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An investigation of microRNAs as markers of human vascular dysfunction in kidney disease

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Declaration

I declare that the work carried out during my PhD and the studies described in this thesis are my own work, unless otherwise stated, under the supervision of Dr James Dear and Dr Neeraj (Bean) Dhaun. Support was provided by Dr Al Ivens for RNA sequencing analysis and blood samples were collected by the clinical staff at The Royal Infirmary of Edinburgh. This thesis has not previously been submitted for any other degree or qualification.

Signed:

Kathleen Maria Scullion

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Whit's fur ye'll no go by ye. – Scottish proverb

Abstract

Vascular dysfunction commonly co-exists with kidney disease, which results in a substantially increased risk of cardiovascular disease (CVD). A severe, acute form of this vascular-renal phenotype is seen in those presenting with systemic vasculitis associated with autoantibodies to neutrophil cytoplasmic antigens (ANCA) - a rare inflammatory disorder. The most frequent severe manifestation of ANCA vasculitis involves endothelial injury giving rise to rapidly progressive glomerulonephritis in the kidney. Despite current treatments, overall survival remains poor with many patients suffering chronic inflammation, a major contributor to the development and progression of both CVD and chronic kidney disease (CKD). Indeed, those with CKD have a substantially higher chance of dying from CVD than of progressing to end-stage renal disease (ESRD). Furthermore, those who respond to treatment remain at risk of further disease relapses.

microRNAs (miRs) are selectively expressed in certain tissues and cell states, which makes them potential biomarker candidates. In the circulation, the miRs are bound to Argonaute 2 proteins or encapsulated in extracellular vesicles (ECVs). This makes them resistant to RNase degradation providing further support for their potential use as novel biomarkers.

miR-126 is enriched in endothelial cells and is a regulator of vascular integrity and angiogenesis. miR-126 was measured in patients with active vasculitis and once in treatment-induced remission. At disease presentation, miR-126 was at a low concentration and increased with successful treatment. Patients with vasculitis - both active disease and in remission states - had lower circulating miR-126 than in healthy volunteers.

miR-126 was also measured in patients with ESRD to establish whether it was also a marker of chronic vascular dysfunction. Circulating levels were measured in patients receiving haemodialysis before and after the treatment.

Before haemodialysis, miR-126 was at a low level in circulating blood and increased after the treatment. The levels of miR-126 in patients with CKD were also measured and were at a higher concentration than patients with ESRD. These data suggest that miR-126 has a potential role as a biomarker of vascular health and could track the progression of vascular disease.

Using small RNA sequencing, the miR profiles of patients with active vasculitis and once in remission were established. These results were analysed in order to determine appropriate miRs for biomarker discovery. Using the data obtained, the miR hits were measured in larger patient cohorts for validation. This did not result in the discovery of a specific miR biomarker for the identification of acute vascular dysfunction in humans.

ECVs were isolated from healthy volunteers and patients with active vasculitis. Uptake of ECVs was established in human and mouse macrophages, as well as renal proximal tubules *in vitro*. This did not result in increased cytokine production in the cultured macrophages or renal cells. This suggests that uptake is possible, however, the ECV cargo does not activate immune and renal cells *in vitro*.

These findings, therefore, show that circulating miRs are potential markers of acute and chronic human vascular dysfunction. miR-126 was a successful marker for differentiating between patients with active disease and treatment-induced remission. The miR profile of these patients did not differ as determined by RNA sequencing. In addition, ECVs can be isolated from patient blood and taken up by recipient macrophage and renal proximal tubule cells, however, this does not result in a phenotypic change. These data suggest that miR-126 has a potential role as a biomarker of vascular health and could track the progression of vascular disease and response to treatment in acute and chronic vascular dysfunction.

Lay abstract

Blood vessel damage and kidney disease often occur in tandem, resulting in a greater chance of developing heart disease. ANCA vasculitis is a rare disease which causes blood vessel inflammation in any blood vessel in the body. If left untreated, the kidneys can be damaged and can progress to chronic kidney disease which may require dialysis due to reduced kidney function. Overall survival in ANCA vasculitis is low and the patient has a chance of relapsing. This means that better disease markers for diagnosis are required, as well as determining whether the treatment plans are working well for the patient and reducing the chance of disease relapse.

The studies in this thesis aimed to define the role of small pieces of genetic material, known as microRNAs, as markers of the blood vessel damage. If this marker proves to be successful, it can be used as a diagnostic test in a clinical environment. The first study compared patients with ANCA vasculitis before and after they were treated for the condition and found that the level of microRNA-126 was substantially lower when they first presented with the disease, and this increased after treatment. Healthy volunteers had high levels of microRNA-126.These results were compared to currently used clinical tests, and there were evident correlations.

The same microRNA was measured in patients with chronic kidney disease and patients on dialysis for end-stage renal disease. This showed that microRNA-126 is at a low level in chronic kidney disease patients and even lower in patients before their dialysis session. After dialysis, the amount of microRNA-126 increases, but not to the same level as healthy individuals. From this, it can be concluded that there is a lot of promise for microRNA-126 as a marker for vessel health and reaction to treatment in acute and chronic disease.

To examine whether there were other microRNAs that could be used as biomarkers of ANCA vasculitis, patient samples were sequenced. This showed that multiple markers were different between active disease and remission. This suggests that the blood samples taken from patients have potential biomarkers of active disease in ANCA vasculitis.

Finally, a study was carried out using the blood taken from patients with active ANCA vasculitis to discover whether the contents of the sample causes inflammation and damage in cells. This was carried out using immune cells that were produced from mice and healthy individuals, as well as kidney cells. The study demonstrated that there was minimal inflammation and damage in the cells tested and therefore the mechanism behind the vessel inflammation in ANCA vasculitis remains unclear.

Abbreviations

AAV	ANCA associated vasculitis
ADMA	Asymmetric dimethylarginine
Ago2	Argonaute 2
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANCA	Autoantibodies to neutrophil cytoplasmic antigens
ANOVA	Analysis of variance
ARG1	Arginase 1
AUC	Area under the curve
BAFF	B-cell activating factor
BLyS	B lymphocyte stimulator
BMDMΦ	Bone marrow derived macrophages
BVAS	Birmingham vasculitis activity score
CAD	Coronary artery disease
CKD	Chronic kidney disease
CRP	C-reactive protein
Ct	Cycle threshold
CVD	Cardiovascular disease
DAPI	4′,6-diamidino-2-phenylindole
DBP	Diastolic blood pressure
DILI	Drug induced liver injury
ECVs	Extracellular vesicles
eGFR	Estimated glomerular filtration rate
EGPA	Eosinophilic granulomatosis with polyangiitis
ENT	Ear, nose and throat
ESRD	End-stage renal disease
FBS	Foetal bovine serum
FLPM	Full-length perfect match
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtration rate

GGT	Gamma-glutamyl transpeptidase
GPA	Granulomatosis with polyangiitis
HD	Haemodialysis
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-10	Interleukin 10
IL-1B	Interleukin 1 Beta
IL-6	Interleukin 6
IRI	Ischemic reperfusion injury
KIM-1	Kidney injury molecule 1
LAMP-2	Lysosome associated membrane protein 2
LFTs	Liver function tests
MAP kinase	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDRD	Modification of Diet in Renal Disease
miR	microRNA
MPA	Microscopic polyangiitis
MRC1	Mannose receptor 1
mRNA	Messenger RNA
NETs	Neutrophil extracellular traps
NGS	Next generation sequencing
NIH	National Institutes of Health
NPM1	Nucleophosmin 1
NTA	Nanoparticle tracking analysis
NTN	Nephrotoxic nephritis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
PFA	Paraformaldehyde
PI3KR2	Phosphoinositide-3-kinase regulatory subunit 2

PLG	Plasminogen
PWV	Pulse wave velocity
qRT-PCR	Quantitative real time PCR
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SENS	Sensitivity
SPRED1	Sprouty-related, EVH1 domain-containing protein 1
T2DM	Type 2 diabetes mellitus
TNFa	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor

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Chapter 1 – Introduction

Vascular dysfunction

The endothelium plays a fundamental role in vascular function through the regulation of vascular tone and maintenance of vascular homeostasis.[1] The endothelium is a single layer of squamous endothelial cells that line the interior of all blood and lymphatic vessels in the body. It forms an interface between circulating blood or lymph in the lumen and for the rest of the vessel wall to control the flow of substances and fluid to the tissue. The molecular and functional properties of the endothelial cells will depend on the type of vessel or organ that they are present in. They are specialized depending on their microenvironment and signals including metabolic stimuli and growth factors, as well as cell matrix and cell-cell interactions.[2]

Endothelial dysfunction is a systemic pathophysiological disorder. It is found in the presence of all traditional cardiovascular risk factors, including arterial hypertension, diabetes mellitus, obesity, and hypercholesterolaemia.[3] In addition, it has been found to correlate with markers of low-grade chronic inflammation, such as C-reactive protein (CRP), which is a risk marker of cardiovascular diseases (CVD).[4] Previous studies have reported that gold standard measures of renal function, including glomerular filtration rate (GFR), correlated with vascular dysfunction in patients with moderate renal impairment.[5, 6] Vascular dysfunction is a key research focus for a range of CVDs, including kidney disease.

ANCA vasculitis

Vasculitis is the umbrella term used to describe aggressive rare inflammatory diseases of blood vessels. This encompasses small, medium and large vessels throughout the body. There are a multitude of causes of vasculitis and the clinical manifestations can be dependent on the site, type and size of vessels involved.[7] Vasculitis can present with inflammation in any vessel from any organ system and this will influence the resulting disease characteristics. Patients can present with non-specific symptoms such as fever, malaise, and

weight loss. Progression of the disease depends on the specific vessels involved. Due to this, vasculitis is challenging to diagnose and has a high relapse and mortality rate. Vasculitis can potentially lead to life-threatening complications within days to weeks. Clinically, there is an unmet need for new diagnostic biomarkers to improve patient outcome by accelerating diagnosis and allowing for enhanced clinical guidance for treatment strategies.[8]

Systemic vasculitis associated with autoantibodies to neutrophil cytoplasmic antigens (ANCA) are a group of systemic autoimmune diseases that affect small to medium-sized blood vessels throughout the body.[9] Based on clinical diagnosis, ANCA associated vasculitis (AAV) is subcategorised into microscopic polyangiitis (MPA), granulomatosis with polyangiitis (Wegener's, GPA), and eosinophilic granulomatosis with polyangiitis (Churg-Strauss, EGPA). In 90% of GPA and MPA cases, the patient's ANCA status is positive.[10] Differentiating between these conditions is challenging due to their clinical and pathological similarity which results in under-reporting of the disease. The annual incidence of the condition is estimated to occur in approximately 20-30 patients per million of population.[11] However, this is thought to be an under-estimation due to an ageing population with more people reaching old-age than previous generations,[12] as well as increased knowledge and awareness of the disease.[13] Overall, the disease is characterised by necrosis and infiltration of leucocytes in the small arteries resulting in endothelial injury[14]. In severe cases, the inflammation can lead to glomerulonephritis in the kidney[15] and haemorrhage in the lung.[16] ANCA vasculitis is the most frequent cause of progressive glomerulonephritis globally.[17] Even with current treatment strategies, the survival rate after the diagnosis of ANCA vasculitis is lower in comparison to unaffected cohorts with a 2.6 mortality ratio when compared to age and sex-matched counterparts.[18, 19] Patients suffer from chronic inflammation, which is a major contributor to the development of cardiovascular disease and chronic kidney disease.[20] Following successful treatment for the disease, the relapse rate remains at approximately 50% within 5 years.[21]

In the majority of ANCA vasculitis cases, patients present with renal involvement. In GPA, renal involvement occurs in 70% of cases. Almost 100% of MPA cases have renal involvement.[22] Without successful immunosuppressive treatment, renal sclerotic lesions can develop which may progress to chronic kidney disease. The patient is often asymptomatic until advanced renal failure occurs.[23] When ANCA vasculitis is localised in the kidney, this can result in glomerulonephritis. Early diagnosis of ANCA vasculitis and tracking response to treatment remains challenging. Currently used biomarkers of the disease are shown in Table 1. With renal involvement, serum creatinine is commonly utilised to measure renal function. However, this is inaccurate as the kidney is often severely damaged before the function is impaired to a measurable degree.[24] Following treatment for the disease, creatinine may decrease yet it is not known whether inflammation continues after renal function improves. In those without renal involvement, there is not a reliable marker for disease activity. Measurement of circulating ANCA is useful for initial diagnosis[25] but it is limited as a measure of disease activity over time. Currently, there are no biomarkers that are specific or reliable enough for small vessel inflammation. The identification of such markers would not only allow early implementation of appropriate treatments but may also identify those patients with grumbling disease activity despite therapy, identify those who might safely stop long-term, potentially toxic, immunosuppression, and even predict disease relapses.

ANCA negative patients are the minority of AAV patients, with clinicians being reluctant to give a definitive diagnosis without ANCA positivity.[26] ANCA is a useful diagnostic biomarker in the correct clinical context and can be effective for patients with single organ involvement or atypical presentation. As a biomarker of disease activity, ANCA has been assessed by taking serial measurements in patients with AAV in order to predict disease relapse. There have been conflicting opinions on whether this is an effective strategy.[27] In a study of adult patients with GPA, a reduction in ANCA did not correlate with a

reduced period till remission, and higher ANCA was not linked to disease relapse.[28]

Biomarker	Description
ANCA	In clinical practice, it has a role as a diagnostic marker.[29] ANCA is more useful in patients with renal involvement as persistent ANCA or rising titre is insufficient to change treatment plans. Pathogenic autoantibodies targeting antigens expression in neutrophil granules and surface.[30]
B-cells	Dysregulation leads to the activation of the inflammatory response and autoantibody production. They are non-specific and unable to differentiate infection from inflammation.[31]
Haematuria/Proteinuria	Demonstrates renal and glomerular damage or inflammation. This is a biomarker for renal involvement.[32]
BAFF (BLyS)	Inverse relationship with disease activity. It promotes B-cell differentiation and survival.[33]
IL6	A pro-inflammatory cytokine which correlates with disease activity; however, it lacks disease specificity.[34]
Anti-hLAMP2 autoantibodies	Autoantibodies that are directed towards a glycoprotein expressed in neutrophil granules and have potential as a diagnostic marker of AAV. Titres decrease rapidly after successful treatment and increase with relapse. The technique requires further development and standardisation.[35]

Anti-PLG autoantibodies	Autoantibodies that are directed towards plasminogen and have a potential role as a diagnostic biomarker of AAV. This biomarker is observed in active disease and is yet to be examined for disease relapse. There are correlations with glomerular lesion severity.[36, 37]
Anti-moesin autoantibodies	This biomarker is at the research stage for AAV. Directed towards the heparin-binding protein moesin. There is no research currently on relapse.[38]
ECVs	Proposed role as a biomarker as they are involved in cell communication.[39]
MCP-1	Circulating monocytes and tissue-resident macrophages are attracted to this chemokine. It is a biomarker for renal involvement. Higher levels are associated with disease relapse and a poor overall prognosis.[40]
sCD163	Urinary biomarker - Higher in active disease with renal involvement when compared with remission.[41]
sCD25	Urinary biomarker - The molecule is cleaved from activated T cells. When combined with sCD163, this can be used as a biomarker of renal involvement.[42]

Table 1. Summary of currently used biomarkers of ANCA vasculitis.
Adapted from [10, 43].

The role of inflammation in vascular disease

The pathophysiology of vascular inflammation comprises an intricate interaction between immune cells, including neutrophils, monocytes and macrophages and endothelial cells, vascular smooth muscle cells and extracellular matrix. Vascular injury is linked with the recruitment of pro-inflammatory cells by cytokines.[44] These cytokines are fundamental for cell signalling and the promotion of systemic inflammation. Activated macrophages are tissue destructive in vessel wall inflammation and are the principal producers and up-regulators of inflammatory reactions. This is an essential part of vasculitis and the subsequent systemic inflammation.[45] Macrophages are tissue-resident phagocytic cells and are fundamental for host defence. They process debris and foreign matter in all organs in the body. Macrophages are responsible for regulating the inflammatory response. Macrophage pattern recognition receptors enable them to interact with pathogens permitting them to recognise and phagocytose infected cells. In addition, macrophages are capable of secreting defence-relevant inflammatory mediators and cytokines.[46] Macrophages bridge the innate and adaptive immune response, aided by their antigen-presenting capabilities.

Macrophages play a major role in the pathogenesis of many CVDs.[47] The mechanisms underpinning macrophage differentiation and activation in vascular inflammation have not been fully characterised. Macrophages pathology is determined by their functional phenotype. Depending on the environment, macrophages are capable of polarising into distinct functional subpopulations. Traditionally, these populations were simplified into a functional dichotomy of classically activated (M1) or alternatively activated (M2) macrophages. Classically activated macrophages produce a plethora of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, IL-23, IL-27 and TNFα.[48] These cytokines are considered to be tissue destructive. In contrast, alternatively activated macrophages are fundamental for tissue repair and resolution of inflammation and are therefore considered to be anti-inflammatory. They express cytokines such as IL-10, mannose receptor and

arginase 1 as well as exhibiting a protective effect by subduing immune cell recruitment[49] and pro-inflammatory cytokine production.[50] The overall macrophage phenotype is considered pivotal for determining their role in disease pathology. Recently, the traditional macrophage classification has evolved to describe the macrophage complexity more accurately. They are now defined based on established markers and activation stimuli which cause the differentiation.[51] Patients with chronic inflammation are at an increased risk of cardiovascular morbidity and mortality. The link between vascular dysfunction and CVD has been well studied, however the mechanistic links between inflammatory diseases, endothelial dysfunction and CVD has not been fully understood.

The mechanism of AAV

The mechanism for ANCA causing AAV involves disproportionate ANCA-mediated leucocyte activation which results in small vessel injury. When leucocytes are primed with inflammatory cytokines, including tumour necrosis factor (TNF-α) or microbial products *in vitro*, they express proteinase 3 (PR3) and myeloperoxidase (MPO) on their surface. PR3 and MPO-ANCA can subsequently activate primed monocytes and neutrophils. They bind to the antigens expressed on the surface of the cells or by Fc-receptor engagement[52] and this then initiates signal transduction cascades. Activated neutrophils degranulate, which causes a release of proteases and enzymes, including elastase, PR3 and MPO. There is also an increase in cell adhesions between neutrophils and endothelial cells, which is caused by ANCA inducing an increase of cell adhesion molecules. Chemotactic cytokines, including IL-1, MCP-1 and IL-8 are produced and released when ANCA binds to primed leucocytes. This then attracts more neutrophils and monocytes to the site of vessel inflammation. This directly impacts the chemotactic gradient, which under normal conditions would draw the neutrophils from the vasculature and into the tissue.

A further mechanism of AAV involves the formation of neutrophil extracellular traps (NETs). These are an essential component of the innate immune system, however, in excess, NET formation can damage small blood vessels. A vicious cycle is involved where ANCA stimulates NET production as well as NETs being involved in the production of ANCA.[53] This is key to AAV pathogenesis (Figure 1). ANCA-activated neutrophils release factors that subsequently activate the alternative complement pathway, which produces C5a and increases the level of inflammation by attracting and priming more neutrophils.[54] NETs have been identified in necrotising lesions associated with AAV. In contrast, they were not observed in ANCA-unrelated necrotising vasculitis.

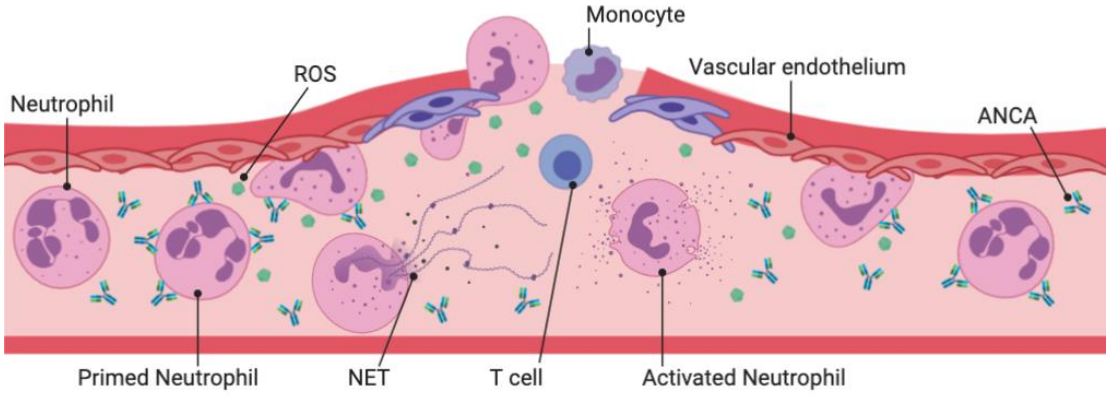


Figure 1. Mechanism of pathogenesis in AAV.
Neutrophils and monocytes are stimulated by ANCA resulting in blood vessel inflammation and damage.

AAV treatment

The traditional and established treatment for AAV is high-dose cyclophosphamide and glucocorticoids. Studies have shown that this can achieve remission in approximately 75% of patients at 3 months and 90% at 6 months.[54] Unfortunately, relapse is common and there is a plethora of undesirable side effects which accompany AAV treatment. To limit these side effects, new therapies are being used at both the induction and maintenance phases, including biologic therapies (such as Rituximab), to reduce toxicity and improve effectiveness.[55]

Chronic kidney disease

Chronic kidney disease (CKD) is the 16th leading cause of years of life lost worldwide.[56] There is a well-defined association between CKD and CVD resulting in higher rates of morbidity and mortality. CKD is a critical independent risk factor for CVD.[57] The risk of CVD increases with declining GFR and patients with CKD, that do not require dialysis, have a greater chance of dying from CVD than progressing to ESRD.[57]

CKD typically evolves over many years, beginning with a prolonged period of silent disease.[58] This makes disease diagnosis challenging and complicates any planning for the patient's treatment options. Biomarkers of kidney function remain the gold standard for CKD assessment, with GFR being a fundamental clinical test.[59] Estimated GFR (eGFR) is based on the Modification of Diet in Renal Disease study (MDRD) equation,[60] which is 175 x (standardised serum creatinine)$^{-1.154}$ x (age)$^{-0.203}$ x 0.742 [if female] x 1.212 [if Black]. Early diagnosis is normally based on abnormal results for proteinuria and serum creatinine which leads to further investigation. Although this is clinically useful for identifying renal disease, these tests are limited and cannot allow for a specific diagnosis of the type of renal injury. A major issue with using serum creatinine for CKD diagnosis is that it is confounded by muscle mass and tubular secretion.[61] This is also a challenge for tracking disease progression. A major issue is that there is a substantial loss of kidney function before creatinine concentration increases. In the context of acute kidney injury, creatinine also has sub-optimal sensitivity and specificity. Serum creatinine does not rapidly report a sudden change in renal function.[24]

The stages of CKD are shown in Table 2.[62] Stages 1 and 2 of CKD are considered to be mild with a GFR of 60 mL/min/1.73m^2 or more. Moderate CKD is defined as a GFR of 15 to 59 mL/min/1.73m^2. Once the patient's GFR is below 15 mL/min/1.73m^2 they have progressed to end-stage renal disease (ESRD).

CKD stage	Description	GFR mL/min/1.73m^2
1	Renal damage with normal renal function	≥ 90
2	Mildly decreased renal function	60 – 89
3	Moderately decreased renal function	30 – 59
4	Moderate to severely decreased renal function	15 – 29
5	Renal failure (ESRD)	<15 or haemodialysis

Table 2. CKD stages with GFR progressing from normal renal function to ESRD.[63]

Despite current treatment strategies, CKD progression to ESRD is still a major clinical and financial issue. Estimates suggest that there are currently over two million patients worldwide dependent on dialysis and this number continues to increase annually. By 2030, it has been predicted that this number will double.[64, 65] The primary types of dialysis are haemodialysis (HD) and peritoneal dialysis (PD). In 2003, 67.5% of patients requiring renal replacement therapy received HD and 29.2% PD.[66] Even with current treatment, patients on HD have high morbidity and mortality rates. In Europe, patients who commenced HD treatment between 2002 and 2006 had a survival rate of 78.7% and 65.8% after 1 and 2 years, respectively.[67, 68] Dialysis adequacy is measured clinically with the Kt/V index for a single treatment. For this measurement, K is the dialysis urea clearance, t is the total treatment time and V is the body volume that the urea is distributed.[69] Kt/V has some limitations, especially for women and smaller patients in regard to the quantity of dialysis received. This can result in women receiving unnecessarily high dialysis dosing.[70] ESRD require robust biomarkers to track disease progression and response to dialysis.

Endothelial dysfunction has been suggested as a possible explanation for the increased CVD risk in CKD.[71, 72] A longitudinal study of ESRD patients demonstrated that all-cause mortality was independently associated with endothelial dysfunction.[73] There are limited data available for endothelial function in moderate and early stages of CKD, as most studies focus on ESRD. This would allow for interventions to prevent CVD deaths at earlier stages of CKD.

Biomarkers of disease

The National Institutes of Health (NIH) working group has defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention”.[74] Biomarkers are fundamental for the development of medical therapeutics and disease diagnostics. Due to the

complexity and diversity of biomarkers, there have been limitations in understanding chronic diseases. The FDA have developed a definition for the various categories of biomarker in order to optimise the concepts for research and medical advancements.[75] Multiple definitions may apply to a single biomarker and validation and evidence must be available for each.

Diagnostic biomarkers are intended for detection and confirmation of a disease, as well as identification of disease subtypes. This class of biomarker is expected to evolve with advancements in precision medicine. The main clinical significance of this class of biomarker is detecting the presence of a disease, as well as defining the classification or subtype of the disease. This requires a high level of precision, reliability and reproducibility, as well as being clinically affordable. It is essential to define the rate of false discovery in the patient population and ensure that it is not affected by the presence of confounding factors, such as comorbidities, age, smoking status and sex.[76]

Monitoring biomarkers are used to measure the effect of a medical product, environmental factor, disease status or medical condition by performing serial measurements. They have a critical role in a clinical care setting. In cardiovascular disease, monitoring biomarkers are essential, including blood pressure and cholesterol control, for evaluation of the treatment strategy. A challenge with this category of biomarker is developing a comprehensive understanding of what the minor changes signal for the patient clinically and how it should influence their treatment.[77]

Pharmacodynamic and response biomarkers change in response to exposure to a medical intervention or environmental factor. This category of biomarker is often used clinically and for the development of therapeutics. An example application for this type of biomarker is in clinical trials. Using pharmacodynamic and response biomarkers allow for the treatment success to be quantified. This can guide whether to continue developing a treatment.

Predictive biomarkers determine the positive or negative outcome for a patient following exposure to a medical product or environmental agent. Validation for this type of biomarker is challenging. Patients are ordinarily randomised into one or two treatment groups (or a placebo group) and the outcomes are compared for the presence, and level, of the biomarker. This demonstrates that a treatment is directly related to a clinical outcome and reflected in predictive biomarker.

Prognostic biomarkers predict the probability of a clinical event, disease relapse or disease progression in patients with a defined disease or condition. This is considered a separate category to the susceptibility and risk category, which is more suitable for examining a healthy state to disease, rather than disease progression. Additionally, this class of biomarker is not affected by therapeutic intervention. They are often utilised in clinical trials to define entry and exclusion criteria in high-risk patient populations. This is essential for how statistical power is calculated based on the possibility of future events, as opposed to sample size. In a clinical care setting, it allows for evaluation of the possible length of treatment for a patient or their length of stay in hospital.

Safety biomarkers are measured pre- or post-medical or environmental exposure to measure the probability or severity of toxicity. This is used as a measure of renal, hepatic and cardiovascular toxicity. This biomarker can be used for measuring the extent of an environmental exposure in the general population and clinically to assess the therapeutic toxicity in a patient population. A challenging aspect of this biomarker is defining the risk of toxicity of a treatment and the benefits for the patient receiving the treatment.

Susceptibility and risk biomarkers establish whether an individual will possibly develop a disease who does not currently present with the condition. There are similarities for this classification with the prognostic biomarkers, however, there is a crucial difference in that this category relates to a disease or condition which the individual does not yet have. The main scope for

susceptibility and risk biomarkers are in examining the epidemiology of a disease.

MicroRNAs as biomarkers

The first microRNA (miR) identified was lin-4 in *Caenorhabditis elegans* in 1993[78, 79] which subsequently led to the discovery of thousands of miRs and revolutionised molecular biology. miRs are a class of RNA molecules that are ~22 nucleotide long, noncoding strands, which are involved in translational repression and cleavage.[80] They play a role in post-transcriptional regulation of gene expression and RNA silencing. miRs are essential for the regulation of many protein-coding genes in a variety of organisms.[81] They can exhibit organ specificity, which makes them a significant emerging class of biomarker for many diseases. As miRs are conserved in sequence across species they can be used in the pre-clinical setting as well as being translated for use in patients.[82] Collectively, miRs play an important role in medicine, toxicology, pharmacology and cardiovascular disease.

miR biogenesis begins with RNA polymerase II/III producing a primary transcript, known as a pri-miR. Pri-miRs contain a double-stranded hairpin loop with single-stranded sequences at both the 5' and 3' end. To ensure efficient processing, they must then be cleaved by Drosha, an RNase III superfamily enzyme.[83] This forms a ~80 nucleotide precursor miR, known as a pre-miR. Following cleavage, they are exported from the nucleus to the cytoplasm by Exportin 5, a nucleocytoplasmic transporter. The pre-miR is further cleaved by Dicer to generate a miR duplex with two ~22 nucleotide mature miR strands. The mature miR can be bound to Argonaute 2 (Ago2) and incorporated into the RNA-induced silencing complex (RISC). It can then facilitate in post-transcriptional regulation of mRNA.[84]

Many miRs have conserved sequences between species, which allows for research carried out in animal models to be translated for human use. There

is an increasing volume of research suggesting that miRs are viable diagnostic or prognostic markers for diseases such as cancer and diabetes. In cancer, some miRs are even considered to be tumour suppressor genes.[85] Numerous miRs have a tissue-specific expression profile, which indicates that they have the potential to be disease markers in the future. miRs are known to be expressed at elevated levels in some tissues and cell states, which make them an attractive biomarker candidate as the progress of the disease can be directly monitored. Samples can be taken which are minimally invasive to monitor circulatory miRs before and after therapeutic intervention. miRs can be found in tissue and in circulation. In the circulatory system, the strands are frequently attached to Ago2 proteins or encapsulated in extracellular vesicles (ECVs). This makes them somewhat resilient and resistant to RNase degradation providing further support for their potential use as novel biomarkers[86] (Figure 2).

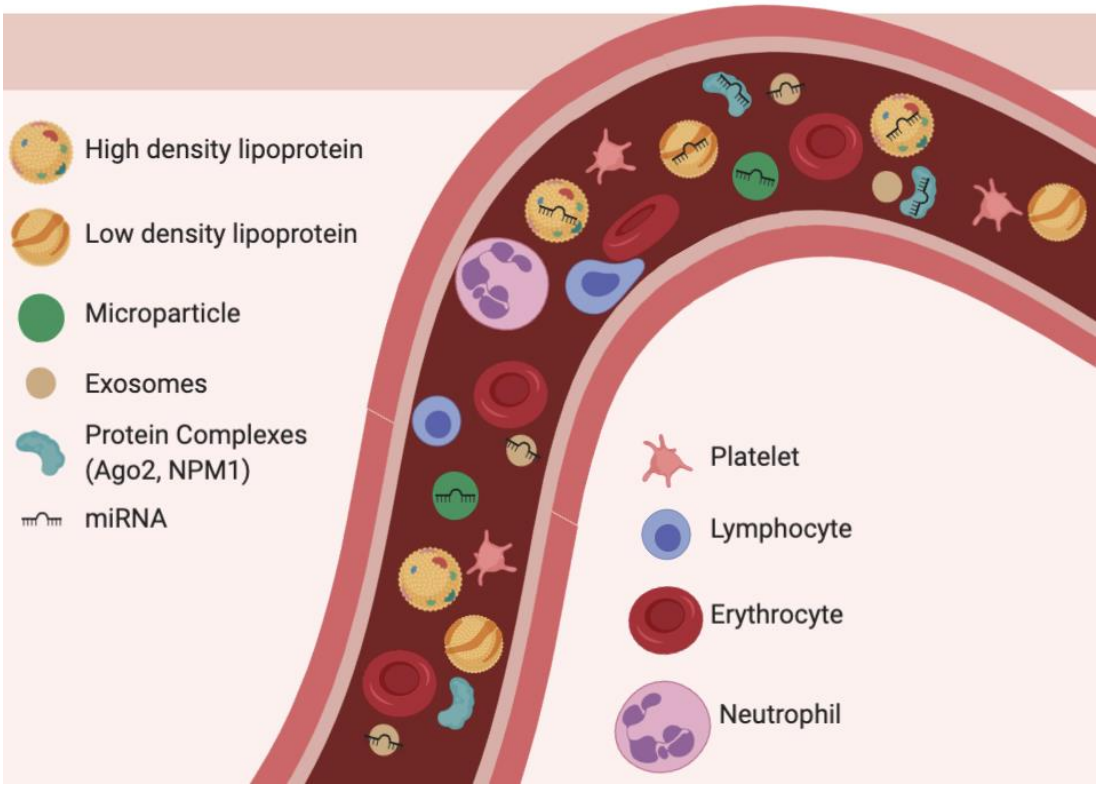


Figure 2. miR stability and transportation in the blood.
To reduce RNase degradation, miRs are encapsulated in microparticles, exosomes, high- and low-density lipoproteins. They are also attached to protein complexes, including Ago2.

Extracellular vesicles

Almost every cell type releases membrane-bound vesicles. ECVs were first described in 1967 and were observed in a diverse range of biological fluids. including blood, saliva and urine. They were considered to be systems for elimination of excess lipid and protein stores in the cell.[87] In cell injury, the ECV cargo is altered which makes them a viable area for biomarker discovery, as a 'liquid biopsy' as it is possible to differentiate between healthy and injured cells.

ECVs can be categorised depending on their size and biogenesis. Exosomes are considerably smaller than other vesicles (20 – 100 nm in diameter) as well as differing in their chemical properties. Exosomes are released into the extracellular space and transfer cytoplasmic and membrane proteins as well as miRs and mRNA. They play an essential role in an array of cellular processes, such as mediating signalling between cells,[88] making exosomes a

promising reservoir for biomarker discovery. Microvesicles are another unique class of ECV. They are released from the cell surface as a result of budding on the cell membrane. They are generally considered to be between 50 and 1000nm in diameter. Their contents are similar to exosomes, including miRs, mRNA and proteins. Since there is cross-over in the size profile between exosomes and microvesicles, there is difficulty in characterising them definitively. For simplicity, both vesicle population will be referred to as ECVs (as per consensus statement guidance).[89] Figure 3 shows the biogenesis of ECVs and their uptake by recipient cells.

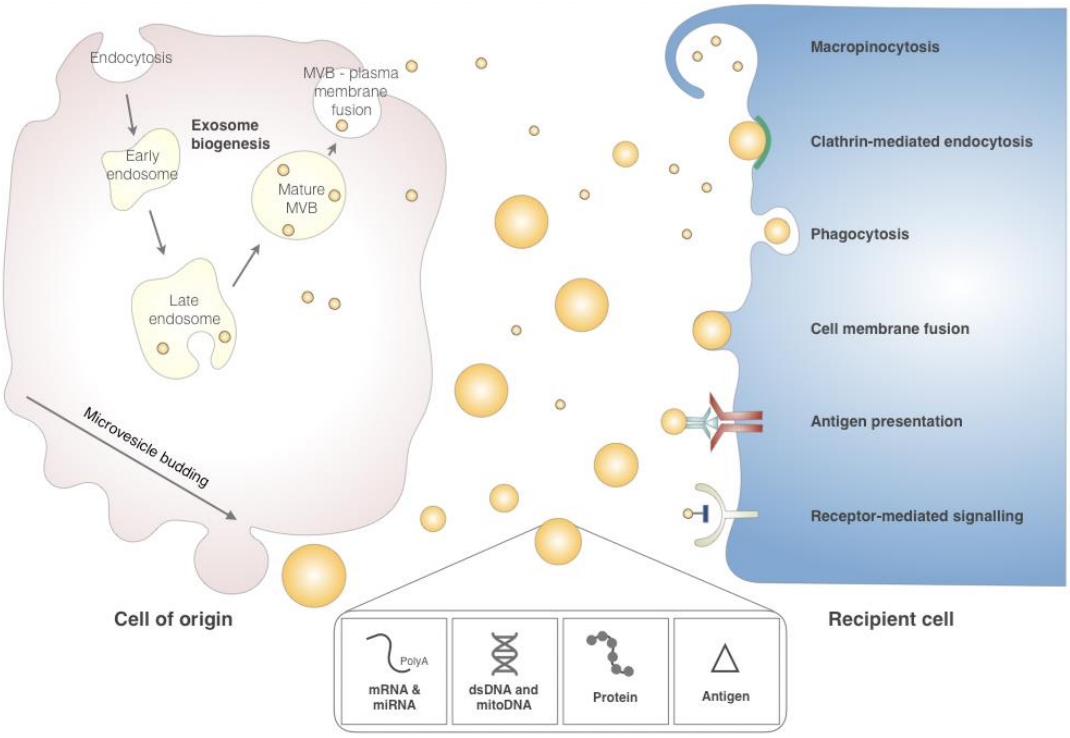


Figure 3. ECV biogenesis and the methods of uptake by recipient cells.
Courtesy of Dr Emma Morrison.

Sequencing technologies

The most commonly used techniques for profiling miRs are sequencing, microarrays and qPCR. miRs have been identified in a variety of diseases using RNA sequencing (RNAseq). Due to their tissue-specific expression patterns, it is possible to compare the miRNome profile for multiple patient or

treatment cohorts in order to identify the unique signature for a disease. Using next-generation sequencing, tissue-specific miR expression patterns can be measured in targeted diseases. miRs have been discovered using this technique in many diseases, including cancer. One advantage of small RNAseq is that it allows for the discovery of novel miRs that were not detectable with traditional screening and profiling methods.[90] Samples are prepared and sequenced to a known depth, allowing for a detailed analysis of the miR expression and subsequent hit validation to compare between active disease and remission.

miR-126

A miR of interest in cardiovascular disease is miR-126. It is located within the 7th intron of the epidermal growth factor like domain 7 (EGFL7) gene, which resides on human chromosome 9. It is endothelial cell-enriched and present throughout the vasculature. miR-126 is fundamental for maintaining the vascular structure and to regulate many properties in endothelial cells, including cell migration, formation of the cytoskeleton, capillary network stability and cell mortality.[91] It has been shown to positively regulate VEGF-dependant PI3 kinase and MAP kinase signalling.[92] This is done through directly targeting negative regulators of the VEGF signalling pathway (Figure 4). miR-126 increases the proangiogenic actions of VEGF and FGF and stimulates blood vessel formation by repressing the expression of Spred-1, an intracellular inhibitor of angiogenic signalling.

Studies in renal ischemic reperfusion injury (IRI) have demonstrated that miR-126 has the potential to act as an angiomiR to modulate mobilisation of haemopoietic stem cells. This in turn promotes vascular integrity and renal recovery following IRI.[93] In addition to renal injury, miR-126 has been shown to play a role in attenuating atherosclerosis.[94] Studies have shown that atherosclerosis is attenuated by miR-126 delivery by apoptotic bodies.[95]

As a biomarker, miR-126 has been shown to have potential diagnostic and prognostic ability in type 2 diabetes mellitus (T2DM). For T2DM, studies have shown a relationship with circulating miR-126 and associations with pre-diabetic syndrome.[96] The study was conducted in a cohort of patient's with impaired glucose tolerance (IGT), impaired fasting glucose (IFG), newly diagnosed T2DM patients and healthy individuals. The study found that miR-126 was at a lower concentration in circulation in the T2DM patients than in the healthy controls. After six months of diet and exercise strategies, the concentration of miR-126 increased for the IGT and IFG groups. For patients with T2DM, a combination of diet and exercise with insulin increased the concentration of miR-126. This study suggested a role for miR-126 as a marker for pre-diabetes and T2DM, and the patient's response to treatment.

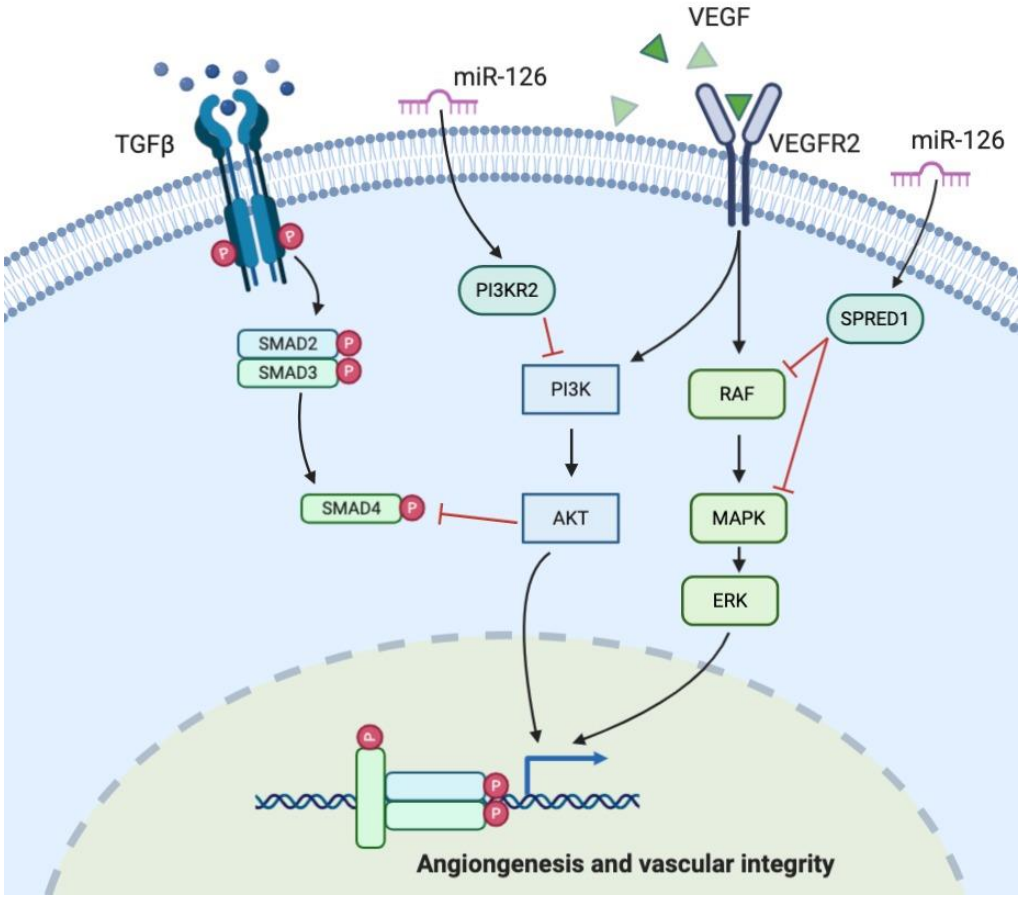


Figure 4. miR-126 regulates angiogenesis and vascular integrity.
miR-126 positively regulates VEGF-dependant PI3 kinase and MAP kinase signalling.

In addition, miR-126 has been shown to be a potential biomarker for coronary artery disease (CAD). One study compared stable CAD patients with healthy volunteers and demonstrated that miR-126 was lower in the patient cohort.[86] This was also observed in a panel of other endothelial cell-enriched miRs (members of the miR-17 cluster). There was also differential expression for inflammatory marker miRs (e.g., miR-155). This study suggested that miR-126 has potential as a biomarker of health and disease and the necessity to perform larger-scale studies to determine the use of circulating endothelial or vascular miRs in cardiovascular disease.

A recent study examined the association between circulating miR-126 and CKD.[97] In the study, over a 6-year period, 601 CKD patients (with stages G1 to G5 and G5D) were compared with healthy controls to ascertain the differences in circulating miR-126 between groups. From CKD stage G2, miR-126 levels were reduced in circulation with disease severity. In patients with a lower concentration of miR-126 than the median, a lower overall survival rate was observed in the follow-up period. In the study, miR-126 independently correlated with eGFR, haemoglobin levels, platelet counts and age. Further development of the potential role of miR-126 in the pathophysiology of CKD is necessary. In addition, this study did not examine the effect of maintenance haemodialysis on circulating miR-126.

Aims and hypotheses

The overarching hypothesis for my thesis is that miRs are clinically relevant biomarkers for acute and chronic vascular dysfunction.

Study 1 (Chapter 3): This study aimed to determine the potential application of miR-126 as a marker of acute vascular dysfunction in patients with ANCA vasculitis. The circulating concentration was compared in healthy volunteers and patients at disease presentation and once in clinically defined treatment-induced remission. These results were then compared against currently used clinical measures of vascular health to determine the validity of miR-126 as a biomarker of vascular dysfunction in acute injury. We hypothesised that miR-126 would be lower in disease and increase with treatment, reflecting the patient's vascular health.

Study 2 (Chapter 4): This study aimed to examine this association in chronic disease, specifically CKD and ESRD with haemodialysis. The concentration of circulating miR-126 in the patient cohorts was directly compared against clinical data which is used currently for diagnosis and patient evaluation. The hypothesis for this study was that miR-126 would have an inverse relationship with disease severity. In CKD, miR-126 would be low and the concentration would be further reduced in ESRD.

Study 3 (Chapter 5): As ANCA vasculitis has a high relapse rate, this study aimed to identify potential circulating miR biomarkers, using small RNA sequencing, to identify disease relapse, in order to prevent additional vascular damage occurring. We hypothesised that patients with active disease would have a panel of differentially expressed miRs in comparison to treatment-induced remission.

Study 4 (Chapter 6): As miRs are known to be transported in circulation in ECVs, this study aimed to determine the role of the miRs as mediators of vascular dysfunction. We hypothesised that ECVs isolated from plasma

samples from patients with active vascular dysfunction would result in a heightened immune response in macrophages and renal damage in human proximal tubule cells *in vitro*.

Chapter 2 – Methods

Patient sampling

All studies were approved by the local research ethics committee and performed in accordance with the Declaration of Helsinki.[98] The study, entitled "MicroRNA signatures of disease activity in ANCA-associated vasculitis", REC reference 13/ES/0126 (formerly 10/S1402/33) was approved by The Tissue Governance committee in June 2015. Informed consent was obtained from all participants. Patients presenting with ANCA vasculitis were recruited from the Vasculitis Service at the Royal Infirmary of Edinburgh. The subjects were aged 18-80, male or female and were sero-positive for ANCA vasculitis and had organ-threatening disease, which required immunosuppression. Patients were considered sero-positive for MPO if their antibody level was > 5.0 IU/mL and PR3 positive with antibody levels > 3.0 IU/mL. All patients gave consent for inclusion in the study. In patients with ANCA vasculitis, data collected included demographics, disease activity and damage assessments, medications, co-morbidities and laboratory results at each assessment and severe adverse events since starting treatment.

Serum and plasma samples were collected at disease presentation and once deemed to be in treatment-induced remission, determined by the Birmingham vasculitis activity score (BVAS) scale (Table 3).[99-101] At disease presentation and remission, 2 EDTA plasma (9 ml each) and 2 serum (7.5 ml each) blood samples were collected from each subject. Pulse wave velocity (PWV) was measured in the clinic for a selection of patients. PWV is the gold standard measurement for quantifying arterial stiffness. It is the velocity at which the pressure waves, which are produced by systolic contractions of the heart, propagate the arterial tree.[102] PWV gives an overall measurement of arterial stiffness, however, this measurement is influenced by factors including age, smoking and blood pressure,[103] Measurements are conducted in a clinical setting by trained staff, therefore it is not always possible to measure it for every patient.

Plasma and serum samples are commonly used in clinical biomarker studies. Plasma samples were used for the biomarker studies due to its stability and reproducibility. Previous studies have demonstrated that results are more robust in biomarker studies using plasma, rather than serum.[104]

Healthy volunteers

Consent was obtained from all healthy volunteers for inclusion in the study. Consenting adults were recruited who reported no medical conditions and no medical complaints. In healthy volunteers, 1 EDTA plasma (9 ml each) and 1 serum (7.5 ml each) blood sample were collected from each subject.

Blood sample processing

After blood samples were drawn, plasma and serum sample tubes were immediately centrifuged at 4ºC for 15 minutes at 1100 x *g*. The centrifuge speed had an impact on the concentration of miRs measured in human blood (Figure 5). This was particularly evident for miR-126-3p and miR-126-5p as the miR concentrations were lower as the centrifuge speed increased. This is an important factor for biomarker studies as sample processing may result in incorrect findings due to technical variation. Once spun, the serum or plasma layers were removed and stored at -80ºC. Freeze thawing was avoided in order to preserve sample integrity.

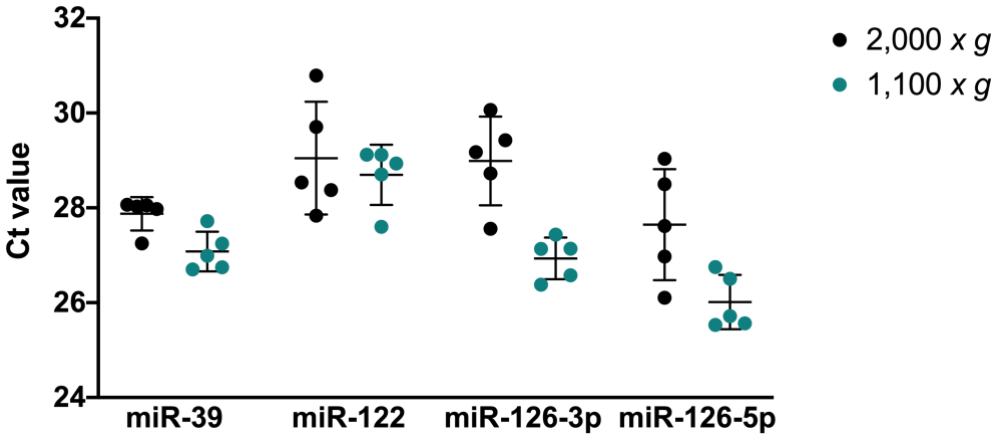


Figure 5. Effect of centrifuge speed on miR concentrations in human blood samples.
The concentration of miRs in blood spun at 2,000 x g and 1,100 x g were compared for a selection of miRs.

Category	*Symptom*	*Persistent*	*New/worse*
General	Myalgia	1	1
	Arthralgia/arthritis	1	1
	Fever ≥38^{o}C	2	2
	Weight loss ≥2 kg	2	2
Cutaneous	Infarct	1	2
	Purpura	1	2
	Ulcer	1	4
	Gangrene	2	6
	Other skin vasculitis	1	2
Mucous membranes / eyes	Mouth ulcers	1	2
	Genital ulcers	1	1
	Adnexal inflammation	2	4
	Significant proptosis	2	4
	Scleritis/Episcleritis	1	2
	Conjunctivitis/Blepharitis/Keratitis	1	1
	Blurred vision	2	3
	Sudden visual loss		6
	Uveitis	2	6

	Retinal changes (vasculitis/thrombosis/exudate/haemorrhage)	2	6
Ear, nose and throat	Bloody nasal discharge/crusts/ulcers/granulomata	2	4
	Paranasal sinus involvement	1	2
	Subglottic stenosis	3	6
	Conductive hearing loss	1	3
	Sensorineural hearing loss	2	6
Chest	Wheeze	1	2
	Nodules or cavities		3
	Pleural effusion/pleurisy	2	4
	Infiltrate	2	4
	Endobronchial involvement	2	4
	Massive haemoptysis/alveolar haemorrhage	4	6
	Respiratory failure	4	6
Cardiovascular	Loss of pulses	1	4
	Valvular heart disease	2	4
	Pericarditis	1	3
	Ischaemic cardiac pain	2	4

	Cardiomyopathy	3	6
	Congestive cardiac failure	3	6
Abdominal	Peritonitis	3	9
	Bloody diarrhoea	3	9
	Ischaemic abdominal pain	2	6
Renal	Hypertension	1	4
	Proteinuria >1+	2	4
	Haematuria ≥10 RBCs/hpf	3	6
	Creatinine 125-249µ/L (1.41- 2.82mg/dl)*	2	4
	Creatinine 250-499 µ/L (2.83- 5.64mg/dl)*	3	6
	Creatinine ≥500 µ/L (≥5.66mg/dl)*	4	8
	Rise in serum creatinine >30% or fall in creatinine clearance >25%		6
	**Can only be scored on the first assessment*		
Nervous system	Headache	1	1
	Meningitis	1	3
	Organic confusion	1	3
	Seizures (not hypertensive)	3	9
	Cerebrovascular accident	3	9

	Spinal cord lesion	3	9
	Cranial nerve palsy	3	6
	Sensory peripheral neuropathy	3	6
	Mononeuritis multiplex	3	9
Other	As required		

Table 3. BVAS scale for defining active vasculitis.
The clinical symptoms are graded using a defined scoring system.[99, 101]

RNA extraction

Plasma and serum samples (50μL) underwent RNA extraction using the miRNeasy serum/plasma kit (Qiagen, Venlo, Netherlands). The manufacturer's instructions were followed. After thawing, 50μL of the serum/plasma sample was diluted with 150μL RNase free water. Five times the volume (1mL) of Qiazol lysis reagent was added and mixed by vortexing before incubating at room temperature (15 – 25°C) for 5 minutes. A spike-in exogenous control was added, 3.5μL of *C. elegans* miR-39, at 1.6 x 10^8 copies/μL. Following this, 200μL chloroform was added before shaking the sample to mix and incubating at room temperature for 3 minutes. The samples were centrifuged for 15 min at 12,000 x *g* at 4°C. The upper aqueous phase was removed and mixed with 1.5 volumes of 100% ethanol. The samples were passed through a RNeasy MinElute spin column at ≥ 8000 x *g* for 15 s at room temperature. The column was then rinsed with 700μL Buffer RWT and centrifuged for 15 s at ≥ 8000 x *g.* This was repeated with 500μL Buffer RPE, a mild washing buffer. The column was then rinsed with 500μL of 80% ethanol for 2 min at ≥ 8000 x *g.* The column was dried, and the RNA was eluted in 14μL RNase-free water by spinning for 1 minute at full speed. Eluted RNA was stored at -80^{o}C until required.

Reverse transcription PCR

After RNA extraction, 5μL of each elute was reverse transcribed into cDNA with a single-step cDNA synthesis reaction using the miScript II RT Kit (Qiagen, Venlo, Netherlands). The manufacturer's instructions were followed. The template RNA was thawed on ice. The reverse transcription master mix was prepared as detailed in Table 4. The RT master mix contained all necessary components for first-strand cDNA synthesis except the template RNA. The miScript buffer varied depending on whether the template RNA was from mRNA or miR as the reaction was optimised for each specific starting material. For each reaction, 5μL of RNA template was mixed with the RT

master mix. To reverse transcribe the product, the samples were incubated at 37ºC for 60 minutes followed by 95ºC for 5 minutes to inactivate the miScript Reverse Transcriptase. The cDNA was then diluted 10-fold in nuclease-free water and stored at -20ºC until required.

Component	**Volume/reaction**
5x miScript HiSpec Buffer (for miRs) 5x miScript HiFlex Buffer (for mRNAs)	4μL
10x miScript Nucleics Mix	2μL
RNase-free water	7μL
miScript Reverse Transcriptase Mix	2μL
Template RNA	5μL
Total volume	**20μL**

Table 4. miScript master mix components for reverse transcription.

Quantitative PCR

The diluted cDNA was used for quantitative PCR (qRT-PCR) with the miScript SYBR Green Kit (Qiagen, Venlo, Netherlands) using the components listed in Table 5. A master mix was prepared for each primer (detailed in the results chapters) and dispensed into a 384 well white plate. Following this, 1μL of the template cDNA was added to each well and the plate was centrifuged in a bench-top centrifuge at 1500 x *g* for 2 minutes at room temperate.

Component	**Volume/reaction**
2x QuantiTect SYBR Green PCR Master Mix	5μL
10x miScript Universal Primer	1μL
10x miScript Primer Assay	1μL
RNase-free water	2μL
Template cDNA	1μL
Total volume	**10μL**

Table 5. Master mix components for miScript SYBR Green qPCR reaction.

Real-time PCR was carried with a Light Cycler 480 (Roche, Basel, Switzerland) using the recommended miScript cycling conditions for 45 cycles, shown in Table 6. Data collection was performed at the extension step for each qRT-PCR run and the cycle threshold (Ct) value was recorded for each sample in duplicate. In addition, dissociation curve analysis was performed for the PCR products to verify the primer specificity.

Step	**Time**	**Temperature**
PCR Initial activation step	15 minutes	95ºC
3-step cycling		
Denaturation	15 seconds	94ºC
Annealing	30 seconds	55ºC
Extension	30 seconds	70ºC

Table 6. Cycling conditions for qPCR using the Qiagen miScript SYBR Green PCR kit.

Relative quantification

MiR expression was analysed using the ΔCt method.[105] A *C. elegans* miR mimic was used as a spiked-in control. This allowed for the Ct values for the miRs of interest to be normalised to the spiked-in control. The data obtained were translated by the $2^{-\Delta Ct}$ method.

Absolute quantification

To measure the expression of the miRs of interest, absolute quantification was performed. The concentrations of the miRs of interest were determined by measuring the Ct values for miR mimics at known concentrations, using serial dilutions, by qRT-PCR. From this, a standard curve was prepared to compare the Ct values for the miRs of interest. This process was carried out for miR-126 and miR-122 (Figure 6).

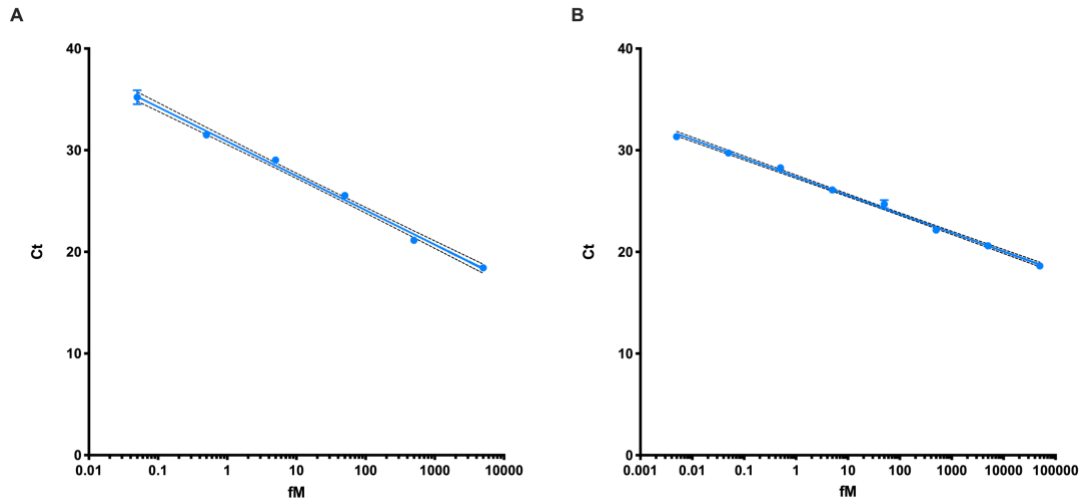


Figure 6. Standard curves from miRs of interest.
(A) miR-122 and (B) miR-126 standard curves for absolute quantification in patient samples. The solid blue line shows the semilog and the dotted line shows the 95% confidence interval (CI).

Data and statistical analyses

Data were stored and statistically analysed using GraphPad Prism 8 software (La Jolla, California, United States) unless stated otherwise. For NTA results, data from three triplicate videos were analysed by calculating the AUC for particles sized between 20-100nm. Throughout, unless otherwise stated, a Wilcoxon test was used for paired samples and for unpaired samples, a Mann-Whitney test. For correlations, normality tests were performed (D'Agostino-Pearson omnibus test) in order to establish the distribution, followed by either a Pearson or Spearman test for the significance. Unless otherwise stated, data were expressed as mean ± standard deviation (SD) and $P < 0.05$ were seen as significant throughout. Alternative methods of statistical analysis employed in further chapters will be discussed.

Chapter 3 – miR-126 is a marker of vascular dysfunction in ANCA vasculitis

Introduction

Vascular dysfunction and kidney disease commonly co-exist, and their presence substantially increases the risk of CVD. ANCA vasculitis is a rare disease, primarily affecting the vasculature, which often causes renal injury, resulting in rapidly progressive glomerulonephritis. It represents a tractable model of acute vascular and renal injury in humans. It is an inflammatory disorder characterised by leucocyte infiltration and necrosis of the small arteries. Even with current treatment strategies, the survival rate after the diagnosis of AAV is lower in comparison to unaffected cohorts with a 2.6 mortality ratio, with more deaths observed than expected, when compared to age and sex-matched counterparts,[18, 19, 106] with many patients suffering chronic inflammation, a major contributor to the development and progression of both CVD[107] and CKD.[20] Furthermore, those who respond to treatment remain at risk of further disease relapses[108] and organ damage.

There is an unmet clinical need for biomarkers that specifically report vascular health and injury. In vasculitis, it is a clinical challenge to evaluate the level of vascular damage and the response to treatment. In AAV patients with renal involvement, the measurement of renal function using serum creatinine is often inadequate because a high degree of renal damage can occur before renal function is impaired to a detectable level.[109] Although serum creatinine may decrease with treatment, inflammation can potentially continue even after renal function has improved. Biomarkers that can specifically report small vessel damage would allow for early therapeutic interventions, as well as help to detect patients at risk of disease relapses.

The miR that was evaluated in this study, miR-126, is endothelial cell-enriched and present throughout the vasculature. miR-126 is located in the 7th intron of the EGFL7 gene, which encodes a secreted inhibitor of smooth muscle cell migration, on chromosome 9 in humans.[110] It has an established role as a regulator of vascular integrity and angiogenesis.[111] Circulating miR-126 has been described as a potential biomarker for vascular disorders such as

diabetes[112, 113] and myocardial infarction[114], with lower circulating concentrations being associated with disease and poor vascular prognosis.[115] Low levels of miR-126 causes reduced vessel formation due to the modulation of endothelial cells to vascular growth factors and leucocyte adhesion.[116] Due to its known role in vascular integrity and endothelial cell enrichment, miR-126 is an attractive biomarker in acute vascular injury. It is also well established as a biomarker of various cardiovascular diseases.

Aims and hypothesis

The hypothesis for this chapter was that miR-126 was a biomarker of vascular integrity in the circulation and that it would be altered by vascular injury. miR-126 would track treatment-induced improvement in vascular function in acute injury in patients with ANCA vasculitis. This study aimed to determine the circulating concentration of miR-126 in healthy volunteers and patients with vasculitis. The concentration of miR-126 was measured in vasculitis patients with active disease and treatment-induced remission. These results were assessed in the context of the currently used clinical measures of vascular and renal health.

Methods

Patients

To estimate the healthy reference interval for circulating miR-126 consenting adults were recruited who reported no medical conditions and no medical complaints. Blood was drawn under normal conditions. In healthy volunteers, 3 EDTA plasma (2.7mL each) and 3 serum (4.9mL each) blood samples were collected from each individual.

For this study, 130 individuals were recruited. This included 60 healthy volunteers and 70 patients with AAV. Patients were recruited from the Vasculitis Service at the Royal Infirmary of Edinburgh, UK. The inclusion criteria for this study were patients who presented with sero-positivity for ANCA vasculitis, as defined by ANCA antibody titres. Patients were considered sero-positive for MPO if their antibody level was > 5.0 IU/mL and PR3 positive with antibody levels > 3.0 IU/mL There were no defined exclusion criteria for this study. Plasma and serum samples were used from patients at entry into the study and at a subsequent follow-up visit when the condition was considered to be at the remission stage after successful treatment. Samples were taken with consent and stored immediately at -80^{o}C after being spun in a workbench centrifuge at 1,100 x *g* for 10 minutes at 4^{o}C. The study was approved by the local research ethics committee and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants. The study, entitled “MicroRNA signatures of disease activity in ANCA-associated vasculitis”, REC reference 13/ES/0126 (formerly 10/S1402/33) was approved by The Tissue Governance committee in June 2015. Clinical data were obtained by clinical staff at the Edinburgh Renal Unit at the Royal Infirmary, Edinburgh.

RNA extraction

Plasma samples (50µL) underwent RNA extraction using the miRNeasy serum/plasma kit (Qiagen, Venlo, Netherlands). Further details are given in Chapter 2.

Reverse transcription PCR

After RNA extraction, 2.5µl of each elute was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen, Venlo, Netherlands). The manufacturer's instructions were followed. The cDNA was then diluted 10-fold in nuclease-free water.

Quantitative PCR

The diluted cDNA was used for qRT-PCR with miScript SYBR Green Kit (Qiagen, Venlo, Netherlands). Real time PCR was carried with a Light Cycler 480 (Roche, Basel, Switzerland) using the recommended miScript cycling conditions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. For paired samples, a Wilcoxon test was used and for unpaired samples, a Mann-Whitney test. For correlations, normality tests were performed (D'Agostino-Pearson omnibus test) in order to establish the distribution, followed by either a Pearson or Spearman test for the significance of the correlation.

Results

Patient's clinical characteristics

For this study, 70 patients with active vasculitis were recruited and followed up. Clinical measurements of the patient's kidney, liver and inflammatory status were recorded pre-treatment, at initial disease presentation, and post-treatment, once remission had been achieved (Table 7). Renal function improved after treatment as determined by creatinine and eGFR. There was an improvement in liver function for ALP and GGT, however, there was no change for ALT. Additionally, CRP was reduced greatly post-treatment, however, albumin and haemoglobin slightly increased.

Characteristics	Pre-treatment (*n* = 70)	Post-treatment (*n* = 70)	P value
Age, years	62 ± 14 (27 - 82)	-	-
Sex, M/F	43/27	-	-
Organs involved	2 ± 1 (1 - 6)	-	-
- Kidney	44	-	-
- Lung	33	-	-
- ENT	17	-	-
- Nerve	13	-	-
- Eyes	9	-	-
eGFR, mL/min/1.73m^2	46 ± 34 (4 - 124)	57 ± 29 (9 - 121)	0.04
Serum creatinine, μmol/L	200 ± 177 (54 - 962)	150 ± 144 (64 - 1000)	0.03
ALT, U/L	20 ± 15 (6 - 83)	20 ± 10 (4 - 48)	0.94
ALP, U/L	96 ± 62 (13 - 338)	67 ± 18 (39 - 122)	0.0002
GGT, U/L	55 ± 44 (11 - 182)	33 ± 26 (10 - 148)	0.0009
Albumin, g/L	28 ± 7 (14 - 41)	37 ± 4 (24 - 46)	<0.0001
CRP, mg/L	82 ± 75 (3 - 275)	6 ± 15 (0 - 108)	<0.0001
Haemoglobin, g/L	107 ± 23 (62 - 157)	124 ± 28 (2 - 170)	<0.0001
Urine protein:creatinine mg/mmol	141 ± 188 (0 - 948)	107 ± 146 (0 - 531)	0.82
Urine creatinine, μmol/24hrs	12 ± 20 (3 - 109)	8 ± 4 (3 - 20)	0.90

Table 7. Clinical data for vasculitis patients pre- and post-treatment.
The data are shown as mean ± SD with range. Significance of numerical data between groups was ascertained using a 2-tailed paired t-test.

Circulating miR-126 concentration in AAV

Plasma samples were obtained from healthy volunteers and patients with AAV pre- and post-treatment. The concentration of circulating miR-126 was measured for each cohort in order to ascertain its ability as a marker of acute vascular damage (Figure 7). A baseline measurement for miR-126 was obtained in the healthy control group. The data demonstrated that the healthy baseline concentration of miR-126 was substantially higher than for the AAV patient cohort pre-treatment. After the patients were deemed to be in treatment-induced remission, post-treatment, the concentration of miR-126 increased. The data establish that miR-126 is higher in health and reduced with acute vascular dysfunction. Even after treatment, miR-126 does not increase to the normal concentration.

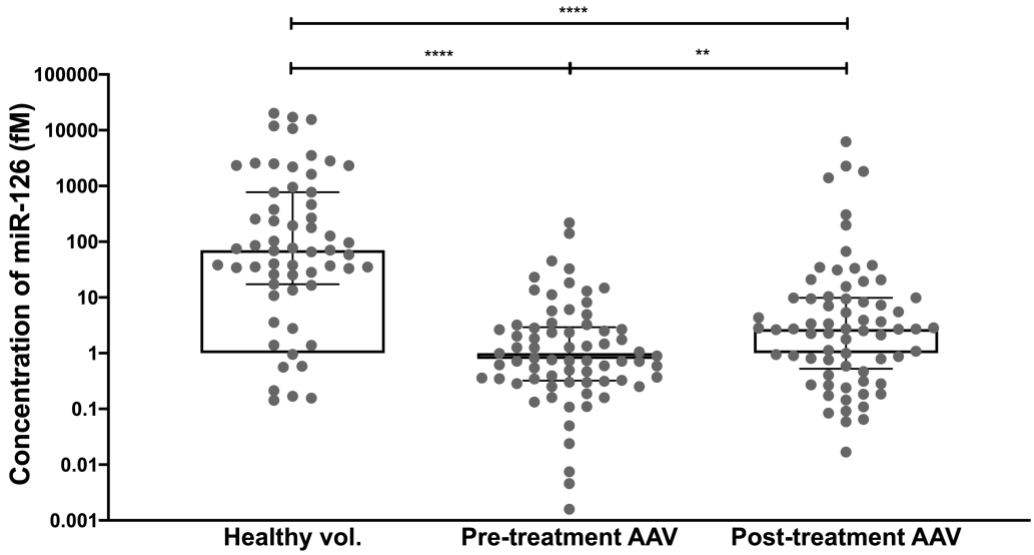


Figure 7. Circulating miR-126 in acute vascular injury.
*The concentration of miR-126 in healthy volunteers (n = 60) and AAV patients (n = 70 paired) pre- and post-treatment. Bars show median with IQR, **p<0.01, ****p<0.0001 (Mann-Whitney test for unpaired analysis and Wilcoxon test for paired analysis).*

Circulating miR-126 in AAV subgroups

To determine whether disease subclass influenced the circulating concentration of miR-126 in patients with AAV, the date was further analysed by clinical subgroups, as shown in Figure 8. This illustrated that for MPO and PR3, there was not a significant difference in miR-126 concentration between

the subtypes. This was also demonstrated in the analysis of AAV patients with renal involvement versus those without.

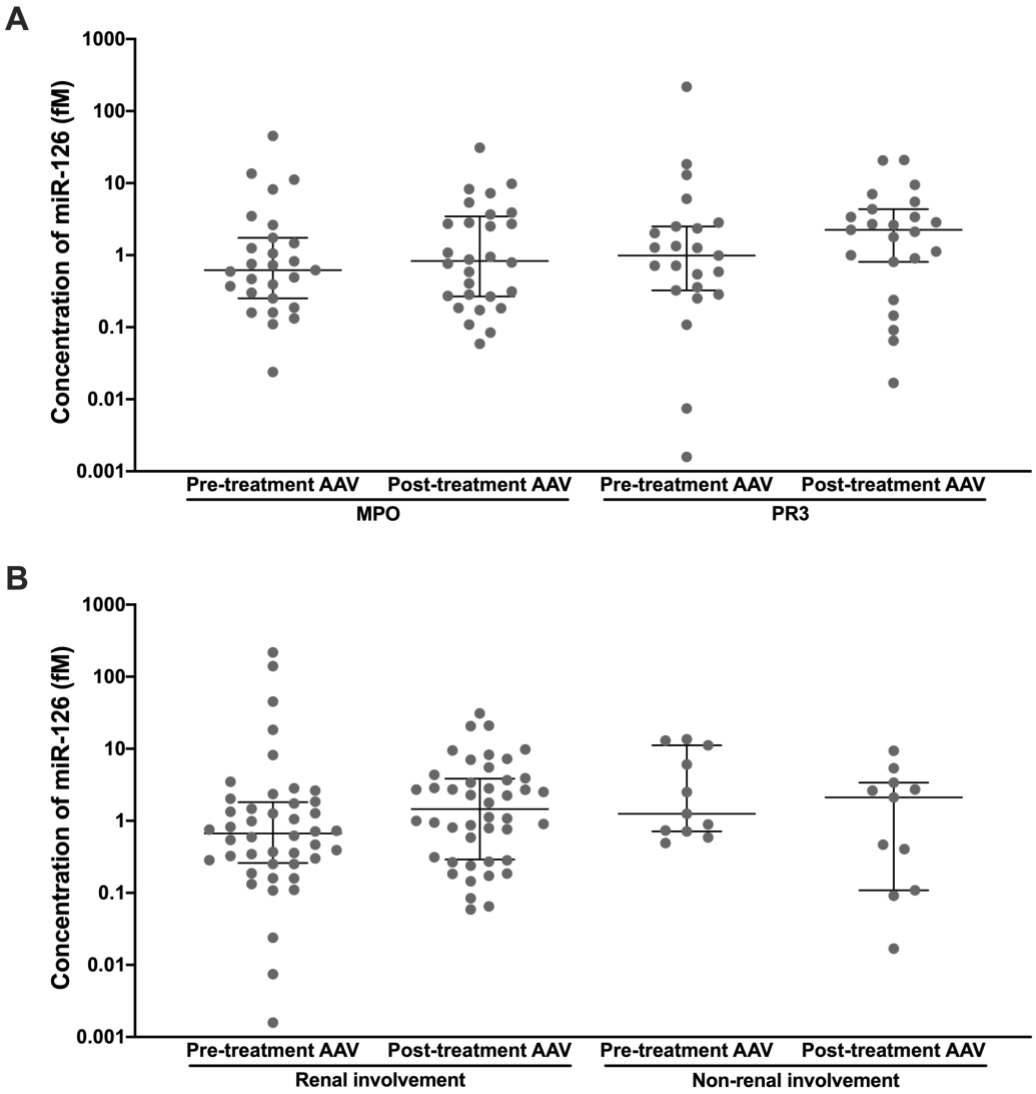


Figure 8. Analysis of circulating miR-126 is AAV subgroups.
The concentration of miR-126 in patients diagnosed as (A) MPO positive (n = 28) and PR3 positive (n = 23) pre- and post-treatment. (B) Patients classified as having renal involvement (n = 44) and non-renal involvement (n = 11) pre- and post-treatment. Bars show median with IQR (Mann-Whitney test for unpaired analysis and Wilcoxon test for paired analysis).

Evaluation of miR-126 as a biomarker in AAV

The sensitivity and specificity of circulating miR-126 as a biomarker for AAV was quantified using receiver operating characteristic (ROC) curve analysis. The sensitivity and specificity were compared for healthy volunteers versus

pre-treatment patients (Figure 9A) and compared for patients pre-treatment versus post-treatment (Figure 9B). The AUC was measured for each analysis to evaluate miR-126 as a biomarker of disease.

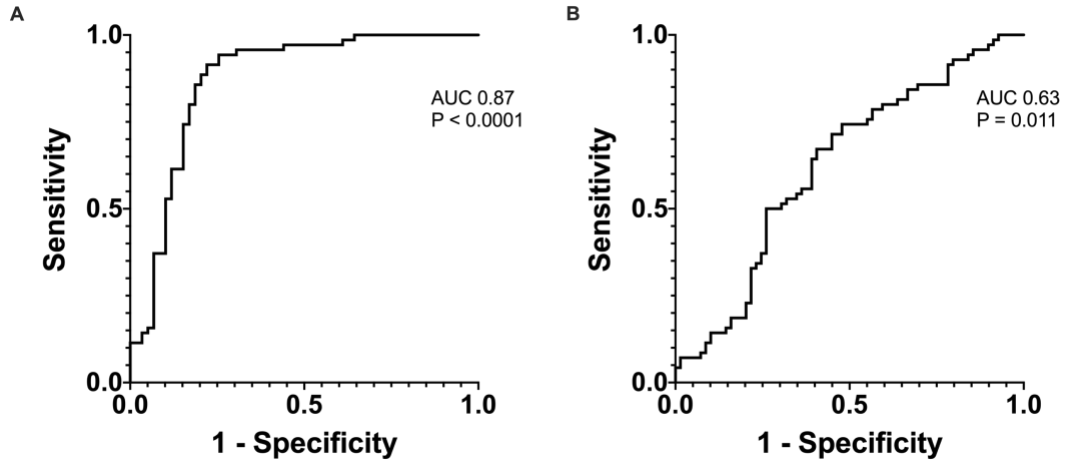


Figure 9. ROC curve analysis for miR-126 as a biomarker for AAV.
Sensitivity and specificity for (A) healthy volunteers versus pre-treatment patients with active disease and (B) pre-treatment versus post-treatment. The ROC curve analysis demonstrates the area under the curve (AUC) and statistical significance.

miR-126 as a marker of vascular damage

PWV is the gold standard measurement for quantifying arterial stiffness. It is also an independent predictor of future adverse cardiovascular outcomes in patient groups[117]. PWV was measured pre- and post-treatment and the change in stiffness was evaluated against the change in circulating miR-126 in a subgroup of AAV patients (Figure 10). There was a clear inverse relationship between arterial stiffness, PWV, and miR-126.

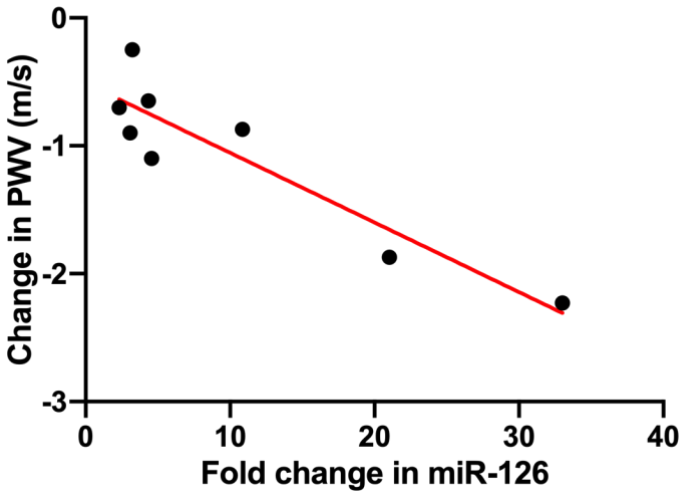
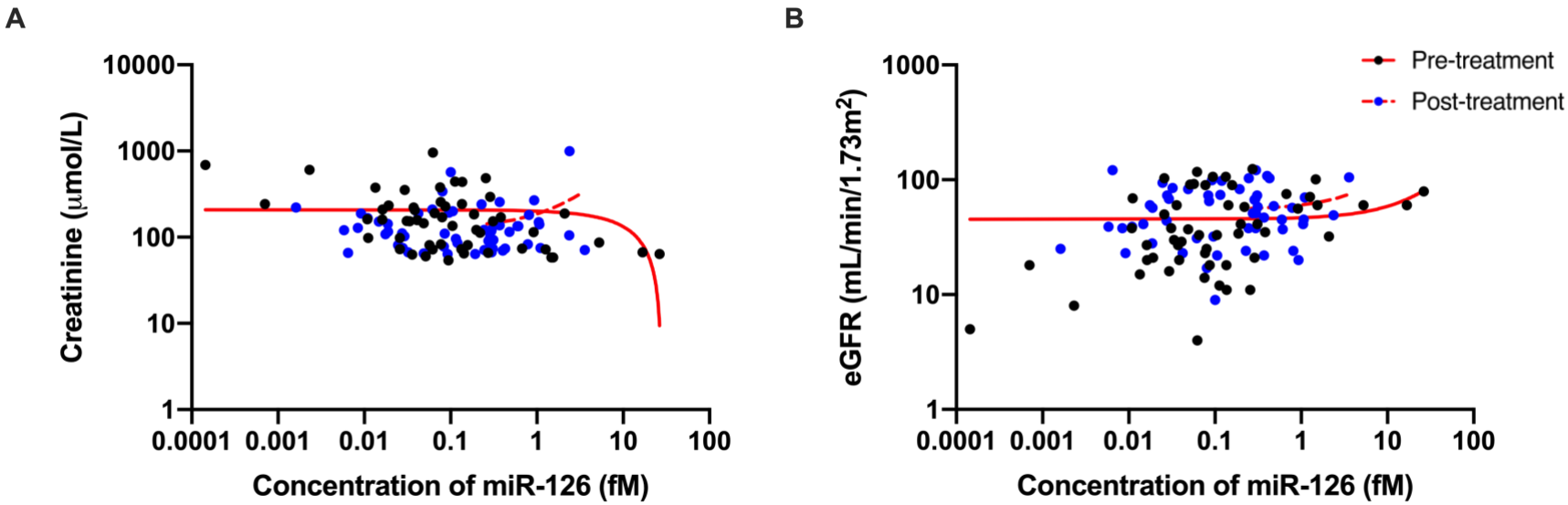


Figure 10. Clinical measures of arterial stiffness correlate with circulating miR-126 in patients with AAV.
The fold change in miR-126 has an inverse relationship with the change in arterial stiffness from pre- to post-treatment (n = 8). The red line shows the linear regression for the correlation. The change in PWV is the value pre-treatment minus the post-treatment value, expressed in m/s.

miR-126 as a marker of renal function in AAV

Vascular dysfunction commonly co-exists with kidney disease, which results in a substantially increased risk of cardiovascular disease. As renal damage is often a severe manifestation of AAV, renal function is measured at disease presentation, as well as follow-up appointments at the clinic. In order to ascertain the relationship between miR-126 and renal function during acute vascular injury, the clinical data were assessed (Figure 11). Creatinine had a weak negative correlation pre-treatment, and this was inverted to a positive correlation post-treatment. This was not reflected in the eGFR results, as no relationship was demonstrated in the AAV patient cohort. This suggests that miR-126 is not a biomarker of renal function.



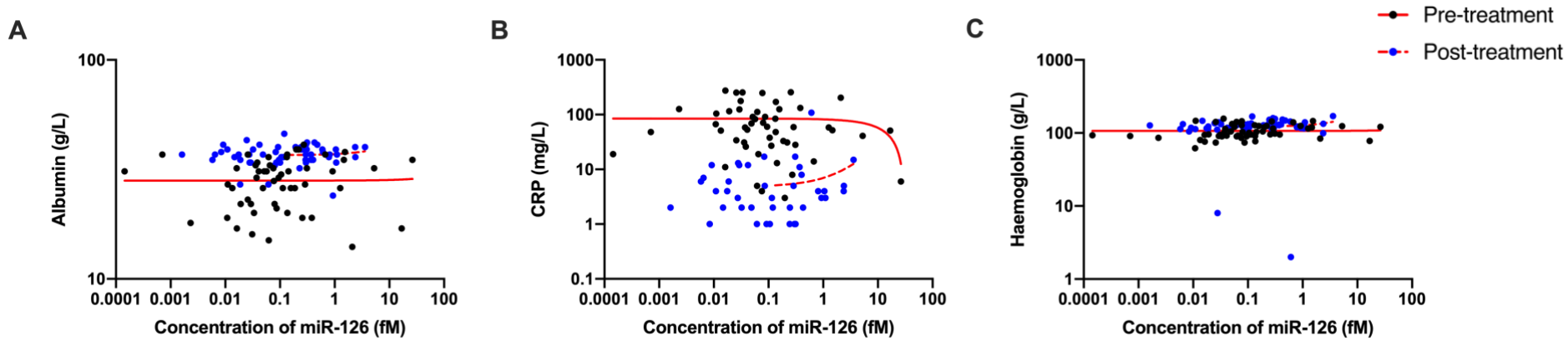
C

	Creatinine Pre-treatment	*Creatinine Post-treatment*	*eGFR Pre-treatment*	*eGFR Post-treatment*
Analysis	Spearman	Pearson	Pearson	Spearman
P-value	0.006	0.04	0.21	0.51
r	-0.37	0.28	0.17	0.09
95% CI	-0.58 to -0.10	0.01 to 0.51	-0.10 to 0.42	-0.19 to 0.36
R squared	-	0.08	0.03	
Number of pairs	54	55	54	54

Figure 11. The concentration of circulating miR-126 in relation to clinical measures of renal function in patients with AAV.
The solid red line shows the linear regression pre-treatment and dotted line for post-treatment. Individual patient data points are shown in black for pre-treatment and blue for post-treatment. (A) Creatinine demonstrates a weak negative relationship pre-treatment and a weak positive relationship post-treatment with circulating miR-126, however, (B) eGFR does not correlate with miR-126. (C) Correlation analysis for creatinine and eGFR based on the normality of the data.

miR-126 does not correlate with systemic inflammation

A major component of vascular damage in AAV is vascular inflammation. This is characterised by leucocyte infiltration into the target blood vessel and as a result, the endothelium is damaged. At disease presentation, and once in treatment-induced remission, clinical markers of inflammation are measured. The results of these tests were compared against circulating miR-126 (Figure 12). The clinical markers measured were albumin, CRP and haemoglobin. These tests all provide information relating to systemic inflammation. Overall, there was no correlation between clinical tests and miR-126. There was a slight positive relationship post-treatment with miR-126 and haemoglobin.



D

	Albumin Pre-treatment	*Albumin Post-treatment*	*CRP Pre-treatment*	*CRP Post-treatment*	*Haemoglobin Pre-treatment*	*Haemoglobin Post-treatment*
Analysis	Spearman	Spearman	Spearman	Spearman	Pearson	Spearman
P-value	0.20	0.43	0.18	0.55	0.92	0.04
r	0.18	0.11	-0.19	-0.08	0.01	0.27
95% CI	-0.10 to 0.44	-0.17 to 0.37	-0.45 to 0.10	-0.35 to 0.20	-0.25 to 0.28	-0.001 to 0.51
R squared	-	-	-	-	0.0002	-
Number of pairs	53	54	51	55	54	55

Figure 12. Clinical markers of systemic inflammation in AAV in relation to circulating miR-126.
The solid red line shows the linear regression pre-treatment and dotted line for post-treatment. Individual patient data points are shown in black for pre-treatment and blue for post-treatment. (A) Albumin, (B) CRP and (C) Haemoglobin do not correlate with miR-126 pre-treatment in AAV. Post-treatment, miR-126 shows a slight positive correlation with haemoglobin. (D) Correlation analysis based on the normality of the data.

A liver enriched miR is unchanged in vasculitis

A biomarker of vascular health must be specific and therefore non-vascular miRs should not be altered by disease state in AAV. Since an endothelial cell marker, miR-126, changed markedly after treatment in AAV, a liver enriched marker, miR-122, was measured in the patients (Figure 13). miR-122 was measured in the same vasculitis patient cohorts as described above. For AAV patients, the level of miR-122 was comparable pre- and post-treatment. The miR profile in these patients was not changed overall, suggesting that the change in miR-126 is specific, rather than the disease state altering the miRNome in circulation.

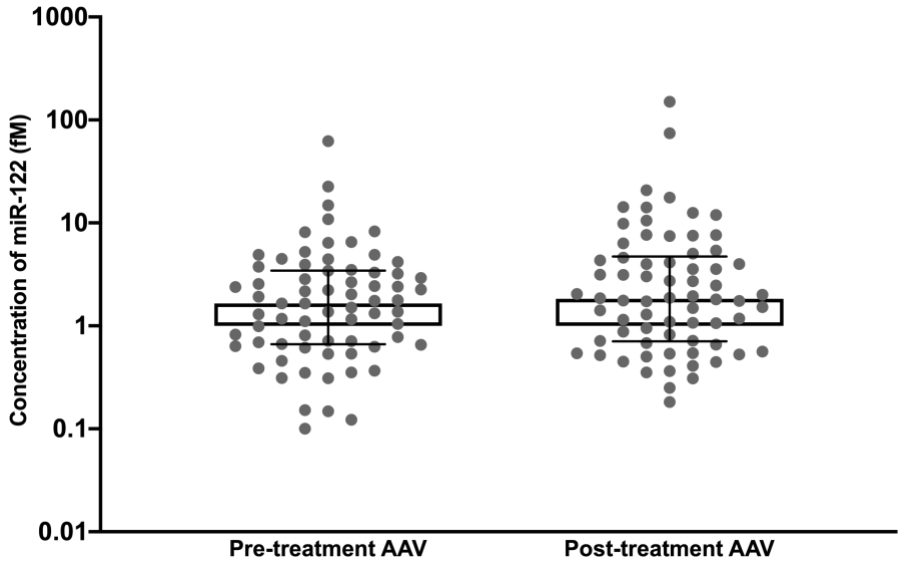


Figure 13. Circulating miR-122, a marker of liver dysfunction, is not altered in AAV between active disease and remission.
The concentration of miR-122 was measured in plasma samples in AAV patients pre- and post-treatment (n = 70 paired). Bars show median with IQR, Wilcoxon test for paired analysis.

Discussion

The risk of cardiovascular disease increases substantially in patients with vascular injury and kidney disease. Endothelial dysfunction often co-exists with cardiovascular disease, meaning that the development of a biomarker to report vascular health is required. This study examined the potential role of miR-126 as a circulating biomarker of vascular dysfunction in acute vascular dysfunction, specifically in ANCA vasculitis as a model of severe injury. This study demonstrated that circulating miR-126 is at the highest concentration in healthy individuals and is reduced in vasculitis. After successful immunosuppression, the concentration of miR-126 increases, however, this is not to the same concentration range for healthy individuals, which may reflect their continuing vascular damage. These data were supported by the patient's PWV results, the gold standard measurement for arterial stiffness. There was no clear relationship between miR-126 and clinical measures of kidney function or inflammatory status. Circulating miR-126 did not distinguish between AAV subclass or reflect whether there was renal involvement or not. This supported the hypothesis that miR-126 is a biomarker of vascular health and injury in patients with acute vascular dysfunction.

This study demonstrated a significant correlation between treatment-induced changes in endothelial cell-enriched miR-126 and pulse wave velocity, a clinical measure of arterial stiffness that often co-exists with endothelial dysfunction.[118] In contrast, there was no correlation between the change in miR-126 and changes in measures of renal injury/repair in those with ANCA disease. This was also the case for measures of inflammatory status in the patient cohort. Clinically, a circulating biomarker that reports vascular health could have widespread utility. Further studies would allow for miR-126 to be measured in a range of other systemic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus to ascertain the clinical potential as a biomarker.

Identifying ANCA vasculitis early and assessing its response to treatment remain important clinical challenges. In those with renal involvement, the measurement of renal function using serum creatinine is often inadequate because substantial renal damage can occur before function is impaired to a detectable extent.[109] Furthermore, although serum creatinine may decrease with treatment, it remains unclear whether histological inflammation continues once renal function has stabilised. Additionally, currently there are no good measures of disease activity in those with extra-renal vasculitis alone. Biomarkers specific to small vessel inflammation would not only allow early implementation of appropriate treatments but also help identify those patients with grumbling disease activity despite therapy and potentially predict disease relapses.

Identifying a reliable and sensitive biomarker to predict disease relapse remains an important research focus. Vasculitis often continues as a 'grumbling' disease or results in a relapse of the disease even after treatment-induced remission has been achieved. A study of 1,804 patients found that relapse occurred in 201 patients (38%) and 133 patients (25%) died.[119] The study concluded that a creatinine level >200 μmol/L at disease presentation was associated with a lower rate of disease relapse. This is potentially a problematic approach as severe renal and vascular damage has occurred at this level. With a successful minimally invasive biomarker of vascular injury, it would be possible to track the disease progression to relapse and alter the treatment strategy appropriately in order to prevent further damage occurring.

In clinical care, miR-126 represents a potential biomarker in ANCA vasculitis. From the FDA guidelines,[75] as outlined in Chapter 2, a biomarker may have multiple definitions with validation and evidence available for each. This study suggests that miR-126 has the potential to clinically act as monitoring biomarker, as well as a prognostic biomarker. As a monitoring biomarker, miR-126 could be used to assess the effect of a medical product, such as therapeutic interventions, in the treatment and maintenance of remission.

Serial measurements could be performed to evaluate the effectiveness of the treatment strategy. To utilise it for this purpose, the minor changes in the concentration would need to be defined to understand the slight changes in the patient's treatment responses. As a prognostic biomarker, miR-126 could be used to predict the probability of disease relapse or disease progression with pre-diagnosed ANCA vasculitis. In a clinical care setting, it allows for evaluation of the possible length of treatment for a patient or their length of stay in hospital.

Limitations

Alongside currently used markers of vascular health, and experimental approaches such as PWV, miR-126 has the potential to supplement the diagnosis of acute vascular dysfunction and response to treatment. One limitation of this study is the small cohort of patients that had PWV measured. It would have been optimal for all patients to have their arterial stiffness measured and compared before and after treatment, however, there was limited availability for this measurement. This would provide a more detailed relationship between the two measurements. In addition, ADMA, an endogenous inhibitor of nitric oxide synthase, was not measured in the vasculitis patient cohort. This is considered an independent risk factor of cardiovascular disease and therefore would give insight into the potential role of miR-126 as a biomarker of vascular health.

A further limitation of this study is that the healthy controls that were included in this study were younger overall in comparison to the vasculitis group. In European studies, the mean age for AAV diagnosis is 60 years old[120] with a peak prevalence for the disease between 65-74 years old.[121] As many individuals aged 60 years and over have pre-existing medical conditions or taking regular mediation, it was not possible to age match the controls. It would have improved the clinical relevance of the study by having age-matched controls as it would have been beneficial to develop a healthy baseline range

in order to compare against the vasculitis patient’s results in active disease and remission.

Conclusions

Previous studies have demonstrated that the circulating concentration of miR-126 in healthy individuals is high. With vascular damage, the concentration is reduced. This study aimed to examine the potential of miR-126 as a biomarker of acute vascular dysfunction in a human model of the disease, AAV. miR-126 was at a low level at disease presentation in ANCA vasculitis patients. After successful immunosuppression, treatment-induced remission was achieved and this increased the circulating concentration of miR-126 in the patient cohort, however, this was still not to the same level as the healthy baseline cohort. This may be representative of their continuing vascular damage as a result of the disease. miR-126 correlates with clinical measurements of vascular health, such as arterial stiffness. This suggests a role of miR-126 as a marker of vascular function. There was not a distinct relationship between other clinical measurements, consistent with its role as a marker of vascular health and not inflammation or renal function.

Chapter 4 – miR-126 is a marker of vascular dysfunction in chronic kidney disease

Introduction

CKD affects millions of people worldwide. Its incidence is growing exponentially, with the disease being responsible for more than 25 million deaths per year.[122] The incidence of cardiovascular events is markedly higher in CKD patients than for the general population.[123] CKD patients with stage 5 of the condition are five times more likely to develop CVD than the general population. ESRD is diagnosed when the patient has between 10 to 15% of renal function remaining and they have progressed to the most severe form of CKD, requiring maintenance HD, or kidney transplantation.

Currently, disease progression from CKD to ESRD, and the associated vascular disease, is monitored using parameters for renal and vascular function. The standard assessment of renal function involves measurement of serum creatinine concentration, blood urea, urinalysis and renal biopsy when indicated.[124] ESRD patients have chronic vascular dysfunction and arterial stiffness, as demonstrated by measurement of arterial PWV. Increased PWV, and therefore stiffer arteries, in comparison to healthy controls, has been identified as an independent predictor of cardiovascular mortality in this patient cohort.[125, 126] Studies have demonstrated that this is also the case for CKD patients, even with mild to moderate renal impairments.[127] Arterial stiffness increases with disease severity and CKD stage.[128, 129]

There is an unmet clinical need for biomarkers that specifically report vascular health in chronic renal disease to enhance early diagnosis, prognosis and monitor response to treatment. The optimal biomarker should be minimally invasive, sensitive, specific and stable. A significant emerging class of biomarker are miRs. They can be relatively organ-specific, and they can be used in a pre-clinical setting as well as being translated for use in patients because their sequence is conserved across species. Currently no known miRs are able to diagnose specific forms of renal disease.[130] Many studies have attempted to define appropriate biomarkers of renal injury in large patient cohorts. miR-126 has a crucial role in vasculature formation,[91, 131]

inflammation[132] and endothelial stress response.[92] It is enriched in endothelial cells and can be detected in body fluid samples in humans and other species.

Aims and hypothesis

The hypothesis for this chapter is that miR-126 will be at a low circulating level in CKD. For patients with ESRD, miR-126 will be at a lower level than moderate CKD. This would reflect the vascular damage for each patient group. This study aims to assess the circulating levels of miR-126 in healthy controls, patients with CKD and ESRD. miR-126 will be measured in ESRD patients before and after a session of haemodialysis to examine the effect on miR levels. This will be evaluated in comparison to clinical data obtained as measures of vascular and renal health.

Methods

Patients

For this study, 105 individuals were recruited. This included 60 healthy volunteers (as described in Chapter 3), 30 patients with moderate CKD and 15 patients with stable ESRD on maintenance HD from the outpatient dialysis unit at the Royal Infirmary of Edinburgh, UK. The study was approved by the local research ethics committee and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants.

ESRD patients

ESRD patient samples were taken from a previously published study[133]. The inclusion criteria for ESRD patients were that they were age 18 years or over and being treated with HD for more than 3 months. All of the included patients were treated with HD three times per week for 4-5 hours per session. The data collected included patient demographics, cause of ESRD, duration of dialysis and current medications.

CKD patients

Patients with CKD stages I–V (estimated glomerular filtration rate 7–57 ml/min/1.73m^2 calculated using the MDRD equation) were included. Briefly, subjects were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh. The inclusion criteria were male or female CKD patients age 18 years or over with an eGFR of <60 and not undergoing dialysis treatment. Patients were excluded with a renal transplant or patients with systemic vasculitis.

Blood collection

In healthy subjects and CKD patients, blood was collected in EDTA plasma tubes and centrifuged at 1,100 x *g* for 15 min at 4°C and then immediately frozen at -80°C. Two blood plasma samples were collected from each HD

patient, one immediately before and one after a single dialysis session, directly from dialysis needles and processed the same as the healthy and CKD samples.

Statistical analysis

For paired samples, a Wilcoxon test was used and for unpaired samples, a Mann-Whitney test. For correlations, normality tests were performed in order to establish the distribution, followed by either a Pearson or Spearman test for the significance of the correlation.

Results

Patient characteristics

A cohort of 30 male and female patients with varying degrees of CKD and 15 age-matched patients with ESRD undergoing maintenance HD were recruited into this study. The patient's demographic characteristics are shown in Table 8. There was some variation between groups for gender. Kidney and liver function data are presented with more severe disease in the ESRD (pre-HD) group, which conforms to the expected disease phenotype.

Characteristics	CKD (*n* = 30)	Pre-HD (*n* = 15)	Post-HD (*n* = 15)
Age, years	59.8 ± 15.6 (26 - 82)	58.9 ± 12.14 (34 - 82)	-
Sex, M/F	21/9	8/7	-
eGFR, mL/min/1.73m^2	24.48 ± 14.12 (7 - 57)	8.00 ± 2.89 (2 - 12)	28.15 ± 10.74 (13 - 53)
Serum creatinine, μmol/L	216.97 ± 133.01 (76 - 654)	660.46 ± 199.15 (360 - 1067)	225.00 ± 79.98 (97 - 426)
ALT, U/L	19.48 ± 8.44 (9 to 50)	14.54 ± 6.12 (7 - 25)	-
ALP, U/L	97.24 ± 37.82 (40 - 182)	100.39 ± 42.66 (52 to 178)	-
CRP, mg/L	5.25 ± 2.36 (2 - 7)	14.44 ± 20.68 (2 to 77)	-
Haemoglobin, g/L	123 ± 16 (83 - 160)	122 ± 12.51 (94 - 139)	-

Table 8. Clinical data obtained for CKD patients and ESRD patients pre- and post-haemodialysis (HD).
The data are shown as mean ± SD with range.

Circulating miR-126 in patients with CKD

Plasma samples were obtained from healthy volunteers and patients with renal disease. In the patient samples, the concentration of miR-126 was measured (Figure 14). A baseline for healthy volunteers was obtained from individuals without renal dysfunction or cardiovascular disease. For patients with moderate CKD, the mean was lower than for healthy volunteers. In ESRD patients, the circulating concentration of miR-126 was substantially lower than in moderate CKD and health. The concentration increased after a single session of maintenance haemodialysis. Even after dialysis, the concentration of miR-126 did not increase to the same concentration as moderate CKD or health and a significant difference remained.

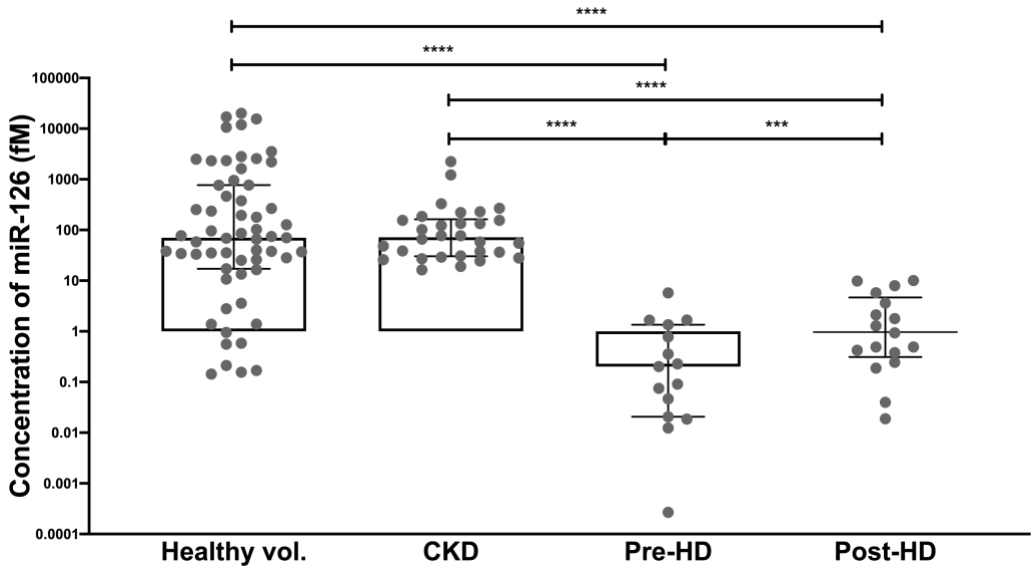
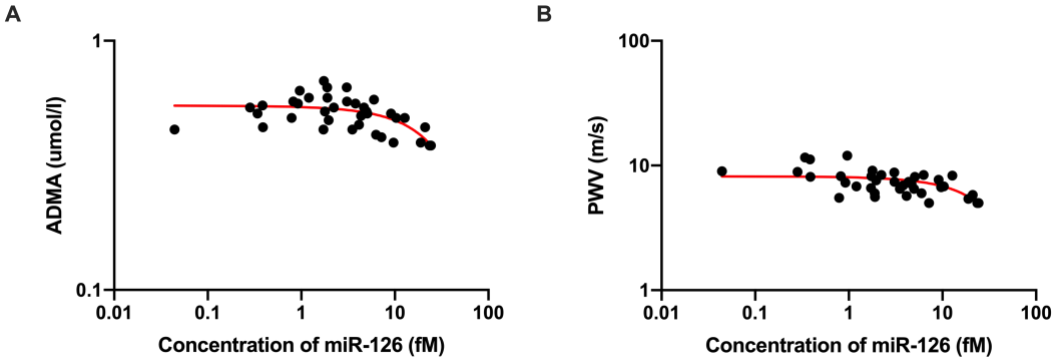


Figure 14. Circulating miR-126 in chronic kidney disease and ESRD.
*The concentration of miR-126 in healthy volunteers (n = 60), CKD patients (n = 30) and ESRD patients (n = 15) pre- and post-haemodialysis (HD). Bars show median with IQR, ***p<0.001, ****p<0.0001 (Mann-Whitney test for unpaired analysis and Wilcoxon test for paired analysis).*

Clinical measures of vascular health correlate with circulating miR-126 in moderate CKD

Clinically, measuring vascular health is an essential parameter to determine a patient's overall cardiovascular risk. For patients with CKD, there was a range of plasma miR concentrations, which may reflect differences in vascular health across patients**.** miR concentrations were correlated against a range of well-

recognised measures of vascular function**.** Consistent with the data from patients with ANCA vasculitis, miR-126 correlated strongly with PWV (Figure 15). In addition, miR-126 associated with circulating asymmetric dimethylarginine (ADMA). Low levels of these markers suggest vascular health, and miR-126 is higher in an inverse relationship.



C

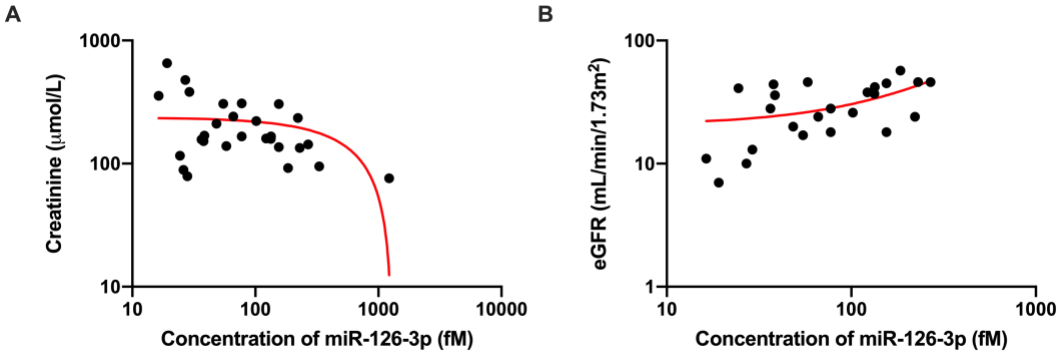
	ADMA	PWV
Analysis	Pearson	Spearman
P-value	0.0002	0.0005
r	-0.58	-0.54
95% CI	-0.76 to -0.31	-0.74 to -0.26
R squared	0.33	-
Number of pairs	37	37

Figure 15. Clinical measures of cardiovascular health correlate with circulating miR-126 in patients with moderate CKD.
Red line shows the linear regression for correlations. (A) ADMA and (B) PWV inversely correlated with miR-126 in CKD patients. (C) Correlation analysis for ADMA and PWV based on the normality of the data.

Circulating miR-126 and clinical measures of renal function

After establishing the circulating concentration of miR-126 in renal patients and healthy volunteers, I examined the relationship between currently used clinical parameters for renal function in patients with CKD. In the CKD patient cohort, renal function was recorded at the clinic on the day that the plasma sample was taken for this study. The relationship between circulating miR-126 and currently used measures of renal function was examined (Figure 16). There

was no correlation between creatinine and miR-126, however, eGFR had a positive relationship. For higher levels of eGFR (a marker of renal function), miR-126 was at a higher level than for patients with reduced renal function.



C

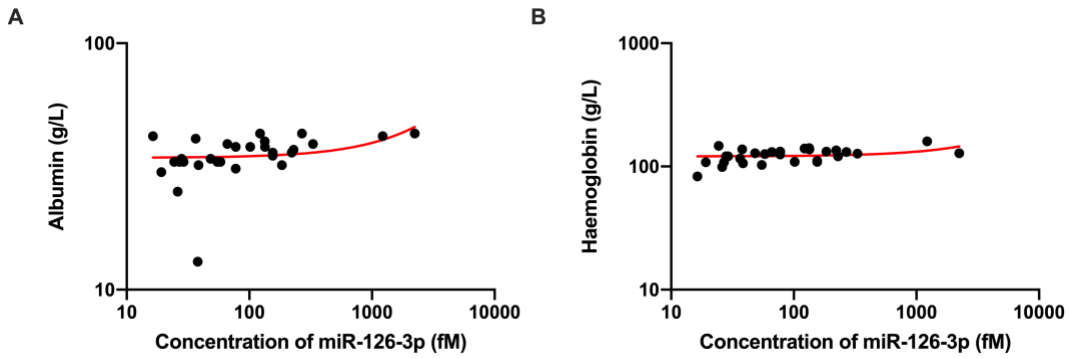
	Creatinine	eGFR
Analysis	Spearman	Pearson
P-value	0.07	0.01
r	-0.35	0.52
95% CI	-0.65 to 0.04	0.15 to 0.77
R squared	-	0.27
Number of pairs	28	24

Figure 16. Clinical measures of human renal function in patients with moderate CKD in relation to miR-126.
Red line shows the linear regression for correlations. (A) Creatinine does not demonstrate a clear relationship with miR-126 however, (B) eGFR correlates with miR-126 in CKD patients. (C) Correlation analysis for creatinine and eGFR based on the normality of the data.

miR-126 as a marker of systemic inflammation in CKD

Clinically, markers of systemic inflammation are measured in patients with CKD. To determine whether miR-126 was a marker of inflammation in this patient cohort, the clinical results were compared with the miR-126 concentration (Figure 17). Albumin is a commonly measured parameter for systemic inflammation as inflammation reduces its rate of synthesis[134]. Albumin is also lost in nephrotic syndrome and produced less in liver disease and malnutrition. It had a positive correlation with miR-126 in CKD patients.

Conversely, there was no correlation between miR-126 and haemoglobin, a marker which can be reduced in inflammation.



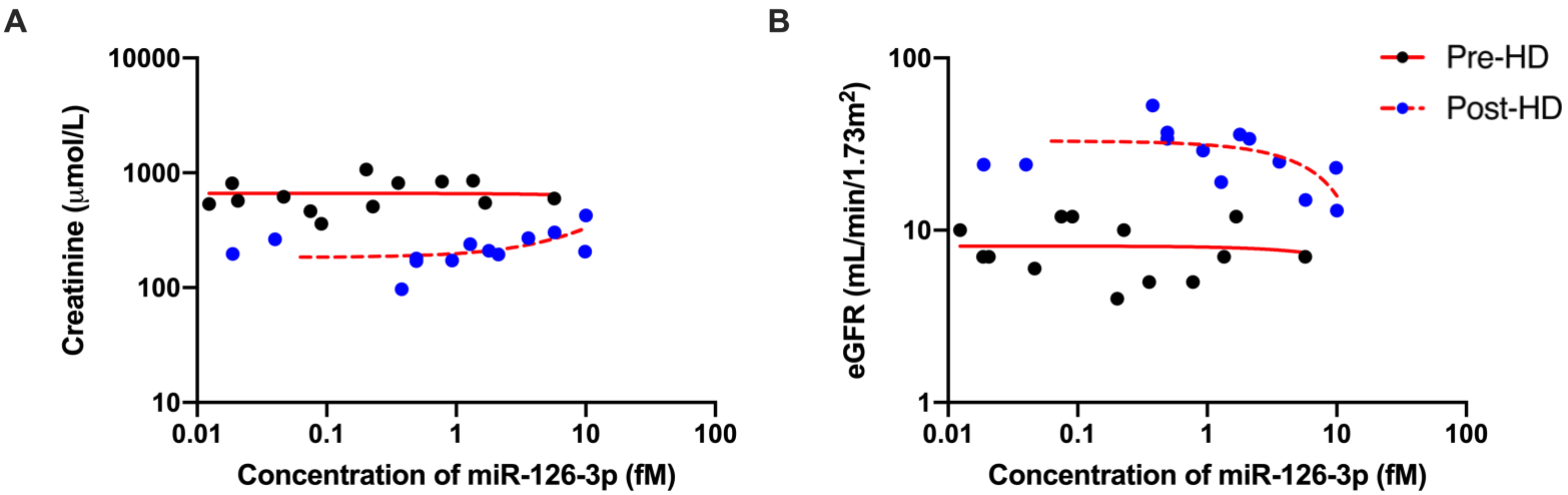
C

	Albumin	Haemoglobin
Analysis	Spearman	Pearson
P-value	0.004	0.11
r	0.51	0.30
95% CI	0.17 to 0.75	-0.07 to 0.60
R squared	-	0.09
Number of pairs	29	29

Figure 17. Inflammation markers in circulation for patients with moderate CKD in relation to miR-126.
Red line shows the linear regression for correlations. (A) Albumin correlates with miR-126, however, (B) haemoglobin does not demonstrate a clear relationship with miR-126 in CKD patients. (C) Correlation analysis for albumin and haemoglobin based on the normality of the data.

miR-126 as a marker of renal function in ESRD

By definition, patients undergoing HD for ESRD have significantly impaired renal function in comparison to a healthy cohort and patients with moderate CKD. Two routinely used markers for renal function, creatinine and eGFR were evaluated with miR-126 pre- and post-HD (Figure 18). Creatinine levels did not correlate with miR-126 concentration pre-HD, however, there was a trend towards a relationship for post-HD. These results are similar for eGFR with no correlation pre-HD but an inverse relationship post-HD.



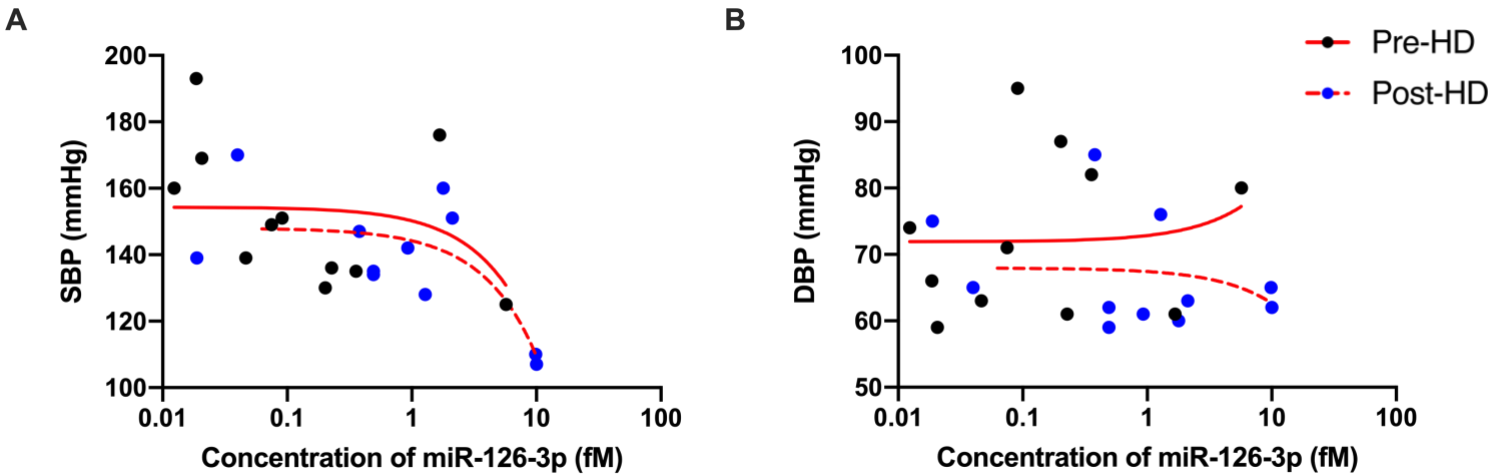
C

	Creatinine Pre-HD	Creatinine Post-HD	eGFR Pre-HD	eGFR Post-HD
Analysis	Pearson	Spearman	Pearson	Pearson
P-value	0.93	0.04	0.83	0.04
r	-0.03	0.57	-0.07	-0.57
95% CI	-0.57 to 0.53	0.01 to 0.86	-0.60 to 0.50	-0.85 to -0.02
R squared	0.001	-	0.004	0.32
Number of pairs	13	13	13	13

Figure 18. Circulating miR-126 in relation to clinical measures of renal function in patients undergoing maintenance HD for ESRD.
The solid red line shows the linear regression pre-HD and dotted line for post-HD. Individual patient data points are shown in black for pre-HD and blue for post-HD. Pre-HD, (A) creatinine and (B) eGFR do not demonstrate a clear relationship with miR-126, however, there is a positive correlation for creatinine and inverse correlation for eGFR post-HD in CKD patients. (C) Correlation analysis for creatinine and eGFR based on the normality of the data.

Blood pressure is altered with HD treatment in ESRD patients

It is well established that hypertension is considered an independent risk factor for CKD progression to ESRD. Previous studies have attempted to examine the role of the endothelium in vascular dysfunction with hypertension. As patients with impaired renal function are likely to have issues with blood pressure regulation, we compared ESRD patient's blood pressure immediately before the HD session and after it (Figure 19). This was compared against the concentration of miR-126 in the circulation. For systolic blood pressure (SBP), there was no correlation pre-HD, however, there was a notable inverse correlation post-HD in matched patients. Conversely, there was no correlation for miR-126 pre- or post- HD for diastolic blood pressure (DBP).



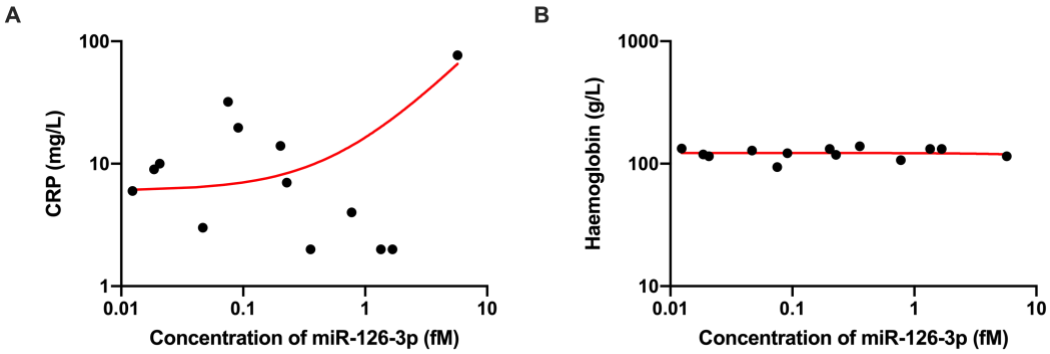
C

	SBP Pre-HD	SBP Post-HD	DBP Pre-HD	DBP Post-HD
Analysis	Pearson	Pearson	Pearson	Pearson
P-value	0.32	0.007	0.70	0.48
r	-0.33	-0.76	0.13	-0.24
95% CI	-0.78 to 0.33	-0.93 to -0.29	-0.51 to 0.68	-0.73 to 0.42
R squared	0.11	0.57	0.02	0.06
Number of pairs	11	11	11	11

Figure 19. Circulating miR-126 in relation to blood pressure measurements in patients undergoing maintenance HD for ESRD.
The solid red line shows the linear regression pre-HD and dotted line for post-HD. Individual patient data points are shown in black for pre-HD and blue for post-HD. (A) SBP does not correlate pre-HD, however, there is an inverse relationship following the treatment. (B) DBP does not demonstrate a clear relationship with miR-126 pre- or post-HD. (C) Correlation analysis for SBP and DBP based on the normality of the data.

miR-126 is not a marker of systemic inflammation in ESRD

Inflammatory markers are routinely measured in ESRD patients undergoing maintenance HD. We compared two of these markers, before the HD session, with the plasma concentration of miR-126 (Figure 20). CRP is a routinely measured marker of chronic inflammation and this did not show a correlation with miR-126. Similarly, haemoglobin, a marker of systemic inflammation, did not correlate in the ESRD patient cohort.



C

	CRP	Haemoglobin
Analysis	Spearman	Pearson
P-value	0.50	0.82
r	-0.20	-0.069
95% CI	-0.69 to 0.41	-0.60 to 0.50
R squared	-	0.005
Number of pairs	13	13

Figure 20. Inflammation markers in circulation for patients with ESRD undergoing maintenance HD in relation to circulating miR-126.
Red line shows the linear regression for correlations. (A) CRP and (B) haemoglobin do not correlate with miR-126 in ESRD patients. (C) Correlation analysis for CRP and haemoglobin based on the normality of the data.

Liver enriched miRs are downregulated in ESRD

In order to determine whether the increase in miR concentration after dialysis was specific to endothelial cell-enriched miRs, a well characterised liver specific miR was measured (Figure 21). miR-122 was measured in the same patient cohorts as described above. Healthy volunteers and CKD patients had

comparable levels of circulating miR-122, however, this was reduced in ESRD pre-HD. With paired samples, post-HD, the concentration of miR-122 significantly increased.

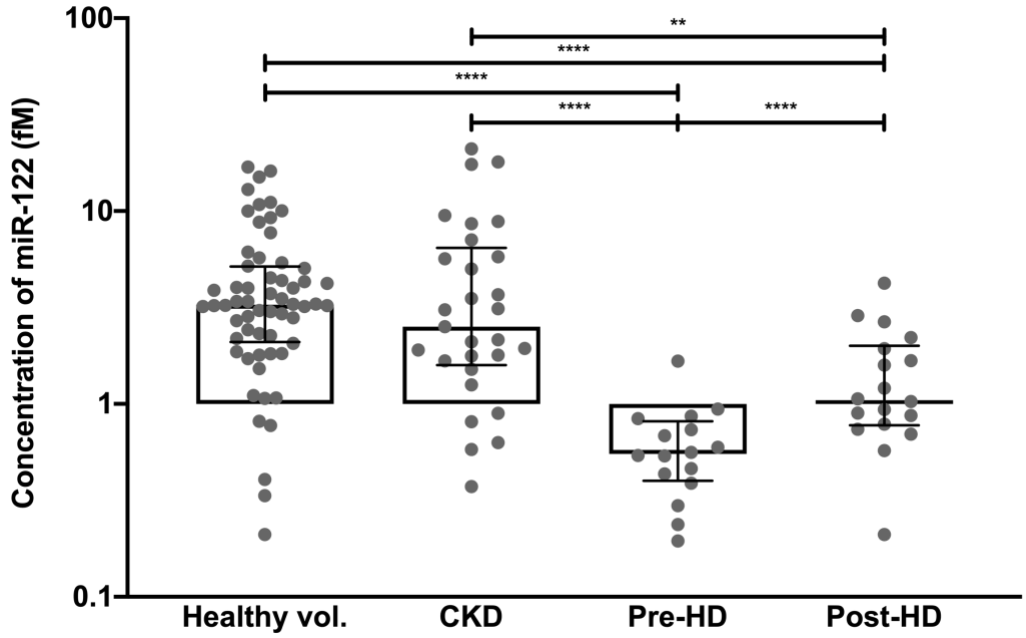


Figure 21. Circulating miR-122, a marker of liver dysfunction in health, CKD and ESRD.
*The concentration of miR-122 was lower in ESRD and increases with HD. miR-122 was measured in plasma samples in healthy volunteers (n = 60), CKD patients (n = 30) and ESRD patients (n = 15) pre- and post-HD. Bars show median with IQR, **p<0.01, ****p<0.0001 (Mann-Whitney test for unpaired analysis and Wilcoxon test for paired analysis).*

Discussion

Endothelial dysfunction is often a precursor for future cardiovascular events, therefore determining an appropriate biomarker to track this is essential. This study demonstrated that circulating miR-126 is highest in healthy individuals and is lower in patients with reduced renal function. In ESRD, miR-126 is lower than for moderate CKD, and the concentration increases following maintenance HD, therefore a reflection of the patient's vascular phenotype. This corresponds with gold-standard measures of vascular health, including PWV and ADMA. This suggests that miR-126 has a potential role in reporting vascular health and injury in human cohorts with chronic vascular dysfunction, including CKD and ESRD. In contrast, there was not a clear relationship implicating miR-126 as a biomarker of inflammation, blood pressure or the functional capacity of the kidneys.

Clinical importance of miR-126 as a biomarker of vascular integrity

Previous studies have suggested that HD will reduce the concentration of certain miR species. Potentially, this could be a consequence of miR clearance in the dialysate fluid.[135] However, in patients with CKD, the total circulating miR concentration is reduced.[133] This has previously been demonstrated by Neal. et al. They concluded that this was due to a global reduction in circulating miRs as a result of increased RNase activity in CKD patient's blood.[136] The findings of this study are consistent with this as miR-126 and liver enriched miR-122 were both reduced pre-HD and increased after treatment. Further studies to directly measure the RNAse activity in the samples would allow for additional conclusions to be drawn about this hypothesis.

Recent studies have demonstrated that miR-126 is at a lower level in circulation in CKD patients.[97, 137] After HD, the concentration of miR-126 increases which could suggest an improvement in vascular health, or alternatively, acute vascular damage caused by the HD procedure. Haemodialysis is an invasive procedure which will cause localised endothelial

cell disruption. A study by Kosch et al. detailed the effect of HD on acute vascular damage in a cohort of ESRD patients.[138] They demonstrated that haemodialysis did not have a direct effect on endothelial function acutely. This, therefore, suggests that the increase in miR-126 after maintenance HD could potentially be the result of improved vascular function.

This study suggests that in moderate CKD, miR-126 inversely correlates with a currently used clinical measure of vascular integrity and cardiovascular risk, ADMA. ADMA is an inhibitor of NO synthase and subsequent reduction in NO availability.[139, 140] Clearance of ADMA during haemodialysis may have a beneficial role in vascular health by reducing the inhibition of NO synthase, and thus improve vascular integrity.[138]

Additionally, this study demonstrated that miR-126 inversely correlated with PWV, the gold standard measure of arterial stiffness. This is an independent predictor of cardiovascular mortality in mild to moderate CKD.[127] Arterial stiffness increases as CKD progresses in severity and stage.[128, 129] This supports the potential for miR-126 to act as a biomarker in patients with impaired renal function and chronic vascular damage.

Renal function was measured in patients with CKD and ESRD. For creatinine, a measurement of the kidney's filtration efficiency, there was not a detectable correlation with miR-126. Interestingly, the CKD cohort exhibited a correlation with miR-126 and eGFR. eGFR is calculated using the MDRD equation, which is calculated based on the serum creatinine concentration, age, sex and ethnicity. This suggests that there are perhaps demographic factors which may influence the concentration of miR-126 in patient cohorts. ESRD patients had inverted results in comparison to CKD patients for renal function. This is indicative of the patient's diminished renal function for this extreme phenotype of renal and vascular dysfunction.

This study did not find any definitive relationships between the concentration of miR-126 and general measures of patient health. For inflammation, there was some correlation between albumin and miR-126, however, this was not apparent for haemoglobin in CKD and there was not a correlation for haemoglobin or CRP in ESRD, pre- or post-HD. This suggests a degree of specificity for reporting vascular health and injury, rather than a general marker of chronic inflammation or disease.

Limitations

The results of this study suggest that miR-126 potentially has a role as a biomarker of vascular integrity. It shows an inverse correlation with clinical markers of vascular health. A limitation of this study is the absence of PWV and ADMA measurements for the ESRD groups. By obtaining these data, it would be possible to link the vascular health of patients with CKD and ESRD, and the subsequent changes in vascular health following HD. This would allow for a direct comparison of disease severity, and stage, in relation to the obtained miR-126 correlations.

In addition, as this study was focused on clinical samples from patients, there is a high level of variation, due to the heterogeneity of the disease. By increasing the size of the patient cohorts, it would be possible to define a baseline measurement for healthy controls, patients with moderate CKD and ESRD.

Conclusions

In health, miR-126 is at a high level and with vascular injury, there is a reduced concentration in circulating blood. In this study, we evaluated this in patients with moderate CKD and those with ESRD undergoing maintenance HD. miR-126 is lower in CKD patients in comparison to health and this is further reduced in patients with ESRD. After a single session of HD, the concentration of miR-126 increases, however, this is not to a near health level, reflecting their vascular health. miR-126 correlates with clinical measurements of vascular health, such as arterial stiffness. For CKD and ESRD patients, there is no clear relationship between other clinical measurements, confirming its role as a marker of vascular health and not inflammation or renal function.

Chapter 5 – Small RNA sequencing in ANCA vasculitis

Introduction

Studies have demonstrated roles for miRs in the pathogenesis of multiple cardiovascular diseases.[141] Circulating miRs are potentially a reservoir for biomarker discovery as they provide insights into the complex regulatory functions of the tissue of origin.[81] They are considered stable in circulation as they are able to avoid degradation by endogenous RNase activity.[85] Due to miRs role in the negative interference of post-transcriptional gene regulation, they have potential as novel biomarkers for health and disease, as well as therapeutic targets.[142, 143]

Many studies have reported the importance of miRNome small RNA sequencing to track disease progression and treatment monitoring.[144, 145] Technologies such as small RNA sequencing have allowed for the screening and discovery of a large amount of differentially expressed miRs that are involved in the development of cardiovascular disease.[146, 147] From this, it is possible to identify and target these miRs for development of novel biomarkers of disease.

ANCA vasculitis is a term used to describe a spectrum of conditions.[148] These include GPA (formerly known as Wegener's granulomatosis), MPA and EGPA (formerly known as Churg-Strauss syndrome). The disease manifestations of GPA and MPA are variable at presentation but both primarily cause small vessel inflammation and damage. There is a high degree of variation in terms of disease severity in vasculitis. Substantial disease heterogeneity exists which has implications for treatment decisions. This is a key factor for determining the appropriate strategies for initiation and maintenance of treatment. The disease state is altered by disease severity, clinical manifestations, ANCA specificity and patient factors.

As ANCA vasculitis is a rare and complex condition, the aim of this study was to identify novel markers of the disease. This would build on the miR-126 findings reporting vascular dysfunction and examine the miRNome as a whole.

In doing this, we sought to determine a novel miR biomarker at disease initiation as well as using the marker to track improvement with therapeutic interventions. In addition, this biomarker could be used to monitor the patients in order to identify those at risk of disease relapse.

Aims and hypothesis

The hypothesis for this study was that, when patients with active disease are compared to when they are in remission, differentially expressed miRs will be identified by small RNA sequencing.

Methods

Sample preparation

The study was approved by the local research ethics committee and performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants. For this study, 15 patients with AAV were recruited for paired small RNAseq analysis. Patients were recruited from the Vasculitis Service at the Royal Infirmary of Edinburgh, UK. Patients who presented with sero-positivity for ANCA vasculitis were included. Plasma samples were used from patients at entry into the study and at a subsequent follow up visit when the condition was considered to be at the remission stage, as defined by the BVAS scale, after successful treatment.

Samples were taken and stored immediately at -80^{o}C after being spun in a workbench centrifuge at 1,100 x *g* for 10 minutes at 4^{o}C. Clinical data were obtained by clinical staff at the Edinburgh Renal Unit at the Royal Infirmary, Edinburgh.

Power of RNAseq study

ANCA vasculitis is a clinically complex disease with a spectrum of disease severity and tissues targeted by inflammatory cells. This study aimed to determine a biomarker of disease remission. Initially, a power calculation was performed to determine the number of paired samples required for the study (Figure 22). We used our existing small RNA sequencing data from human serum to model the sample size effect on the ability to obtain 80% power corrected for false discovery.[149] Across a range of sample sizes there was an inflection point at n = 15 (around 50% of genes discovered with 80% power).

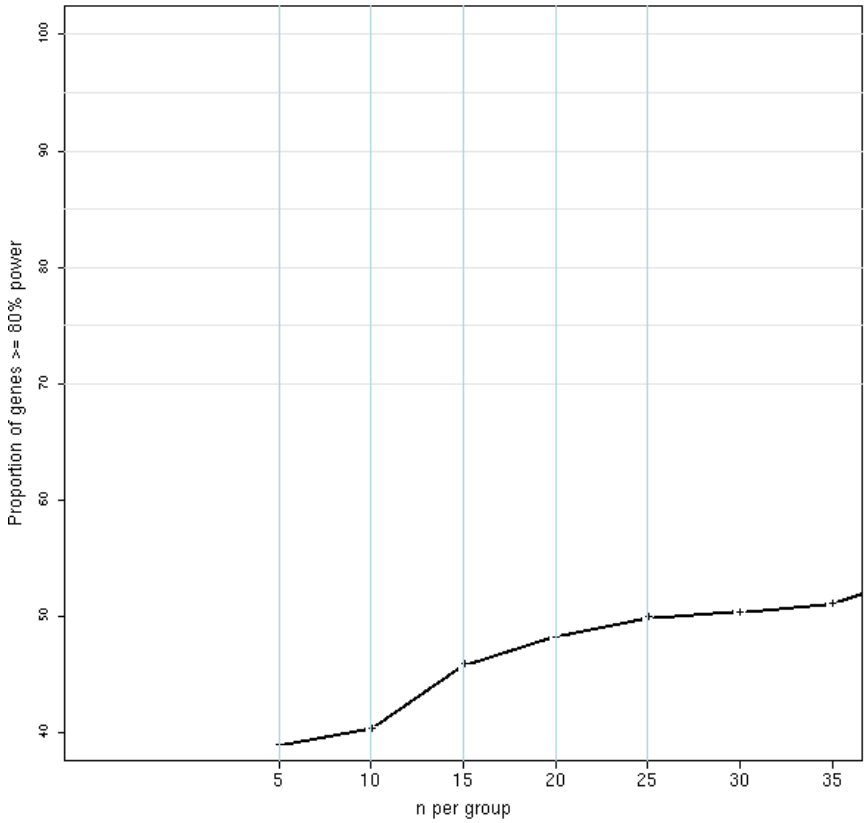


Figure 22. Small RNAseq power calculations to achieve 80% power.
The plot shows the proportion of genes ≥80% power versus n per group.

Sample sequencing

Small RNA sequencing was performed by BGI Genomics (Hong Kong) using the BGISEQ-500 system. Small RNA encompasses all RNA molecules that are less than 200 nucleotides long. The system used captured small RNA and miR species from the plasma samples. Using the BGISEQ-500 platform, at least 20 million clean reads per sample were generated. Samples were sequenced to produce 50 base pair long sequences. They were then processed initially by BGI to remove the primer sequences. To produce sufficiently large datasets, some of the patient samples required multiple sequencing attempts.

RNA sequencing analysis

Initial RNAseq analysis was performed by Dr Al Ivens. The raw sequences were pre-processed and assessed for quality using FASTQC to ensure that

there were not any recognised primers present. The sequences were then aligned to version hg19 of the Homo sapiens genome (obtained from Ensembl). Only FLPM alignments to the reference genome were assessed for the initial analysis. Alignments to the reference genome were performed using bowtie (version 0.12.5) and miRDeep2 (version 2.0.0.4) software. FLPM sequences were used for miRDeep2 analysis. Raw aligning sequences were obtained for 1335 different mature miRs. The raw counts data were then filtered to include only sequences with an average of ≥ 5 reads (836 loci).

Pairwise comparisons of pre-treatment and post-treatment was performed on the normalised tag counts using linear modelling and differences were identified as the fold change in the group comparison. Significance values were controlled for false discovery, yielding a more rigorous adjusted P value.

PCR validation of sequencing results

Total RNA was extracted from 50μl of each patient sample using the miRNeasy Serum/Plasma kit (Qiagen) according to the manufacturer's instructions and detailed in Chapter 2.

The RNA from patient samples was then used to generate cDNA with the miScript II Reverse Transcription kit (Qiagen) according to the manufacturer's instructions and detailed in Chapter 2.

Real-time quantification was performed using the SYBR green miRNA kit (Qiagen) using a Lightcycler 480 machine (Roche). Details of the pre-designed miScript Primer Assays (Qiagen) are described in Table 9.

Primer	Mature miR target	Catalogue number	Sequence
Hs_miR-4732-5p_1	hsa-miR-4732-5p	MS00037506	5'UGUAGAGCAGGGAGCAGGAAGCU
Hs_miR-181c*_1	hsa-miR-181c-3p	MS00008848	5'AACCAUCGACCGUUGAGUGGAC
Hs_miR-3150_1	hsa-miR-3150a-3p	MS00020678	5'CUGGGGAGAUCCUCGAGGUUGG
Hs_miR-375_2	hsa-miR-375	MS00031829	5'UUUGUUCGUUCGGCUCGCGUGA
Hs_miR-483-5p_1	hsa-miR-483-5p	MS00009758	5'AAGACGGGAGGAAAGAAGGGAG
Hs_miR-3192_2	hsa-miR-3192-5p	MS00041895	5'UCUGGGAGGUUGUAGCAGUGGAA
Hs_let-7b_1	hsa-let-7b-5p	MS00003122	5'UGAGGUAGUAGGUUGUGUGGUU
Hs_miR-365_2	hsa-miR-365a-3p	MS00031801	5'UAAUGCCCCUAAAAAUCCUUAU

Table 9. miScript Primer Assays used for qRT-PCR validation of miR expression changes in vasculitis patients pre- and post-treatment.

Results

Patient characteristics

For this study, male and female patients were recruited at disease presentation with AAV and subsequently followed up (*n* = 15 paired). The patients selected were confirmed to have AAV, well-defined renal involvement and an adequate volume of plasma for sequencing. Plasma samples were sequenced to identify small RNAs in active disease and once in treatment-induced remission to identify markers of the disease progression and determine the patient's remission status. The patient's demographic characteristics are shown in Table 10.

Characteristics	Pre-treatment (*n* = 15)	Post-treatment (*n* = 15)	P value
Age, years	67 ± 10 (53 - 81)	-	-
Sex, M/F	8/7	-	-
Organs involved	3 ± 1 (1 - 6)	-	-
- Kidney	15	-	-
- Lung	11	-	-
- ENT	6	-	-
- Nerve	4	-	-
- Eyes	5	-	-
eGFR, mL/min/1.73m^2	31 ± 22 (8 - 92)	44 ± 23 (22 - 108)	<0.0001
Serum creatinine, μmol/L	233 ± 141 (81 - 605)	146 ± 55 (70 - 257)	0.002
ALT, U/L	24 ± 22 (6 - 83)	17 ± 7 (5 - 32)	0.25
ALP, U/L	127 ± 77 (52 - 338)	66 ± 15 (39 - 103)	0.007
GGT, U/L	67 ± 60 (14 - 182)	21 ± 5 (11 - 28)	0.02
Albumin, g/L	25 ± 7 (17 - 39)	36 ± 4 (27 - 41)	<0.0001
CRP, mg/L	114 ± 86 (11 - 275)	4 ± 4 (0 - 12)	0.0002
Haemoglobin, g/L	90 ± 18 (62 - 125)	123 ± 11 (99 - 143)	<0.0001

Table 10. Clinical demographics for vasculitis patients pre- and post-treatment that were included in RNAseq analysis.
The data are shown as mean ± SD with range. Significance of numerical data between groups was ascertained using a 2-tailed paired t-test.

RNAseq quality control

Quality control was conducted on the 15 paired samples following small RNA sequencing. Figure 23 shows the boxplots for the 836 miRs with ≥ 5 reads in each sample. This illustrates all samples scaled down to have an identical number of reads aligning to miRs. It also shows the quantile normalised for the samples to normalise the distributions between patient samples. Due to abnormal boxplot distributions, five paired samples were removed from further analyses, resulting in ten paired patient samples. As this study was designed as paired analysis, if either the pre- or post-treatment sample distribution was abnormal, both samples were removed from further analysis. This allowed the group membership balance to be maintained for group wise interpretation. The paired patient samples removed at this stage were AN, AP, JS, PM and VT.

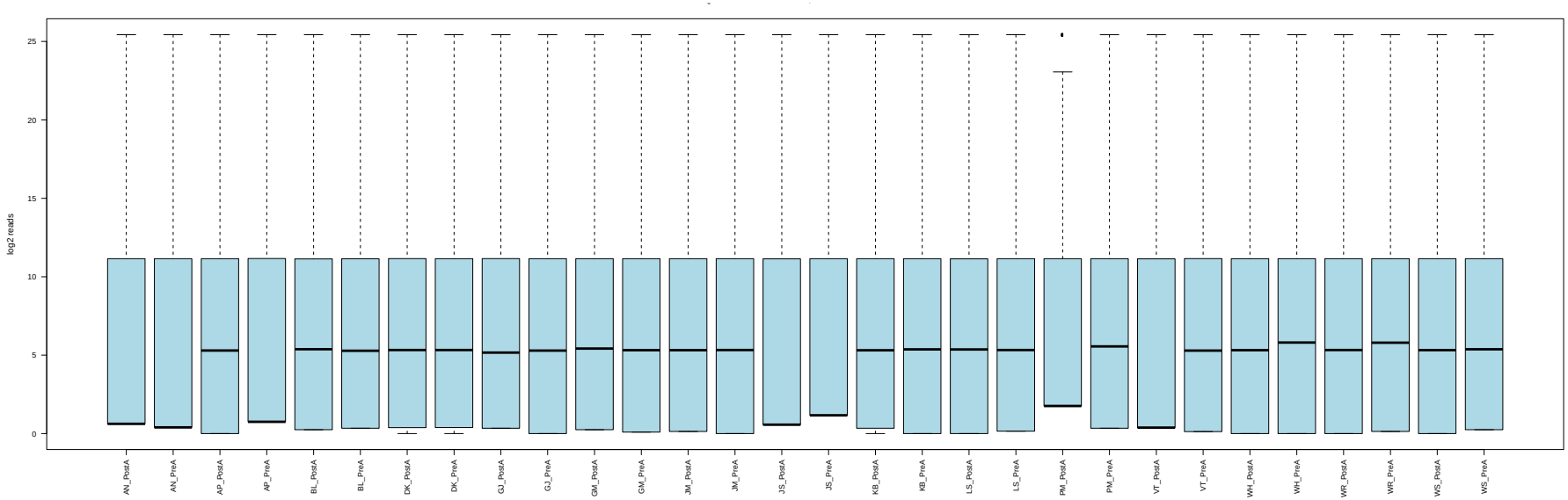


Figure 23. Quantile normalised boxplots for the 836 miRs with an average of ≥ 5 reads.
The x-axis shows the patient samples (pre- and post-treatment) and the y-axis shows the log2 reads for each sample (n = 15 paired).

Analysis of individual comparisons by RNAseq

Following quality control for the patient samples, further analysis was conducted to determine the degree of relatedness for the remaining paired samples (*n* = 10 paired). To do this, principal component analysis (PCA) was performed (Figure 24). This demonstrated that there was low level of similarity between the cohorts. The individual patient samples for pre- and post-treatment were more closely related overall, which suggested that it would be more appropriate to measure miR expression in individual samples, rather than treatment cohorts.

Further analysis of the individual patient's miR expression was conducted. A heatmap was produced to illustrate the miR expression hits for post- relative to pre-treatment (Figure 25). The analysis from the 10 paired patient samples showed that there were not any significantly differentially expressed mature miR markers of vascular health and disease in this cohort. There was a high level of variation within the sequenced group and this was illustrated in the heatmap of the results. There was not a consistent miR change.

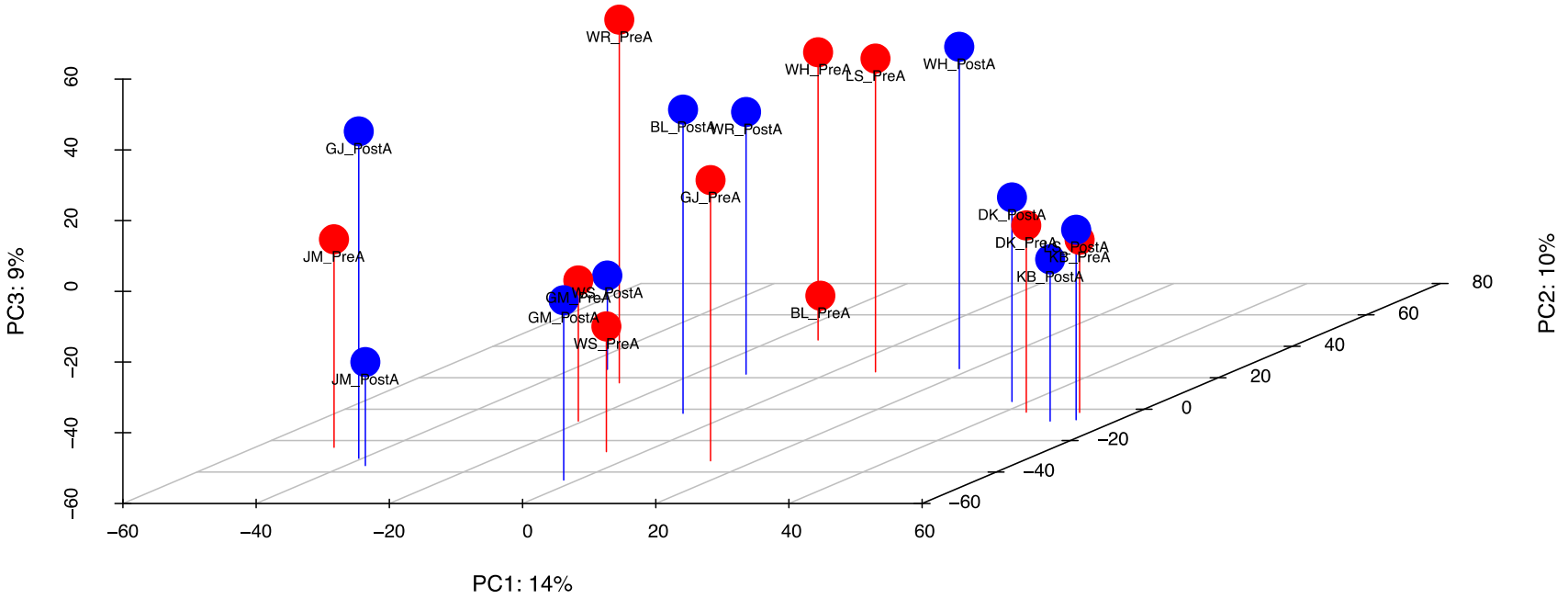


Figure 24. Principal component analysis of the relatedness of the pre and post-treatment samples from RNAseq.
The axes represent the genetic variability of the samples which were sequenced. Pre-treatment samples are shown in red and post-treatment are shown in blue. The axes represent the degree of variability between samples.

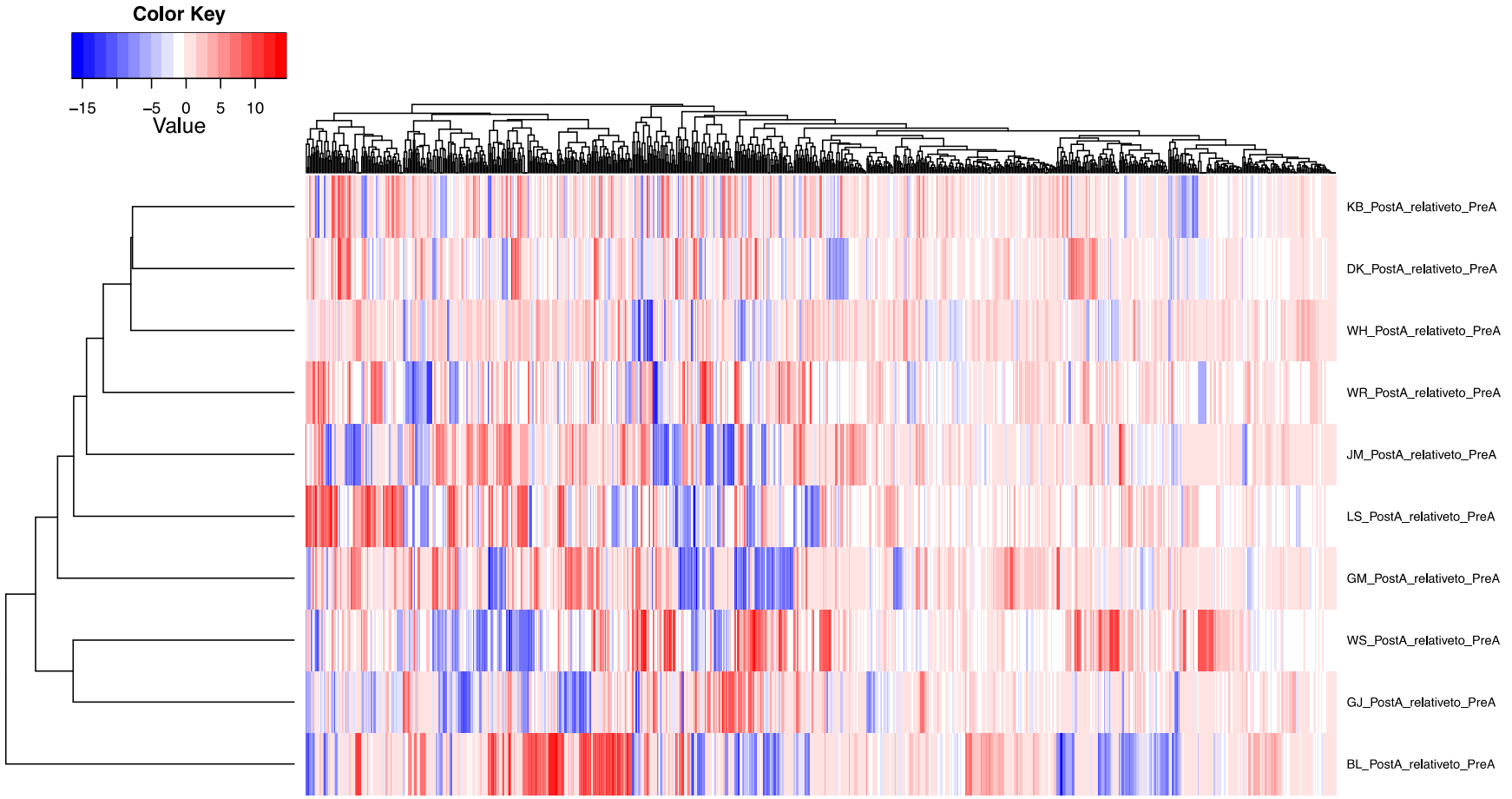


Figure 25. Differential expression changes post-treatment relative to pre.
The x-axis represents the 836 mature miRs identified with RNAseq and the y-axis represents the paired cohort comparison between the treatment groups for each individual patient.

Summary of raw data

In total, 836 mature miRs were identified in the pre- and post-treatment paired samples. Circulating miRs post- relative to pre-treatment were analysed for variation in their fold change between the two disease states (Figure 26). For the fold change profile, without statistically filtering the data, 453 miRs were upregulated post-treatment and 383 miRs were downregulated. For >2-fold change, 129 miRs were upregulated and 124 were downregulated.

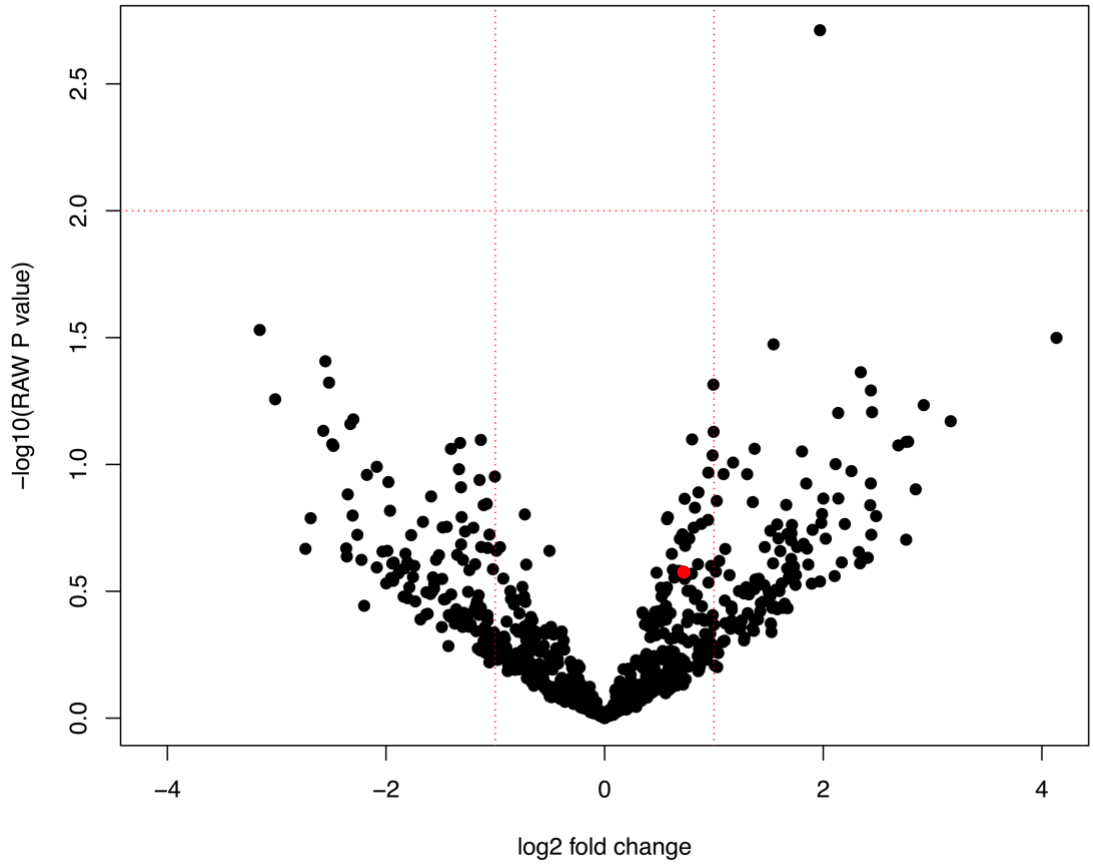


Figure 26. Volcano plot of group comparisons post-treatment relative to pre-treatment.
The plot shows -log(10) raw P value versus log2 fold change for the group comparison. Red lines transecting the x- and y- axis demonstrate ± 1-fold change and P-value 0.05 respectively. The red dot illustrates the expression of miR-126 in this analysis.

The 9 miRs exhibiting the largest differential expression changes between the two groups are shown in Table 11. The 9 mature miRs were statistically significant (5 up-regulated and 4 down-regulated). Within the significant features, hsa-let-7b, hsa-mir-3150a, hsa-mir-3192, hsa-mir-375 and hsa-mir-

4732 were up- regulated. Within the significant features, hsa-mir-181c, hsa-mir-365a and hsa-mir-483 were down-regulated.

Mature miR	Log fold change	Raw P value	Average expression
hsa-let-7b-5p	1.0	0.05	22
hsa-miR-181c-3p	-3.2	0.03	4.8
hsa-miR-3150a-3p	4.1	0.03	3.7
hsa-miR-3192-5p	2.3	0.04	1.6
hsa-miR-365a-3p	-2.5	0.05	2.9
hsa-miR-365b-3p	-2.5	0.05	2.9
hsa-miR-375	1.5	0.03	16
hsa-miR-4732-5p	2.0	0.002	11
hsa-miR-483-5p	-2.6	0.04	13

Table 11. Circulating miRs exhibiting the largest differential expression (fold change) in the post- relative to pre-treatment groups.
The average expression is the average log2 signal intensity of the probe set across all arrays, as a method to normalise the data.

The 9 miRs of interest were further analysed to assess their differential expression in patients pre- and post-treatment. Figure 27 demonstrates the expression patterns between the treatment groups. In addition, the heatmap illustrates the variation within the treatment groups. The 9 miRs were, has-let-7b-5p, hsa-miR-181c-3p, hsa-miR-3150a-3p, hsa-miR-3192-5p, hsa-miR-365a-3p, hsa-miR-365b-3p, hsa-miR-375, hsa-miR-4732-5p and hsa-miR-483-5p.

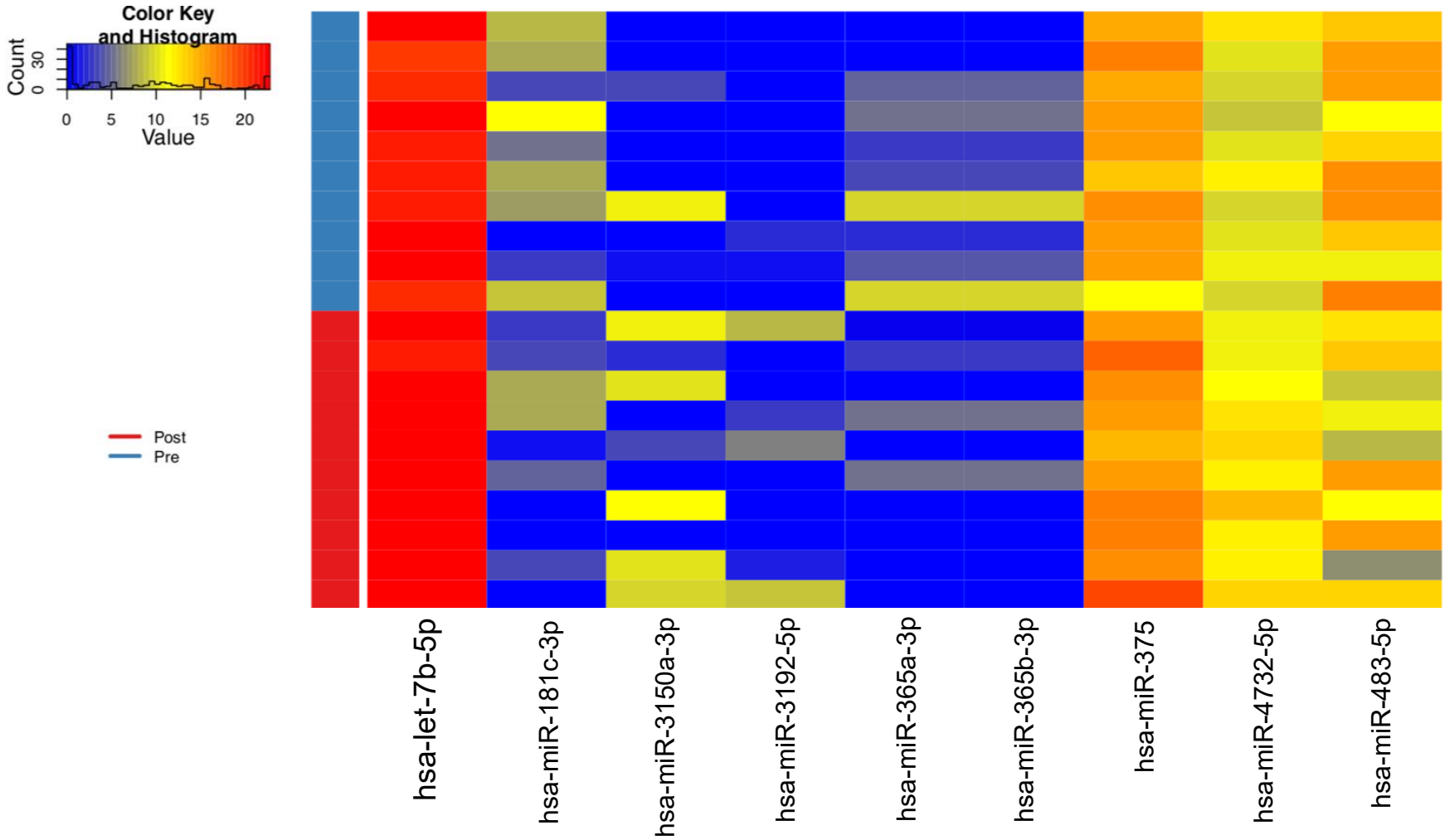


Figure 27. Heatmap for the differential expression changes of miRs of interest.
The x-axis represents the 9 miRs, identified with small RNAseq, which demonstrated >1-fold change and P-value <0.05.

Validation of RNAseq hits by qRT-PCR

The process of miR validation was performed using qRT-PCR. Samples were collected and processed following the conditions described above. The same 10 patient's samples (from pre- and post-treatment) were used to validate the miRs of interest from the RNAseq analysis. Further analysis was not carried out on miR-365b-3p as it was a replicate of miR-365a-3p. The qRT-PCR validation was performed and analysed using raw Ct expression (Figure 28) and relative expression using the $2^{-\Delta Ct}$ method using *C. elegans* miR-39 as an exogenous normaliser for miR expression (Figure 29). Neither raw Ct nor relative expression demonstrated the differences between the treatment groups observed with RNAseq. There was not a significant difference for pre- and post-treatment for any of the validated miRs of interest using qRT-PCR, therefore the miRs of interest in the patient samples could not be validated using this method. The primer sequences were validated between qRT-PCR and RNAseq to ensure that there were no discrepancies which would produce conflicting results.

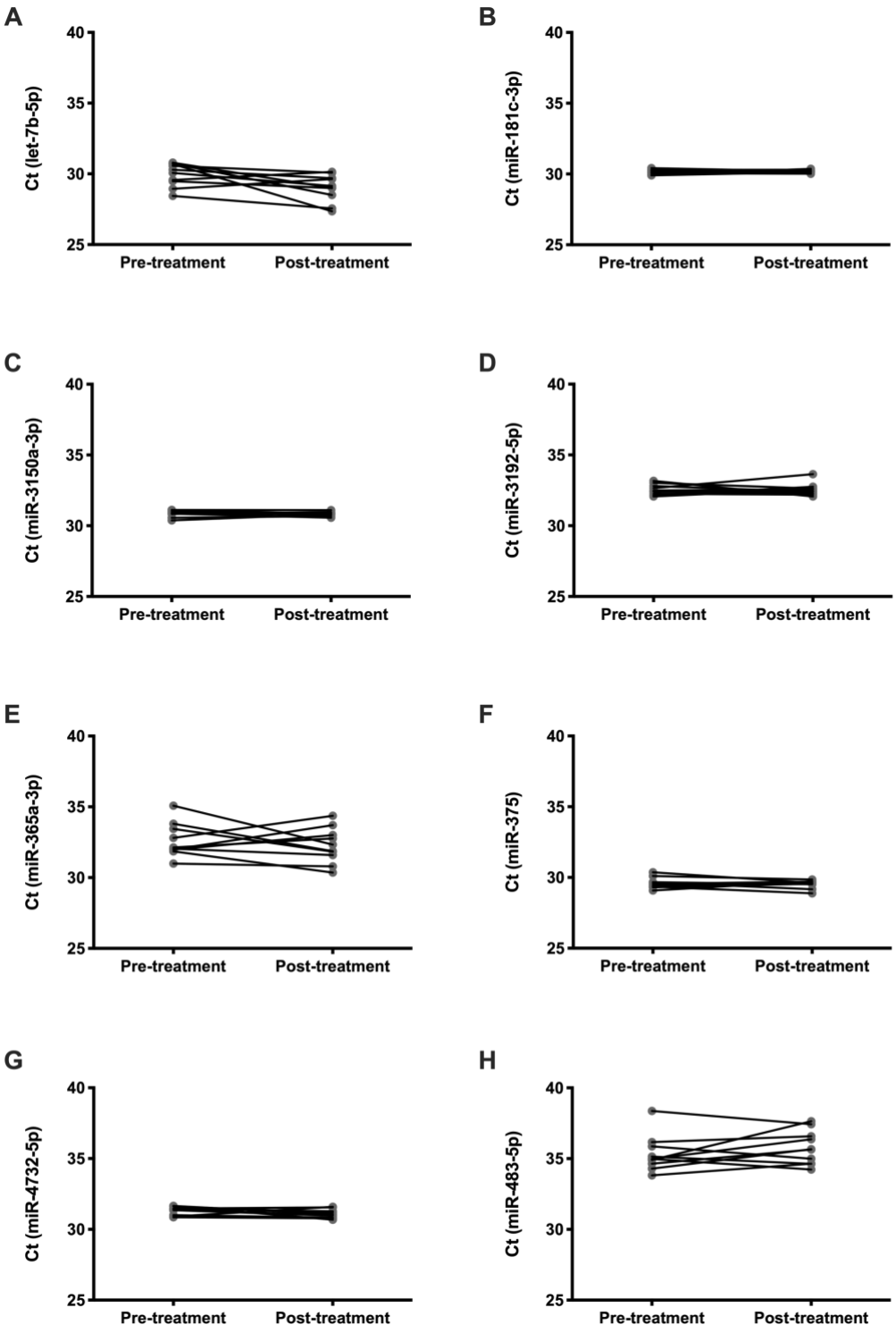


Figure 28. Plasma qRT-PCR validation of miR expression changes.
The change in miR expression was measured in a panel of miRs, identified as hits in RNAseq. The miRs measured pre- and post-treatment were (A) let-7b-5p, (B) miR-181c-3p, (C) miR-3150a-3p, (D) miR-3192-5p, (E) miR-365a-3p, (F) miR-375, (G) miR-4732-5p and (H) miR-483-5p. Significance was determined using a Wilcoxon test for paired analysis.

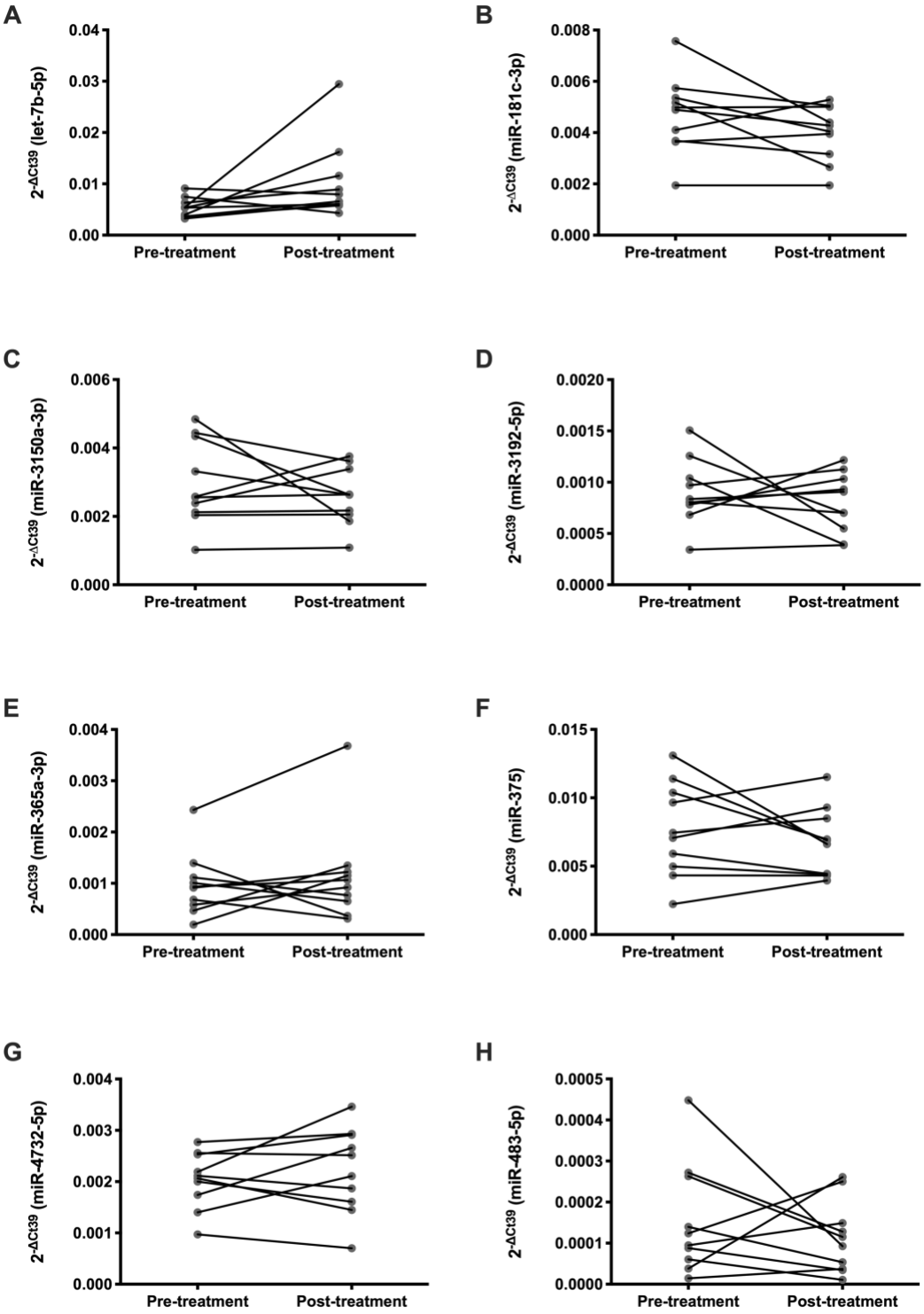


Figure 29. Relative quantification for plasma qRT-PCR validation of miR expression changes. *The change in miR expression was measured in a panel of miRs, identified as hits in RNAseq. The miRs measured pre- and post-treatment were (A) let-7b-5p, (B) miR-181c-3p, (C) miR-3150a-3p, (D) miR-3192-5p, (E) miR-365a-3p, (F) miR-375, (G) miR-4732-5p and (H) miR-483-5p. Data are expressed as 2-ΔCt (normalising the miR of interest to miR-39). Significance was determined using a Wilcoxon test for paired analysis.*

Discussion

Discovering novel biomarkers for health and disease is essential for the development of medical therapeutics and disease diagnosis.[75] The main objective of this study was to determine a panel of miR biomarkers to differentiate between active ANCA vasculitis and once treatment-induced remission has been achieved. Using paired patient samples at both stages of the disease, small RNA sequencing identified 9 differentially expressed miRs in the patient samples. These hits were explored with qRT-PCR, which did not align with the expression differences. Analysis of variance between the sample groups demonstrated that there was a high degree of heterogeneity in the patient groups.

Small RNAseq identifies differential expression in vasculitis

BGISEQ-500 is a novel platform for next generation sequencing (NGS) for large scale DNA and RNA sequencing.[150, 151] It has been previously validated in small RNAs and shows high technical reproducibility.[152] Using this technology, 9 hits were identified as miRs which were differentially expressed between pre- and post-treatment vasculitis in whole patient cohorts. The 9 hits chosen were significantly different between the two groups and therefore appropriate to assess for biomarker discovery. After further analysis, miR-375b-3p was discovered to be a replicate of miR-375a-3p and therefore removed from further validation. The data were also assessed as individual patient's pre- versus post-treatment to determine whether differences existed for each patient at the two disease states, which would allow for a greater understanding of the various forms of the disease. By interpreting the data in this way, there were not any significantly differentially expressed miRs for the two disease groups. This suggested that there were not any suitable miR biomarkers to differentiate between active disease and treatment induced remission in the small cohort of ANCA vasculitis patients that were recruited. Notably, miR-126 was not differentially expressed in the RNAseq analysis. This implies that the RNAseq had some limitations and did not identify all of

the potential microRNA expression differences between the two patient cohorts. The suitability of miR-126 as a marker in AAV is discussed in Chapter 3.

RNAseq validation

In order to develop a biomarker of disease, validation of RNAseq hits is required.[153] For miRs, a widely used and robust method of validation is qRT-PCR. RNAseq data for fold-changes in expression correlate with qRT-PCR expression.[154] This study did not show this relationship. The miR hits for differential expression between the treatment groups were not confirmed by qRT-PCR. The primer sequences were validated between the two technologies and there were no discrepancies detected. Due to this, it was not possible to further validate the hits. This is potentially explained by the differences in sensitivity between RNAseq and qRT-PCR. Further development of this relationship is required in larger patient cohorts. An additional explanation is that the miRs of interest were expressed at a low level in the RNAseq and this was reflected in the high Ct values with qRT-PCR. This could suggest that the miRs may have been undetectable with qRT-PCR.

Heterogeneity of ANCA vasculitis

As ANCA vasculitis represents a spectrum of disorders, including GPA, MPA and EGPA, there is an array of clinical manifestations.[155] In addition, the underlying immune dysfunction can lead to a variety of tissues being targeted.[156] In turn, the patient presents with symptoms representing their organ involvement, often including more than one. This is a fundamental issue in defining an appropriate biomarker for ANCA vasculitis.[43] In addition, there is a variable disease course which may be mild or systemic and life threatening. Due to this, there are challenges in defining an appropriate marker to comprehensively diagnose and track ANCA vasculitis. This is demonstrated in the complex heterogenous data obtained in this study.

Small RNAseq biases

There are a multitude of known small RNAseq biases which may affect the interpretation of the data and makes reliable quantification complicated. One of the challenges for quantifying miRs is the presence of isoforms, known as isomiRs, which are produced during biogenesis. The isomiRs show variations in stability, localisation, and functionality. The slight differences in length at the 5′ termini, 3′ termini, or both, creates difficulties for small RNAseq and validation with qRT-PCR.[157] Other important biases result from the RNAseq library preparation. These are based on inconsistencies associated with adapter ligation, cDNA synthesis, and amplification and are fundamentally dependent on library preparation and pre-processing methods, which are not as well controlled.[158] These biases may have had a crucial impact on the results obtained for the study. Further analysis and comparison on the small RNAseq would be required if the study were to be repeated.

Limitations

The study would have been more powerful if more paired samples had undergone RNAseq analysis. Due to resource limitations, it was not possible to sequence as many samples as would be ideal. The power calculation was performed based on data previously obtained by the group in patients with myocardial injury. As this cohort is considerably different clinically, this was potentially a limitation to the power of the study. In addition, due to abnormal boxplot distributions for 5 patients, the number included in the study was reduced from 15 to 10. This had a significant impact on the power of the study and was suboptimal for a human cohort.

To improve the overall design of this study, it would also have been of interest to include health age and sex-matched volunteers for comparison. This would have allowed for a greater understanding of the miRNome differences between systemic vascular inflammation and health individuals. As ANCA vasculitis is an extreme phenotype, it would have been possible to define miRs in active

disease and health, as shown in Chapter 3 with miR-126. With additional time and resources, this would be a valuable study to conduct. Overall, defining a suitable novel miR biomarker of ANCA vasculitis had multiple challenges, therefore it was not possible to define one in this study.

Conclusions

In this study the circulating miR profile did not change in human subjects between active vasculitis (pre-treatment) and in remission (post-treatment). There is potential for novel miR biomarker discovery, however, these were not detected with the platform used. This is potentially a consequence of vasculitis being a very heterogeneous disease with varying target organs and degrees of vascular damage.

Chapter 6 – Extracellular vesicle uptake in renal and immune cells *in vitro*

Introduction

In AAV, patients suffer from chronic inflammation. This is a major contributor in the development of cardiovascular disease, including ischemic heart disease, cerebrovascular events, thromboembolisms[159] and peripheral arterial disease.[160] In addition, patients are at increased risk of developing chronic kidney disease.[22] AAV is characterised by necrotising vasculitis with neutrophil and monocyte infiltration, resulting in vessel injury.[15] The inflammatory cells are recruited to the site of the vascular damage by cytokines. Pro-inflammatory cytokines are fundamental for cell to cell communication[161] and damage the vasculature and cause inflammation in the vessel wall.[162] They are major producers and up-regulators of inflammatory reactions. This is a key component of the pathogenesis of vasculitis and systemic inflammation.[163] Macrophages are tissue-resident phagocytic cells that are fundamental for host defence from infection. Immune cells are present throughout the body in all organs, where they identify and remove debris and foreign matter. Macrophages are responsible for regulating the inflammatory response as well as tissue repair.

Previous studies have shown that ECVs can directly mediate immune cell responses. This is due to their ability to transport miRs, proteins and other components to facilitate cell to cell communication.[164] This means that ECVs can be released from a damaged cell and affect the physiology of neighbouring recipient cells. This is can be the result of the ECVs inducing intracellular signalling after binding to cell receptors or presenting new properties after gaining new receptors, enzymes or genetic material.[165] Immune cell ECVs have been shown to regulate the vascular endothelium. The vascular inflammatory status can determine the secreted ECVs and therefore facilitate ECVs derived from the endothelium to differentially modulate target cell responses. This process has been demonstrated for both a pro- and anti-inflammatory response.[166] In the kidney, ECVs are released into urine from all regions of the nephron.[167] They have been implicated in the pathogenesis of acute kidney injury by transferring TGF-β1 mRNA into fibroblasts which

resulted in cell activation.[168] This makes them an interesting area of research to determine their role in immune and renal cells during active AAV.

Aims and hypothesis

The hypothesis for this chapter is that in ANCA vasculitis, circulating ECVs directly mediate pathophysiological changes in the vasculature, inflammatory cells and in the kidney. ECVs isolated from patients with vasculitis, before and after treatment, will play differing functional roles when applied to macrophages *in vitro*, by activating a defined inflammatory response. In addition, ECVs will cause cell damage in renal proximal tubules *in vitro.* This study aimed to determine the effect of ECVs from patients with ANCA vasculitis on murine macrophages, human macrophages and renal tubular cells *in vitro*.

Methods

ECV isolation

Vesicles were obtained from plasma taken from healthy volunteers and vasculitis patients at disease presentation and once in disease remission after successful therapeutic treatment, as described in Chapter 3. ECVs from human blood plasma were isolated following the Thery et al. protocol for purification of ECVs from viscous fluids.[169] Briefly, the plasma was diluted with an equal volume of PBS before undergoing 30 minutes of centrifugation at 2,000 x *g* at 4°C to pellet dead cells. The supernatant was removed and added to ultracentrifuge tubes and centrifuged at 12,000 x *g* for 45 minutes at 4°C to pellet the cell debris. The supernatant was transferred to new ultracentrifuge tubes and centrifuged for 2 hours at 110,000 x *g*. The pellet was resuspended in 1mL of PBS before being diluted to obtain 4mL of resuspension. This was filtered through a 0.22µm filter (Merck Millipore, Livingston, United Kingdom). The filtered solution was centrifuged at 110,000 x *g* for 1 hour and 10 minutes at 4°C. The pellet was resuspended in PBS and again centrifuged at 110,000 x *g* for 1 hour and 10 minutes at 4°C. The supernatant was poured off and the pelleted ECVs (including exosomes) were resuspended to give a final suspension in PBS.

ECV quantification

ECVs were sized and quantified with NTA using the NanoSight LM 10 instrument (NanoSight Ltd, Amesbury, United Kingdom). The equipment visualises particles in liquids (10 to 1000 nm) and relates the rate of Brownian motion to particle size. The system settings were optimised and kept constant for all samples. Each video record was analysed to give the mean, mode, median and estimated concentration for each particle size. All sample analysis was carried out at a 1:1000 dilution of the sample in distilled water in accordance with the manufacturer's guidance. Measurements were in triplicate. Measurements were plotted and the AUC calculated for ECVs of

exosomal size (20 - 100 nm) to determine the concentration of vesicles in each sample. After isolation, samples were stored at -20^{o}C until required.

ECV fluorescent labelling

The pelleted ECVs were labelled with Cell Tracker 655 (Invitrogen, Paisley, United Kingdom) following the manufacturer's protocol. The ECVs were incubated with Qtracker 655 Cell Labelling dye (Invitrogen) in media without FBS, to avoid the addition of vesicles from the serum, for 45 minutes at 37^{o}C before removing excess dye with two PBS washes and centrifugation at 110,000 x *g* for 1 hour and 10 minutes. A separate stock of unlabelled vesicles was stored for use in experiments which did not require fluorescently labelled ECVs, including qPCR.

Bone marrow-derived macrophages from mice

Bone marrow-derived macrophages (BMDMΦ) were derived from the femur and tibia of C57BL/6, male or female, 6-12 weeks old mice. The mice were culled by schedule 1 killing and de-trousered before being washed with 70% ethanol to limit contamination of the sample with fur and dander. The femur was exposed by cutting through the rectus femoris and biceps femoris muscles that surround the bone. All tissue was removed from the bones to enable detachment of the hip and knee joint from the femur and tibia. The epiphysis of the femur and tibia were removed using bone cutters and a syringe containing media (DMEM/F12 media with added FBS (10%), L929 (20%), penicillin and streptomycin (Gibco, Paisley, United Kingdom), with a 25$^{1/2}$ gauge needle, was inserted into the diaphysis of the bone and flushed through. The bone marrow was then suspended in media and incubated at 37^{o}C with 5% carbon dioxide.

Following bone marrow extraction from the mouse, the primary cells were grown over 8 days to differentiate into BMDMΦ cells. They were passaged every 48 hours. After 8 days of growth, the cells were counted and seeded to

$5x10^5$ per mL in 12 well tissue culture-treated plates to allow for adhesion of the cells to the wells for 24 hours. Cells were washed with PBS warmed to 37ºC to remove the remaining FBS before co-culturing with ECVs.

Vesicles isolated from human plasma were added at $1x10^9$ per mL. For cells used in qPCR and ELISA, ECVs were left unstained. For immunofluorescent imaging and flow cytometry, vesicles were stained with Qtracker 655 Cell Labelling dye as detailed above. ECVs were diluted to $1x10^9$ per mL in media without the addition of FBS and incubated at 37ºC and 5% CO_2 for 0 to 18 hours. The duration of serum starvation was kept constant throughout the study for all groups.

Flow cytometry

Cell fluorescence was measured by flow cytometry using a 5LSR Fortessa cytometer (BD Biosciences, Oxford, United Kingdom). BMDMΦ cells were exposed to dye loaded ECVs, as described, before detachment using a cell scrape with PBS. Detached cells were transferred to FACS tubes before being centrifuged at 300 x *g* for 5 minutes at 4ºC. The supernatant was removed before blocking with 10% mouse serum for 20 minutes on ice. The cells were incubated for a further 30 minutes with 1:2000 dilution of FITC anti-mouse/human CD11b antibody (Cat number: 101206, Biolegend, London, United Kingdom) in darkness. PBS was added after the incubation and centrifuged at 300 x *g* for 5 minutes at 4ºC. The supernatant was removed and 200µl formalin added. Samples were stored in darkness at 4ºC and analysed within 2 hours. FACS was run using an optimised protocol for QDots with excitation at 655nm and FITC at 488nm. For all samples, 10,000 events were used as the threshold value. Unstained and single stained samples were used for controls for use in analysis with FlowJo software. The gating strategy used for flow cytometry was optimised by Dr Wilna Oosthuyzen and Dr Emma Morrison previously. Gates were set using unstained cells and cells stained with DAPI alone. The populations of interest were identified using the FSC/SSC parameter. FITC positive single cells were measured and those

containing QDot 655nm labelled vesicles. The results are presented as the percentage of total fluorescent cells.

Immunofluorescence

Cultured cells were dispensed into separate clear 12 well plates with coverslips placed at the bottom of each well to facilitate growth. Initially, wells were washed twice with PBS to remove media and fixed with 4% *paraformaldehyde* (PFA) for 15 minutes. The wells were washed twice with PBS and 0.3% triton added to permeabilize the membranes. Wells were again washed after 10 minutes with PBS (twice). Cell filamentous actin was stained using Alexa Fluor 488 Phalloidin antibody (Cat number: A12379, Molecular Probes, Paisley, United Kingdom). The antibody was added at 1:500 dilution at room temperature in darkness. The samples were washed with PBS before mounting with DAPI, Fluoromount-G (SouthernBiotech, Cambridge, United Kingdom). Once dried, the slides were stored in darkness at 4^{o}C until imaging.

Image capture and analysis

Images were captured using a confocal laser scanning microscope (LSM 710, Zeiss, Cambridge, United Kingdom). Exposure, brightness and magnification were optimised and kept constant for all slides. Images were collected with a 64x oil immersion objective lens and acquired using ZenPro Software (Zeiss). Image analysis was performed using ImageJ software.

RNA extraction

Total RNA was extracted following the manufacturer's protocol using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA concentration was measured using a Nanodrop. Samples were diluted to give a consistent RNA concentration and stored at -80^{o}C.

Reverse Transcription PCR

Following extraction, each elute of RNA was reverse transcribed into cDNA with miScript II RT Kit (Qiagen) according to the manufacturer's instructions.

Quantitative PCR

A standard curve was created for quantification of gene expression. The qPCR was performed using SYBR Green (Qiagen) with murine primers (listed in Table 12) or human specific QuantiTect Primer assays (Qiagen). The plate was centrifuged at 2000 x *g* for 2 minutes at room temperature to collect all liquid in the bottom of the wells to remove experimental variation. The qPCR was carried out on a Lightcycler480 (Roche). Dissociation curves were utilised to ensure the amplification of a single product.

Primer	**Sequence**
Gapdh - Forward	ACT GGC ATG GCC TTC CG
Gapdh - Reverse	CAG GCG GCA CGT CAG ATC
Arg1 - Forward	GTG GAT GCT CAC ACT GAC ATC A
Arg1 - Reverse	GGT TGC CCA TGC AGA TTC C
Il1b - Forward	AGT TGA CGG ACC CCA AAA GAT
Il1b - Reverse	GGA CAG CCC AGG TCA AAG G
Il6 - Forward	CCA CGG CCT TCC CTA CTT
Il6 - Reverse	TTG GGA GTG GTA TCC TCT GTG A
Il10 - Forward	CCC AGA AAT CAA GGA GCA TTT G
Il10 - Reverse	CGC ATC CTG AGG GTC TTC A
Mrc1- Forward	TCA TTG GAA CAT CCA CTC TGG
Mrc1- Reverse	CAG CGC TTG TGA TCT TCA TTA TAG
Tnfa - Forward	CAC AAG ATG CTG GGA CAG TCA
Tnfa - Reverse	TCC TTG ATC GTG GTG CAT GA

Table 12. Primers used for qPCR for murine inflammatory genes.
Forward and reverse primers were used for all genes.

Human macrophage isolation

Human peripheral blood monocytes were isolated from healthy human blood by dextran sedimentation followed by percoll gradient centrifugation. Monocytes were seeded onto glass coverslips in 12 well plates at $2x10^6$ cell per mL in serum-free Iscove's modified dulbecco's medium (IMDM; Gibco). After 1 hour, cells were washed to remove non-adherent cells. Adherent cells were cultured for 7 days in IMDM with 10% donor serum. After this time, cells were co-cultured with ECVs as detailed above.

RPTEC/TERT1 cells

REPTEC/TERT1 cells are commercially available epithelial cells immortalised with pLXSN-hTERT retroviral transfection. Cells were grown in DMEM:F12 media (ATCC, Middlesex, United Kingdom) in $75cm^2$ flasks. Once confluent, cells were seeded into 12 well plates at $1.5x10^5$ cells per mL and left for 72 hours to adhere. Cells were incubated with isolated ECVs following the protocol detailed above.

Statistical analysis

All analysis was performed using GraphPad Prism software (GraphPad, La Jolla, United States). Statistical significance was calculated using a one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-test for comparison to healthy controls and for all FLOW analysis. For pre- and post-treatment, a paired T-test was used.

Results

Human ECV uptake by murine bone marrow-derived macrophages

After ECV isolation by differential ultracentrifugation, NTA was used to measure the concentration of ECVs isolated from human plasma samples from healthy volunteers. A representative trace obtained from the NTA analysis is presented in Figure 30A, showing the particle size concentration distribution. ECVs isolated from healthy volunteers were fluorescently labelled using Cell Tracker 655 then added to BMDMΦ for various incubation periods to determine the optimal time required for uptake. Figure 30B demonstrates the number of CD11b positively stained macrophages, as determined by the pan-macrophage marker[170]. There was no significant difference in positively marked macrophage single cells at the measured time points between 0 and 18 hours. Figure 30C displays the number of single cells with successful uptake of ECVs. In Figure 30D, CD11b positive cells took up QDot tracker labelled ECVs in a time-dependant manner with a maximum level of uptake after 18 hours of co-culturing.

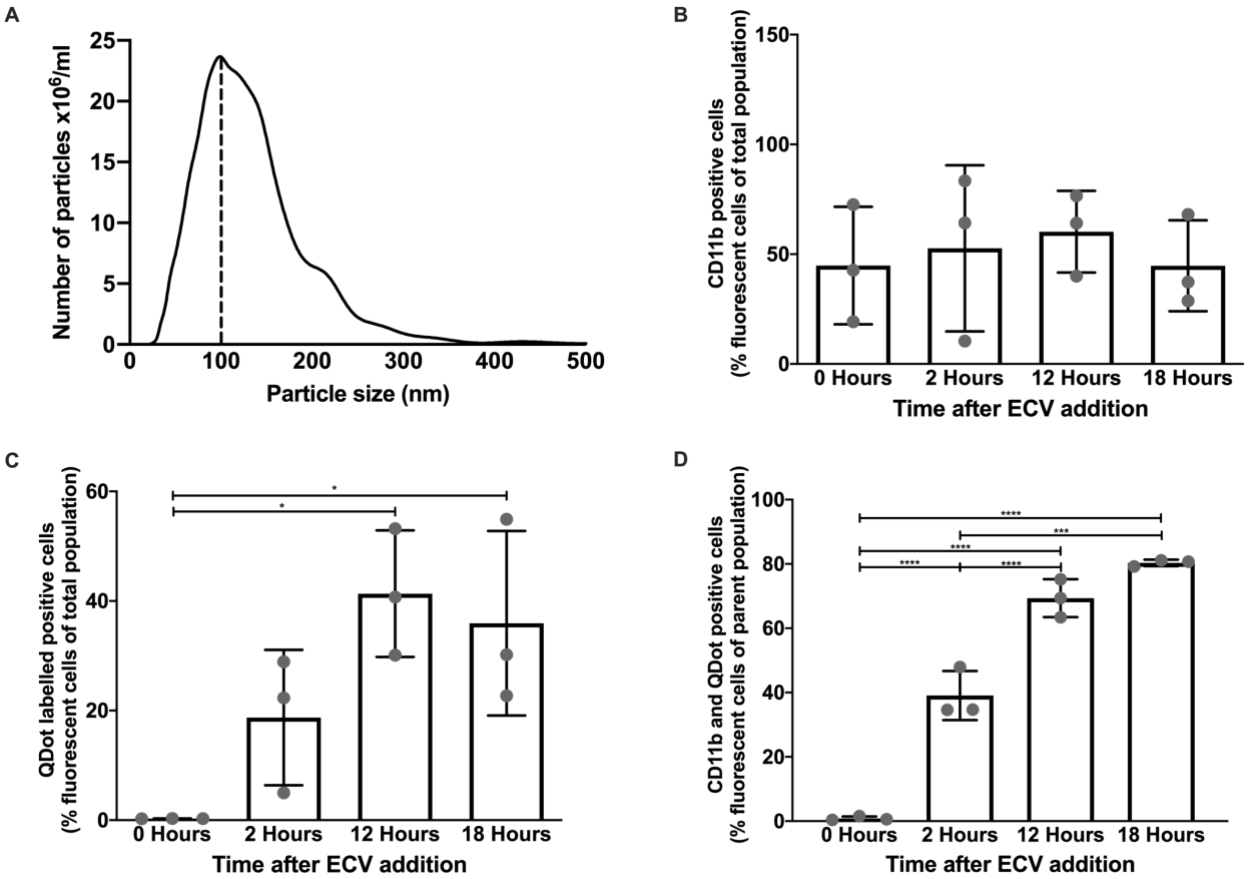


Figure 30. ECVs isolated from human plasma can be taken up by BMDMΦ in a time-dependant manner.
*(A) Representative NTA trace of ECVs isolated from plasma. The line represents the threshold between ECVs considered to be of exosomal size. Flow cytometry was used to quantify the uptake of ECVs into recipient CD11b cells (B) Quantification of CD11b cells in BMDMΦ cell culture. (C) Single cells taking up Qdot tracker labelled ECVs and (D) the percentage CD11b positive single cells with QDot tracker labelled ECVs at varying time points ranging from 0 to 18 hours. Bars show mean with SD (n=3 per group with 10,000 events each) *p<0.05 ***p<0.001, ****p<0.0001 (One-way ANOVA with Bonferroni's multiple comparisons test).*

In order to visualise the capabilities of BMDMΦ cells to take-up ECVs isolated from human plasma, ECVs from healthy volunteers were labelled and added to cells for 18 hours before fluorescent imaging. The images obtained, shown in Figure 31, are representative of the control cells (incubated without the addition of labelled ECVs) and with the ECVs from healthy volunteers and patients with active vasculitis, pre-treatment. This demonstrated that labelled ECVs can successfully be taken up by the BMDMΦ after 18 hours of incubation. The ECVs can be seen intracellularly near the nuclei. This is also seen in the cells co-cultured with the vasculitis patient's ECVs.

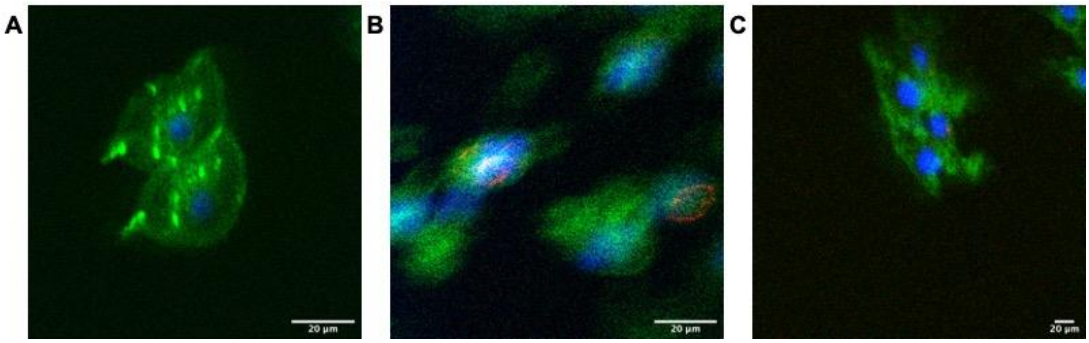


Figure 31. Cultured murine BMDMΦ take up labelled ECVs isolated from human plasma. *Representative images using fluorescent microscopy of BMDMΦ cells (A) cultured without ECVs, (B) BMDMΦ cells with fluorescently loaded ECVs from healthy volunteers and (C) xwith ECVs isolate from patients with active vasculitis. The images show ECVs (red), nuclei stained with DAPI (blue) and cell membrane stained with phalloidin (green). Images are representative of 3 samples for each group. Scale bars are 20µm.*

Human ECVs alter murine macrophage phenotype

ECVs isolated from plasma taken from patients with vasculitis, pre- and post-treatment, were quantified by NTA and the number of ECVs normalised for each individual. ECVs were added to BMDMΦ cells and co-cultured for 18 hours, as determined by flow cytometry. The cellular gene expression - for multiple inflammatory markers - was measured in the macrophages. The relative change in expression for five genes is presented in Figure 3 and 4. The genes were measured in order to ascertain the potential pro- or anti-inflammatory response in the cells after co-culturing. For the classic macrophage response, the cytokines tested were *Tnfa* and *Il1b* (Figure 32). There was not a significant increase in gene expression for either cytokine.

Figure 33 demonstrates the gene expression of cytokines involved in the alternative macrophage response, considered to be anti-inflammatory. *Mrc1* shows an increase in gene expression in macrophages with ECVs from pre- and post-treatment. There was no significant difference in expression for *Il10* and *Arg1* between ECVs from patients with vasculitis and healthy controls.

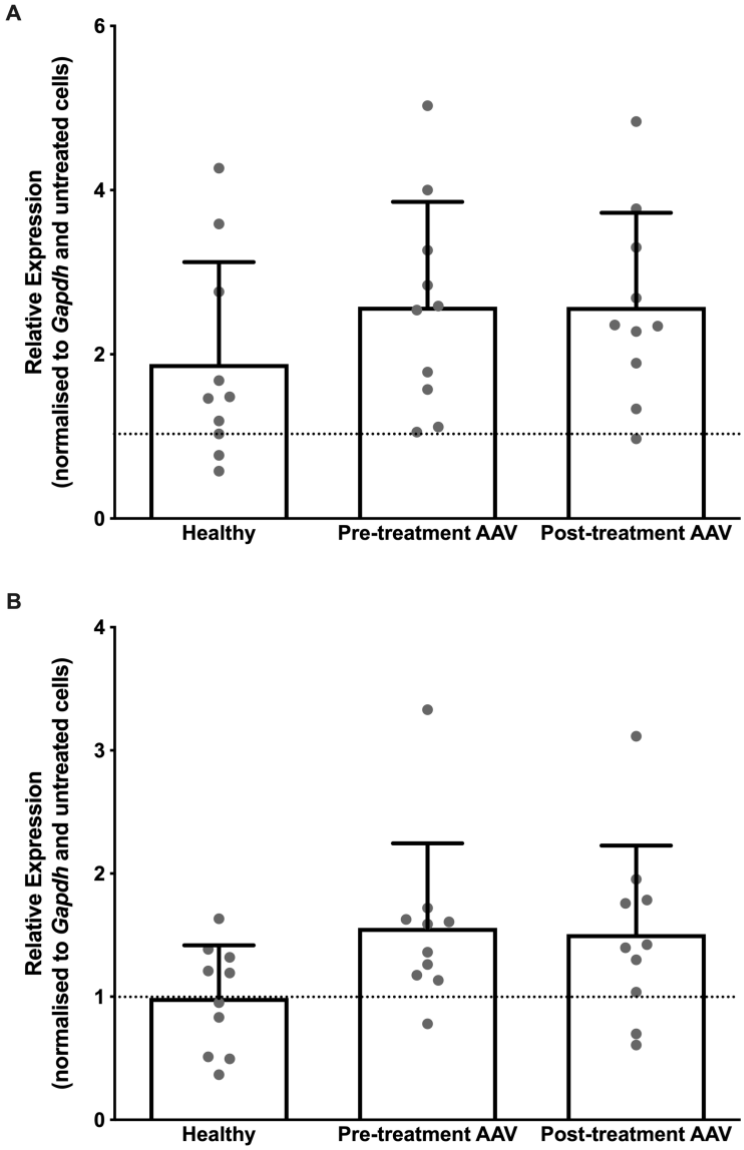


Figure 32. Gene expression to measure the pro-inflammatory response in BMDMΦ cells after ECV uptake.

The fold change of cytokines was measured. The cytokines are involved in the M1 inflammatory response including (A) Il1b and (B) Tnfa. Each point represents the 2-ΔΔCT value, normalised to GAPDH and untreated control cells. The dotted line represents no change in expression in comparison to untreated control cells. Bars show mean with SD (n = 10 per group in triplicate, one-way ANOVA with Bonferroni's multiple comparisons test).

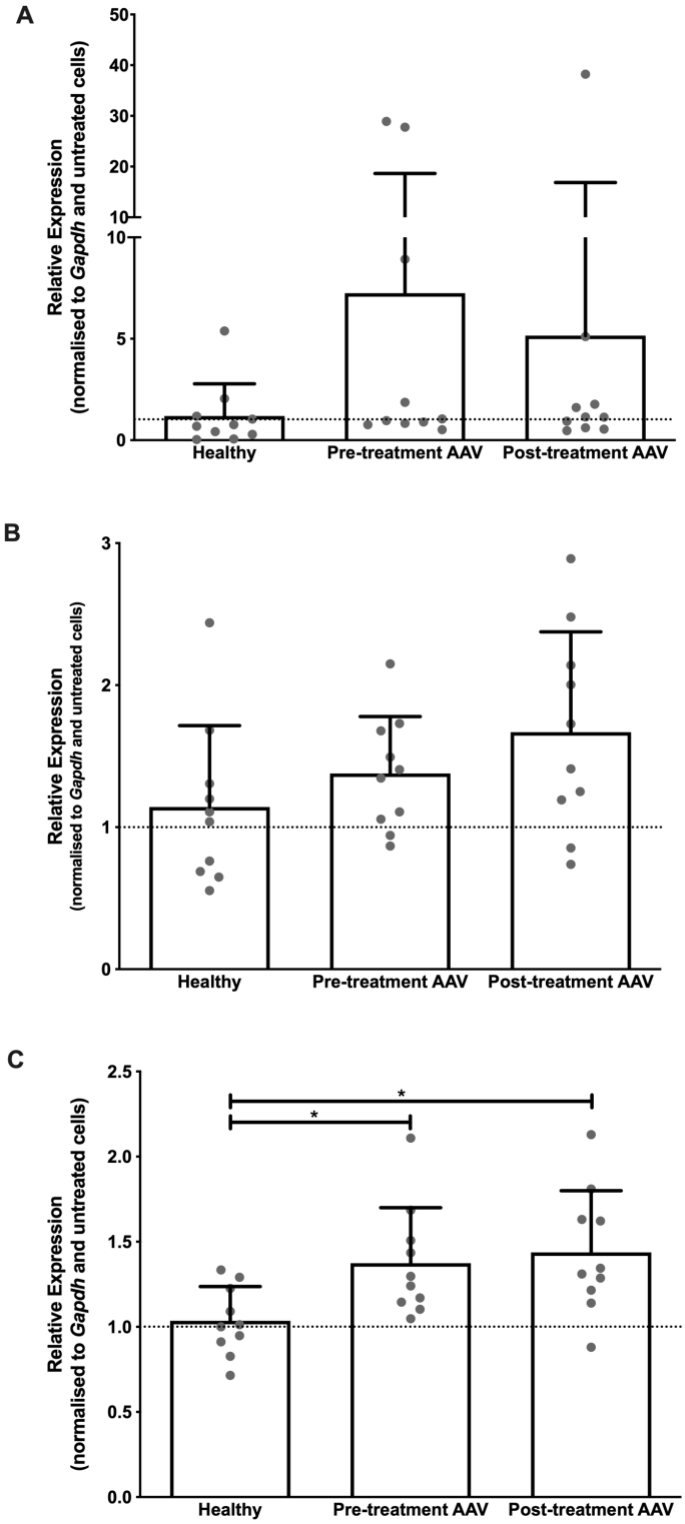


Figure 33. Gene expression to measure the anti-inflammatory response in BMDMΦ cells after ECV uptake.

*The fold change of cytokines was measured. The cytokines are involved in the M2 inflammatory response including (A) Arg1, (B) Il10 and (C) Mrc1. Each point represents the $2^{-\Delta\Delta CT}$ value, normalised to GAPDH and untreated control cells. The dotted line represents no change in expression in comparison to untreated control cells. Bars show mean with SD (n=10 per group in triplicate) *p<0.05 (one-way ANOVA with Bonferroni's multiple comparisons test).*

Human ECVs are taken-up by human primary macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated and cultured from healthy volunteers to assess the capabilities of human macrophages to take-up ECVs isolated from human plasma. ECVs from healthy volunteers were labelled and added to cells before fluorescent imaging. The images obtained, shown in Figure 34, are representative of the control cells (incubated without the addition of labelled ECVs) and with the ECVs after co-incubation for 24 hours and 48 hours. Figure 34B demonstrates that labelled ECVs can successfully be taken up by the primary human macrophages after 24 hours of incubation. There was also uptake of the labelled ECVs at 48 hours (Figure 34C), however, the level of uptake was consistent with the 24-hour incubation period. The ECVs can be seen intracellularly near the nuclei, similar to the uptake in BMDMΦ.

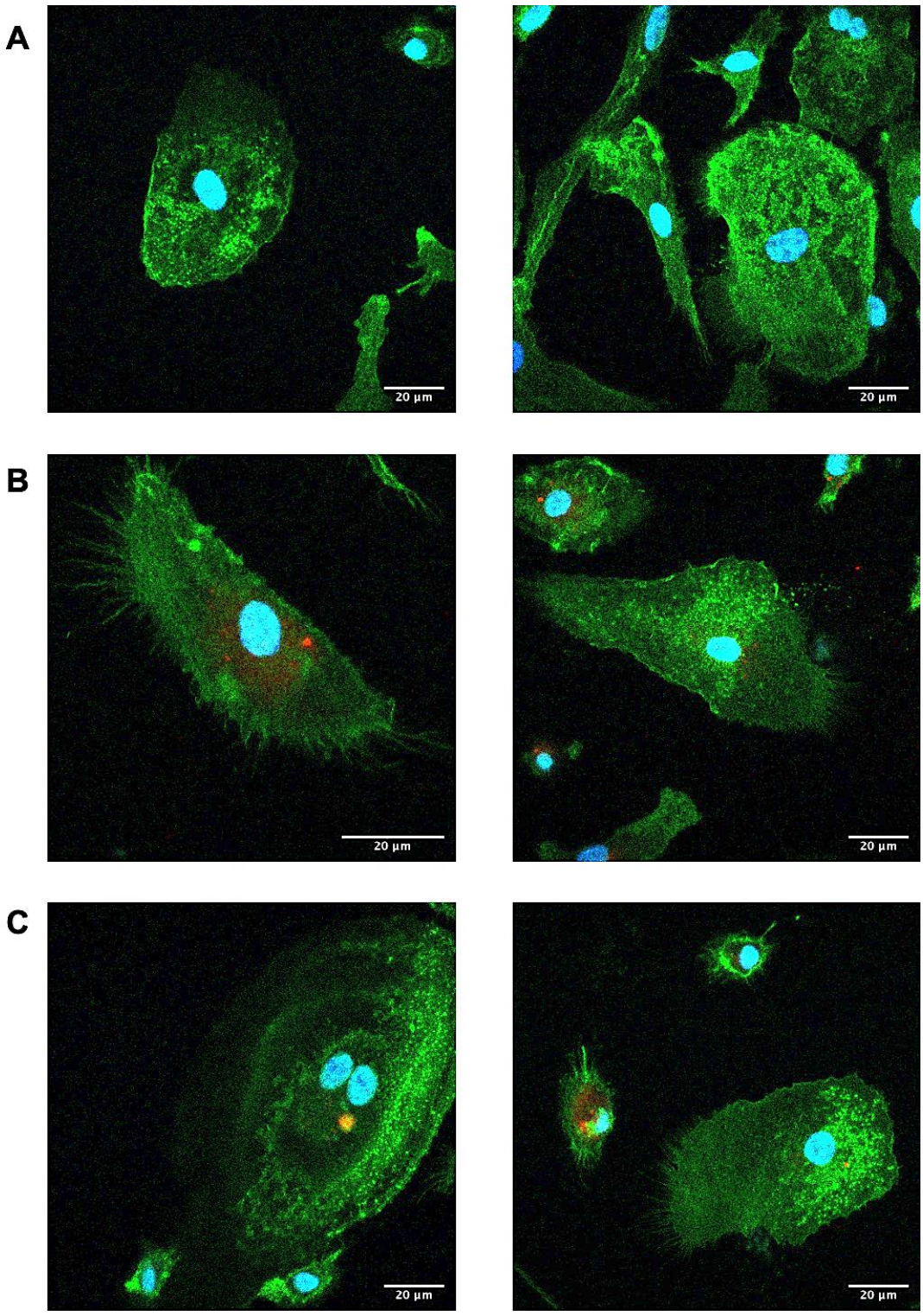


Figure 34. Human primary macrophages take up human ECVs from healthy volunteers.
(A) Fluorescent microscopy of human macrophages without ECVs and (B) with fluorescently loaded ECVs at 24 hours and (C) 48 hours of co-culturing. Fluorescently loaded ECVs (red), nuclei stained with DAPI (blue) and cell membrane was stained with phalloidin (green). Images are representative of 3 samples for each group.

Human macrophages do not have altered phenotypes after ECV uptake

ECVs were isolated from AASV patient's plasma pre-treatment in addition to healthy volunteers. The ECVs were added to human primary macrophages and the gene expression for multiple inflammatory markers were measured. The relative change in expression for the six genes measured is presented in Figure 35 and Figure 36. The genes tested to measure a classic macrophage phenotype were *TNFa, IL1b* and *IL6*, shown in Figure 35. There was not a significant difference in gene expression between the control cells, cells treated with healthy volunteer's ECVs and ECVs from vasculitis patients with active disease.

Cytokines involved in the alternative macrophage phenotype were also measured (Figure 36). These *were IL10, MRC1* and *ARG1*. There was no detectable change in expression between healthy and AASV samples. These data demonstrate that there are no regulatory changes in the healthy and diseased state for ECV uptake in macrophages.

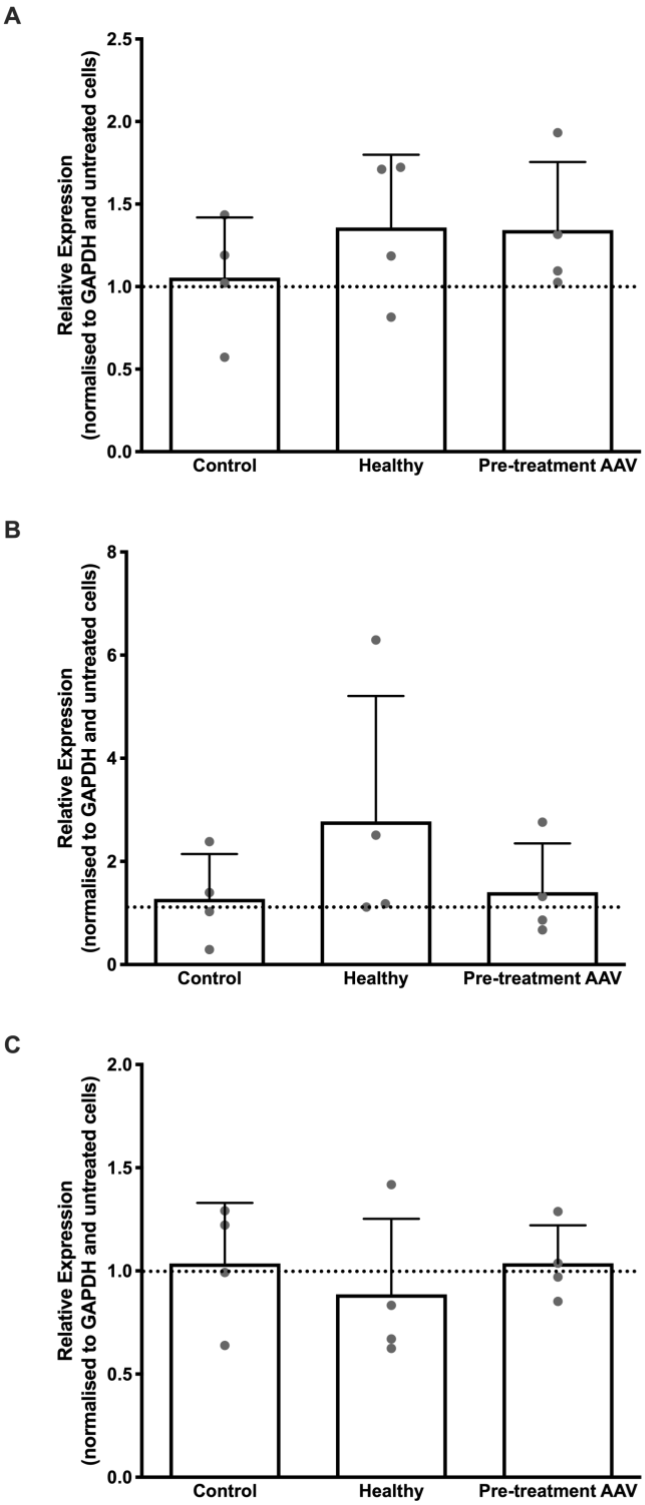


Figure 35. Gene expression to measure the pro-inflammatory response in human PBMCs after ECV uptake.

The fold change of cytokines was measured. The cytokines are involved in the M1 inflammatory response including (A) IL1B (B) IL6 and (C) TNFA. Each point represents the $2^{-\Delta\Delta CT}$ value, normalised to GAPDH and untreated control cells. The dotted line represents no change in expression in comparison to untreated control cells. Bars show mean with SD (n = 4 per group in triplicate, one-way ANOVA with Bonferroni's multiple comparisons test).

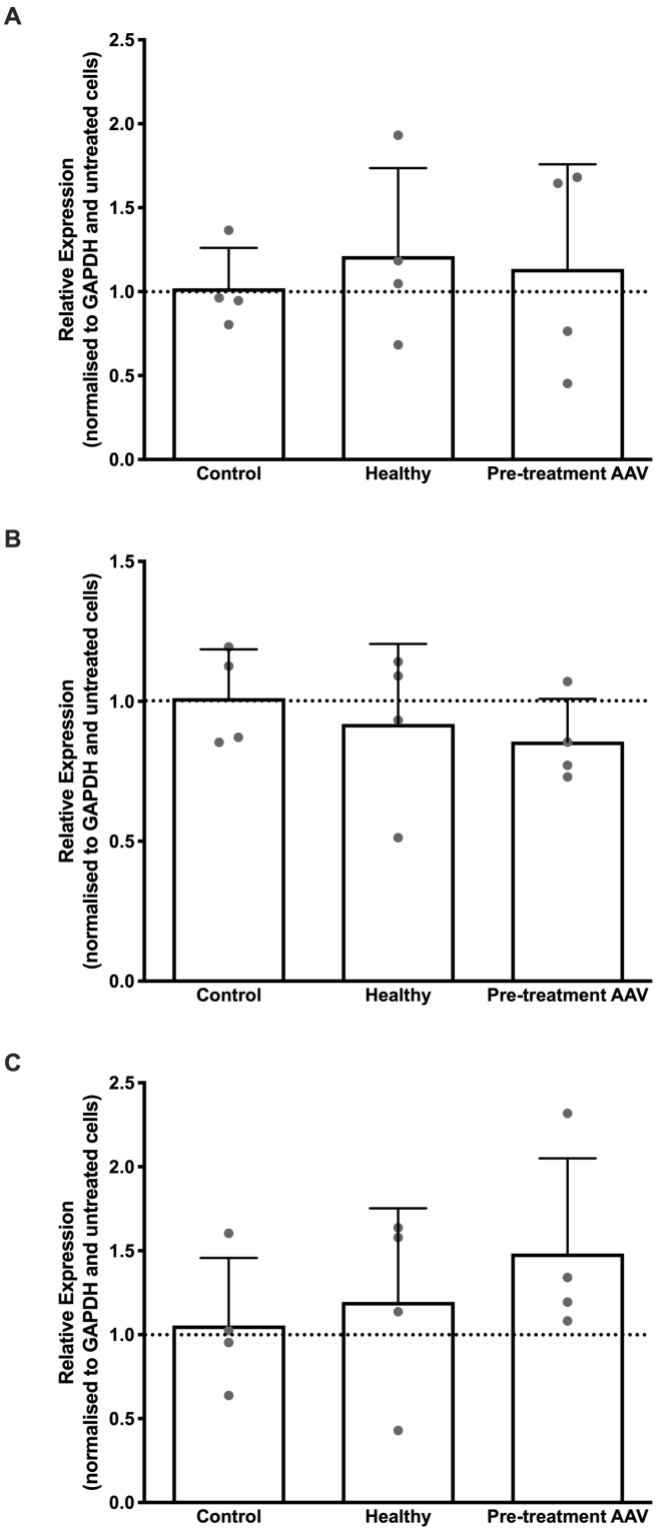


Figure 36. Gene expression to measure the anti-inflammatory response in human PBMCs after ECV uptake.

The fold change of cytokines was measured. The cytokines are involved in the M2 inflammatory response including (A) ARG1, (B) IL10 and (C) MRC. Each point represents the $2^{-\Delta\Delta CT}$ value, normalised to GAPDH and untreated control cells. The dotted line represents no change in expression in comparison to untreated control cells. Bars show mean with SD (n = 4 per group in triplicate, one-way ANOVA with Bonferroni's multiple comparisons test).

RPTEC/TERT1 human proximal tubule cell line uptake of labelled ECVs

Previous studies have demonstrated that ECV uptake is possible in human proximal tubule cells, however uptake has not been compared in ECVs from healthy volunteers and those with acute vascular inflammation. The ability of the human proximal tubule cell line, RPTEC/TERT-1, to take-up ECVs was confirmed by immunofluorescence using ECVs isolated from healthy volunteers' plasma (Figure 37). The labelled ECVs are abundant at 24 and 48 hours and absent in the control cells (cultured without human ECVs). The ECVs are intracellularly localised near to the nuclei.

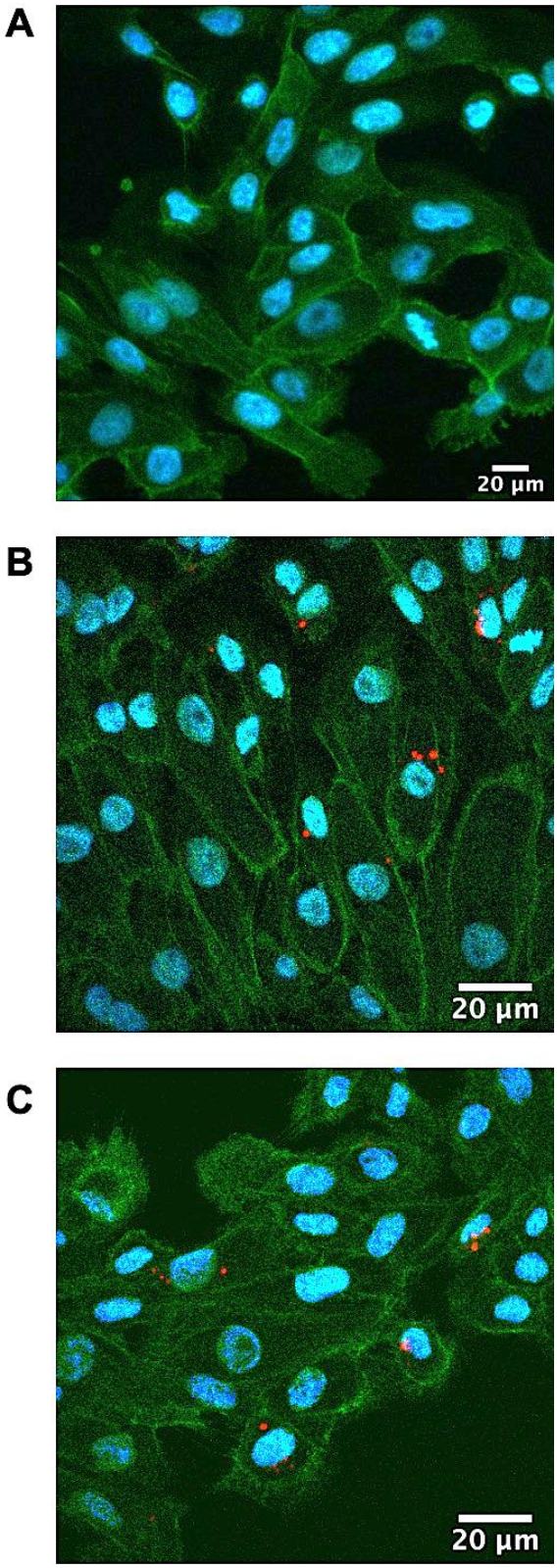


Figure 37. A human proximal tubule cell line can take up human ECVs.
Fluorescent microscopy of RPTEC/TERT-1 cells (A) without ECVs and with fluorescently loaded ECVs (B) at 24 hours and (C) at 48 hours of co-culturing. Fluorescently labelled ECVs (red), nuclei stained with DAPI (blue) and cell membrane was stained with phalloidin (green). Images are representative of 4 samples for each group.

ECVs do not alter a biomarker of renal injury

A biomarker commonly used to reflect human tubular injury is *HAVCR1*. RPTEC/TERT-1 cells were co-cultured with ECVs from healthy controls and patients with active vasculitis. Additionally, the tubular cells were separately treated with two concentrations of cisplatin to confirm cell damage (Figure 38A). Gene expression of *HAVCR1* was not altered by the healthy or active vasculitis ECVs. The cisplatin treatment resulted in a significant difference in HAVCR-1 gene expression between 10μM and 100μM.

HAVCR1 protein expression within the cell supernatant was quantified following RPTEC/TERT-1 co-culturing with ECVs and nephrotoxic cisplatin. This was conducted to confirm the injury response at a proteomic level with active vasculitis ECVs. The ECVs from patient samples had no substantial effect on HAVCR-1 release from cells (Figure 38B). After treatment with 100μM cisplatin, the concentration of HAVCR1 increased in comparison to the control cells, including DMSO treated cells, and the tubule cells cultured with human ECVs.

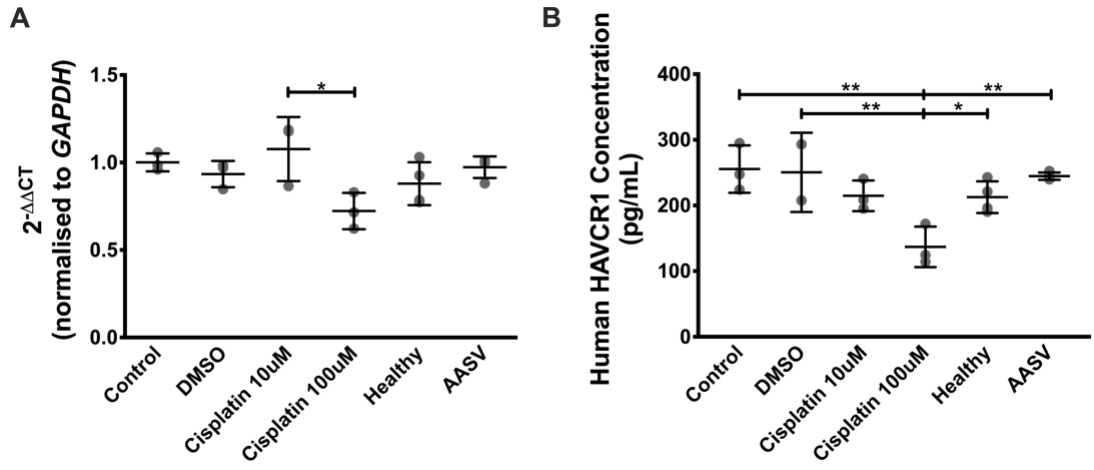


Figure 38. HAVCR1 expression in treated RPTEC/TERT-1 cells.
*Renal proximal tubules treated with a nephrotoxin (10μM and 100μM cisplatin) and ECVs from healthy volunteers and patients with active vasculitis. (A) qRT-PCR analysis of HAVCR1 gene expression. Each point represents the 2-ΔΔCT value, normalised to GAPDH and untreated control. (B) Protein expression in the cell supernatant (n = 3-4 per group in triplicate) *p<0.05, ** p<0.005 (one-way ANOVA with Tukey multiple comparison). Horizontal lines represent the mean values and SD.*

ECVs from AASV patients do not instigate apoptosis

Apoptosis is often a consequence of renal cell injury from nephrotoxic agents. In RPTEC/TERT-1 cells, apoptosis was measured using CASP3 after co-culturing with cisplatin (10 and 100μM) and ECVs isolated from healthy controls and patients with active AAV. Figure 39A demonstrates that at 100μM, cisplatin increases gene expression of CASP3 significantly in comparison to other groups tested, including the cells cultured with ECVs from vasculitis patients. In addition to CASP3, CSF1 was measured in the RPTEC/TERT-1 cells (Figure 39B). CSF1 is involved in macrophage recruitment in the proximal tubule. There was a significant difference in expression of CSF1 in the cells treated with 100μM in comparison to the control groups and the cells treated with human ECVs.

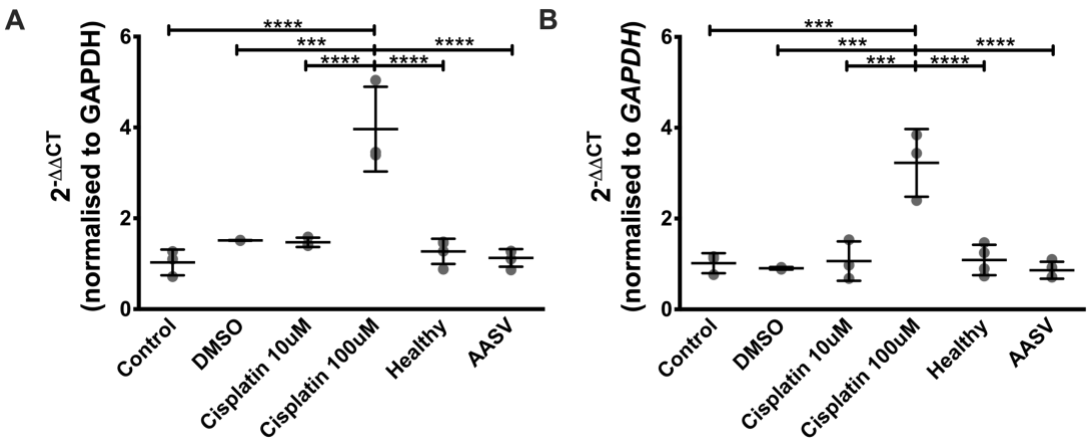


Figure 39. CASP3 and CSF1 gene expression in RPTEC/TERT-1 cells.
*Renal proximal tubules treated with a nephrotoxin (10μM and 100μM cisplatin) and ECVs from healthy volunteers and patients with active vasculitis. Gene expression was measured by qRT-PCR analysis of (A) CASP3 and (B) CSF1. Each point represents the 2-ΔΔCT value, normalised to GAPDH and untreated control supernatant (n=3 per group in triplicate. ***p<0.001, ****p<0.0001 (one-way ANOVA with Tukey multiple comparison). Horizontal lines represent the mean values and SD.*

Discussion

The role of ECVs in cardiovascular disease and inflammation is an emerging area of research, primarily due to their potential use in biomarker discovery for a multitude of cardiovascular diseases.[171, 172] The role of ECVs is to transport miRs, protein and RNA between cells, resulting in an altered phenotype for the recipient cell. The key mechanisms involved in this process have yet to be fully investigated.[173, 174] Research is ongoing to discover the processes involved in ECV development, cargo organisation and the release of vesicles. The hypothesis for this chapter was that in ANCA vasculitis, circulating ECVs contain a pathognomonic cargo of miRs. These vesicles directly mediate pathophysiological changes in the vasculature, inflammatory cells and in the kidney.

This study was conducted to determine whether vesicles could be taken up by inflammatory and renal cells. The primary aim of this was to determine the biodistribution of the vesicles in macrophages, as shown by BMDMΦs and PBMCs, and in the kidney, as shown in RPTECs. In this study, the data obtained established that human ECV uptake is possible in recipient primary murine macrophages as well as primary human macrophages. We have also demonstrated that this uptake is possible in a human proximal tubule cell line. Our data have established that a change in gene expression is induced by ECVs in murine macrophages but not in human macrophages or tubular cells.

Vesicle uptake *in vitro*

The first aim of this chapter was to establish whether macrophages internalise vesicles. This was achieved with murine macrophages utilising two separate detection approaches, flow cytometry and immunofluorescent imaging. Using these techniques, the time period required for uptake of labelled human vesicles by mouse BMDMΦ *in vitro* was evaluated. Using a pan-macrophage marker, CD11b, this study demonstrated that positive macrophages were present at a similar concentration from 0 to 18 hours after ECV addition. This

suggests that labelled vesicles did not affect the overall number of macrophages in culture over the 18-hour time period. As the study required a period of serum starvation, it was expected that there would be a proportion of CD11b negative cells. This study established the optimal time period required for human vesicles to be taken up by the primary cultured mouse macrophages. The data suggest that 12 to 18 hours is the most appropriate experimental time period for vesicle uptake by murine macrophages *in vitro.* Due to this finding, 18 hours was used as the peak time for vesicle incubation with macrophages for the remainder of the study.

Using immunocytochemistry imaging, vesicle uptake was confirmed using Qdot tracker labelled human vesicles isolated from healthy volunteers and from patients with active vasculitis (pre-treatment). From the images obtained, this study has illustrated that ECVs can be engulfed by the murine macrophages and concentrated intracellularly. There was a clear red signal in multiple macrophages from the labelled ECVs. The images obtained were limited due to the macrophage positioning. Further optimisation is required to gather more detailed images of vesicle uptake with cells in a monolayer. The available representative images showed clear signal intracellularly and localised near the nucleus.

To fulfil the second aim for this chapter, ECV uptake was also successfully established in human primary macrophages *in vitro*. By using cells from both species, it allows for a greater overview of the cell diversity in culture. It has been established that there is a high degree of heterogeneity in both mouse and human macrophage lineage,[175] therefore comparing the two cell types optimised the assay for further studies. Additionally, the time required for ECV uptake in human macrophages was determined. The results gathered demonstrate that ECVs are engulfed by the cells after 24 hours and there is a similar amount of labelled ECVs after 48 hours when assessed qualitatively. This is similar to the time course in murine cells. Successful uptake in varying

time frames could be further confirmed by flow cytometry analysis. This would allow for more quantitative results.

For the third aim of this study, in addition to macrophage uptake of ECVs, a human proximal tubule line was co-cultured with healthy human ECVs. Through immunocytochemistry, this again demonstrated successful uptake of vesicles at both 24 and 48 hours. Further studies have been planned to assess the effects of ECVs on the proximal tubule cell line expression of injury markers to mirror the damage caused by AAV with renal involvement. It would also be of interest to compare the injury readouts to AAV patients without renal involvement to ascertain whether there was a key mediator which was causative for kidney damage. Previous studies have shown that HAVCR1, also known as kidney injury molecule 1 (KIM-1), is able to distinguish relapse and remission with renal vasculitis.[176] KIM-1 is detectable in many human kidney diseases, specifically when proximal tubule damage is present[177] and correlates inversely with renal function.[178] In AAV, previous studies have suggested that KIM-1 can distinguish between active AAV with renal involvement and once the disease is in remission,[176,177] and correlates inversely with renal function.[178,177]

Macrophage activation by vesicles

This study demonstrated that ECVs isolated from humans can be taken up by murine macrophages and subsequently alter their phenotype *in vitro.* These effects were measured by qPCR measurement of a panel of cytokines which are secreted by macrophages after either classical or alternative activation. Of the genes studied, *Mrc1* had the most statistically significant expression change. The expression of *Mrc1* was upregulated in macrophages treated with pre- and post-treatment vesicles in comparison to the healthy controls. This is traditionally considered a marker of alternative activation of macrophages, which are considered to have anti-inflammatory and tissue repair roles as well as subduing the recruitment of additional immune cells.

An interesting result is that there is not a detectable change in human macrophage cytokine expression once co-cultured with ECVs from healthy volunteers and AAV patients. A possible explanation for the lack of difference between the test groups is the macrophage source. In future studies, it would be valuable to characterise the healthy volunteer cohort donating PBMCs to determine the possible differences observed between individuals. There are many features that could interfere with the healthy macrophage baseline results, such as infection and lifestyle factors, on their macrophage phenotype and therefore their cytokine production.[82] In addition to this, the age of the blood donor could potentially be of importance for macrophage phenotype. For the previous experiments, macrophages from female donors aged between 23 and 25 were cultured. By using donors of equivalent age to the AAV patients, we could assess the need for compatibility between the cells and ECVs in the study. In European studies, the mean age for AAV diagnosis is 60 years old[179] with a peak prevalence between 65-74 years old.[121] The therapeutic strategies used for the treatment of AAV may also be a caveat, As the treatment strategies are based on immunosuppression, this may have an effect on their immune cell response to ECVs. This is again a limitation of using healthy macrophages for this study.

Another feasible explanation for this is that the cargo of the ECVs were not appropriate to cause a detectable effect on the cells. Possible reasons for this are that the vesicle concentration is not high enough. This is however unlikely as a phenotypic effect has been detected in the murine macrophages and would, therefore, be expected to be sufficient for human cells. Alternatively, the cargo shuttled in the macrophage is not mediating the inflammation. It would also be valuable to sequence the ECV content in the patient groups and measure expression differences in AAV plasma samples and in the ECV fraction to compare the transportation in circulation. To confirm this, future studies will require the addition of a positive control for inflammation to ensure that the cells are capable of eliciting an immune response and that the gene expression is detectable. Further studies could be conducted to confirm

inflammation in the immune cells by measuring cytokine protein expression from the cells. This would allow for further confirmation of whether the cells had been altered by the patient's vesicles.

Human proximal tubule cells

RPTECs were used in this study as an overall measurement for the uptake of vesicles within the nephron. RPTECs are well characterised proximal tubule cells which are therefore downstream of the glomeruli where the most extreme damage is observed in AAV patients with glomerulonephritis. It has been established that RPTEC/TERT1 cells are capable of ECV uptake after 24 hours. To follow on from this, cells were co-cultured with ECVs from healthy controls and AAV patients following the protocol detailed above. As well as the human samples, cisplatin was used a positive control for established tubular damage as it is a known nephrotoxic agent. This was used at two concentrations, as determined by a dose-response curve. After co-culturing, *HAVCR1* expression was measured from the treated cells alongside HAVCR1 protein release into the cell supernatant by ELISA.[180] This demonstrated that gene expression and protein concentration of HAVCR1 was lower at the higher dose of cisplatin than the other tested cells. This was an unexpected result, therefore we explored whether this could be a consequence of apoptosis. With apoptosis, the number of cells would be reduced, therefore the protein and gene expression would decline.

Gene expression was measured for CASP3 and CSF1. For both genes, expression was greatly increased at 100μM cisplatin. This suggests that renal damage has occurred. CASP3 is a marker of cell apoptosis and CSF1 plays a crucial role in macrophage recruitment for damaged cells in the proximal tubule. Cisplatin is known to cause direct damage to renal proximal epithelial cells as demonstrated in RPTEC/TERT1 cells.[181] This, therefore, demonstrates an extreme and targeted form of renal damage, yet the ECV cargo would provide less direct assault. To refine this study, it would be necessary to include alternative controls to provide a spectrum of renal cell

injury. Overall, the finding of this study suggests that ECVs from healthy volunteers and patients with active vasculitis do not carry a cargo that is capable of causing renal cell damage to the same extent as a nephrotoxic agent, such as cisplatin.

Conclusions

In this chapter, ECV signalling in acute vascular dysfunction was assessed *in vitro* with immune and renal cells. It was established that ECVs can be taken up by immune cells in a time-dependent manner and can be visualised intracellularly. There was an immune response in murine macrophages but and no response in human primary macrophages when co-cultured with ECVs from patients with active disease in comparison to the healthy control vesicles. Furthermore, the ECVs were successfully taken up by renal cells but did not cause measurable cell injury in human proximal tubule cells. This is an important finding as this would suggest that the immune and renal cell response is initiated by factors which are not directly related to ECV uptake.

Chapter 7 – General Discussion

The studies presented in this thesis sought to investigate the potential of miRs as markers of human vascular dysfunction. The major findings of my PhD are as follows: that the concentration of miR-126 is reduced in patients with active acute vascular dysfunction and increases once in remission; that miR-126 is low in CKD and ESRD and the circulating concentration increases following haemodialysis; that small RNA sequencing did not identify further miRs as biomarkers of disease relapse in ANCA vasculitis; and that ECVs isolated from patients with active vascular dysfunction did not cause an inflammatory response or renal cell damage *in vitro*.

miR-126 in AAV

The studies in Chapter 3 demonstrated that miR-126 decreases as vascular function declines in patients with acute vascular dysfunction. In patients with active ANCA vasculitis, the concentration of miR-126 is low and increases after successful treatment. It was also shown that in the circulation, miR-126 does not increase to a level seen in healthy individuals even after treatment. This may be representative of their continuing vascular damage as a result of the disease. miR-126 correlated with a gold standard measurement used to assess vascular health and arterial stiffness, PWV. The results suggest that there is a potential biomarker role for miR-126 to detect active disease in ANCA vasculitis. miR-126 has previously been examined as a potential biomarker for atherosclerosis,[92] diabetes[96] and cancers.[182, 183] In addition, studies have suggested a potential role for miR-126 as a therapeutic strategy in chronic myeloid leukaemia.[184]

In patients with ANCA vasculitis, the relapse rate is variable across studies (21% - 89%) for patients within 5 years.[185] It would be of great interest to develop my study further and examine the potential of miR-126 to predict patients that have disease relapses and return to active disease. It is currently very challenging to clinically predict the patients that this will occur in, therefore by using miR-126 to routinely monitor the disease, it could be possible to alter treatment strategies to limit further vascular and organ damage. In contrast, it

could also predict the patients that no longer require such high doses of treatment, which could reduce treatment side effects.[186]

A limitation of the studies in chapter 3 and 4 is that the healthy controls that were included were not age-matched with the patient cohorts. As ANCA vasculitis is a disease that mainly affects individuals between 65 and 74 years old,[187] it is challenging to recruit healthy volunteers in this age group who do not have any co-morbidities and are not taking regular medication. In addition, it was not possible to obtain the healthy volunteer's clinical parameter data, including arterial stiffness, inflammation status and renal function. Having these data would allow for further analysis of correlations with miR-126 and develop the potential for miR-126 as a biomarker of health and acute vascular dysfunction.

miR-126 in CKD

The studies in Chapter 4 support the potential role of miR-126 in vascular dysfunction, specifically in chronic disease. This chapter focused on CKD and ESRD with dialysis treatment. With chronic vascular injury, in moderate CKD, the concentration of miR-126 is reduced in comparison to healthy volunteers. In ESRD patients undergoing HD, miR-126 is further reduced when compared to moderate CKD, and this concentration increased following a single session of HD. miR-126 correlates with clinical measurements of vascular health, such as arterial stiffness. As a biomarker, miR-126 could guide treatment plans for ESRD to optimise the amount of time required for HD to avoid unnecessary sessions or treatment. In addition, miR-126 could be used as a prognostic marker to track CKD patient's disease progression to ESRD.

Developing a biomarker for CKD progression is essential as it is well established that patients with CKD are at an increased risk of developing and dying from CVD. This link is not fully explained by traditional risk factors, such as hypertension and diabetes mellitus.[188] As miR-126 is a potential biomarker of vascular health, it may be valuable to track pharmaceutical dosing and

optimisation of dialysis sessions. For drug dosing, miR-126 could be used to assess the patient's improving vascular health, using drugs such as statins. Statin-based treatments have been shown to reduce CVD risk in CKD patients who do not require HD, therefore tracking this would be valuable to assess the patient's risk of CVD.[189]

Small RNA sequencing for biomarker discovery in AAV

Chapter 5 presents the results of a study using small RNAseq analysis to determine whether there are miR biomarkers which can differentiate between active ANCA vasculitis and remission. This study showed that the patients did not have differences in their miRNome. Additionally, the heterogeneity of the condition was confirmed. Due to the reduction in patient samples that were included in the RNAseq analysis, because of abnormal boxplot distributions, the study was underpowered. As the number of paired samples were reduced from 15 to 10, this consequently reduced the proportion of genes with ≥ 80% power from approximately 50 to 40 with the loss of these paired samples. This was a major limitation for this study. It would be beneficial to repeat this study with a larger sample size, and if possible, include patients that had active disease relapse.

ECVs as markers and mediators of AAV

In Chapter 6, the studies examined whether ECVs isolated from patients with active ANCA vasculitis contained a pathognomonic cargo which would cause inflammatory and renal damage. ECVs were taken up by immune cells over 18 hours and imaged intracellularly in murine and human-derived macrophages. There was not a defined immune response in the immune cells. An additional study examined ECV uptake in renal epithelial proximal tubule cells *in vitro*. Uptake was observed by fluorescent microscopy, however, there was no renal cell damage identified qualitatively. Together, this suggests that the immune and renal cell response is initiated by alternative factors which are not directly mediated by ECV uptake.

A limitation of this study was that the phenotypic changes in immune cells were not shown in control samples. To do this, macrophages would be stimulated with exogenous LPS/IFNγ to elicit a classical phenotype (M1) and IL4/IL13 for an alternative phenotype (M2). The gene expression for the cells could then be compared from macrophage and ECVs alone versus cells that are pre-stimulated. This would confirm that the macrophages could be successfully stimulated to elicit a switch of macrophage phenotype and compare this to switching mediated by ECVs during active vascular dysfunction.

Future work

From the results obtained in the studies presented in this thesis, further questions have been raised and additional areas identified to examine. Details of a selection of these are discussed below.

1. **miR-126 as a therapeutic target in AAV**

Building on the results obtained in Chapter 3, it would be of interest to determine if miR-126 is a mediator of ANCA vasculitis and translates the human data into a mouse model. As miR-126 is a key vascular regulator, it is proposed that it is a mediator and potential treatment for vasculitis. This study would determine the effect of miR-126 treatment in a mouse model of vasculitis.

To do this, the study could examine the effect of systemic delivery and antagonism of miR-126 in nephrotoxic nephritis mouse (NTN), a widely accepted and reproducible model of human rapidly progressive glomerulonephritis that reflects the histological changes observed in ANCA vasculitis.[190] Nephrotoxic nephritis would be induced in C57/BL6 male mice at 10-12 weeks of age. NTN can be initiated in mice by intravenous injection of sheep anti-glomerular basement membrane nephrotoxic serum as previously described.[190] For *in vivo* treatment, mice would be randomised and administered miR-126 mimic or antagomiR-126. Mice would be administered

miR and antagomiR in a preventative (pre-NTN) and therapeutic (post-NTN) manner. For a preventative strategy, antagomiR treatment would be delivered by IV injection 3 days before the initial NTS injection.

As a readout of the treatment, tissue sampling could be conducted, including blood, urine and histology. The drug delivery would be confirmed in the blood, kidney and the vasculature by direct measurement of miR, using qRT-PCR for gene expression and in-situ hybridisation. Vascular function would be assessed by wire myography of aortic and mesenteric vessels[191] and kidney injury by histology and assessment of proteinuria as described.[192] These studies would define the role of miR-126 in the vascular and renal dysfunction seen in NTN.

2. miR-126 in transplantation

Following the measurement of miR-126 in dialysis patients, a future study that would be beneficial would be examining miR-126 in renal transplant patients. This would directly compare the circulating concentration of miR-126 in patients with ESRD and their potentially improved vascular and renal function following a kidney transplant. Previous studies have shown conflicting evidence for the changes in overall vascular health and arterial stiffness in patients with renal transplants.[193] By measuring miR-126 is the transplant patient's blood, it could potentially be used to monitor the changes in vascular integrity from CKD to ESRD with HD to recovery following transplantation.

3. Small RNAseq in patients with vascular dysfunction

To improve and adapt the RNAseq study described in this thesis, it would be beneficial to increase the ANCA vasculitis cohorts pre- and post-treatment to add power to the study. To further assess the miRNome differences in patients with vascular dysfunction, it would be advantageous to expand the study to include both acute and chronic vascular damage. This would include the patients described in this thesis with managed and moderate CKD, ESRD with maintenance dialysis and also healthy individuals. In doing this, a direct

comparison of each phenotype of vascular dysfunction could be compared and further biomarkers identified for diagnostic and prognostic uses.

Final conclusion

Through a series of *in vitro*, *ex vivo* and clinical studies, this thesis has explored and shed light on miRs as potential biomarkers of acute and chronic vascular dysfunction. MiR-126 is a promising marker for acute and chronic vascular dysfunction, as demonstrated in patients with ANCA vasculitis, CKD and ESRD. RNAseq did not determine alternative miRs to compliment miR-126 is ANCA vasculitis. Additionally, the transfer of ex vivo ECVs from patients with active vascular dysfunction did not affect murine and human-derived macrophages or immortalised renal proximal tubule cells. Further experiments are required to fully understand the diagnostic and prognostic value of miR-126 in acute and chronic human vascular dysfunction.

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