Section

In total 49 patient samples were used for RNA within this section. 21 of the patients were female whilst 28 were male. The patient ages ranged from 20 to 88, with an average age of 60.6 and a standard deviation of 14.5. RNA was extracted from the PBMCs of 20 healthy donors, of which 12 were female and 8 were male. The ages of the healthy donors ranged from 27 to 56 years with an average age of 37.5 and a standard deviation of 9.9 years. Overall the ages of the healthy donors were significantly less than that of the fistuloplasty patients ($p = <0.0001$) (Figure 3.26).

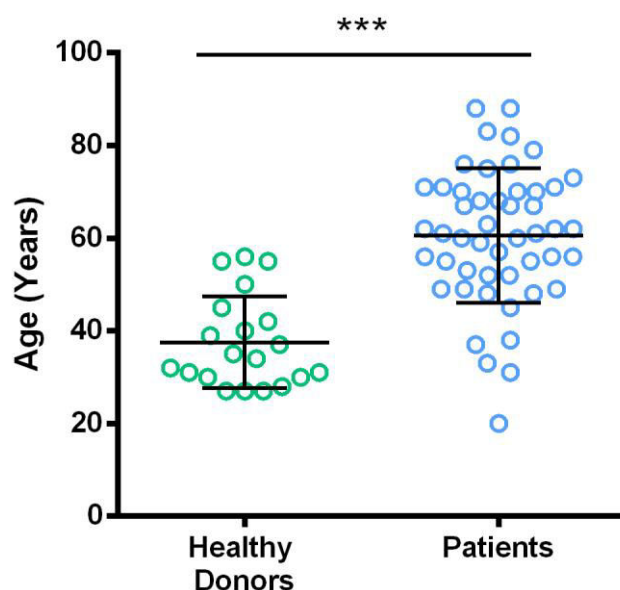


Figure 3.26: The ages of the healthy donors and the patients used within this section. Healthy donors' ages are shown in green, patients' ages are shown in blue. Healthy donors were significantly younger than patients. Results are expressed as mean $\pm$ SD. ($n = 20$ healthy donors and 49 patient samples). Significant differences were determined an unpaired t test. *** $p < 0.001$.

3.4.4 There are is a no Significant Shift in Monocytes Gene Expression Post Fistuloplasty

Simply comparing the pre and 1-2 days post fistuloplasty samples by paired analysis revealed that there were no genes with a greater than 1.75-fold change in either direction, and a p value less than 0.05 that were differentially expressed between the pre and post fistuloplasty samples (Figure 3.27).

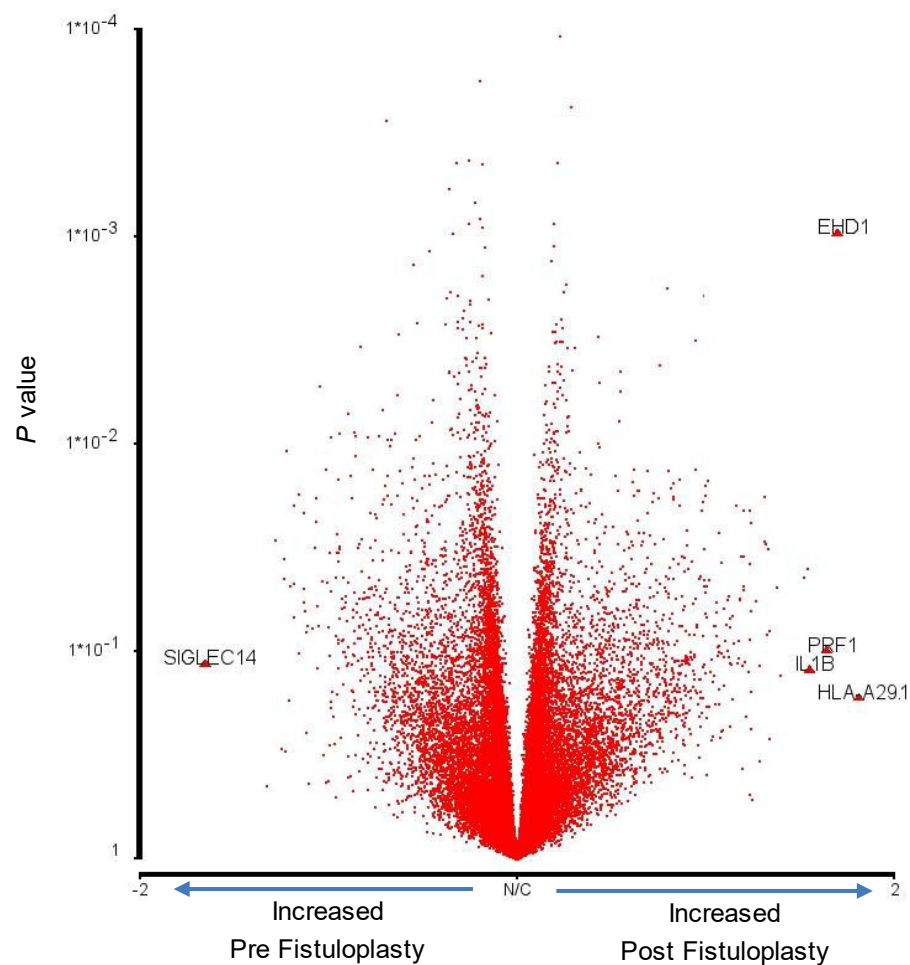


Figure 3.27: Volcano plot showing monocyte gene expression changes after a fistuloplasty procedure.

There were no genes with a greater than 1.75-fold change in either direction and a p value less than 0.05 that were differentially expressed between the pre and post fistuloplasty samples (n = mean gene expression of 16 pairs of samples).

3.4.5 There is a Weak Association between Pre Fistuloplasty Monocyte Overall Gene Expression and Patient Outcome

I then separated the 16 pre fistuloplasty monocyte samples into two groups based on whether the samples were derived from patients who had a 'good' or 'poor' post-intervention primary patency outcome. Poor outcome was defined as having a post-intervention primary patency of less than six months (i.e. the patients had thrombosis, a radiological or surgical re-intervention, or abandonment of their AVF within the first six months of the fistuloplasty procedure occurring), whilst all good outcome was defined as any patients who had surpassed this six-month cut off period. Under this definition, nine patients out of the sixteen-patient cohort were said to have had a good outcome whilst seven patients were said to have a poor outcome.

Whilst this definition is arbitrary, interestingly, principle component analysis, PCA (Figure 3.28) revealed that the monocyte samples from eight out of nine patients with a good outcome seemed to have little gene expression variance with the exception of one outlier patient. The overall association between gene expression and good or poor outcome was found to be close to significance (Pseudo $R^2 = 0.139$, Permutation $p = 0.08$).

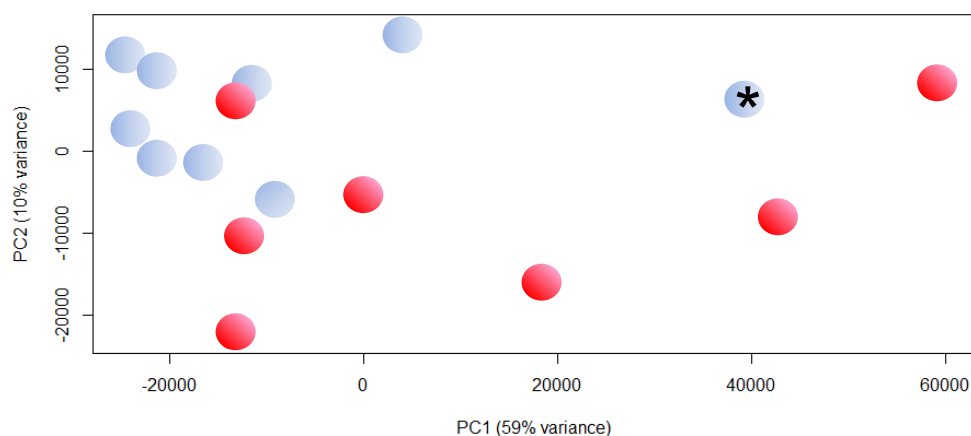


Figure 3.28: Principle component analysis allowing the visualisation of the gene expression states of the pre fistuloplasty samples. Blue spheres represent patients which had a good outcome and red spheres represent patients which had a poor outcome. Astrix (*) represents a notable outlier sample (n = 16 pairs).

An Analysis of Variance (ANOVA) analysis comparing the gene expression of monocytes from patients which had a good outcome with patients who had a poor outcome following their fistuloplasty procedure revealed that there were 83 genes with a 1.75-fold change, and a p value less than 0.05 differentially expressed between the groups (see Appendix C – Microarray ANOVA Analysis for the full list). Genes of interest which were down regulated in patients with a poor outcome included calcium-binding protein (S100) A4, S100A9 and the Calreticulin gene (CALR). Genes upregulated in patients with a poor outcome included Vanin 1 (VNN1) and haemopoietic cell kinase (HCK) (Figure 3.29).

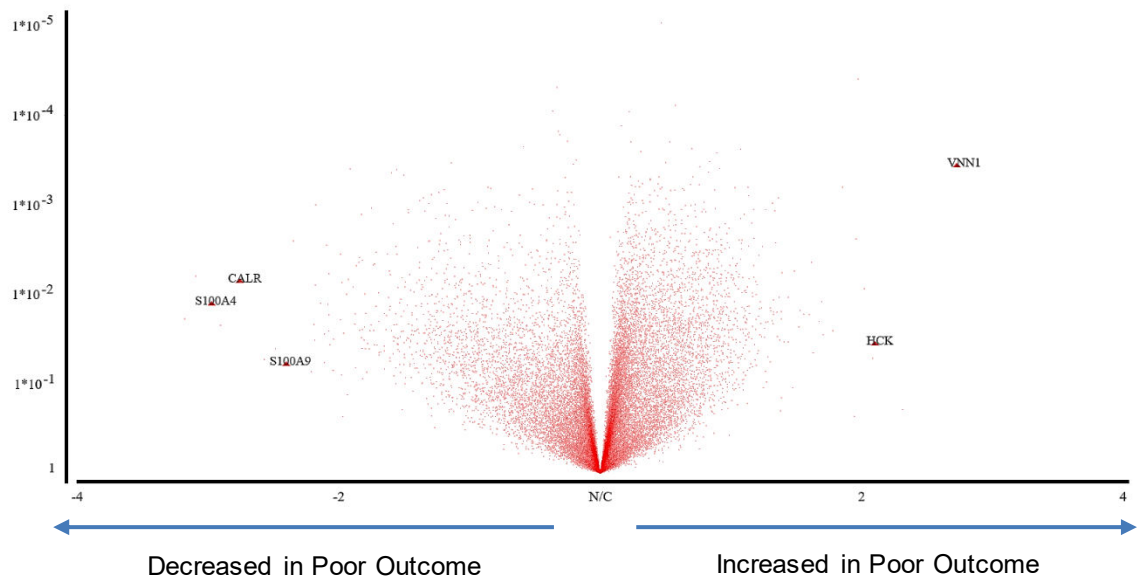


Figure 3.29: Volcano plot comparing the pre fistuloplasty derived monocytes of patients who had a good outcome with those who had a poor outcome. X axis depicts fold change. Y axis depicts p value. There were 83 genes with a 1.75-fold change, and a p value less than 0.05 differentially expressed between the groups (n = 9 good outcome vs. 7 poor outcome).

This analysis revealed that after separating patients based on 'good' or 'poor' post-intervention primary patency outcome, the most prominently affected gene was VNN1. Specific examination of the VNN1 gene revealed that monocytes from eight out of nine patients which had a good outcome had a lesser mean expression of VNN1 than those patients who had a poor outcome (Figure 3.30). Interestingly, the one exception to this was the VNN1 expression level in the patient found to be an outlier in the PCA. As previously described, these monocytes were derived from a patient that had a good

outcome yet had similar gene expression to patients who had a poor outcome. This separation of patient outcome based on VNN1 expression was not apparent when examining post fistuloplasty derived samples.

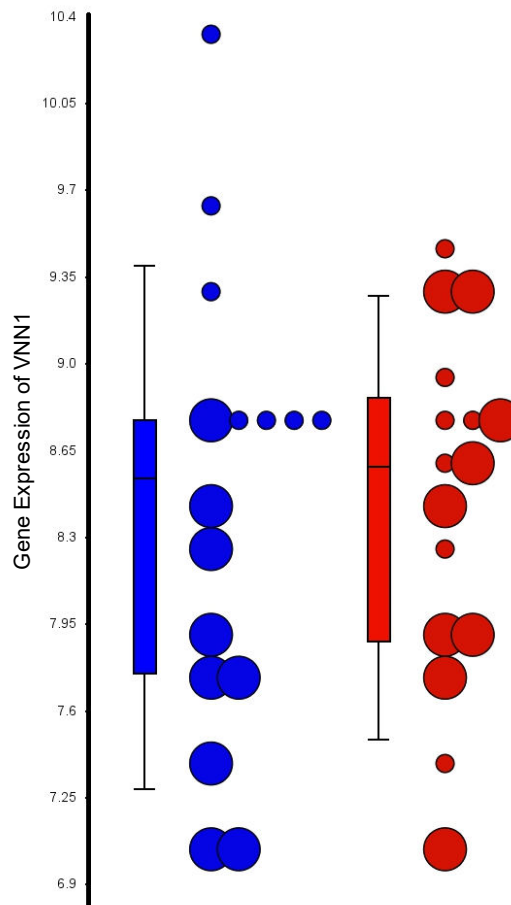


Figure 3.30: The mean gene expression of VNN1 in patient monocytes. Blue represents pre fistuloplasty samples, red represents post fistuloplasty samples. Large circles represent patients which had a good outcome after their procedure, small circles represent patients which had a poor outcome after their procedure (n = 16 pairs).

As dividing the patients into 'good' and 'poor' outcome groups is arbitrary, I next attempted to associate gene expression and the number of days it took for the fistulas to reach the end of post-intervention primary patency. As the microarray generated very large amounts of data from relatively so few patients, a typical univariate and multivariate data cox regression analysis associating individual genes with outcome would be inappropriate. As an alternative to this, in order to assess whether gene expression in pre fistuloplasty monocytes associated with patient outcome, Multivariate

Distance Matrix Regression (MDMR) was carried out. MDMR is a two-step statistical procedure. Firstly the similarity or dissimilarity between each pair of patient's outcome data is computed and then the similarity or dissimilarity between each pair of patient's monocyte gene expression profile is computed. Thus a distance matrix is created in which pair-wise distance values relating to both outcome data variation and overall monocyte gene variation is reflected. No significant association was found between monocyte gene expression and the number of days it took for the fistulas to reach the end of post-intervention primary patency (Figure 3.31) ($p = 0.114$).

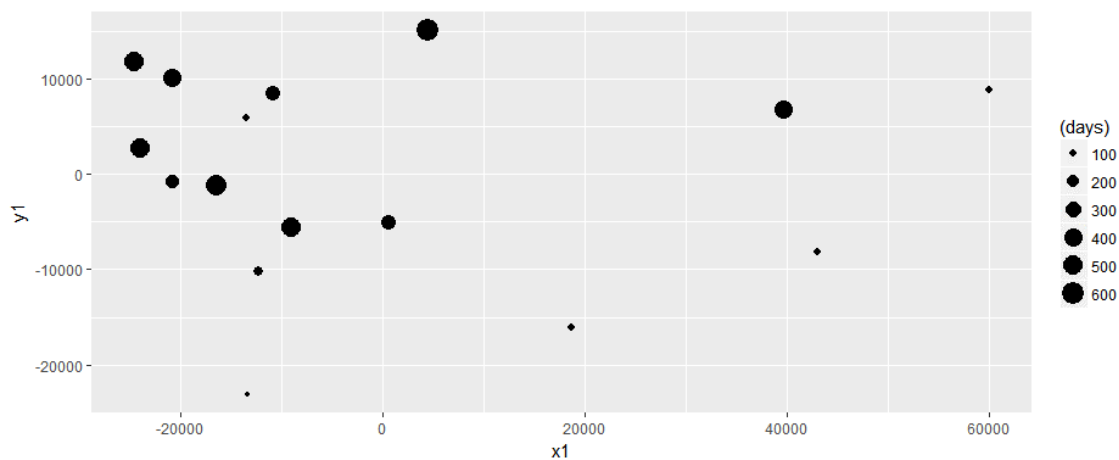


Figure 3.31: MDMR analysis showing the association of the distance matrix of genes with number of days it took for the fistulas to reach the end of primary patency. No significant association was found between gene expression and the number of days it took for the fistulas to reach the end of primary patency ($p = 0.114$).

3.4.6 Calreticulin Pre Fistuloplasty Gene Expression is likely to be Associated with Post-intervention Primary and Cumulative patency

Whilst not statistically significant, the genes with the strongest p values in combination with fold change identified as potentially being of potential importance by the microarray were S100A4, S100A9, CALR and VNN1. As a result, the expression of these genes pre fistuloplasty were analysed further using RT-PCR. VNN2 was also included in the panel due to its similarities with VNN1 in both structure and function [185]. Given that a genetic biomarker for patient outcome would be more useful if it could be identified in PBMCs rather than only in CD14 positive bead isolated cells, RT-PCR for gene expression was run on both CD14 positive bead isolated cells and

PBMCs. Initially a univariate cox regression test was carried out on CALR, S100A4, S100A9, VNN1 and VNN2 pre fistuloplasty gene expression against post-intervention primary patency and post-intervention cumulative patency. A hazard ratio of less than one would indicate a positive correlation with outcome whilst a correlation of greater than one would indicate a negative correlation with outcome. Table 3-28 and Table 3-29 reveal that no statistically significant associations were with post-intervention primary patency or post-intervention cumulative patency respectively.

Table 3-28: Univariate cox regression exploring for associations with the pre fistuloplasty gene expression of CALR, S100A4, S100A9, VNN1 and VNN2 in CD14 bead isolated cells and PBMCs with post-intervention primary patency outcome. n = 49 PBMC donors, n = 39 CD14 bead isolated cells donors.

Gene	Cell type	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
CALR	CD14 bead isolated cells	0.105	0.889	0.770	1.025
CALR	PBMCs	0.262	1.099	0.932	1.297
S100A4	CD14 bead isolated cells	0.909	1.008	0.876	1.160
S100A4	PBMCs	0.905	0.992	0.868	1.134
S100A9	CD14 bead isolated cells	0.343	0.904	0.733	1.114
S100A9	PBMCs	0.678	1.014	0.948	1.086
VNN1	CD14 bead isolated cells	0.927	0.991	0.821	1.197
VNN1	PBMCs	0.759	0.981	0.866	1.110
VNN2	CD14 bead isolated cells	0.620	0.965	0.838	1.111
VNN2	PBMCs	0.857	1.012	0.890	1.150

Table 3-29: Univariate cox regression exploring for associations with the pre fistuloplasty gene expression of CALR, S100A4, S100A9, VNN1 and VNN2 in CD14 bead isolated cells and PBMCs with post-intervention cumulative patency outcome. n = 49 PBMC donors, n = 39 CD14 bead isolated cells donors.

Gene	Cell type	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
CALR	CD14 bead isolated cells	0.421	1.125	0.845	1.497
CALR	PBMCs	0.280	1.144	0.897	1.459
S100A4	CD14 bead isolated cells	0.258	1.174	0.889	1.549
S100A4	PBMCs	0.939	0.993	0.820	1.202
S100A9	CD14 bead isolated cells	0.479	1.121	0.816	1.541
S100A9	PBMCs	0.102	1.095	0.982	1.220
VNN1	CD14 bead isolated cells	0.330	1.143	0.873	1.496
VNN1	PBMCs	0.998	1.000	0.839	1.193
VNN2	CD14 bead isolated cells	0.732	1.044	0.815	1.338
VNN2	PBMCs	0.458	1.073	0.891	1.293

I then sought to carry out multivariate analysis in order to seek for patterns and relationships between several variables simultaneously. I included patient clinical variables (gender, ethnicity, patient age, type of native fistula, log age of fistula, history of previous interventions and previous thrombosis) in case gene associations were being made undetectable due to a larger influence of a clinical variable on the data set. The multivariate analysis carried out manually was a forward analysis as further explained in the methods chapter, section 2.8. The exploratory 'stop' p value was set to 0.15 due to the relatively weak *p* values found within the univariate analysis.

Table 3-30 shows the forward stepwise multivariate cox regression analysis carried out investigating the association of patient clinical variables and pre fistuloplasty gene expression levels of various genes in CD14 bead isolated cells and PBMCs with post-intervention primary patency. The variables identified using this exploratory method was the following: the gene expression of CALR in CD14 bead isolated cells, the log age of the patient's fistula, the type of native fistula, the gene expression of CALR, VNN2 and S100A9 in PBMCs in the respective order.

Table 3-30: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and pre fistuloplasty gene expression levels of various genes in CD14 bead isolated cells and PBMCs with post-intervention primary patency.

The 'start' step denotes p values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was the gene expression of CALR in CD14 bead isolated cells as it had the lowest p value. 'Step 1' shows the p values of the first multivariate cox regression analysis carried out in which each variable (both clinical and gene expression) was combined with var1. The protein found to have the lowest p value here denoted as variable 2 (var2), and in this case is the log age of the patient's fistula. 'Step 2' shows the p value of the second multivariate cox regression carried out in which each protein was combined with var1 (the gene expression of CALR in CD14 bead isolated cells) and var2 (log age of the patient's fistula) to find the protein with the next lowest p value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a p value of less than 0.15. Light blue cells highlight the protein found to have the lowest p value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest p value. $n = 49$ PBMC donors, $n = 39$ CD14 bead isolated cells donors.

	0	Step 1	Step 2	Step 3	Step 4	Step 5	STOP
Gender	0.278	0.478	0.241	0.704	0.592	0.886	0.533
Ethnicity	0.309	0.665	0.841	0.618	0.788	0.98	0.606
Patient Age	0.720	0.677	0.634	0.32	0.779	0.476	0.382
Type of Native Fistula	0.581	0.208	0.113	X	X	X	X
Log Age of Fistula	0.201	0.040	X	X	X	X	X
Previous Interventions	0.600	0.804	0.537	0.986	0.738	0.844	0.833
Previous Thrombosis	0.902	0.620	0.515	0.495	0.544	0.515	0.742
CALR CD14	0.105	X	X	X	X	X	X
CALR PBMC	0.262	0.132	0.160	0.109	X	X	X
S100A4 CD14	0.909	0.410	0.257	0.545	0.31	0.409	0.693
S100A4 PBMC	0.905	0.515	0.597	0.504	0.325	0.949	0.158
S100A9 CD14	0.343	0.829	0.836	0.485	0.515	0.609	0.255
S100A9 PBMC	0.678	0.261	0.285	0.274	0.546	0.057	X
VNN1 CD14	0.927	0.621	0.487	0.612	0.572	0.701	0.568
VNN1 PBMC	0.759	0.815	0.729	0.758	0.8	0.901	0.771
VNN2 CD14	0.620	0.941	0.685	0.826	0.666	0.642	0.716
VNN2 PBMC	0.857	0.640	0.932	0.963	0.106	X	X

A matrix of regression coefficients table was then carried out in order to assess for co-linearity between variables identified as potentially associating with post-intervention primary patency (Table 3-31). The gene expression levels of VNN2 and S100A9 in PBMCs both strongly correlated each other and with the gene expression level of CALR in PBMCs ($p < 0.001$) and can be visualised in Figure 3.32. The gene expression of S100A9 in PBMCs also correlated with the gene expression of CALR in CD14 bead isolated cells as well, but to a lesser extent ($p = 0.001$). As a result of these co-linearities, the gene expression of S100A9 and VNN2 in PBMCs were excluded from the final model. Interestingly, the gene expression of CALR in CD14 bead isolated cells did not correlate with the gene expression of CALR in PBMCs.

Table 3-31: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-30.
Variables highlighted yellow are * $p < 0.05$, orange ** $p < 0.01$.

	CALR CD14	Log Age of Fistula	Type of Native Fistula	CALR PBMC	VNN2 PBMC	S100A9 PBMC
CALR CD14						
Log Age of Fistula	0.920					
Type of Native Fistula	0.674	0.475				
CALR PBMC	0.184	0.525	0.311			
VNN2 PBMC	0.230	0.172	0.482	< 0.001		
S100A9 PBMC	0.001	0.812	0.319	< 0.001	< 0.001	

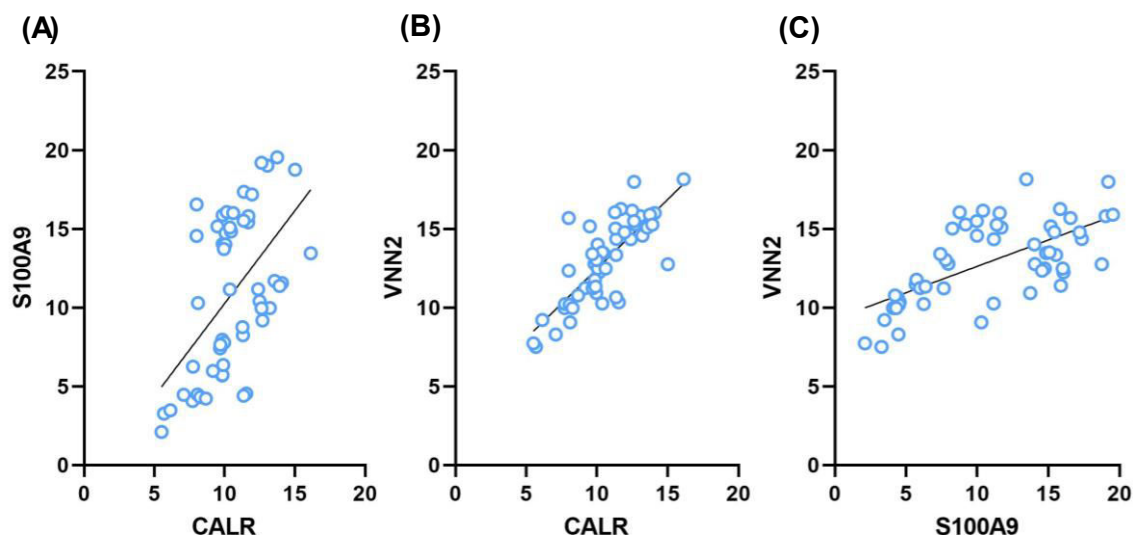


Figure 3.32: The correlations between the gene expression of CALR, VNN2 and S100A9 in PBMCs were very strong. n = 39 paired samples.

(A) Correlation of the PBMC gene expression of CALR with S100A9. R squared value 0.2919.

(B) Correlation of the PBMC gene expression of CALR with VNN2. R squared value 0.6154.

(C) Correlation of the PBMC gene expression of S100A9 with VNN2. R squared value 0.4215.

Thus the preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and genes included the gene expression of CALR from both CD14 bead isolated cells and PBMCs, the log age of the fistula and the type of the fistula itself with a final p value of 0.05 (Table 3-32). A higher log age of the fistula associated with a good post-intervention primary patency outcome (hazard ratio of 0.441) whilst the type of native fistula the patient had associated with a poor outcome (hazard ratio of 1.431), though the 95% confidence interval for this finding was large. Appendix B – Kaplan-Meier Curves for Patient Clinical Nominal Data shows a Kaplan-Meier curve for fistula type with outcome. Patients with radio-cephalic fistulas did better than patients with basilic vein transposition and brachio-cephalic fistula. Interestingly, the higher gene expression of CALR in CD14 bead isolated cells associated with good outcome (hazard ratio of 0.963) whilst the higher gene expression of CALR in PBMCs associated with a poor outcome (hazard ratio of 1.150). This is further discussed in the chapter discussion, but it should be reiterated here that the matrix of regression coefficients shown in Table 3-31 highlights that this finding is not as a result of an inverse correlation between CALR gene expression in monocytes and PBMCs.

Table 3-32: Table summarising a preliminary model for predicting post-intervention primary patency outcome based on the patient clinical variables and genes.

Omnibus Tests of Model Coefficients				
Significance		0.050		
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
CALR CD14	0.065	0.963	0.738	1.009
Log age of Fistula	0.056	0.441	0.190	1.022
Type of Fistula	0.152	1.431	0.876	2.339
CALR PBMC	0.109	1.150	0.969	1.364

Likewise to before, a multivariate cox regression analysis was carried out again using the same variables, but this time assessing for associations with post-intervention cumulative patency (Table 3-33). The variables identified using these exploratory methods were the following: patient age, patient gender, gene expression of CALR and S100A4 in PBMCs in the respective order. A matrix of regression coefficients table was then carried out in order to assess for co-linearity between variables identified as potentially associating with post-intervention cumulative patency, but no associations were found (Table 3-34).

Table 3-33: Forward stepwise multivariate cox regression analysis showing the association of patient clinical variables and pre fistuloplasty gene expression levels of various genes in CD14 bead isolated cells and PBMCs with post-intervention cumulative patency.

The 'start' step denotes *p* values of the initial univariate cox regression analysis carried out in which the first variable (var1) was selected. Var1 in this case was patient age as it had the lowest *p* value. 'Step 1' shows the *p* values of the first multivariate cox regression analysis carried out in which each variable (both clinical and gene expression) was combined with var1. The protein found to have the lowest *p* value here denoted as variable 2 (var2), and in this case is the log age of the patient's gender. 'Step 2' shows the *p* value of the second multivariate cox regression carried out in which each protein was combined with var1 (the patient's age) and var2 (the patient's gender) to find the protein with the next lowest *p* value (var3). This process is continued until the 'stop' step is reached in which there are no proteins with a *p* value of less than 0.15. Light blue cells highlight the protein found to have the lowest *p* value which was selected for the next step. The purple cell in the stop column indicates the protein with the lowest *p* value. n = 49 PBMC donors, n = 39 CD14 bead isolated cells donors.

	0	Step 1	Step 2	Step 3	STOP
Gender	0.433	0.099	X	X	X
Ethnicity	0.629	0.297	0.260	0.410	0.351
Patient Age	0.040	X	X	X	X
Type of Native Fistula	0.165	0.231	0.341	0.461	0.702
Log Age of Fistula	0.550	0.378	0.229	0.162	0.240
Previous Interventions	0.141	0.163	0.429	0.529	0.191
Previous Thrombosis	0.336	0.531	0.390	0.271	0.370
CALR CD14	0.421	0.636	0.931	0.986	0.986
CALR PBMC	0.280	0.182	0.125	X	X
S100A4 CD14	0.258	0.316	0.533	0.599	0.607
S100A4 PBMC	0.939	0.948	0.839	0.150	X
S100A9 CD14	0.479	0.625	0.875	0.719	0.896
S100A9 PBMC	0.102	0.117	0.223	0.702	0.260
VNN1 CD14	0.330	0.358	0.424	0.898	0.924
VNN1 PBMC	0.998	0.955	0.586	0.969	0.813
VNN2 CD14	0.732	0.740	0.882	0.964	0.927
VNN2 PBMC	0.458	0.381	0.199	0.800	0.456

Table 3-34: A correlation matrix showing correlation coefficients between variables of interest previously identified in Table 3-33.

	Patient Age	Gender	CALR PBMC	S100A4 CD14
Patient Age				
Gender	0.079			
CALR PBMC	0.958	0.417		
S100A4 CD14	0.901	0.245	0.929	

Thus the preliminary model for predicting post-intervention cumulative patency outcome based on the patient clinical variables and genes included patient age, gender and the gene expression of CALR and S100A4 in PBMCs (Table 3-35). Patient age, gender and CALR gene expression in PBMCs negatively associated with post-intervention cumulative patency (hazard ratios of 1.060, 3.125 and 1.544 respectively), whilst gene expression of S100A4 in PBMCs associated with better post-intervention cumulative patency outcome (hazard ratio of 0.791).

Table 3-35: Table summarising a preliminary model for predicting post-intervention primary cumulative outcome based on the patient clinical variables and genes.

Omnibus Tests of Model Coefficients				
Significance		0.020		
Variables in the Equation				
Variable	Significance	Hazard Ratio (HR)	Lower 95% Confidence Interval for HR	Upper 95% Confidence Interval for HR
Patient Age	< 0.001	1.060	1.012	1.109
Gender	0.022	3.125	1.094	8.927
CALR PBMC	0.046	1.544	1.034	2.305
S100A4 PBMC	0.150	0.791	0.575	1.088

3.4.7 S100A9 Relative Gene Expression in PBMCs is Significantly Higher in Patients with CKD than in Healthy donors.

The pre fistuloplasty PBMC gene expression levels of CALR, S100A4, S100A9, VNN1 and VNN2 were compared to that of in healthy donors (Figure 3.31). Interesting, the relative gene expression of S100A9 was found to be significantly higher in the patient samples than in healthy donors ($p = 0.007$).

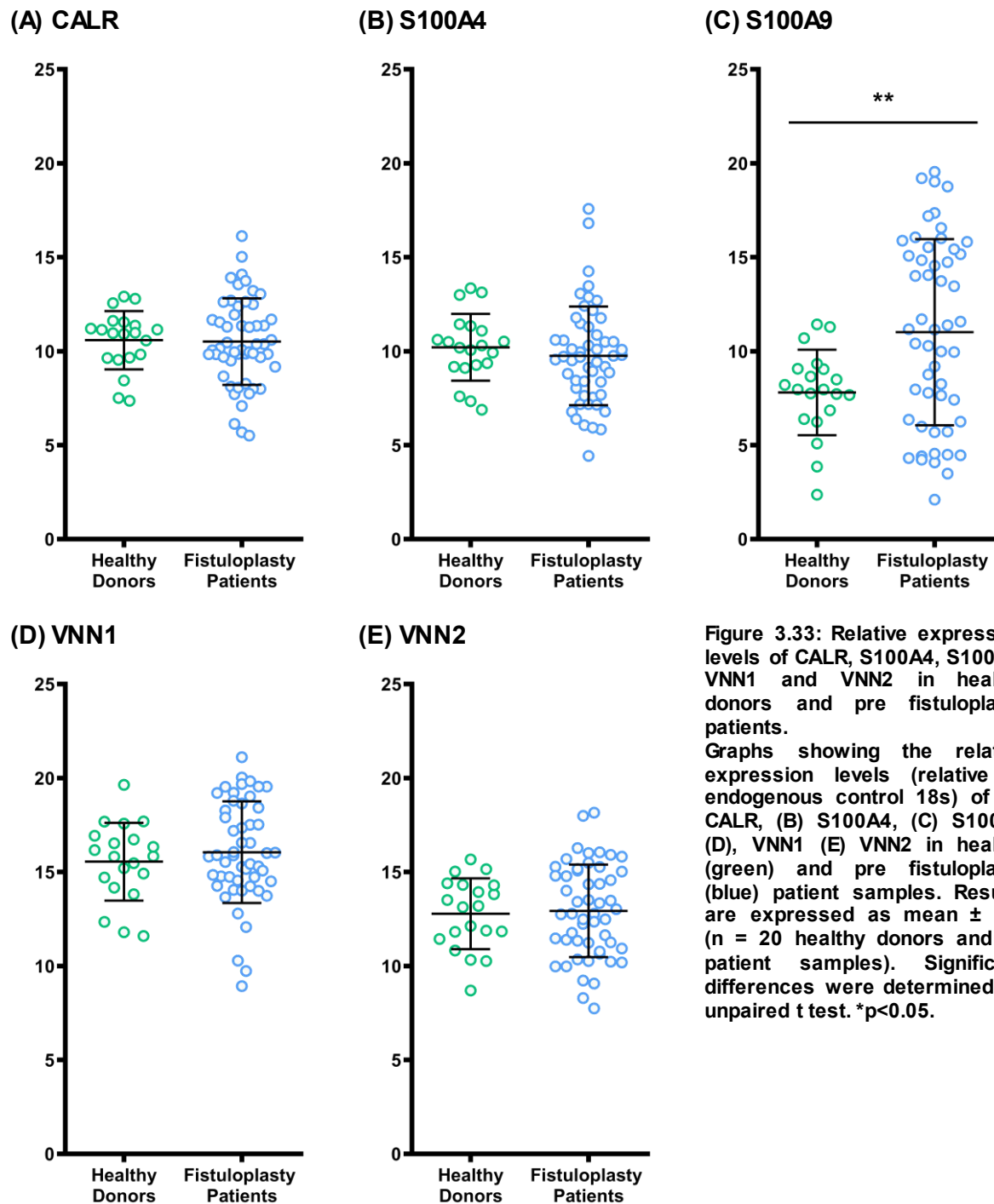


Figure 3.33: Relative expression levels of CALR, S100A4, S100A9, VNN1 and VNN2 in healthy donors and pre fistuloplasty patients.
 Graphs showing the relative expression levels (relative to endogenous control 18s) of (A) CALR, (B) S100A4, (C) S100A9 (D), VNN1 (E) VNN2 in healthy (green) and pre fistuloplasty (blue) patient samples. Results are expressed as mean $\pm$ SD. (n = 20 healthy donors and 49 patient samples). Significant differences were determined an unpaired t test. * $p < 0.05$.

3.4.8 Discussion

To explore the gene expression changes in monocytes after a fistuloplasty procedure, a microarray was performed on 16 pairs of pre and post fistuloplasty CD14 positively selected cells. Comparing the pre and post fistuloplasty samples by paired analysis revealed that there were no genes that were differentially expressed between the two groups which had a greater than 1.75-fold change in either direction and a p value less than 0.05. Initially, whilst disappointing, the results indicate that the overall gene expression response to the fistuloplasty procedure was very heterogeneous thus reflecting the large patient variation within the cohort. Given the results from section 3.3.5 on how there is an increase in CD16 expressing monocytes post fistuloplasty, it may have been expected to see some down regulation in typical classical monocyte genes (e.g. CD14) and an upregulation of typical non classical monocyte genes (e.g. CD16) post fistuloplasty. A limitation of the technique carried out was that CD14 positive bead isolation depletes non classical monocytes and much of intermediate monocytes and so perhaps this would explain why this effect was not seen.

The pre fistuloplasty patients were subsequently divided into a 'good' and 'poor' outcome group, and this rendered the individuals within the comparative groups more homogenous. Indeed, clustering could be observed within the PCA for the patients who had a good outcome following their fistuloplasty and the association between gene expression and outcome based on their arbitrary definitions was very close to significance with an adjusted p value of 0.08. Clustering for the patients who had a poorer outcome, however, still could not be seen indicating that the influence of monocytes for more negative outcome is more heterogeneous. Hence, it comes as no surprise that when the arbitrary definition of 'good' and 'bad' outcome was removed and replaced with the number of days for post-intervention primary patency loss no significant association was found between gene expression and the number of days it took for the fistulas to reach the end of primary patency with MDMR analysis.

Another factor to consider is, and one which is a major limitation of all analysis in this thesis aimed at examining associations of particular variables with patient outcome, is that the outcome data, (both post-intervention primary patency or post-intervention cumulative patency), in itself is an approximation. For instance, the main reason for primary patency to end in patients was because of a radiological procedure, most likely another fistuloplasty procedure. The date in which a patient has a fistuloplasty can be dependent on hospital waiting lists and bookings are frequently rescheduled. Hence, a patient may have reached post-intervention primary patency at 100 days, but perhaps their procedure had been cancelled many times before this and in reality they could have reached post-intervention primary patency as early as at 50 days. This also may highlight why clustering could be observed in the PCA analysis when using arbitrary definitions whilst the MDMR analysis found no significance.

The microarray indicated that pre fistuloplasty S100A4, S100A9 and CALR gene expression in CD14+ cells were associated with a post-intervention primary patency of more than 6 months, whilst VNN1 in CD14+ cells was associated with post-intervention primary patency of less than 6 months. S100A4 and S100A9 encode for S100 proteins, which are small calcium binding proteins predominantly expressed in the myeloid lineage cells known to be important to a variety of both extracellular and intracellular functions including cell growth and differentiation. They are also considered to be damage-associated molecular pattern molecules (DAMPs). CALR encodes for calreticulin (other known as calregulin), which is a multifunctional calcium binding protein and is also considered a DAMP. It is primarily found in the endoplasmic reticulum where it functions as an important element of the antigen presentation machinery by aiding in the assembly of MHC class I molecules. It is also noted to be important in cell migration and adhesion, being one of the few cytoplasmic proteins that can directly interact with integrins. When calreticulin is exposed on the surface of a cell it becomes an 'eat me' signal to phagocytes, thus calreticulin is also important for the phagocytic removal of apoptotic cells [186] [187]. In contrast, VNN1 encodes for

pantetheinase, an enzyme which hydrolyses pantetheine into pantothenic acid and cysteamine. VNN2 and VNN3 are the two other human genes within the vanin family. VNN2 is structurally very similar to VNN1 and also has pantetheinase enzymatic activity, whilst VNN3 has been suggested to simply be a pseudogene in humans [188].

Martinez *et al* using high-throughput RNA sequencing (RNA-seq) found that the gene expression of S100A8, S100A9 and VNN2 (as well as Colony Stimulating Factor 3 Receptor (CSF3R)) and FPR1 (Formyl Peptide Receptor 1)) were upregulated in pre-access veins that developed NIH and thus failed to mature [155]. The RNA-seq was carried out on a digested anastomotic area of AVF segments collected from 12 failed and 12 successfully matured AVFs during a second-stage transposition or salvage procedure, with patients in both groups having similar demographics and a clinical history. It is therefore interesting that both the microarray carried out here on CD14 expressing cells and an RNA-seq experiment carried out on digested AVF tissue found that calcium binding protein genes and vanin genes were associated NIH development in AVF tissue. Of all the genes, pre fistuloplasty VNN1 gene expression in particular had the strongest influence on dataset as patients could be separated on outcome based on this gene expression alone. Indeed, VNN1 has previously been linked to NIH. Dammanahalli *et al* found that VNN1 knockout mice had reduced NIH formation following carotid ligation and that VNN1 knockout SMCs had reduced proliferation, migration and MMP-9 activity in response to PDGF [189].

It is important to consider that the findings from the microarray findings are limited from potentially large false discovery rates, which I had not corrected for. Hence, the findings from the microarray were tentative, and were limited to representing hypotheses to further explore. I chose to next investigate the pre fistuloplasty gene expression of CALR, S100A4, S100A9, VNN1 and VNN2 association with post-intervention primary patency and post-intervention cumulative patency in both CD14 positively bead isolated cells and PBMCs. Whilst this step could not remove the issue

of false discovery due to the inclusion of samples from the microarray, it did enable us to investigate a much larger patient cohort. The univariate cox regression analyses carried out yielded no significant results and so a multivariate cox regression analysis with patient clinical variables included was carried out.

For post-intervention primary patency, the final model consisted of gene expression of CALR in both PBMCs and CD14 bead isolated cells as well as the log age of the fistula and type of fistula, with a higher log age of the fistula associating with a good outcome and a basilic vein transposition fistula being worse than a radio-cephalic fistula. For post-intervention cumulative patency, the final model consisted of patient age, gender and the gene expression of S100A4 and CALR in PBMCs, with a higher patient age and being female associating with a negative outcome. The advantage of multivariate testing is that it allows for the relationship between multiple variables to be studied, thus highlighting significances which may have otherwise had been missed. It is, however, by definition an exploratory analysis requiring large n numbers to find significances, hence with so few samples it comes as no surprise that the 95% confidence intervals of both model's variables to be large. Whilst strong conclusions from such an analysis should be avoided, the association of the relative gene expression of CALR in PBMCs with both post-intervention primary and cumulative patency (with a higher relative gene expression associating with a negative outcome) does, however, give more confidence that this gene is indeed important for post-intervention AVF patency.

For post-intervention primary patency, higher relative gene expression of CALR in PBMCs associated with a worse outcome whilst higher relative gene expression of CALR in CD14 expressing cells associated with a better outcome. Indeed in the microarray analysis carried out on CD14 expressing cells, CALR expression also associated with a better outcome. Whilst the difference in associations with outcome based on whether the gene was isolated from CD14 expressing cells or a mixed PBMC population initially seems conflicting, it is important to note that CALR is expressed by

monocytes, but is expressed more so in NK cells, B cells, CD4+ T cells and almost 3 times as more in CD34+ cells (expression data available on biogps.org). Thus it comes as no surprise that the levels of CALR expression in monocytes and PBMCs do not correlate as the majority of CALR expression would not be from monocytes in a mixed PBMC population.

Calreticulin has previously been linked to NIH formation, however, whether it attenuates or escalates disease, is controversial. Zimmerman *et al* observed that there was an increase in calreticulin expression in regions of vascular remodelling in mice with carotid artery ligation. Knocking down the calreticulin in this model resulted in significantly inhibited NIH formation and collagen deposition demonstrating that calreticulin increases disease. Zimmerman *et al* also observed that there was reduced the cellularity within the NIH lesion at 21 days following injury in mice with calreticulin knocked down, suggesting that in addition to regulating extracellular matrix production, calreticulin may also regulate cellular proliferation [190]. Indeed knockdown of calreticulin in a separate study has been shown to inhibit PDGF-BB stimulated bronchial SMC proliferation [191].

In contrast to Zimmerman *et al*, Dai *et al* found that calreticulin inhibits NIH at sites of balloon induced injury in mice iliofemoral arteries using a model in which calreticulin was infused to arteries prior to balloon injury [192]. The key differences in these models was that Zimmerman *et al* investigated the effects of calreticulin internal to the vessel, whilst Dai *et al* investigated the effects of calreticulin on the endothelial cell surface, and thus this model focused more so on the effect of calreticulin on peripheral vascular cells. Importantly, Dai *et al* found that there was a decrease in monocyte invasion in arterial sections taken from the sites of balloon injury after calreticulin infusion [192]. Hence, a possibility as to why higher CALR in CD14 isolated cells may have associated with better post-intervention primary patency outcome is because calreticulin may limit monocyte extravasion into injured sites.

The relative gene expression of S100A4, (other known as fibroblast-specific protein 1 (FSP-1)), relative gene, in PBMCs was found to associate with good post-intervention cumulative patency outcome in the multivariate regression and in the microarray. Whilst S100A4 expression in PBMCs in relation to NIH has not been published before, S100A4 has previously been linked to NIH development. Cheng *et al* used a model in which mice had fluorescently labelled S100A4 proteins in order to investigate the effects of S100A4 to NIH formation in AVGs. They found that bone marrow derived S100A4 expressing cells, in a homing mechanism dependent on stromal cell-derived factor-1 from platelets, accumulate in the AVG, and knocking down S100A4 expression prevented NIH formation [193]. Hence, in the study presented by Cheng *et al*, S100A4 expression by bone marrow derived cells was aiding to NIH formation. It has been known for a long time that S100A4 can bind to cytoskeletal proteins, such as non-smooth muscle myosin-IIA, to stimulate cell motility [194]. Perhaps a higher motility or inflammatory potential for PBMCs is important for post-intervention cumulative patency. In any case, such a finding would need to be explored further in order to conclude its significance.

The relative gene expression of CALR, S100A4, S100A9, VNN1 and VNN2 in PBMCs were also investigated in healthy donors to be compared to patient samples. There was no significant difference in the relative gene expression of CALR in pre fistuloplasty patients with that of healthy donors, but if patient samples were to arbitrarily be divided into 'good' and 'bad' outcome again it is likely that a difference would be seen. Interestingly, S100A9 gene expression in PBMCs was significantly higher in patients than in healthy donors. As a DAMP of inflammation, S100A9 is significantly increased in many types of inflammation such as in sepsis [195], gout [196] and arthritis [197] and so perhaps it would come as no surprise that it would be increased in CKD. One major limitation of this experiment was that the healthy donors were significantly younger than the patients and the gene expression of S100A9 is also known to increase with aging

[153]. Malícková *et al* previously showed that the concentrations of plasma calprotectin in patients undergoing haemodialysis were significantly higher than in healthy controls [198] and Pepper *et al* had shown that patients with anti-neutrophil cytoplasm antibody associated vasculitis had higher monocyte and neutrophil cell surface S100A8-S100A9 heterodimer expression than in healthy controls, and this reflected disease activity [154]. The finding of S100A9 being significantly higher in patients than in healthy controls, however, remains novel as the cellular expression and secretion of calprotectin in PBMCs may not always be proportional to the amount of the mRNA produced by the cell.

Chapter 4 – Discussion

4.1 Future Work

4.1.1 Introduction

The work presented within this thesis has provided us with a good foundation to build on. The next sections within this thesis detail how some of this work could be further developed.

4.1.2 Applying Thesis findings to the Randomised PAVE Clinical Trial Patient Cohort

The PAVE clinical trial aimed to determine whether the use of paclitaxel coated balloons rather than standard high-pressure balloons during the fistuloplasty procedure could reduce the restenosis recurrence rate in patients on haemodialysis [45]. 212 patients were recruited to the trial and research blood samples were processed from much of these patients pre and post fistuloplasty. Much of the experiments carried out within this thesis can be applied to the pre and post samples of patients who had paclitaxel coated balloons. It would be interesting to see if, for instance, patients who had paclitaxel balloon treatment would still have this shift of monocytes to a CD16 expressing phenotype and increase of TNF- α 1-2 days post fistuloplasty.

The non-randomised patients used within this thesis often had multiple lesions within their fistula. The patients randomised for the PAVE clinical trial all had one lesion, or lesions within close proximity of one another that could be treated with one fistuloplasty balloon. Henceforth, post-intervention target lesion patency (the time from the fistuloplasty date to the end of the treated segments primary patency) data has also been collected from these patients and may serve as a more orderly post-intervention outcome to use.

At the time of writing this thesis, the outcome of the PAVE clinical trial has not yet been published. Regardless of outcome of the trial and the potential laboratory data from experiments on randomised patients, the findings from these experiments would still be relevant to both understanding the mechanism by which restenosis occurs following a fistuloplasty procedure and the paclitaxel coated balloons mechanism of action. This is highlighted in Table 4-1:

Table 4-1: Table to show that regardless of the PAVE trial outcome or the data from laboratory experiments, the findings would still be relevant to both understanding the mechanism by which restenosis occurs following a fistuloplasty procedure and the paclitaxel coated balloons mechanism of action.

		PAVE trial meets primary endpoint	PAVE trial does not meet primary endpoint
Changes within monocyte subsets and plasma proteins post fistuloplasty observed in plain balloon treated patients are different in paclitaxel balloon treated patients	Finding	The mechanism of action of paclitaxel coated balloons could include inflammatory systemic effects, which impacts on changes in monocyte subsets post procedure.	The use of paclitaxel coated balloons leads to inflammatory systemic effects, which impacts on changes in monocyte subsets post procedure, but this effect did not translate into a clinical difference in the trial.
	Significance	This would help us better understand the mechanism of action of paclitaxel coated balloons in patients undergoing a fistuloplasty procedure.	This would be useful in understanding why PAVE failed the primary endpoint, and this could be due a number of other factors including patient heterogeneity and magnitude of the effects.
Changes in monocytes and plasma proteins post fistuloplasty observed in plain balloon treated patients are similar in paclitaxel balloon treated patients	Finding	The mechanism of action of paclitaxel coated balloons does not include effects on changes in monocyte subsets post procedure.	The changes in inflammatory systemic effects and monocyte subsets are important for restenosis
	Significance	This would highlight that the changes in monocytes could still be relevant to the overall mechanism of restenosis, but that paclitaxel coated balloons predominantly have a local effect only.	This would help us better understand the potential reasons for why the paclitaxel coated balloons had little clinical effect.

4.1.3 Further investigating the Histopathology of Venous Stenosis in Dysfunctional AVFs

A major limitation of this chapter was the limited number of excised fistula samples gathered. AVF1 was notably different from AVF2-4 in terms of having lots of monocytes and CD34+ cells and therefore it would be interesting to see whether this fistula tissue was an outlier, or whether there are many more AVF tissues which have a similar staining pattern. With more tissue, clinical data, such as a history of previous thrombosis, would become more relevant as patterns in histology can then be better correlated with clinical observations. As previously mentioned the excision of non-thrombotic fistulas is not a common surgical procedure and so in order to obtain more fistula samples, gathering samples from other UK sites may have to be explored.

Within this thesis, H&E, modified elastin staining and immunohistochemistry for vWF, SMA, SMMHC, vimentin and desmin was carried out on the four vein samples. I propose more immunohistochemistry stains should be carried out, namely CD16, MPO, TNF- α , IL-6, PF4, S100A4, S100A9, calreticulin, VNN1 and VNN2 based on the findings within the other chapters within this thesis. For instance, it was noted that 1-2 days post fistuloplasty, monocytes shift to a CD16 expressing phenotype. AVF1 had a high number of monocytes, but the subset of monocytes within this tissue remains unknown. Serial section immunohistochemistry staining for CD68 in addition to the CD16 staining would therefore be interesting as, for example, a region rich in monocytes but without CD16 staining would highlight that these monocytes were of the classical subset. Moreover, plasma levels of TNF- α , IL-6 and MPO were found to significantly change 1-2 days post fistuloplasty and it would be interesting to see if these could be detected within the NIH lesion of the tissue.

A preliminary experiment found that in AVF3, TNF- α was present in the intima but not within other area of the tissue (Figure 4.1). Similar findings in many more AVF tissue with old NIH lesions would challenge the conventional notion that TNF- α cytokine

secretion is most important simply during the acute inflammation stage of NIH. Moreover, plasma levels of PF4, MPO and S100A9 were associated with post-intervention patency hence it would be interesting to see if these factors becomes internalised within the vessel. S100A9 was also indicated by the microarray as potentially being important in post-intervention patency alongside S100A4, calreticulin, VNN1 and VNN2. Similarly, Martinez *et al* using RNA-seq had found that the gene expression of S100 proteins and vanin proteins was upregulated in pre-access veins that failed [155], giving more reason to investigate the staining of these proteins within the vessel.

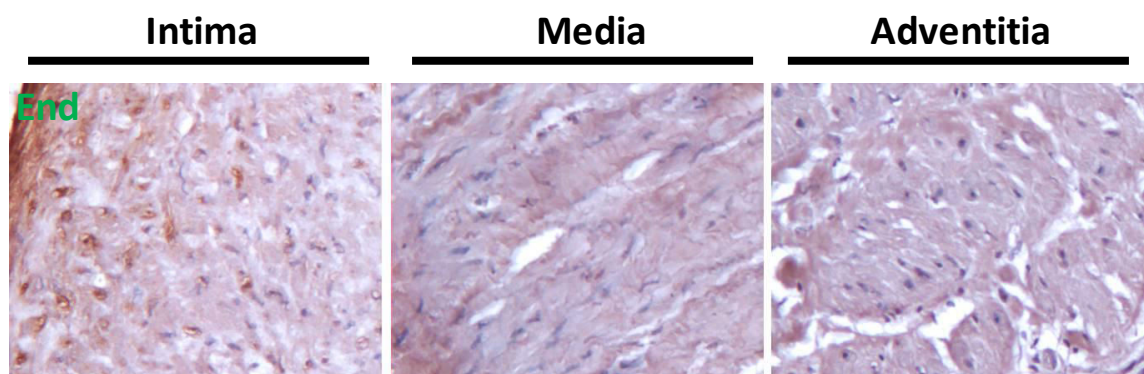


Figure 4.1: Immunohistochemistry for TNF- α in AVF3 (X400 magnification). 'End' indicates the endothelial layer. Note that the staining for TNF- α staining is only present within the intima layer of the AVF tissue.

4.1.4 Further investigating changes in Plasma Proteins following a Fistuloplasty Procedure

It was observed that whilst plasma levels of CRP did not significantly change 1-2 days post fistuloplasty, CRP plasma protein levels decreased at 0 hours post fistuloplasty and increased 2 hours post fistuloplasty. Likewise, whilst MPO significantly decreased 1-2 days post fistuloplasty, it increased 3 hours post fistuloplasty. Hence, there are acute changes in plasma protein levels and the changes which occur more acutely may be more relevant to patient outcome. Thus it would be interesting to assay more plasma proteins in a time course experiment. A protein that would be particularly relevant to focus on would be M-CSF, as M-CSF is likely to be important in monocyte subset conversion [174].

With a larger number of patient samples, it is more likely that a clinically relevant plasma biomarker for post intervention outcome can be found. If this biomarker is found using all patient samples from patients who had a plain balloon fistuloplasty, it would be important to see whether patients who had a paclitaxel coated balloon would have different plasma levels of this protein. Plasma levels of IL-6, TNF- α and MPO significantly changed 1-2 days post fistuloplasty in patients who had a plain balloon treatment. Hence it would also be interesting to see if this inflammatory effect would also be blunted in paclitaxel treated patients.

4.1.5 Further investigating changes in Leukocytes following a Fistuloplasty Procedure

1-2 days post fistuloplasty I found that whilst there was no increase in monocytes, there was a population shift in monocyte subsets towards a CD16 expressing phenotype. It is therefore quite possible that in the other leukocyte populations I had studied there may also be a shift in population phenotype. As previously discussed in section 3.3.7, there is a subset of CD4⁺ T cells known to lack the CD28 co-stimulatory receptor that increases with dialysis, is significantly more pro-inflammatory and increases with atherosclerosis [171]. Likewise, there is a subset of neutrophils known to be more inflammatory [172]. It would be novel to carry out experiments specifically designed to observe if these subsets increase post fistuloplasty with flow cytometry.

4.1.6 Further investigating the changes in monocytes following a Fistuloplasty Procedure

In this report I noted that monocytes shift towards a CD16 expressing phenotype 1-2 days post fistuloplasty. Whether this affect would also be seen in patients who were treated with a paclitaxel coated balloon should be further explored.

A major limitation of the microarray carried out on the monocytes is that the monocyte subsets were not separated. Sorting monocytes into classical and CD16⁺ monocytes

and then subsequently comparing the groups may have yielded better results. Recently, Hamers *et al* using whole genome transcriptional analysis found that non-classical monocytes can be further defined into subpopulations, with 45 genes differentially expressed and one of these subsets being of particular importance in atherosclerosis development [199]. Hence, using bulk RNA sequencing, it would be interesting to clarify if the increase in CD16⁺ monocytes I observed post fistuloplasty were an expansion of the CD16⁺ monocytes at baseline, or whether they represented an expansion of a different subpopulation which would not be evident using conventional flow cytometry techniques. If the transcriptional profile CD16⁺ monocytes post fistuloplasty is very different to those at baseline, then this would highlight the emergence of a new unique population and warrant also further investigating the classical monocytes for heterogeneity.

4.1.7 Developing a Triple-cell Vascular-immune Co-culture Model to carry out Functional Experiments

A major limitation of this project is that no model was used to test hypotheses using functional experiments. For instance, the multivariate analysis found that higher plasma levels of S100A9 is likely to associate with worse post-intervention patency outcome, but S100A9 action in directly inducing NIH was not explored. The lack of functional experiments in this thesis is not through a lack of trying as many models were tested throughout the course of this thesis with little success.

Initially, a saphenous vein organ culture model as first validated by Porter *et al* was tried [200]. In this model, freshly excised saphenous vein from coronary artery patients were cultured in a static culture for two weeks, during which the vein would develop NIH. The notion was that the culture media could be supplemented with proteins found to be of interest in order to validate whether they had a direct impact on NIH development. Initially the vein was cut into ring structures and promisingly NIH developed during the period of two weeks (Figure 4.2A); however the model stopped

soon after working despite there being no change in the media or materials used. This is possibly due to the surgical team changing the storage solution of the saphenous vein from saline to autologous heparinised blood during the course of this thesis, which is known to better preserve against NIH development [201]. Rather than collected saphenous vein, I then collected gonadal veins from healthy donors donating a kidney and cut the vein in square pieces in order to optimise the amount of tissue used (Figure 4.2B). The degree of NIH which developed using this vessel under baseline conditions, however, was minimal and very variable. The tissue obtained was also very small meaning it would be suboptimal for carrying out multiple experiments. It should be noted that for both the saphenous vein model and the gonadal vein model, various culture media and other experimental conditions were explored. Finally, a mouse model for NIH development within an aorta and inferior vena cava AVF was briefly tested [202]. This mouse AVF model was selected over other mouse models as the creation of the AVF was technically less challenging; Rather than suturing of vessels together, a 25 G needle would be used to puncture both walls of the aorta and inferior vena cava and the two vessels would connect naturally. Successful formation of the AVF was difficult to establish as the model required a post-operative Doppler ultrasound, which was both technically very challenging to carry out and infeasible within the own establishment.



Figure 4.2: Figures showing examples of a saphenous vein and gonadal vein culture experiments previously carried out.

(A) Modified elastin staining of circular ring saphenous vein explants at baseline, cultured for 10 days and cultured for 14 days showing the growth of NIH overtime, which could no longer be replicated.

(B) A gonadal vein explant pinned down on a mesh with vein culture media. Veins cultured in this method would grow very variable and minimal amounts of NIH at baseline conditions.

As a result, I propose carrying out a vascular-immune co-culture model for carrying out future functional experiments. There are many vascular-immune co-culture models in the literature, but many are technically demanding and often require specialised equipment such as custom cell culture scaffolds or perfusion equipment. Recently, Noonan *et al* proposed a simple triple-cell two-dimensional model to study immune-vascular interplay in atherosclerosis, which could well be adapted to studying NIH development [203]. In this model, primary human umbilical vein smooth muscle would be cultured on the underside of a transwell insert, which is then inverted when they are adherent. Once the SMCs are confluent, primary human umbilical vein endothelial cells would be cultured on the upper side of the transwell insert, and once would be confluent, an immune compartment, such as non-classical monocytes would added to the bottom of the culture well (Figure 4.3).

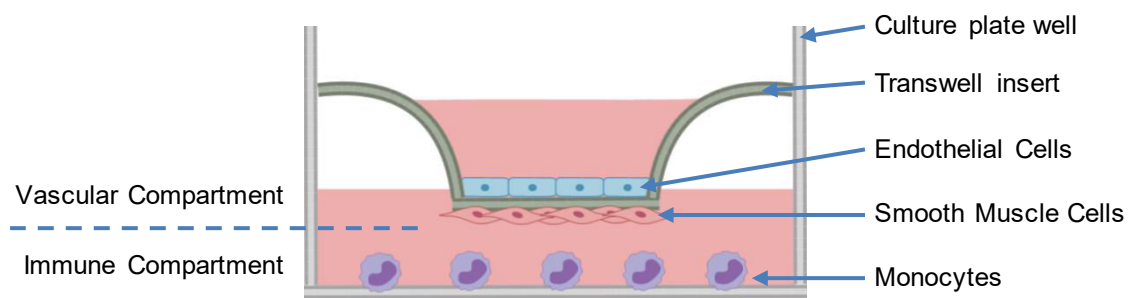


Figure 4.3: A schematic of triple-cell vascular-immune co-culture model proposed to be used. The model was developed by Noonan *et al* and the schematic is drawn with reference to this work [203].

Asides from the adaptability of the model, an additional benefit it is that each cell compartment within the model can easily be collected without cell sorting or digestion, thus allowing for further analysis with techniques such as flow cytometry or RT-PCR. For instance, RT-PCR for proliferating cell nuclear antigen (PCNA) on SMCs or endothelial cells would help demonstrate if these cells are proliferating more and smooth muscle myosin heavy chain expression in SMCs would help demonstrate if these cells are more of a contractile or synthetic phenotype. The culture media of this model can also easily be supplemented with proteins of interest, such as MPO and S100A9 to verify whether these proteins cause changes in the vascular compartment. Pre and post fistuloplasty monocytes effect on the vascular compartment of the system can also be investigated using this model. For instance, the increase in post fistuloplasty CD16 expressing monocytes may result in an increase of PCNA in endothelial cells and SMCs and a decrease of contractile phenotype markers in SMCs.

4.2 Summary & General Discussion

With an ageing population, a rise in diabetes, hypertension and obesity, the prevalence of CKD continues to be an ever growing public health problem [2]. Haemodialysis is the most common form of RRT [204] and the AVF is the ideal form of providing vascular access for long term haemodialysis because of its overall superior patency and mortality rate compared to AVGs and CVCs [10]. The main determinant of the effectiveness of a patient's haemodialysis is the patency their fistula. As a result vascular access failure is a crucial cause of morbidity in patients on haemodialysis. Indeed, a survey in the UK found that patients on haemodialysis occupy 320,000 bed days per year, with nearly a third of those admissions being related to vascular access complications [33]. The cost of this is great, with an estimated \$1 billion alone being spent in the US on vascular access and its problems [34]. Stenotic lesions caused by NIH commonly occur in fistulas resulting in patients requiring a fistuloplasty procedure. Restenosis, however, is a common problem associated with this treatment, with nearly half of all patients requiring a repeat procedure within their first year of initial procedure [27] [29] [205]. This need for repeat procedures following a fistuloplasty is both difficult for patients and expensive for the NHS. It is becoming increasingly apparent that whilst the fistuloplasty treats stenotic lesions, it also may accelerate the process of restenosis in mechanisms which has poorly been defined.

This thesis therefore initially sought to investigate some of the biological changes that occurs post fistuloplasty in order to better understand how restenosis occurs in the AVF. There are some studies about post angioplasty changes in patients with coronary artery disease in the literature, but not all these findings may be relevant to patients undergoing a fistuloplasty procedure as patients with CKD have physiological changes due to uraemia, oxidative stress, the fact that the NIH nearly always occurs in the venous section of the access circuit and the altered haemodynamic within the AVF. The aim of this thesis was not to compare patients on dialysis with healthy donors, but a few experiments were nonetheless carried out of which the findings should be noted

as it further highlights the difference between patients with CKD and other patient populations. This included the finding that there are less CD4⁺ T cells in patients on dialysis and that the relative gene expression of S100A9 in PBMCs is significantly higher in patients on dialysis when compared to healthy controls. Given that S100A9 is a DAMP and CD4⁺ T cells are a key component of the adaptive immune response, this overall highlights the well reported phenotype of patients on dialysis having immune exhaustion coupled with chronic inflammation.

In order to investigate the biological changes that occurs post fistuloplasty I focused on three key areas: plasma proteins, leukocytes and gene expression within monocytes. Using ELISAs and Luminex assays I found that of the 41 proteins assayed in 30 pairs of patient plasma samples pre and post fistuloplasty, only IL-6, TNF- α and MPO were found to have significantly changed 1-2 days post fistuloplasty, with IL-6 and TNF- α significantly increasing, and MPO significantly decreasing. Using a time course experiment, I found that plasma MPO initially increased 3 hours post fistuloplasty before decreasing suggesting that there was an acute increase in neutrophil and monocyte inflammatory activity resulting in a release of MPO, and a systemic subsequent decrease due to MPO binding to the site of injury. The time course experiment also suggested a gradual rise in TNF- α levels, with the highest values at 1 day post fistuloplasty, whilst for IL-6 although significance was not reached, there is a notable trend of IL-6 increasing 2-3 hours post fistuloplasty followed by declining from this peak 1 day, though remaining elevated when compared to baseline levels.

The increase in plasma IL-6 and TNF- α correlates with the changes I observed in monocyte subsets. I found that monocytes shift to a CD16 expressing phenotype 1-2 days post fistuloplasty and the time course experiment I had carried out shows that I had not missed a much earlier peak in monocyte numbers. It has previously been reported that classical monocytes generate IL-6 when in close proximity with endothelial cells resulting in a down regulation of E-selectin on endothelial cells. In

contrast CD16 expressing monocytes generate high levels of TNF- α when in close proximity with endothelial cells, which results in an up regulation of E-selectin on endothelial cells [184]. Given that in my findings IL-6 plasma levels peaked earlier than TNF- α level, which continued to raise 1-2 days post fistuloplasty, this shows that my cytokine findings may reflect the shift of classical monocytes to a CD16 expressing phenotype.

Monocytes have long been considered an important cell type in the systemic response to vascular injury. Mori *et al* showed that monocytes infiltrate the intima after carotid artery angioplasty in rabbits and that MCP1 blockade is therapeutic and prevented NIH, confirming a key role for monocytes in the processes occurring in the injured vessel formation [112]. Depletion of monocytes with clodronate prevented NIH formation in two different animal models [113] [114]. As a result, I had carried out a microarray to investigate for post fistuloplasty changes within monocytes. Simply comparing the pre and post fistuloplasty samples by paired analysis revealed that there were no genes with a greater than 1.75-fold change in either direction, and a *p* value less than 0.05 that were differentially expressed between the pre and post fistuloplasty samples. This, however, did not mean that monocyte gene expression did not change post fistuloplasty. Rather, my findings showed that there was a shift in monocyte gene expression post fistuloplasty but the gene signature varied significantly from patient to patient.

I had also obtained four AVF excised tissues for histological investigation as understanding the differences in the composition of cellular phenotypes within the NIH lesion can provide valuable information on how cells migrate, proliferate and transform. I found that the primary composition of secondary failure NIH lesions of the AVF is of myofibroblasts with a few contractile SMCs, much like how primary failure NIH lesions are [53]. The extent of fibrosis within the lesion, however, varied from patient to patient and did whether there were CD34+ cells or monocytes and macrophages present

within the NIH. Subtle differences in NIH composition leads us to conclude that it is possible that different vascular injuries before and after the fistuloplasty procedure may have resulted in the predominance and acquisition of different cellular phenotypes seen within the lesion.

In addition to investigating some of the biological changes that occurs post fistuloplasty, I also sought to find biomarkers that would indicate the patient's post-intervention outcome. No particular leukocyte major subset or platelets associated with outcome, but higher haemoglobin levels associated with better post-intervention outcome in both univariate and multivariate cox regression analysis. Damaged kidneys have compromised erythropoietin production, and thus haemoglobin deficiency is common in patients who have CKD. Many patients are given treatment for haemoglobin deficiency, which includes the administration of erythropoietin. It is possible that haemoglobin is a general marker for health rather than it directly participating in NIH formation, but it is also possible that hypoxia caused for anaemia may have a direct role.

Interestingly, both pre and post levels of non-classical monocytes associated with worse post-intervention primary patency outcome. Functional studies need to be carried out to state with certainty that CD16⁺ monocytes have a direct role in promoting restenosis, but the hypothesis of this thesis that they facilitate an over reparative response. This is fitting with the observation that CD16⁺ monocytes have been associated with restenosis after peripheral percutaneous transluminal angioplasty even at 3, 6 and 12 months post procedure, a length of time coinciding with the time for restenosis to occur [115].

None of the proteins investigated had a hazard ratio with a magnitude deemed clinically important, which is quite possibly as a result of not having adequate numbers of patient samples for such a large analysis. Despite this, the plasma proteins MPO,

PF4 and S100A9 consistently significantly associated with two different post-intervention outcomes in both univariate and multivariate analyses. PF4 is notably released from activated platelets, whilst both MPO and S100A9 are largely secreted by activated neutrophils and monocytes when in contact an inflamed endothelium or extracellular matrix proteins. Classical monocytes main role is to promote the first line of antimicrobial defence and so the expression of MPO and S100A9 is highest in the classical subset than in CD16+ monocytes [206]. These potential biomarkers signify that higher amounts of platelet, neutrophil and monocyte activation have adverse consequences.

The microarray's identification of gene expression of S100A9 in monocytes as potentially being important for outcome and the observation that S100A9 gene expression is significantly higher in patient PBMCs in comparison to healthy donors adds weight to the hypothesis that S100A9 might be an important molecule in promoting restenosis post fistuloplasty. The microarray and multivariate analysis carried out using RT-PCR also associated calreticulin with post-intervention outcome. Both S100A9 and calreticulin are well known DAMPs. Whilst S100A9 can bind to TLR4, both S100A9 and calreticulin can also bind to scavenger receptors to trigger sterile inflammatory reaction. Higher relative gene expression of calreticulin in PBMCs associated with a loss of post-intervention patency. Conversely, lower relative gene expression of calreticulin in CD14+ cells associated with a loss of post-intervention patency. The discrepancy here can by part be explained by how monocytes do not express the majority of calreticulin in PBMCs. Notably, calreticulin infusion has been associated with a decrease monocyte arterial invasion [192], hence there is a possibility that calreticulin may limit monocyte extraversion into injured sites.

4.3 Hypothesised Model from Results

Based on my findings, therefore, my hypothesis is that the systemic inflammatory response to the fistuloplasty procedure is paralleled with the conversion of classical to intermediate and non-classical monocytes. There is an initial inflammation phase, as first described by Forrester *et al* [47] as highlighted by an increase in both TNF- α and IL-6 plasma levels. Activated neutrophils and classical inflammatory monocytes release IL-6, but also MPO during this phase, some of which becomes absorbed by the endothelial layer. Between 3 hours and 1 day post fistuloplasty, beyond the acute inflammation phase, there is a shift of classical monocytes to intermediate followed by non-classical monocytes, which corresponds to a reduction in IL-6 production and a continuous increase in TNF- α production. This response is thought to contribute to restenosis following treatment through the CD16⁺ monocytes facilitating an over reparative response. Increased platelet activity resulting in more PF4 production and increased DAMPs release such S100A9 or calreticulin activity further exuberates this wound healing process. In monocytes, however, high expression of calreticulin inhibits monocytes extravasation the vessel. This helps attenuate an exaggerated wound healing response as, once in the vessel, monocytes can differentiate into macrophages, which promote the proliferation and migration of SMA producing cells. Alternatively, they monocytes may differentiate into fibrocytes, which have to capacity to differentiate into myofibroblasts. Overall this is a hypothesised model suggests that monocytes in the context of patients undergoing a fistuloplasty procedure need to be further explored in order to have a better understanding as to how restenosis develops following a fistuloplasty procedure. Figure 4.4 summarises this model.

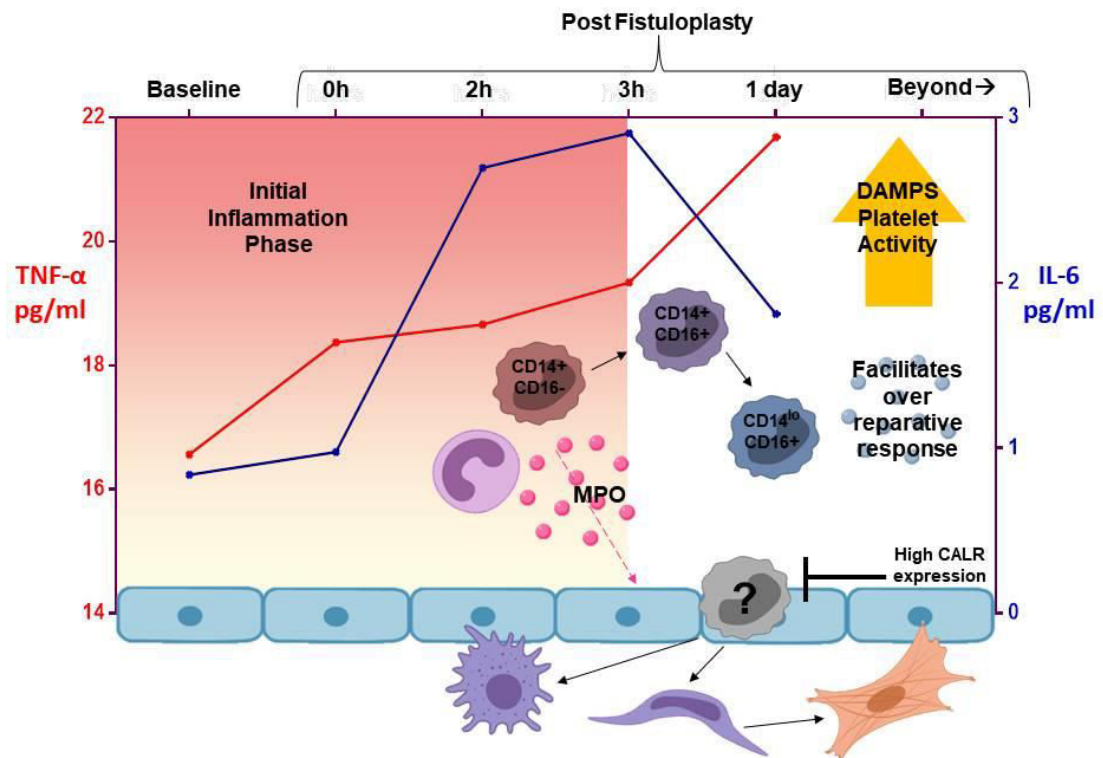


Figure 4.4: Hypothesised Model from Results.

Graphs show the plotted means of the plasma levels of TNF- α (red, right Y axis) and IL-6 (blue, left Y axis) from six patients before, 0 hours, 2 hours, 3 hours and 1 day post fistuloplasty. TNF- α plasma levels continue to increase post fistuloplasty, peaking at 1 day post fistuloplasty. In contrast, IL-6 levels increase post fistuloplasty, but, despite remaining higher than baseline levels, decrease 1 day post fistuloplasty. Hence the initial acute inflammation phase of NIH development is thought to last at least three hours, but less than 24 hours. This is thought to correspond to the changes seen to monocyte subsets post fistuloplasty. During the inflammation phase, activated classical monocytes and neutrophils release MPO, of which some is absorbed into the endothelial layer. Between 3 hours and 1 day post fistuloplasty, there is a shift of classical monocytes to intermediate followed by non-classical monocytes. Non-classical monocytes exuberates NIH development by contributing to an over reparative immune response. Increased platelet activity resulting in more PF4 production and increased DAMPs release such S100A9 or calreticulin activity further exuberates this wound healing process. In monocytes, however, high expression of calreticulin inhibits monocytes extravasation the vessel. Once in the vessel, monocytes can differentiate into macrophages, which promote the proliferation and migration of SMA producing cells. Alternatively, they monocytes may differentiate into fibrocytes, which have to capacity to differentiate into myofibroblasts.

4.4 The Potential Wider Impact

There is a debate about whether maintaining an AVF is the appropriate vascular access option in some patients where there may be a competing risk of death, and indeed in many patients fistulas are never used due to death occurring before the patient's dialysis initiation. A cut off point using a simple risk variable such as age is not adequate to determine candidacy for AVF creation [207]. There is also no clear definition as to when it is best for a fistula with stenosis to have fistuloplasty procedure or to be abandoned instead as currently there is no way to predict what patient outcome will be post fistuloplasty. Hence, biomarkers that can predict restenosis risk prior to the fistuloplasty procedure occurring would be desirable. The pre fistuloplasty potential biomarkers presented here if further explored could have potential to help here with clinical decisions; however, it is important to note that even when in combination with other biomarkers, the biomarkers presented here only moderately add to the prediction of a risk in an individual and so they would be of limited value for the risk stratification of individual patients.

Often a biomarker has to have a very large hazard ratio and strong association with outcome if it is to provide a basis for individual patient stratification. The post fistuloplasty and change biomarkers presented here may be more useful if they can serve as a predictor for the efficacy of interventions. For instance, if post fistuloplasty non-classical monocytes associate with post-intervention primary patency, then patients who had a larger increase in non-classical monocytes may require more clinical monitoring post-intervention. Equally, such a marker would be useful as a predictor for the efficacy of new interventions. For instance, if non-classical monocytes were found to decrease in response to a new drug coated balloon or systemic drug prior during a clinical trial, one might be more confident that the treatment will be effective in preventing restenosis.

More importantly, the biomarkers suggested here could help devise new treatments to target to complicated pathway leading to restenosis. For instance, if further studies highlight that non-classical monocytes are perpetrators of NIH, then new therapies can directly target this cell type. Glucocorticoids, a class of corticosteroids which are widely used immunosuppressive and anti-inflammatory drugs, have been shown to be able impact monocyte subsets as early as in 1998 when a group based in the University of Munich showed that glucocorticoids selectively depleted CD16+ monocytes in patients with multiple sclerosis whilst increasing classical monocytes [208]. The same group also showed that healthy volunteers who were infused with high doses of glucocorticoids daily for 3 consecutive days had a selective depletion of CD16+ monocytes of about 95% on day 5 [209]. The mechanism of depletion remained elusive and whether results similar to the patients with multiple sclerosis results can be replicated in other inflammatory conditions is controversial. For instance, in patients with autoimmune uveitis, (a condition that causes inflammation of the pigmented layer of the eye), had increased numbers of intermediate monocytes, which was directly linked with dosage of the glucocorticoid therapy [210]. As such there are currently no interventions available that are known to deplete CD16+ monocytes in patients undergoing a fistuloplasty procedure, but the data here suggests there could be an important role for these in preventing restenosis.

Ultimately, even if the main impact of this work would be to stimulate discussion, this too would be considered a success as there has been little etiological research in preserving vascular access in patients undergoing haemodialysis in the last decade. This thesis should serve as a reminder that there is still much more work to be done in this field and any research which can reduce the need for re-interventions would be very valuable.

Appendix A – Patient Clinical Data Summary

Overall blood samples were taken from 75 patients undergoing a fistuloplasty who were between the ages of 20 to 88 with a mean age of 61 and a standard deviation of 14. 45% of patients were female. 48% of patients were white, 37% of patients were black, 7.5% of patients were Asian and 1 patient was listed as 'other' for ethnicity. The most common type of native fistula patients had was a brachio-cephalic fistula (52%), followed by a radio-cephalic fistula (24%), and a basilic vein transposition fistula (23%). Only 2 patients had a history of previous thrombosis in their fistula whilst 16 patients had previous interventions in their fistula. The age of the fistula from date of creation to date of procedure ranged from 12.75 years to 0.08 years, with a mean of 2.37 years and a standard deviation of 2.23 years.

Table 0-1: Table to show the demographic data and clinical data of the patients undergoing fistuloplasty procedures, which donated blood samples.

Patient Number	Gender	Patient Age (Nearest Year)	Ethnicity	Type of Native Fistula	Age of Fistula a (Years)	Previous Thrombosis?	Previous Interventions?
1	M	62	White	Radio-cephalic	12.75	No	No
2	F	48	Black	Basilic vein Transplantation	2.72	No	No
3	F	59	Black	Brachio-cephalic	0.24	No	No
4	F	70	Asian	Brachio-cephalic	4.40	No	Yes
5	F	56	Asian	Basilic vein Transplantation	4.80	No	Yes
6	M	61	Black	Basilic vein Transplantation	0.98	No	No
7	M	57	Black	Radio-cephalic	1.68	No	No
8	F	67	White	Brachio-cephalic	4.51	No	Yes
9	M	68	Black	Radio-cephalic	3.52	No	No
10	M	83	Black	Basilic vein Transplantation	0.21	No	No
11	M	70	White	Brachio-cephalic	4.02	No	No
12	M	60	Asian	Basilic vein Transplantation	0.31	No	No
13	M	79	White	Brachio-cephalic	1.85	Yes	Yes
14	F	76	White	Brachio-cephalic	5.86	No	Yes
15	M	20	Asian	Radio-cephalic	1.76	No	No
16	M	52	Black	Radio-cephalic	0.58	No	No
17	M	60	Black	Radio-cephalic	0.08	No	No
18	M	56	Black	Brachio-cephalic	0.36	No	No

Patient Number	Gender	Patient Age (Nearest Year)	Ethnicity	Type of Native Fistula	Age of Fistula a (Years)	Previous Thrombosis?	Previous Interventions?
19	M	82	Black	Basilic vein Transplantation	4.82	No	Yes
20	F	70	White	Brachio-cephalic	1.19	No	No
21	M	67	White	Brachio-cephalic	1.40	No	No
22	M	73	Black	Brachio-cephalic	5.83	No	No
23	M	68	Black	Brachio-cephalic	0.54	No	No
24	F	41	Black	Brachio-cephalic	4.51	No	Yes
25	F	71	Asian	Basilic vein Transplantation	0.53	No	No
26	F	62	Black	Brachio-cephalic	2.31	No	No
27	F	45	Black	Radio-cephalic	1.81	No	Yes
28	M	75	White	Brachio-cephalic	1.44	No	No
29	F	49	Black	Basilic vein Transplantation	1.04	No	Yes
30	M	48	Black	Basilic vein Transplantation	0.25	No	No
31	F	55	Black	Brachio-cephalic	2.32	No	No
32	F	37	White	Radio-cephalic	0.41	No	No
33	M	52	Black	Radio-cephalic	4.47	No	No
34	F	33	White	Brachio-cephalic	2.84	No	Yes
35	F	49	Black	Basilic vein Transplantation	4.12	No	Yes
36	F	63	White	Brachio-cephalic	0.64	No	No
37	M	53	White	Basilic vein Transplantation	0.59	No	No
38	M	62	Black	Radio-cephalic	0.37	No	No
39	F	38	Asian	Basilic vein Transplantation	1.10	No	No
40	M	31	White	Brachio-cephalic	1.49	No	No
41	F	71	Black	Brachio-cephalic	1.22	No	No
42	M	88	White	Brachio-cephalic	1.20	No	No
43	M	67	White	Radio-cephalic	3.41	No	No
44	M	76	Asian	Basilic vein Transplantation	0.91	No	No
45	F	74	Black	Brachio-cephalic	3.85	No	Yes
46	M	61	Black	Brachio-cephalic	1.48	No	No
47	F	49	White	Brachio-cephalic	0.35	No	No
48	M	55	Black	Brachio-cephalic	7.07	Yes	Yes
49	M	71	Black	Basilic vein Transplantation	2.54	No	No
50	F	71	Asian	Basilic vein Transposition	1.11	No	No
51	M	71	Black	Radio-cephalic	0.98	No	No
52	M	59	White	Radio-cephalic	0.95	No	No
53	M	59	White	Brachio-cephalic	0.60	No	No
54	F	48	White	Radio-cephalic	5.56	No	No
55	M	86	White	Basilic vein Transposition	2.48	No	No
56	M	56	White	Brachio-cephalic	0.96	No	No
57	M	74	White	Brachio-cephalic	2.68	No	Yes
58	M	67	Asian	Brachio-cephalic	2.69	No	Yes

Patient Number	Gender	Patient Age (Nearest Year)	Ethnicity	Type of Native Fistula	Age of Fistula a (Years)	Previous Thrombosis?	Previous Interventions?
59	F	78	White	Brachio-cephalic	4.59	No	Yes
60	F	41	White	Radio-cephalic	0.71	No	No
61	F	54	White	Unknown	?	?	?
62	M	51	White	Brachio-cephalic	2.92	No	Yes
63	M	74	White	Radio-cephalic	1.94	No	Yes
64	F	52	Other	Brachio-cephalic	2.10	No	Yes
65	F	52	Black	Brachio-cephalic	0.78	No	No
66	F	44	White	Brachio-cephalic	?	No	No
67	F	55	White	Brachio-cephalic	0.71	No	No
68	F	80	White	Brachio-cephalic	0.80	No	No
69	M	59	White	Radio-cephalic	8.01	No	Yes
70	M	56	Asian	Brachio-cephalic	1.59	No	No
71	F	88	White	Brachio-cephalic	3.22	No	Yes
72	M	62	White	Radio-cephalic	7.29	Yes	Yes
73	F	80	White	Basilic vein Transposition	1.02	No	No
74	F	76	White	Brachio-cephalic	1.48	No	No
75	M	73	White	Brachio-cephalic	1.65	No	No

Table 0-2: Table to show the post fistuloplasty fistula outcome data of the patients undergoing fistuloplasty procedures, which donated blood samples.

Patient Number	Was the Access Circuit Still Patient?	Number of day s for Post-intervention Primary Patency to be Reached	Reason for the End of Post-intervention Primary Patency	Was the AVF Patent Still?	Number of days for Post-intervention Cumulative Patency/Censoring/End of Follow up to be Reached	Reason for the End of Post-intervention Cumulative Patency
1	Yes	230		Yes	230	Died
2	No	511	Radiological Procedure	Yes	559	Transplant
3	No	109	Thrombosed	No	109	Decision to Abandon
4	No	82	Decision to Abandon	No	82	Decision to Abandon
5	Yes	1177		Yes	1177	Transplant
6	No	124	Thrombosed	Yes	819	Transplant
7	No	180	Surgical Intervention	Yes	484	Transplant
8	No	84	Decision to Abandon	No	84	Decision to Abandon
9	Yes	1353		Yes	1353	End of Follow Up
10	No	114	Thrombosed	No	114	Decision to Abandon

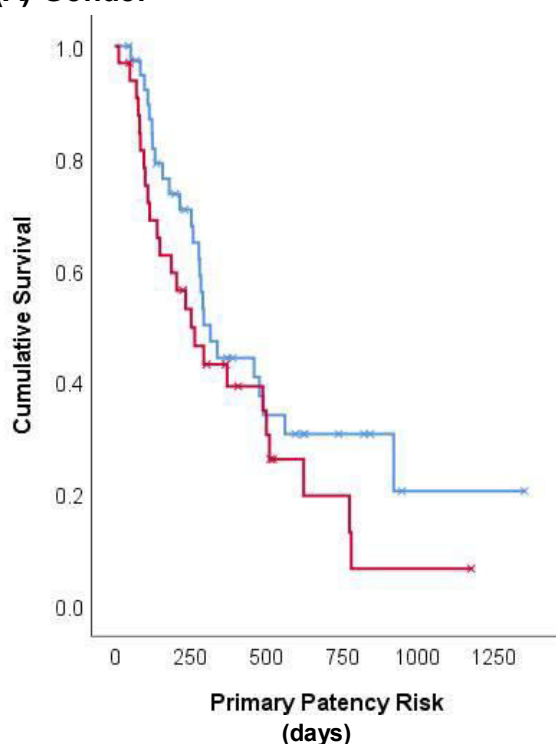
Patient Number	Was the Access Circuit Still Patient?	Number of day s for Post-intervention Primary Patency to be Reached	Reason for the End of Post-intervention Primary Patency	Was the AVF Patent Still?	Number of days for Post-intervention Cumulative Patency/Censoring/End of Follow up to be Reached	Reason for the End of Post-intervention Cumulative Patency
11	No	52	Radiological Procedure	No	584	Thrombosed
12	No	123	Surgical Intervention	No	246	Thrombosed
13	No	253	Decision to Abandon	No	253	Decision to Abandon
14	No	624	Decision to Abandon	No	624	Decision to Abandon
15	No	98	Thrombosed	Yes	1233	End of Follow Up
16	No	339	Thrombosed	Yes	343	Died
17	No	280	Radiological Procedure	Yes	477	Died
18	No	109	Thrombosed	No	109	Thrombosed
19	No	562	Radiological Procedure	No	610	Ligated
20	No	489	Thrombosed	No	489	Thrombosed
21	No	284	Surgical Intervention	Yes	1087	End of Follow Up
22	No	158	Thrombosed	Yes	583	Died
23	No	921	Radiological Procedure	Yes	1082	End of Follow Up
24	Yes	514		Yes	514	Transplant
25	No	50	Radiological Procedure	Yes	474	Died
26	No	371	Radiological Procedure	Yes	996	End of Follow Up
27	No	252	Radiological Procedure	No	315	Decision to Abandon
28	No	84	Decision to Abandon	No	84	Decision to Abandon
29	No	501	Radiological Procedure	No	696	Decision to Abandon
30	No	316	Radiological Procedure	No	487	Thrombosed
31	No	100	Radiological Procedure	No	492	Thrombosed
32	No	140	Radiological Procedure	Yes	509	Died
33	Yes	823		Yes	823	End of Follow Up
34	No	781	Radiological Procedure	Yes	809	End of Follow Up
35	Yes	48		Yes	48	Transplant
36	No	294	Radiological Procedure	Yes	764	End of Follow Up
37	Yes	739		Yes	739	Died
38	Yes	843		Yes	843	End of Follow Up
39	No	204	Radiological Procedure	Yes	316	Transplant
40	Yes	70		Yes	70	Transplant
41	No	71	Thrombosed	No	71	Thrombosed
42	No	291	Thrombosed	Yes	638	End of Follow Up
43	Yes	623		Yes	623	End of Follow Up
44	No	215	Decision to Abandon	No	215	Decision to Abandon
45	No	187	Decision to Abandon	No	187	Decision to Abandon
46	No	294	Radiological Procedure	Yes	414	End of Follow Up
47	Yes	366		Yes	366	Transplant

Patient Number	Was the Access Circuit Still Patient?	Number of day s for Post-intervention Primary Patency to be Reached	Reason for the End of Post-intervention Primary Patency	Was the AVF Patent Still?	Number of days for Post-intervention Cumulative Patency/Censoring/End of Follow up to be Reached	Reason for the End of Post-intervention Cumulative Patency
48	Yes	138		Yes	138	Transplant
49	Yes	371		Yes	371	End of Follow Up
50	No	115	Radiological Procedure	No	591	Thrombosed
51	Yes	522		Yes	522	End of Follow Up
52	Yes	225		Yes	225	Died
53	Yes	45		Yes	45	Died
54	Yes	596		Yes	596	End of Follow Up
55	No	264	Radiological Procedure	Yes	297	Transplant
56	No	234	Radiological Procedure	Yes	738	End of Follow Up
57	Yes	303		Yes	303	Transplant
58	Yes	339		Yes	339	Transplant
59	No	78	Surgical Intervention	Yes	577	End of Follow Up
60	No	48	Radiological Procedure	Yes	395	End of Follow Up
61	No	133	Radiological Procedure	Yes	388	End of Follow Up
62	Yes	407		Yes	407	End of Follow Up
63	No	478	AVF replaced with AVG	No	478	Decision to Abandon
64	No	96	Decision to Abandon	No	96	Decision to Abandon
65	No	460	Radiological Procedure	Yes	932	End of Follow Up
66	Yes	199		Yes	199	Died
67	Yes	627		Yes	627	End of Follow Up
68	No	259	Radiological Procedure	Yes	571	End of Follow Up
69	No	491	Decision to Abandon	No	491	Decision to Abandon
70	Yes	389		Yes	389	End of Follow Up
71	No	149	Surgical Intervention	Yes	382	End of Follow Up
72	No	278	Surgical Intervention	Yes	503	Died
73	No	13	Surgical Intervention	No	46	Decision to Abandon
74	No	775	Radiological Procedure	Yes	1043	End of Follow Up
75	Yes	948		Yes	948	End of Follow Up

Appendix B – Kaplan-Meier Curves for Patient Clinical Nominal Data

Nominal variables are variables which have two or more categories, but which do not have an intrinsic order, for instance ethnicity. Whilst multivariate analysis can show if a nominal variable like ethnicity associates with outcome, it would not be able to show which ethnicity did better specifically. Hence, Kaplan-Meier curves were drawn for the nominal variables studied here (gender, ethnicity and type of native fistula) (Figure 0.1, Figure 0.2, Figure 0.3).

(A) Gender



Number of patients at risk:

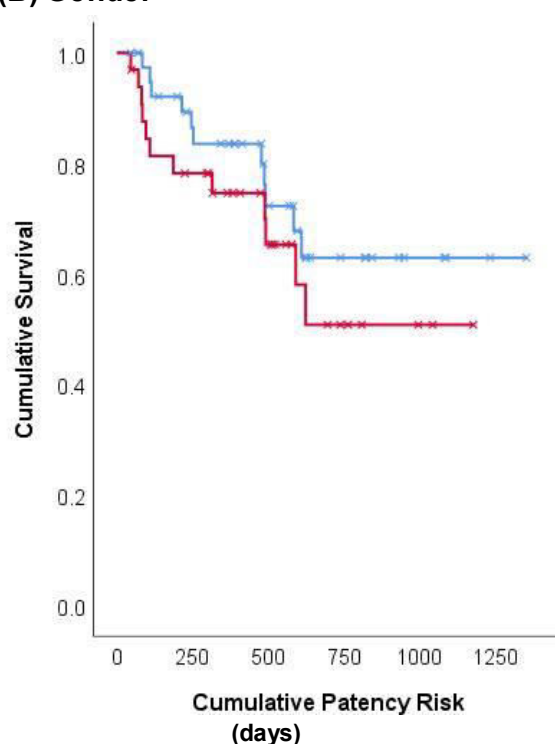
Male (blue)

0 days: 41, 250 days: 24, 500 days: 10,
750 days: 5, 1000 days: 1, 1250 days: 1

Female (red)

0 days: 34, 250 days: 16, 500 days: 8,
750 days: 3, 1000 days: 1, 1250 days: 1

(B) Gender



Number of patients at risk:

Male (blue)

0 days: 41, 250 days: 30, 500 days: 19,
750 days: 9, 1000 days: 4, 1250 days: 1

Female (red)

0 days: 34, 250 days: 24, 500 days: 14,
750 days: 5, 1000 days: 2, 1250 days: 1

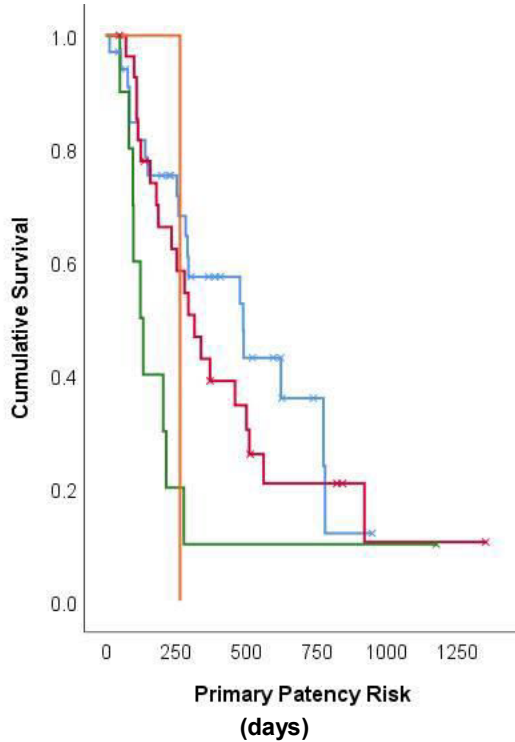
Figure 0.1: Kaplan-Meier curves of time for AVF to reach post-intervention patency in males and females.

(A) Post-intervention primary patency Kaplan-Meier curves.

(B) Post-intervention cumulative patency Kaplan-Meier curves.

Males are represented in blue, females are represented in red. Steps represent survival function. X marks patient censoring.

(A) Ethnicity



Number of patients at risk:

Asian (green)

0 days: 10, 250 days: 9, 500 days: 9,
750 days: 0, 1000 days: 0, 1250 days: 0

Black (red)

0 days: 28, 250 days: 16, 500 days: 8,
750 days: 4, 1000 days: 0, 1250 days: 0

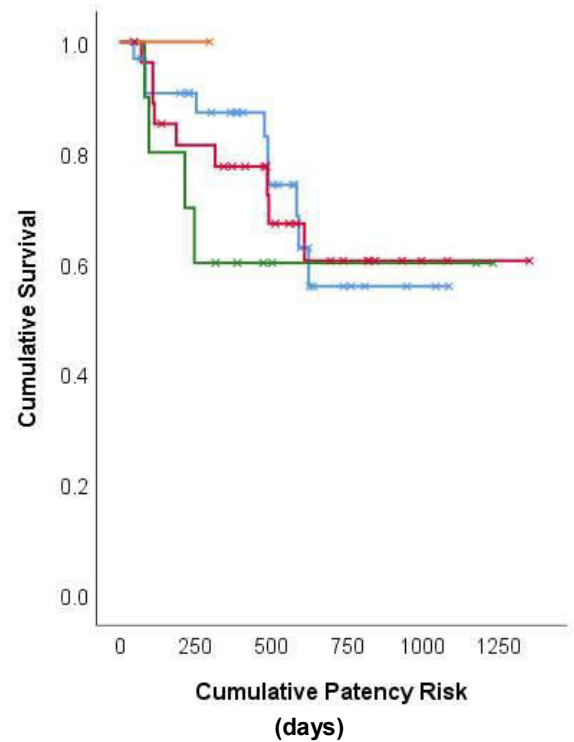
White (blue)

0 days: 36, 250 days: 21, 500 days: 9,
750 days: 3, 1000 days: 0, 1250 days: 0

Other (orange)

0 days: 1, 250 days: 0, 500 days: 0,
750 days: 0, 1000 days: 0, 1250 days: 0

(B) Ethnicity



Number of patients at risk:

Asian (green)

0 days: 10, 250 days: 6, 500 days: 3,
750 days: 2, 1000 days: 2, 1250 days: 0

Black (red)

0 days: 28, 250 days: 21, 500 days: 13,
750 days: 8, 1000 days: 2, 1250 days: 1

White (blue)

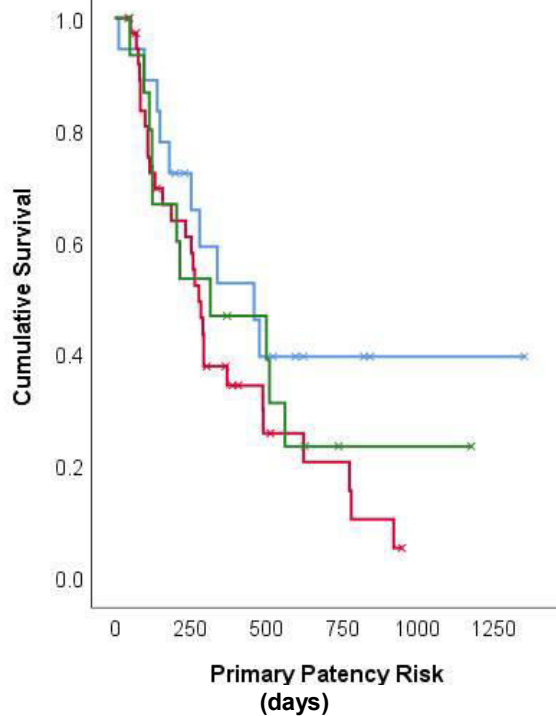
0 days: 36, 250 days: 26, 500 days: 17,
750 days: 5, 1000 days: 2, 1250 days: 0

Other (orange)

0 days: 1, 250 days: 0, 500 days: 0,
750 days: 0, 1000 days: 0, 1250 days: 0

Figure 0.2: Kaplan-Meier curves of time for AVF to reach post-intervention patency in various ethnicities.
(A) Post-intervention primary patency Kaplan-Meier curves.
(B) Post-intervention cumulative patency Kaplan-Meier curves.
Asian is represented in green, black is represented in red, white is represented in blue and other is represented in orange. Steps represent survival function. X marks patient censoring.

(A) Type of Native Fistula



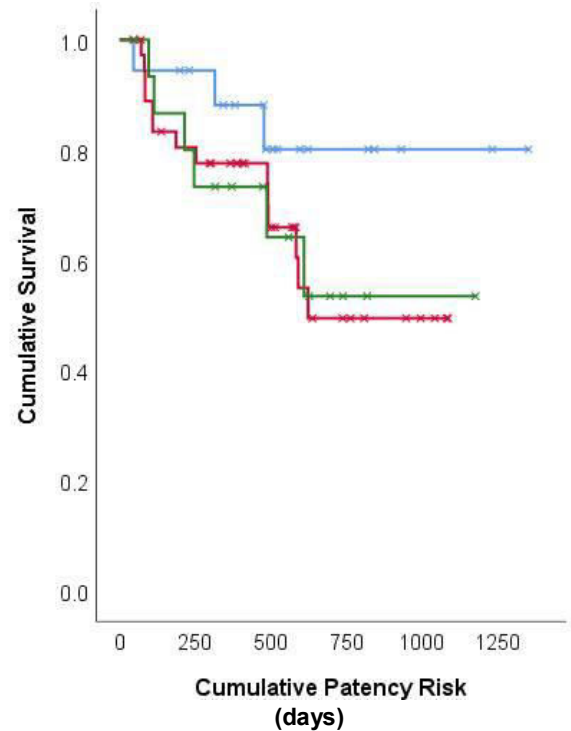
Number of patients at risk:

Basilic vein transposition
0 days: 18, 250 days: 8, 500 days: 6,
750 days: 0, 1000 days: 0, 1250 days: 0

Brachio-cephalic
0 days: 40, 250 days: 21, 500 days: 6,
750 days: 4, 1000 days: 0, 1250 days: 0

Radio-cephalic
0 days: 19, 250 days: 11, 500 days: 6,
750 days: 3, 1000 days: 1, 1250 days: 1

(B) Type of Native Fistula



Number of patients at risk:

Basilic vein transposition
0 days: 18, 250 days: 11, 500 days: 7,
750 days: 2, 1000 days: 1, 1250 days: 0

Brachio-cephalic
0 days: 40, 250 days: 28, 500 days: 17,
750 days: 7, 1000 days: 3, 1250 days: 0

Radio-cephalic
0 days: 19, 250 days: 15, 500 days: 9,
750 days: 5, 1000 days: 2, 1250 days: 1

Figure 0.3: Kaplan-Meier curves of time for AVF to reach post-intervention patency in various native fistulas.

(A) Post-intervention primary patency Kaplan-Meier curves.

(B) Post-intervention cumulative patency Kaplan-Meier curves.

Basilic vein transposition is represented in green, brachio-cephalic is represented in red and radio-cephalic is represented in blue. Steps represent survival function. X marks patient censoring.

Appendix C – Microarray ANOVA Analysis

Table 3: Genes with a 1.75-fold change, and a *p* value of less than 0.05 differentially expressed between patients who had a good outcome following their fistuloplasty procedure and patients which had a poor outcome following their fistuloplasty procedure

GENE	Mean Gene Expression		<i>p</i> value	Fold Change	Gene Expression Description
	Good Outcome	Poor Outcome			
LOC100132394	11.00	9.41	0.01907	-3.01	Decreased in poor outcome
LOC642073	9.82	8.27	0.00624	-2.93	Decreased in poor outcome
S100A4	9.30	7.81	0.01268	-2.80	Decreased in poor outcome
FTL	9.78	8.31	0.02374	-2.77	Decreased in poor outcome
LOC100134364	11.53	10.06	0.01785	-2.77	Decreased in poor outcome
CALR	8.93	7.56	0.00706	-2.60	Decreased in poor outcome
HLA-A29.1	8.89	7.65	0.04067	-2.37	Decreased in poor outcome
LOC100132247	9.51	8.33	0.00255	-2.25	Decreased in poor outcome
HS.575725	9.38	8.29	0.02179	-2.13	Decreased in poor outcome
CST3	8.97	7.88	0.03253	-2.13	Decreased in poor outcome
GLT25D1	8.56	7.47	0.00793	-2.13	Decreased in poor outcome
PIM1	8.24	7.16	0.00101	-2.12	Decreased in poor outcome
LOC730415	9.33	8.26	0.01265	-2.10	Decreased in poor outcome
LOC644039	8.74	7.69	0.02790	-2.08	Decreased in poor outcome
ITGB7	8.09	7.05	0.00285	-2.06	Decreased in poor outcome
MT1A	8.46	7.42	0.01295	-2.06	Decreased in poor outcome
LOC100132673	9.33	8.29	0.02583	-2.05	Decreased in poor outcome
LOC728888	9.94	8.90	0.00593	-2.05	Decreased in poor outcome
HLA-DQA1	8.60	7.56	0.01693	-2.05	Decreased in poor outcome
LOC284393	8.86	7.83	0.03676	-2.05	Decreased in poor outcome
LOC645173	8.24	7.22	0.03176	-2.03	Decreased in poor outcome
LOC648668	8.04	7.05	0.01936	-1.99	Decreased in poor outcome
EEF1D	8.42	7.43	0.00358	-1.99	Decreased in poor

GENE	Mean Gene Expression		p value	Fold Change	Gene Expression Description
	Good Outcome	Poor Outcome			
					outcome
RARRES3	9.22	8.23	0.00739	-1.98	Decreased in poor outcome
LOC728453	8.57	7.59	0.02737	-1.97	Decreased in poor outcome
TMC6	8.79	7.82	0.01004	-1.96	Decreased in poor outcome
ICAM2	7.98	7.02	0.00686	-1.95	Decreased in poor outcome
LOC100134598	11.17	10.22	0.00040	-1.94	Decreased in poor outcome
LOC100133931	9.84	8.90	0.00837	-1.92	Decreased in poor outcome
RPS5	8.18	7.25	0.02167	-1.92	Decreased in poor outcome
MIR612	7.81	6.87	0.01658	-1.91	Decreased in poor outcome
LOC440353	10.14	9.21	0.00481	-1.91	Decreased in poor outcome
HS.553301	9.73	8.81	0.00442	-1.89	Decreased in poor outcome
SLC4A5	8.86	7.94	0.00389	-1.89	Decreased in poor outcome
CAPG	8.53	7.61	0.02540	-1.89	Decreased in poor outcome
CTGLF6	9.07	8.15	0.00129	-1.88	Decreased in poor outcome
KLHL22	9.80	8.89	0.03062	-1.88	Decreased in poor outcome
LOC389342	8.55	7.64	0.04038	-1.88	Decreased in poor outcome
MIR21	9.06	8.15	0.04050	-1.88	Decreased in poor outcome
PSME2	10.64	9.73	0.02482	-1.88	Decreased in poor outcome
ORMDL3	7.97	7.06	0.00418	-1.88	Decreased in poor outcome
MT2A	8.05	7.15	0.03242	-1.87	Decreased in poor outcome
LOC100133678	8.56	7.66	0.02333	-1.87	Decreased in poor outcome
FLJ45337	8.56	7.66	0.01000	-1.87	Decreased in poor outcome
LOC100133045	7.61	6.72	0.04083	-1.86	Decreased in poor outcome
CORO1A	8.33	7.43	0.01641	-1.86	Decreased in poor outcome
LOC646294	8.17	7.28	0.03550	-1.85	Decreased in poor outcome
LOC285176	8.59	7.71	0.04087	-1.84	Decreased in poor outcome

GENE	Mean Gene Expression		p value	Fold Change	Gene Expression Description
	Good Outcome	Poor Outcome			
HLA-DRB4	9.16	8.28	0.02560	-1.84	Decreased in poor outcome
P704P	8.85	7.97	0.02208	-1.84	Decreased in poor outcome
C7orf64	10.68	9.81	0.00109	-1.83	Decreased in poor outcome
LOC729021	8.39	7.53	0.00470	-1.82	Decreased in poor outcome
HS.544637	10.49	9.63	0.02554	-1.82	Decreased in poor outcome
LOC650646	8.62	7.75	0.03678	-1.82	Decreased in poor outcome
LOC729737	9.22	8.36	0.00461	-1.82	Decreased in poor outcome
DYSF	8.04	7.18	0.01364	-1.81	Decreased in poor outcome
OR2W5	7.90	7.04	0.00341	-1.81	Decreased in poor outcome
LOC100134241	8.25	7.40	0.03716	-1.81	Decreased in poor outcome
APOA2	7.80	6.95	0.00964	-1.80	Decreased in poor outcome
LMOD3	11.64	10.79	0.02018	-1.80	Decreased in poor outcome
RIN1	8.40	7.56	0.04813	-1.80	Decreased in poor outcome
LOC387930	8.61	7.76	0.04487	-1.80	Decreased in poor outcome
LOC730187	9.58	8.74	0.04418	-1.79	Decreased in poor outcome
PMVK	7.99	7.16	0.04311	-1.77	Decreased in poor outcome
SFRS15	9.17	8.35	0.03801	-1.77	Decreased in poor outcome
ITPK1	8.46	7.64	0.01646	-1.77	Decreased in poor outcome
ZNF483	9.97	9.16	0.02483	-1.76	Decreased in poor outcome
EIF3G	8.03	7.21	0.01728	-1.76	Decreased in poor outcome
RAET1K	9.24	8.43	0.00357	-1.76	Decreased in poor outcome
SEMA4D	9.52	8.72	0.01093	-1.75	Decreased in poor outcome
HSPA1A	10.23	11.04	0.00442	1.75	Increased in poor outcome
PFKFB4	8.06	8.88	0.04498	1.76	Increased in poor outcome
BATF	7.94	8.77	0.02000	1.79	Increased in poor outcome
HS.407903	10.04	10.89	0.01215	1.80	Increased in poor

GENE	Mean Gene Expression		<i>p</i> value	Fold Change	Gene Expression Description
	Good Outcome	Poor Outcome			
					outcome
STARD8	8.04	8.89	0.02835	1.80	Increased in poor outcome
TSSC4	8.64	9.53	0.02519	1.85	Increased in poor outcome
C9orf102	7.53	8.45	0.00062	1.89	Increased in poor outcome
DDX28	9.00	9.97	0.00004	1.96	Increased in poor outcome
CENTA1	9.65	10.63	0.00233	1.97	Increased in poor outcome
ZNF467	8.81	9.83	0.00853	2.03	Increased in poor outcome
HCK	9.32	10.37	0.03538	2.07	Increased in poor outcome
VNN1	7.81	9.17	0.00036	2.57	Increased in poor outcome

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