Monocyte Subsets in Atrial Fibrillation with Preserved Left Ventricular Function

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Abstract

Introduction

Atrial fibrillation is the commonest arrhythmia in the cardiovascular system. It can have debilitating effects, including life changing thromboembolic stroke. Monocytes and their subsets have proven to be both beneficial and detrimental in heart failure and coronary artery disease. Less is known regarding their role in atrial fibrillation. The aims of this thesis were to study the following parameters in patients with atrial fibrillation and preserved left ventricular function: 1) monocyte subset numbers, 2) monocyte subset expression of surface receptors for inflammation, 3) crosstalk between monocytes and platelets in the formation of monocyte-platelet aggregates (MPAs), 4) markers of cardiac fibrosis, 5) utility of Spironolactone to aid in improved exercise capacity in this cohort of patients.

Methods

Monocyte subsets were analysed by flow cytometry on venous blood samples in 250 patients with permanent atrial fibrillation and preserved left ventricular function who were double blindly allocated to spironolactone or placebo and followed up in clinic at specified time intervals. The subsets were analysed at 12 and 24 months from the treatment allocation. Plasma levels of cardiac markers of fibrosis were analysed by ELISA at baseline, 12 months and 24 months. The patients underwent cardiopulmonary exercise testing, 6-minute walk test and quality of life assessment at set time intervals during the 2-year study period to assess their relationship to monocyte subsets.

Results

CD16 expression on Mon3 subset was associated with a higher Peak VO_2 and CD42 expression on MPA associated with Mon3 with a reduced exercise capacity (p value of 0.001 and 0.026 respectively). Quality of life and hospitalisations were unaffected by monocyte subsets. Spironolactone did not impact on primary and secondary outcomes of the study and monocyte characteristics. The markers of cardiac fibrosis did not explain the mechanistic role by which Mon3 influences exercise capacity in this patient population.

Conclusion

Differences in monocyte cell surface marker expression influence exercise capacity and functional status in patients with atrial fibrillation and preserved left ventricular function. Spironolactone, however, does not affect such study parameters when compared to placebo. Further studies are required to decipher the mechanisms by which monocyte cell surface markers influence exercise capacity.

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Finally, in these unprecedented times I would like to dedicate this thesis also to my close colleagues, friends and indeed loving family members I have lost in this pandemic. To my uncle, Sajjad who passed away during this time, the drive and commitment to completing this thesis is in honour of you and the unconditional love you showed me as a child and also as a nephew growing up into the man. I hope I have made you proud and you will always be in my thoughts and prayers.

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Abbreviations

HF - Heart Failure

AF - Atrial Fibrillation

CCR2- C-C chemokine receptor 2

CX3CR1- CX3C chemokine receptor 1

MDP- Macrophage dendritic cell precursors

CMP- Common myeloid progenitor cells

MI- Myocardial Infarction

Mon1- Monocyte subset 1

Mon2- Monocyte subset 2

Mon3- Monocyte subset 3

Cluster of differentiation 14- CD14

Cluster of differentiation 16- CD16

VCAM- Vascular Cell Adhesion Molecule

ROS- Reactive oxygen species

IL-6- Interleukin 6

VEGF- Vascular endothelial growth factor

ICAM – Intracellular adhesion molecule

MCP-1- Monocyte chemoattractant protein-1

IKKβ- Inhibitor of nuclear factor kappa β kinase

NO- Nitric oxide

TNFα- Tumour Necrosis Factor α

Interleukin 1-β- L-1β

Extracellular Matrix- ECM

TLRs- Toll-like receptors

Heart Failure with Preserved ejection fraction- HFpEF

Coronary artery disease- CAD

Transforming growth factor β- TGF-β

Heart failure with reduced ejection fraction- HFrEF

Renin-angiotensin-aldosterone system (RAAS)

C-reactive protein- CRP

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Chapter 1

Introduction

1.1 Introduction To Atrial Fibrillation

Atrial Fibrillation (AF) is a global epidemic (1). The prevalence of AF has shown a modest increase in men worldwide from 570/100,000 in 1990 to 596/100,00 in 2010. Although AF is less common in women there has been a similar increase in AF prevalence from 360/100,000 in 1990 to 373/100,000 in 2010 (1). The AF prevalence is still much higher in developed regions, but a more pronounced growth in the arrhythmia prevalence is expected in rapidly developing Asian countries (1). At present Asia-Pacific region has the lowest AF rates in both men and women (340/100,000 and 196/100,000 respectively) but in view of the large population in Asia the number of AF patients in the region is even higher than in the United States and Europe (2) (3).

In Europe, AF affects 8 million people, and the number is expected to rise 2.3-fold by 2060 (4). In the UK projections from the Clinical Practice Research Database suggest that AF will affect between 1.3 and 1.8 million people by 2060 (5). In the USA, about 3-5 million people are currently affected by AF and by 2050 this figure is expected to be greater than 8 million people (6). In Australia, Europe and the USA, the current estimated prevalence of AF is about between 1% and 4% (4) . Australia has the highest prevalence of AF (5.4%) followed by Africa (4.6%), Iceland (2.4%) and lowest in Asian countries (0.49%-1.9%) (7). A recent review on AF epidemiology of 58 studies from 5 Asian (China, Japan, South Korea, India and Malaysia) and 8 Middle Eastern countries (Turkey, Bahrain, Qatar, Kuwait, Saudi Arabia, Oman, United Arab Emirates and Yemen) reported the annual incidence of AF to be 5.38 per 1000 person-years (7).

Studies examining the prevalence of AF have shown this to be highest among the Whites compared with Afro-Caribbeans, East Asians and Hispanics, ranging from 42% to 2.5% among the Whites (8, 9), and 21% to 1.7% among Afro-Caribbeans (8, 10) . More than 70% of AF patients in Western Europe, Australia and North America are aged >65 years (11). A different pattern in the average age of AF diagnosis is evident from other regions whereby AF patients are younger. For example, the mean age of AF patients was 57 ± 16 years in the Gulf-SAFE registry from Arabic population (12), 41 ± 13 years in an Ethiopian study (13), <65 years in 43% of the South Korean population detected with AF (14). Results from the RE-LY AF registry which enrolled AF patients from the 164 emergency departments worldwide to evaluate the differences in the presentation and management of AF, also showed some regional variation in terms of age at AF diagnosis; patients from America and Europe countries were on average 10-12 years older than those from Africa, India and the Middle East (15).

The public health burden of AF is expected to grow with the progressive ageing of the world population, the increasing prevalence of risk factors, and the use of advanced screening tools (11). AF is a well-established risk factor for ischemic stroke, carrying a three to fivefold increased risk of stroke (16). The percentage of ischemic strokes associated with AF has been reported to range between 16% and 39%, and although the attributable risk is 17%, it is highly dependent on age ranging from 4.6% in those aged 50–59 years to >20% in those aged >80 years (4) . Although the overall incidence of ischemic stroke is decreasing, in high-income countries, cardioembolic strokes related to AF have tripled during the past 25 years and is expected to continue to climb at a similar rate by 2050, particularly in those aged ≥80 years (17). Risk factors associated with the development of AF overlap with those leading to an increased risk of ischemic stroke. Common risk factors include advanced age, male sex,

hypertension, diabetes, smoking, heavy alcohol consumption, obesity, sleep apnea, elevated inflammatory markers, chronic kidney disease, and the presence of underlying cardiac diseases (4) . Particularly, patients with cardioembolic strokes tend to be, more often, female, older, and have underlying cardiac disease (18).Validated prediction models such as $CHADS_2$ and CHA_2DS_2VASc have been developed to predict the risk of stroke based on some of the major cardiovascular risk factors. The risk of stroke recurrence within the first year is approximately 15%, which can be significantly (~70%) reduced with the use of oral anticoagulant therapy (19). Unfortunately, even when the dysrhythmia is detected early, it is estimated that fewer than 50% of patients with AF receive oral anticoagulation worldwide (11). The elderly are particularly affected as they are frequently perceived as fragile and at increased risk of complications despite the absence of contraindications, while they carry the highest risk for thromboembolism (11).

Despite this, there is a likely underreporting of cases of AF outside North America and Europe, which leaves more people in the developing countries deprived of optimal antithrombotic treatment. The uptake of oral anticoagulation is lower in areas such as China where aspirin is still used as a major method of thromboprophylaxis in AF patients (3).

Incidence rates for AF follow a similar pattern to that of prevalence with a prominent diversity in incidence rates of AF between the developed and developing regions (1). One reason why AF-related strokes still represent a global epidemic is the 'silent' nature of the arrhythmia in many individuals. Opportunistic screening by pulse check has been shown to be both clinically and cost effective for improving detection of AF in the general population of Europe (20).

The diagnosis of AF requires rhythm documentation using an electrocardiogram (ECG) showing the typical pattern of AF: Irregular RR intervals and no discernible, distinct P waves. By accepted convention, an episode lasting at least 30s is diagnostic. Individuals with AF may be symptomatic or asymptomatic ('silent AF'). Many AF patients have both symptomatic and asymptomatic episodes of AF.

My thesis explores the role of AF in impacting upon exercise capacity and quality of life in patients with this debilitating arrythmia despite having a perceived normal left ventricular function. As the above introduction highlights, AF has a significant clinical burden on those patients who are diagnosed with the condition. Although the consequences of AF with regards to stroke and cardiac remodelling have been extensively studied, the impact this arrythmia has on exercise capacity has not been extensively researched despite being commonly reported in outpatient clinical settings. Subsequent text explores the pathophysiology and molecular basis as to how AF affects cardiac function with emphasis on the process of inflammation and cardiac fibrosis.

1.1.1 **Pathophysiology Of Atrial fibrillation**

External stressors such as hypertension, diabetes mellitus and AF itself can stimulate a process of atrial remodelling and subsequent fibrosis which acts as a substrate for AF (along with other cardiac arrhythmias) (21). The structural remodelling that takes place leads to an alteration in the electrical conduction pathway in the atrium leading to a low threshold re-entry circuit and propagation of arrhythmias (22). The formation of myocardial fibrosis is one of the major factors leading to cardiac remodelling in patients with AF (23). Atrial fibrosis is a multifactorial process resulting from complex interactions among cellular and neurohormonal mediators (24).These processes result in redistribution of connective

myocardial tissue due to new conditions of pathological tissue function. The mechanisms of cardiac fibrosis in AF include the incorporation of cardiac fibroblasts and myoblasts, Transforming Growth Factor B, matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases. Cellular and extracellular components are involved in the remodelling process (25).This process can result in heterogeneity of current conduction, shortening of action potentials, depolarization of resting cardiomyocytes, and induction of spontaneous depolarization. Previous in vitro data has demonstrated that atrial fibrosis caused localised regions of conduction slowing, increasing conduction heterogeneity and therefore providing an arrhythmia substrate (26). Thus, atrial fibrosis might be the underlying substrate and directly involved in the occurrence and propagation of focal and re-entry arrhythmia mechanisms. An arrhythmia substrate based on atrial fibrosis may develop as part of AF-related structural remodelling, as well as consequences of other cardiovascular diseases that result in atrial haemodynamic overload and stretch of the affected tissue (27).Thus, treatment aimed at minimising this adverse remodelling pathway should be initiated at the earliest opportunity (28).

The rhythm of AF itself along with the structural remodelling that takes predispose the atrial myocardium to a prothrombotic state (especially within the left atrial appendage) (29). Furthermore, short episodes AF can cause myocardial damage within the atrium which in turn stimulates the release of prothrombotic factors onto the endothelial surface, leading to the aggregation of platelets. This in part explains why even short episodes of AF can confer long term stroke risk (30, 31).

The mechanisms that cause AF are heterogeneous. For example, in patients with structural heart disease there is a prolonged atrial refractory period that acts as the substrate to AF

whereas as patients who develop AF in the absence of ischaemic heart disease often have a shortening of the atrial refractory period due to the downregulation of inward calcium channels and the upregulation of potassium inward currents (32, 33). This alteration in calcium handling by the atrial myocardium in line with atrial remodelling appears to be the most plausible explanation of how changes in autonomic tone can initiate AF (34).

This pathophysiology becomes relevant clinically as early studies demonstrated that fibrillation of the atrium can lead to regional atrial or left atrial appendage (LAA) blood stasis, thrombus formation, and subsequent embolization to the brain. Although recurrent bouts of AF can lead to atrial remodelling and subsequent perpetuation of the dysrhythmia, atrial cardiomyopathies including fibrosis, elevated filling pressures, and chamber dilation have been associated with an increased thromboembolic risk (35). Abnormal prothrombotic markers (e.g., elevated C-reactive protein, D-dimer, plasma vWF, and prothrombin fragments 1and 2) have been linked to progressive atrial and LAA dysfunction as well as vascular events and stroke in patients with AF (35). The combination of blood stasis from dysrhythmia, a procoagulant state, and abnormal atrial substrate together completes the Virchow-Robin triad leading to thrombogenesis. Thus, the pathophysiology underlying AF is complex and involves several factors, including genetic predisposition, abnormal atrial substrate, hematologic and inflammatory derangements. My hypothesis is that inflammation and fibrosis play a key role in the detrimental effects AF has on exercise capacity in this population and proving such would lead to a pathway by which interventional treatment can help such patients.

1.1.2 **Screening For Atrial Fibrillation**

The adverse outcomes associated with AF are preventable by the appropriate and timely introduction of medical therapy. Given the fact that AF related stroke carries with it a poorer outcome than in those patients who suffer non-AF related stroke the appropriate use of oral anticoagulants (OACs) provides a means by which the detrimental thromboembolic effects of AF can be avoided. In this section I briefly outline the importance of detecting AF as my thesis included patients with permanent AF only and thus its delineation from other forms of this common arrythmia is important to decipher.

In an ideal setting AF would be negated by the introduction of effective primary preventative therapies, with the next best option being the early initiation of treatment if and when AF is detected. However, with 30% of AF being found in asymptomatic patients how best to detect this arrhythmia is of growing concern (36). A proportion of patients are fortunate enough to have AF detected by chance, often due to routine medical examinations for other reasons. Screening for AF is already recommended by the European Society of Cardiology (ESC) in all patients ≥65 years contacting health services (37). This is based on the 60% improvement in AF detection compared to routine care over 12 months in the landmark SAFE trial (38). Risk scores that are commonly used to predict stroke in patients with AF (e.g., CHA_2DS_2VASc) are also able to predict AF risk in patients in sinus rhythm, suggesting that they may be used to identify individuals who are more likely both to display AF upon screening and to benefit from treatment (39).

The absence of symptoms does not remove or reduce the risk of associated stroke, with this cohort of patients often found to have a higher CHA_2DS_2VASc score than symptomatic patients (40). Unfortunately for the vast majority of patients with asymptomatic AF the first opportunity to detect this arrhythmia is in the context of an acute stroke (41). One in five ischaemic strokes are attributable to AF of which greater then 20% AF are diagnosed after the stroke event (42). Without question such events could have been avoided with earlier detection and initiation of OAC.

Screening studies report varying rates of newly detected AF, depending on the method of screening and population risk factors. For example, the REHEARSE-AF study recruited 1001 patients with a mean age of 72.6 years and CHA_2DS_2VASc score of 3 and randomised them to twice weekly home ECG screening or usual care(43). In the screening group, there were 19 new cases of AF detected over 1-year follow-up, compared to 5 in the usual care arm at a cost of $10 780 per AF diagnosis. In contrast, the ASSERT-II study used implantable subcutaneous ECG monitoring to detect AF over 16-month follow-up (44). They recruited 256 people in sinus rhythm at baseline with a mean age of 74 years and CHA_2DS_2VASc score of 4.1 and found 34.4% of participants had at least one episode of AF lasting over 5 min.

The need for more proactive screening for AF (e.g., using ECG Holter monitoring) has been much debated as many patients have ‘asymptomatic AF’ and the arrhythmia is often first diagnosed during presentation with stroke. Asymptomatic AF confers a similar stroke and thromboembolic risk as symptomatic AF (45). Prolonged ambulatory ECG monitoring has been associated with higher yields of AF detection, with up to 15.6% of patients found to have AF episodes using a 7-day event loop recorder in selected populations (46, 47). ECG

monitoring for 30 days resulted in a 5-fold increase in detection rate of AF (48). A study conducted by Elijovich et al concluded that up to 20% of patients with cryptogenic stroke had intermittent AF on 30-day ambulatory monitor and required anticoagulation (49).

To further identify the most productive form of monitoring to detect AF in patients after a stroke event a large-scale meta-analysis of over 11,500 patients across 50 studies evaluated the number of new cases of AF detected by various diagnostic methods (50). About 10% of the study data came from Asia. Nearly 8% of AF cases were diagnosed based on presentation ECG during admissions after a previous stroke. In hospital ECG monitoring provided similar yield. By far the greatest yield of new cases of AF was derived from outpatient ambulatory ECG monitoring (telemetry, external and internal loop recorders) which identified AF in upto 16.9% of cases (50). Given that it is crucial to establish anticoagulant therapy before thrombotic events occur the diagnostic focus should clearly be on ambulatory AF detection in primary care settings.

The prospective, multicentre CRYSTAL AF study randomised patients who had a stroke within the last 90 days to conventional monitoring (as decided by the treating physician) vs. use of implantable cardiac monitors (ICM) (51). Of the 441 patients enrolled in the study, AF was detected at a rate of 8.9% at 6 months, 12.4% at 1 year and 30% at 3 years in the ICM arm as opposed to only 1.2%, 2% and 3%, respectively in the standard therapy arm (51). Of those with detected AF more than 92% had the arrhythmia for >6 minutes per day, a suggested threshold for increased risk for stroke risk based the ASSERT study (52). Additionally, the EMBRACE study found superiority of 30-day event triggered recording over 24 hour Holter monitoring in a study of 572 patients with cryptogenic stroke (48). The increased detection of

AF using prolonged methods of monitoring has allowed OAC to be appropriately justified in a larger proportion of patients.

Knowing AF burden also helps risk stratification (53), 5-6 minutes duration having been found to be clinically relevant (54). In one study of 312 patients 5-minute AF burden was associated with a 3.2% risk of stroke (54). Subsequently, different values of AF burden have been proposed to justify OAC use (ranging from 5 minutes to 24 hours) but the ASSERT trial points to a 6 minute AF burden as a clinically justifiable cut off value (52).

Recently the SEARCH AF study analysed the feasibility and cost effectiveness of opportunistic, community-based screening in Australia for patients aged over 65 (40). A structured screening method including a brief history, pulse palpation, and a handheld phone-based ECG recording was taken. A total of 1000 pharmacy customers were screened with newly identified AF in 1.5% of the cohort. The sensitivity and specificity for this automated iECG algorithm was 98.5% and 91.4% respectively. A cost-effective analysis showed that most benefit was observed in relation to quality adjusted life years in those patients in whom anticoagulation adherence was optimal.

The detection and management of AF is a core component of stroke prevention in the AF patient population. A proposed method by which screening, and management of Non valvular AF is shown in **Figure 1**. With an increasingly ageing population with multiple co morbidities, the diagnosis of AF becomes more likely. Most guidelines advocate simple opportunistic pulse check in primary care practices, but more prolonged forms of monitoring increase the yield of AF detection (37). With the introduction of NOACs there appears to be

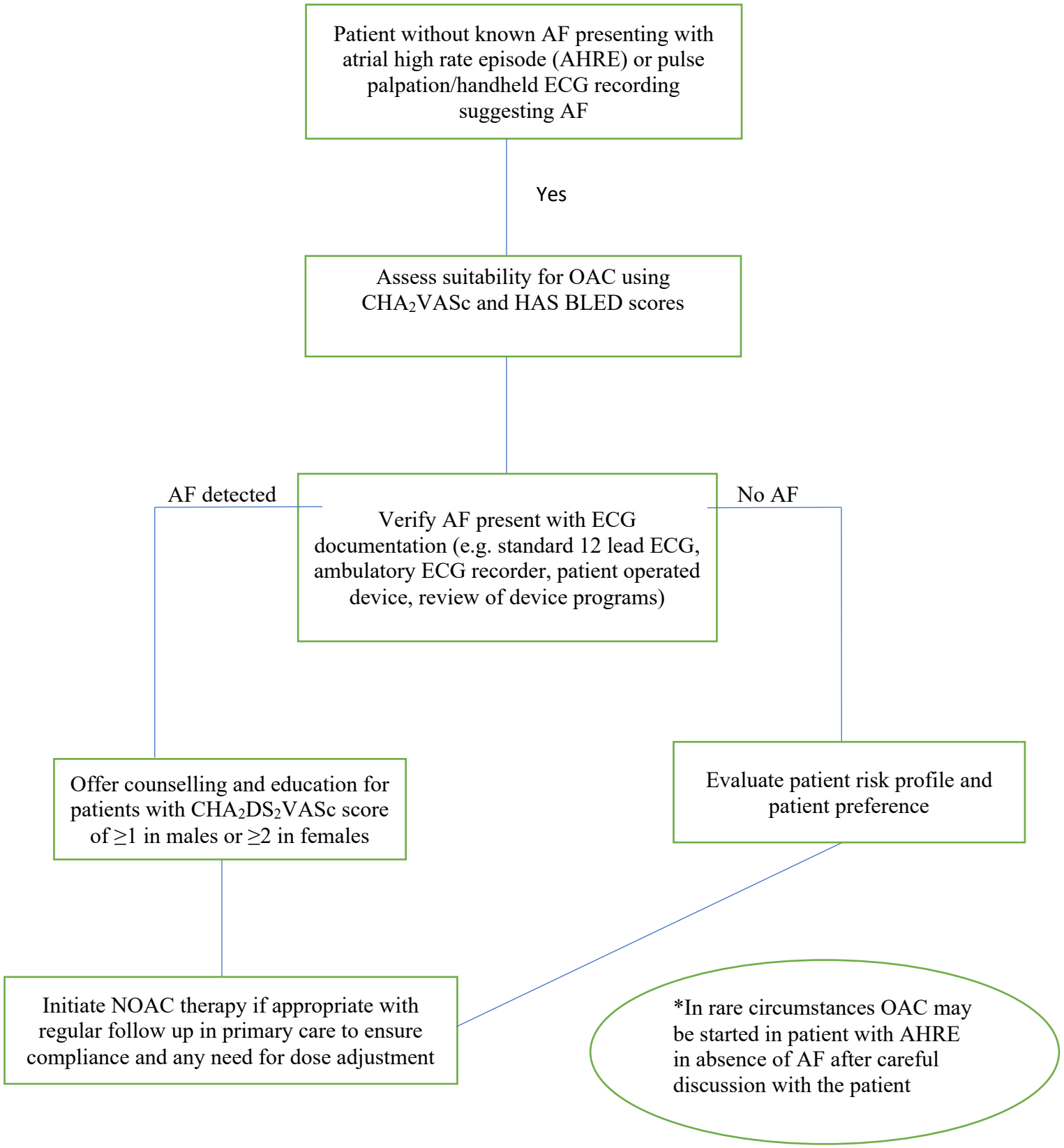
little reason not to offer anticoagulation to all AF patients with ≥1 stroke risk factor, apart from those truly deemed "low risk" using the CHA_2DS_2VASc score.

With regards to Atrial High-Rate Episodes (AHRE) recent studies have provided a higher association with thromboembolic disease (2.6%/year) and mortality (3%/year) compared to their counterparts in whom AHRE were not detected. (55) . In such cases monitoring is often done through pre-existing implanted cardiac devises, for example, pacemakers or cardiac defibrillators. However, AHRE as a lone risk factor to thromboembolic risk without pre-existing risk factors as dictated by the CHA_2DS_2VASc score is less well established (56).

With the fruition of large randomised studies showing a clear benefit for prolonged monitoring in patients at risk of AF along with a cost benefit, guidelines will no doubt begin to incorporate a more structured approach for the detection of asymptomatic AF. However, screening can be potentially harmful. Population-level screening could lead to significant numbers of false-positive results. A range of non-invasive approaches to screening are possible, almost all with a sensitivity and specificity above 90% (57). However, unlike other screening programmes where a positive screening result would prompt further detailed investigation to confirm the diagnosis, a positive AF screening result or confirmatory ECG would directly trigger the initiation of anticoagulant treatment where appropriate. Even if the screening method used had a 95% specificity for AF diagnosis, up to 50 000 people per million screened might be falsely diagnosed (58). These patients might be exposed to unnecessary additional investigations, health anxiety around the implications of their diagnosis and an increased bleeding risk for those started on anticoagulation therapy.

At present pulse palpation and ECG rhythm strip are recommended for primary prevention and short-term monitoring of at least 72 hours in those patients having suffered a TIA or ischaemic stroke (59). Current ESC guidelines do not recommend a definitive length of monitoring for the detection of AF but do state 30 seconds of documented AF on a rhythm strip of capture on a 12 lead ECG is sufficient for the diagnosis (37). Studies alluded to above give more pragmatic approaches to length of monitoring based on patient's risk profile and likelihood of AF.

Figure 1. Proposed Algorithm for the detection and management of Atrial Fibrillation.



1.1.3 **Risk Stratification And Treatment Of Atrial Fibrillation**

Patients recruited into my study to understand the role of inflammation and fibrosis upon exercise capacity in permanent atrial fibrillation will have been assessed for stroke risk and initiated on OAC if deemed appropriate. Thus, I give a brief outline of the recommended methods used to assess such patients and the common treatment options available. In depth explanations of risk stratification and treatment is not relevant to my hypothesis of thesis purpose.

The risk of stroke is not homogenous amongst patients with AF, and numerous risk factors have been proven to provide heightened risk of stroke in this population (60) **(Table 1)**. Many of these risk factors were derived from the non-VKA arms of the historical trials performed 20 years ago, although these trials only randomized <10% of patients screened, and many risk factors were not recorded nor well-defined.

Table 1. Risk factors shown to be well validated and less validated for the incidence of AF

Validated risk factors for incidence of AF	Less validated risk factors for incidence of AF
Age	Height
Male gender	Sleep apnea
Hypertension	Subclinical hyperthyroidism
Valvular Heart Disease	Alcohol consumption
Heart Failure	Chronic kidney disease
Diabetes	Chronic obstructive airways disease
Coronary Artery Disease	Coffee
Genetic Factors	PR interval
Obesity	Biomarkers (ANP, BNP, CRP, IL-6, TNF-α)

With the identification of various stroke risk factors, various risk stratification-scoring systems were formulated to help identify patients that are at significant risk of stroke. These scores were focused on identifying 'high risk' patients to be targeted for an 'inconvenient' drug, warfarin, that required regular anticoagulation monitoring and had various drug/diet interactions. However, risk is a continuum and the artificial division into low, moderate and high-risk strata did not reflect rates of OAC use. Hence the shift of focus to initially identify low risk patients who did not require any antithrombotic therapy, following which OAC can be prescribed to those with ≥1 stroke risk factor (61). However, one must always be aware of the counterbalance of bleeding risk and adverse side effects in such patients. Often those patients who warrant OAC therapy are older, frailer, and with added co morbidity. This in turn can lead to heightened bleeding risk when initiated on OAC therapy. Furthermore, in this

population, drug interactions are more common which in themselves can be detrimental to the patients. Therefore, a careful balance between benefit of stroke prevention vs potential adverse effects of pharmacotherapy must be taken into account.

1.1.4 **CHA_2DS_2VASc Score In Stroke Risk Stratification**

Various studies have shown that the $CHADS_2$ score either performs similarly or less well than the more dynamic CHA_2DS_2VASc (congestive heart failure, hypertension with blood pressure > 140/90, age 65 to 74 or > 75 years, diabetes mellitus, previous stroke/TIA or thromboembolism, vascular disease). A large Swedish cohort study showed a clear advantage of the CHA_2DS_2VASc over the $CHADS_2$ system. In a total of 182 678 patients the c statistics for predicting stroke and thromboembolism were similar at 0.66 and 0.67 respectively. However, the CHA_2DS_2VASc was more effective in identifying truly low risk patients not in need of OAC (62) .

The CHA_2DS_2VASc score extends the older $CHADS_2$ score by considering additional 'non $CHADS_2$' stroke risk factors, such as age 65-75, female sex and vascular disease. For example, increasing age is a powerful driver of stroke risk (63, 64) (65) and the risk rises dramatically from the age of 65 upwards (66). More recent studies suggest that the stroke risk in Asians may start to rise from age >50, such that age 50-64 years confers an ischaemic stroke risk of approximately 1.5% /year (67).

Historically, the CHA_2DS_2VASc score was initially derived from the 2006 Birmingham schema algorithm (68) and the acronym validated using the Euro Heart Survey on AF cohort in 2010 (69). The CHA_2DS_2VASc consistently outperforms the $CHADS_2$ score for

identifying ‘low risk’ patients (45). For example, in the Danish nationwide cohort study, the CHA_2DS_2VASc score was superior to the $CHADS_2$ score for predicting high risk patients, but more importantly offered additional risk refinement of those categorised as ‘low risk’ using the older $CHADS_2$ score (70). Such appealing evidence has led to the CHA_2DS_2VASc schema being adopted in European guidelines as it provides an effective and rapid assessment of stroke risk, which can be accommodated at the patient bed side and busy outpatient settings (37). (**Table 2**) highlights the diverse range of risk stratification tools available that can be used in patient with AF to assess need for OAC).

Table 2. Comparison of risk assessment schemas in patients being considered for thromboprophylaxis in AF related stroke. (The Qstroke score includes additional risk factors of: Ethnicity, deprivation score, smoking status, Tot.chol:HDL ratio, Body Mass Index, Family history of coronary disease, rheumatoid arthritis, valvular heart disease).

Risk Factor	**$CHADS_2$**	**CHA_2DS_2VASc**	**R_2CHADS_2**	**QStroke**	**ATRIA**
Age	≥ 75	65-74; ≥ 75	≥ 75	25-84	≥65
Female	✗	✓	✗	✓	✓
Prev. Stroke/TE events	✓	✓	✓	✗	✓
Hypertension	✓	✓	✓	✓	✓
Heart Failure	✓	✓	✓	✓	✓
Diabetes	✓	✓	✓	✓	✓
Vascular Disease	✗	✓	✗	✓	✗
eGFR<60 ML/MIN/1.73m^2	✗	✗	✓	✗	✗
Moderate/Severe CKD	✗	✗	✗	✗	✓
Atrial Fibrillation	✗	✗	✗	✓	✗
Proteinuria	✗	✗	✗	✗	✓

1.1.5 **HAS BLED Score For Bleeding Risk**

The HAS BLED score (hypertension SBP>160mmHg, abnormal liver/renal function (with creatinine ≥200μmol/L), stroke, bleeding history or predisposition, labile INR (INR in range <60% of the time), elderly (>65), concomitant drugs/alcohol), was developed and validated in 2010 in the Euro Heart survey population and is recommended in the latest ESC guidelines along with the CHA_2DS_2VASc score for the stroke risk stratification of patients with AF (71, 72). The HAS BLED score demonstrates a good predictive ability in this cohort with a c-statistic of 0.72, with excellent prediction of bleeding risk on antiplatelet therapy (c-statistics 0.91) or no antithrombotic therapy (c-statistics 0.85).

In this scoring system patients are stratified into low-moderate risk with a score 0-2, and high risk as score ≥ 3. In a recent meta-analysis by Zhu et al, the HAS BLED score outperformed the $HEMORR_2HAGES$ and ATRIA bleeding scores, with superior net reclassification improvements, most evident in low and high risk groups (73). Similar findings were found in a meta-analysis by Caldeira et al where the HAS BLED score was more sensitive in predicting bleeding risk than both the $HEMORR_2HAGES$ and ATRIA scores, with added benefit due to ease of use (74). (**Table 3**. Comparison of factors included in the HAS BLED score compared to other available risk scores).

Table 3. Comparison of risk factors used in assessment of bleeding risk in AF patients requiring OAC. The HAS BLED score uses an age >65 for elderly, whereas this is ≥75 in the ATRIA schema and >75 for HEMORR$_2$HAGES.

Risk Factor	HAS BLED	HEMORR$_2$HAGES	ATRIA	ORBIT
Hypertension	✓	✓	✓	✗
Abnormal Liver/Renal function	✓	✓	✓	✓
Stroke	✓	✓	✗	✗
Bleeding/Rebleeding risk	✓	✓	✓	✓
Liable INR	✓	✗	✗	✗
Elderly	✓	✓	✓	✓
Concomitant drugs/alcohol	✓	✓	✗	✓
Malignancy	✗	✓	✗	✗
Reduced PLT count/function	✗	✓	✗	✗
Anaemia	✗	✓	✓	✓
High falls risk	✗	✓	✗	✗
Genetic factors	✗	✓	✗	✗

1.1.6 **Anticoagulation In Atrial Fibrillation**

Stroke prevention in patients with non-valvular AF requires careful consideration of the risk versus benefit of starting OAC therapy. Stroke and bleeding risk factors in patients with AF are not homogeneous and risk stratification schemes such as the CHA_2DS_2VASc and HAS BLED scores are well validated and provide a simple, quick, yet concise method of assessing a patient's suitability for anticoagulation, without the necessity of complex composite scores or multiple biomarkers (75, 76).

In 2009 came the introduction of the non-VKA oral anticoagulants (NOACs), which revolutionised the management of stroke prevention in non-valvular AF. Initially referred to as new or novel OACs, or sometimes direct oral anticoagulants (DOACs), the NOAC acronym has been retained to refer to non-VKA oral anticoagulants(77) (78). The 4 major drugs (Dabigatran, Apixaban, Rivaroxaban and Edoxaban) compare favourably with warfarin, showing at least non-inferiority with regards to stroke prevention, with a superior safety profile in relation to major bleeding (79-82). Recent data from ancillary analyses of the major trials show that patients taking NOACs are at 30-50% lower risk of major bleeding than with warfarin (83-85). As yet no head-head trials amongst the NOACS has been undertaken. (**Table 4** highlights the recommendations for NOAC therapy form major international guidelines).

Table 4. Summary of current recommendations for the initiation and management of NOAC therapy.

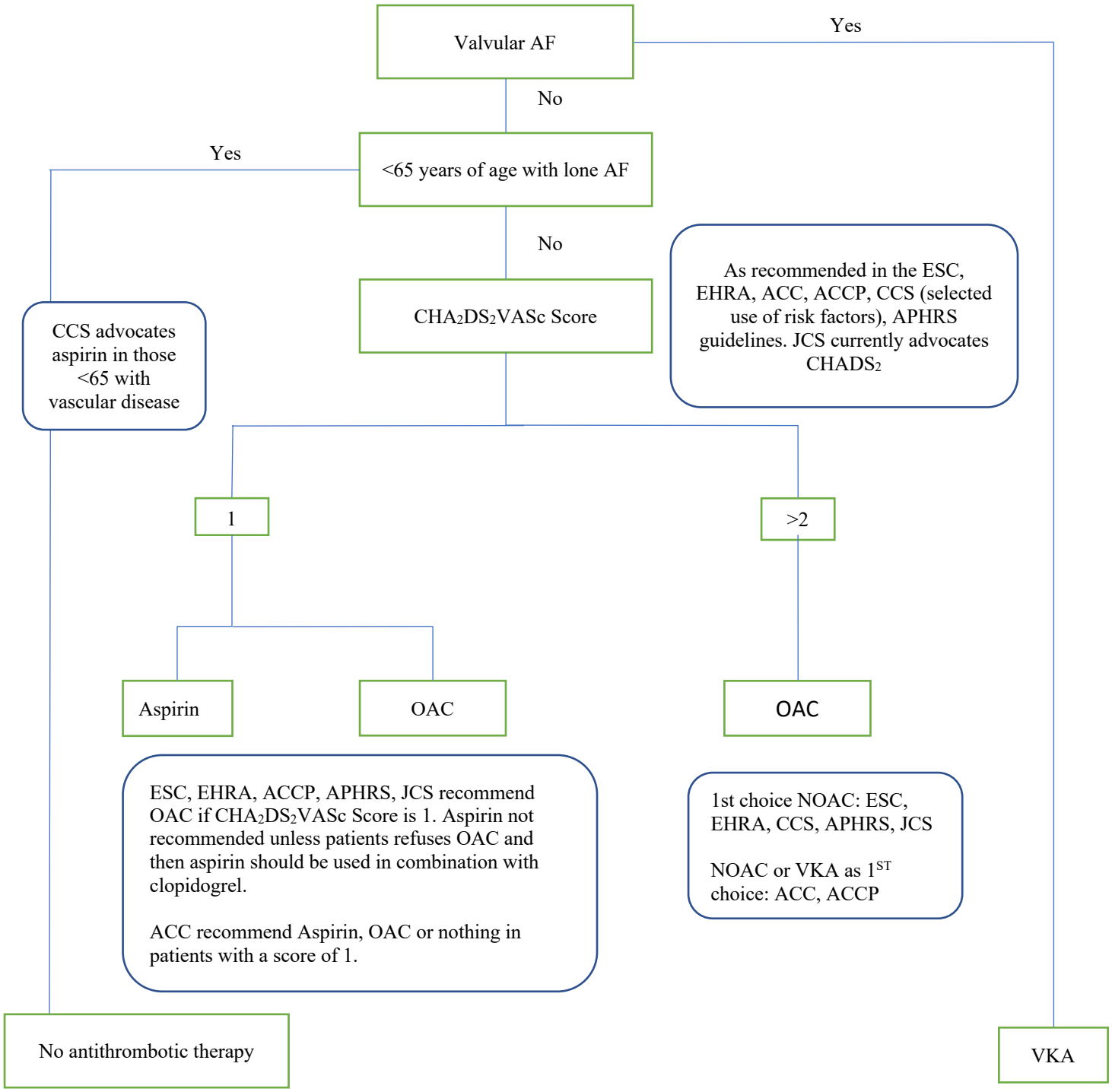
	ESC (59)/EHRA (86)	**ACC/AHA/HRS (87)**	**CCS (88)**	**APHRS (89)**	**JCS (90)**
NOAC VS VKA	NOAC	No preference	NOAC	NOAC	NOAC
Dose Reduction in CKD	Dabigatran not recommended if CrCl<30ml/min 15mg OD Rivaroxaban if CrCl 30-49ml/min. 2.5mg BD Apixaban if serum Creatinine ≥ 1.5mg.dl. 30mg (or 15mg) OD Edoxaban if CrCl<50 ml/min	75mg BD Dabigatran if CrCl 15-30ml/min 15mg OD Rivaroxaban if CrCl 15-50 ml/min 2.5 or 5mg BD Apixaban if CrCl 30-50ml/min	In all patients taking NOAC dose reduction to lower dose recommended irrespective of NOAC if eGFR is 30-50 ml/min/1.73m^2	110mg BD Dabigatran if Cr Cl 30-50 ml/min 15mg OD Rivaroxaban if CrCl 30-50ml/min 2.5mg BD Apixiban if serum Creatinine ≥ 1.5mg. dl.	Statement advising dose adjustment to lower doses in patients with "moderate" renal dysfunction
Management of acute severe bleeding/Emergency Surgery	Consider specific antidote or PCC if antidote unavailable	Not Specified	Not specified.	Not specified	Administration of VII, IX, FFP Consider dialysis for dabigatran Gastric lavage with activated charcoal
Duration of cessation of NOAC therapy for elective procedures	24 hours if low bleeding risk 48 hours if high bleeding risk	24 hours if low bleeding risk 48 hours if high bleeding risk	24 hours if low bleeding risk 48 hours if high bleeding risk	Upto 5 days for major surgery	Upto 4 days with possible heparin bridging
Follow Up Timeline	1st follow up at 1 month	Not Specified	Not formally specified but recommendation of annual renal function monitoring	Not specified	Not specified

Abbreviations: ESC (European Society of Cardiology), EHRA (European Heart Rhythm Association), ACC (American College Cardiology), ACCP (American College of Chest Physicians), CCS (Canadian Cardiology Society), APHRS (Asian Pacific Heart Rhythm Society), JCS (Japan Cardiology Society), NOAC (Non-vitamin K oral anticoagulants), VKA (Vitamin K antagonists), CKD (Chronic Kidney Disease), CrCl (Creatinine Clearance), eGFR (estimated Glomerular Filtration Rate), PCC (Plasma Cell Concentrate), FFP (Fresh Frozen Plasma)

Thus, NOACs provide a better, safer and more convenient anticoagulation option with a greater net clinical benefit (91) . Accordingly, NOACs are now a well-established option (in addition to Warfarin) for the prevention of thrombo-embolic events in non-valvular AF and venous thromboembolism, being given preference over warfarin in many updated clinical guidelines on the management of AF (59, 87, 92). (**Figure 2** brings together anti coagulation guidelines from various countries in the form of a proposed algorithm).

Despite AF being a major risk factor for stroke such patients rarely have lone AF and indeed present with multiple co-morbidities. Heart failure is one of the commonest diagnosis to be found alongside AF and as such carry's significant morbidity and mortality in such patients. However, the study population for my thesis included people with preserved left ventricular function and excluded those with significant cardiovascular and indeed medical co-morbidities that would reduce the likelihood of completing the 2-year study period and indeed impact the primary and secondary outcomes of the study.

Figure 2. Decision tree for antithrombotic therapy in patients with non-valvular atrial fibrillation: A summary of recent guidelines



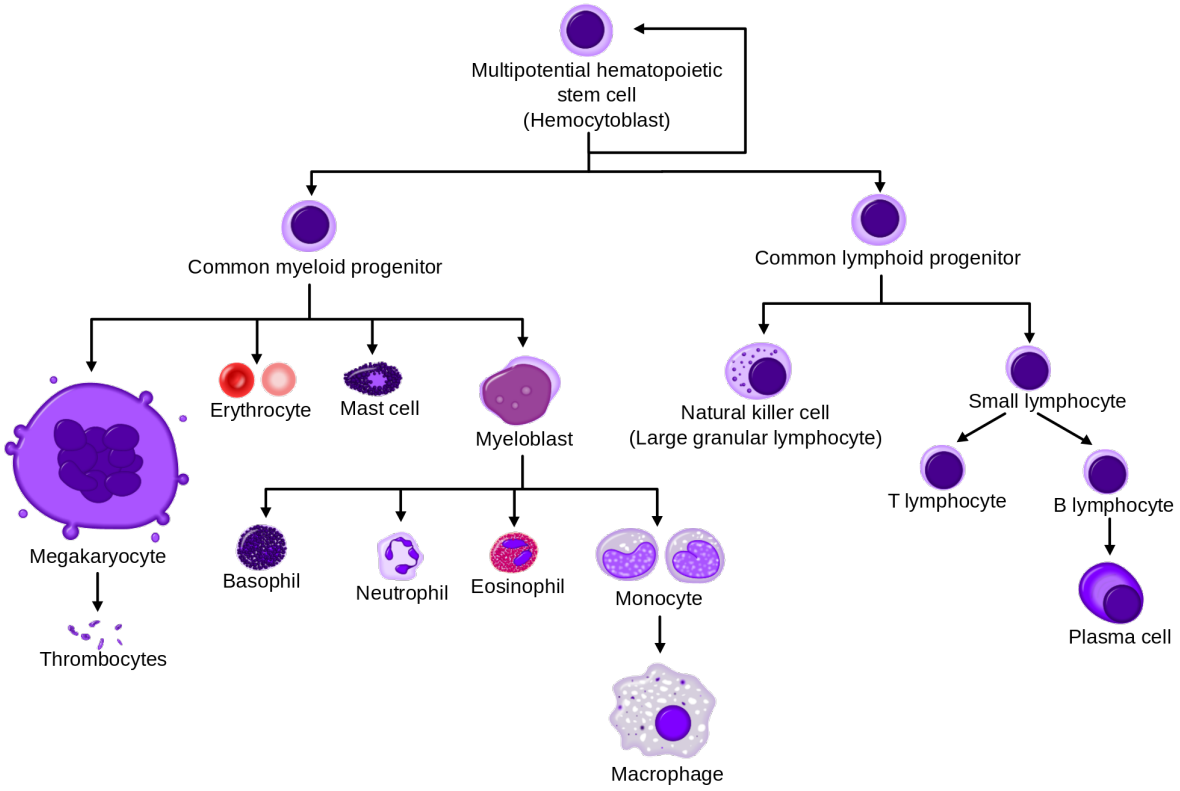
Legend: This figure combines recommendations from current ESC, EHRA, ACC, ACCP, CCS, APHRS and JCS guidelines. The use of purpled boxes explains differences in management between guidelines. ESC (European Society of Cardiology), EHRA (European Heart Rhythm Association), ACC (American College Cardiology), ACCP (American College of Chest Physicians), CCS (Canadian Cardiology Society), APHRS (Asian Pacific Heart Rhythm Society), JCS (Japan Cardiology Society), OAC (Oral anti coagulation) NOAC (Non-vitamin K oral anticoagulants), VKA (Vitamin K antagonists) AF (Atrial Fibrillation)

1.2 **What Do Monocytes Do And Their Relevance In Cardiovascular Disease?**

As part of my study hypothesis, I explored the role of monocytes and their subsets with regards to their impact on exercise capacity in patients with permanent AF and preserved left ventricular function. Monocytes have been studied in a variety of cardiovascular disease states and this section of my thesis gives a background to their functional role, properties and how this brings relevance to my study hypothesis.

Monocytes are a type of white blood cell present in the peripheral circulation. The primary role of monocytes are in the participation of innate immunity and to maintain or replenish different types of macrophages and dendritic cells which aid in phagocytosis of pathogens (93). Monocytes make up to 8% of the peripheral blood white cells and play a central role in the host response to exogenous and endogenous pathogen species such as bacteria and viruses. Additionally, they modulate the inflammatory processes, producing both pro- and anti-inflammatory cytokines and developing macrophages with pro- and anti-inflammatory phenotype (94)**. (Figure 3)**

Figure 3. Presence of monocytes in the inflammatory cascade



Monocytes are derived from macrophage dendritic cell precursors (MDP) that originate from the bone marrow under normal homeostatic conditions. Common myeloid progenitor cells (CMP) derived from the bone marrow are responsible for differentiation of precursor progenitor cells into monocytes (94). MDP mature to form either dendritic cells or macrophages. This process is dependent upon stimulation by cytokines and/or microbial molecules (95). Evidence to date suggests that both monocytes and dendritic cells diverge at a very early multi-potent progenitor stage (94). CMP give rise to the granulocyte-macrophage lineage which in turn give rise to MDP and subsequently, the committed monocyte precursor (96). Control of monocyte/macrophage differentiation is guided by a multitude of transcription factors, the complexity of which is beyond the scope of this section (97).

In the 1970s studies highlighted the increase in monocyte proliferation within the bone marrow in response to inflammatory stimuli, allowing for monocytosis (98). During steady state, circulating monocytes have a half-life of approximately 3 days (99). Monocytes are mobilized from the bone marrow at times of tissue injury and differentiate into macrophages or dendritic cells whilst mounting an immune response. However, they are also implicated in diseases with pro-inflammatory shift such as heart failure and atherosclerosis (100, 101). Multiple animal studies have shown a diverse and complex function of monocytes depending upon the inflammatory environment, central to which is the ability of monocytes to be mobilized to site of injury (102). With regards to atherosclerosis, monocyte-derived "foam cell" macrophages act as a substrate and thus facilitate the progress to myocardial infarction (MI). Monocyte counts are further highly increased in other forms of acute cardiovascular pathology (101, 103, 104).

Overall, monocytes have been used as indicators of prognosis in humans with their high numbers being associated with increased risk of recurrent MI, hospitalisation and cardiac death. Available data indicate that monocyte mobilisation in acute cardiac disease does not simply reflect response to cardiac damage, but its active involvement in the pathological process (105, 106). However, limited evidence is available for the role of monocytes and their subsets in this same regard for patients with AF.

1.2.1 **Mouse Monocyte Heterogeneity**

Research into the role and function of monocytes was initially carried out on mouse models and this laid the foundation for subsequent research into human subsets. Distinct mouse monocyte subsets were initially distinguished based on the differential expression of a

chemokine receptor, CCR2 (receptor to monocyte chemoattractant protein 1). $CCR2^{+}$ monocytes showed a higher migratory and infiltratory capacity compared to $CCR2^{-}$ cells, most recently being studied in post infarct cardiac remodelling (107) (108) (109). Differential expression of an inflammatory monocyte marker, Ly6C, allowed for better mouse subset characterization (110). Analysis of the two principal mouse monocyte subsets ($Ly6C^{+}$ and $Ly6C^{-}$) is commonly used in experimental research; there is accumulating evidence for the existence of a subset with intermediate phenotype, which resemble human "intermediate" Mon2 subset (111, 112). The subsets differ in expression of surface markers, for example: CD11b, CD115 have a high density of CCR2 and only small numbers of CX3CR1 are present on $Ly6C^{+}$ monocytes. In contrast, $Ly6C^{-}$ monocytes virtually lack CCR2, but express high levels of CX3CR1(110).

Ly-$6C^{+}$ monocytes have phagocytic and pro-inflammatory characteristics. In acute MI they accumulate promptly in areas of myocardial injury, along with macrophages providing a pro-inflammatory environment(113, 114). Ly-$6C^{-}$ monocytes on the other hand have been found to have anti-inflammatory properties and this subset promotes post-MI myocardial healing via the processes of myoblast activation, angiogenesis, and collagen formation (111, 115, 116). Overall, the Ly-$6C^{+}$ subset is associated with detrimental effects to myocardium and their high levels in acute phase of MI delay myocardial healing (117).

Functional studies have demonstrated that $Ly6C^{+}$ cells release ROS, nitric oxide (NO), and inflammatory cytokines (e.g., tumour necrosis factor α [TNFα], IL-1β) in response to bacterial infection (95). The subset migration is potentiated via the CCR2 receptor that initiates a change in the ligand for VCAM-1 (VLA 4). Studies have found that $Ly6C^{+}$ monocytes preferentially

migrate into the sites of vascular inflammation and CCR2 is central to this process, also promoting the subset maturation towards M1 macrophage phenotype (108, 118).

In the absence of inflammation, $Ly6C^{+}$ transforms into $Ly6C^{-}$ which predominates in the circulation, binding to vascular endothelium using CX3CR1 receptors (119). In response to bacterial infection $Ly6C^{-}$ cells release anti-inflammatory cytokines (namely IL-10). The response to inflammation triggers the differentiation of monocytes into M2 macrophages, which in turn release anti-inflammatory cytokines central to tissue repair (110, 120, 121).

1.2.2 **Human Monocyte Heterogeneity**

Monocyte subsets were first isolated in 1988 using flow cytometry (122). Expression of Cluster of Differentiation 14 (CD14) (lipopolysaccharide receptor) and cluster of differentiation 16 (CD16) (Fc receptor) is used to define human monocyte subsets. It should be noted that changes in expression of monocyte subsets are limited to cell surface protein expression assessed by flow cytometry, with changes in gene expression being up or downregulated dependent on functional properties (123).

Human monocyte subsets do not follow their mouse counterparts, in which initial studies in this field were undertaken. As such, nomenclature for human and mouse monocytes is not directly interchangeable, and thus should not be directly compared. Human monocytes do not express Ly6C and description of their subsets is primarily based on expression of CD14 (lipopolysaccharide receptor) and CD16 (FC gamma III receptor) **(Table 5)**

Table 5. Phenotypic and functional differences between human and mouse monocyte subsets

	Human (Mon1)	Mouse (Mon1)	Human (Mon2)	Mouse (Mon2)	Human (Mon3)	Mouse (Mon3)
Proportion of total monocytes (%) (124, 125)	85	40-45	5	5-32	10	26-50
Functional properties (124-126)	High phagocytic activity	High phagocytic activity, pro-inflammatory	High phagocytic activity. T- cell proliferation and stimulation, angiogenesis, superior ROS production	High phagocytic activity, pro inflammatory	Low phagocytic activity, high "patrolling" activity (in vivo), T- cell proliferation and stimulation	Low phagocytic activity, patrolling function, tissue repair
Surface markers present (124, 127-129)	CD62L, CCR2, CLEC4D, CLEC5A, IL13Rα1, CXCR1, CXCR2	CCR2, CD11b, CD115, CCR5	CCR2, CD74, HLA-DR, Tie-2, ENG	CCR2, CD11b, CD115	Siglec10, CD43, SLAN	CX3CR1, CD11b, CD115, CCR5
Surface markers absent (124, 126, 130)	CX3CR1, CD123, p2rx1, Siglec10	CX3CR1(low)	CD62L, CXCR1, CXCR2, CLEC4D IL13Rα1	CX3CR1(low)	CCR5, CD62L, CXCR1, CXCR2, CD163, CLEC4D, IL13Rα1	CCR2(low)
Response to LPS (124, 127, 131-133)	IL-10, G-CSF, CCL2, RANTES, IL-6, IL-8	ROS, TNFα,nitric oxide, IL-1β,IL10(low levels), IFN-1, VLA-4, IL-6, CD62L	IL-6, IL-8	ROS, TNFα,nitric oxide, IL-1β,IL10(low levels), IFN-1, VLA-4, IL-6, CCR7, CCR8	TNFα,IL-1β,IL-6, IL-8	IL-10 (High levels)
Increased gene expression (96, 124, 127, 134-136)	Wound healing and anticoagulation, S-100 proteins, scavenger receptors, C type lectin receptors, anti-apoptosis, response to stimuli (CCR2, THBS1, CD163, RNASE4, EDG3, S100A12, CLEC4D, VEGFA, F5, RNASE2, RNASE6, F13A1, CRISPLD2, PLA2G7CES1, EREG, QPCT)	CD177, FN1, Sell, Mmp8, F13a1, Atrnl1, Ly-6c, Chi313,	MHC Class II, presentation and processing (CD14, CSPG2, SLC2A3, CD9, CD163, PLA2G7, MCEMP1, CLEC10A, EVA1, RNASE2, GFRA2, ALDH1A1, GALS2, MARCO, ALOX5AP, S100A12, QPCT, FOLR3, OSM, EGR1, CYP27A1, OLFM1, PAD14, HLADOA, ANG, H19, SCD, calgranulin B, S100A9DDIT4	Inconclusive data	Cytoskeletal arrangement, complement components, pro-apoptosis, downregulation of transcription (FMNL2, CDKN1C, FCGR3A/B)	Vegfc, G0s2, Ikzf3, Tgfbr3, Cd83, Eno3, Tgm2, Itgax, CD36, Dusp16, Slc12a2, Fabp4

Human monocytes are dominated by 'classical' $CD14^{++}CD16^{-}$ (Mon1) monocytes (i.e., 85%). Humans have at least two types of non-classical monocytes. The CD14++CD16+ (Mon2) subset makes up about 6% of monocytes in humans. CD14+CD16++ (Mon3) human monocytes make up 9% of all monocytes (137, 138). There are many significant differences between Mon2 and Mon3 and, overall Mon2 is phenotypically and functionally closer to Mon1 than to Mon3 (discussed in more detail below). Earlier studies analysed these two subsets together and such data need to be interpreted with care.

A consensus opinion on the nomenclature of human monocytes in 2010 classed monocyte subsets as classical ($CD14^{++}CD16^{-}$), "intermediate" ($CD14^{++}CD16^{+}$) and "non-classical" ($CD14^{+}CD16^{++}$) (139). However, to avoid ambiguity the phenotypic definition and numerical designation (i.e., Mon1, Mon2 and Mon3) have been incorporated into the most recent consensus document on monocytes subsets (140).

Although direct correlation between human monocyte subsets is difficult, their differentiation and role in innate immunity is comparable. In fact, both Mon2 and Mon3 have reduced phagocytic activity, reduced production of reactive oxygen species (ROS) along with lower levels of CCR2 expression (141). Several studies have highlighted the presence of raised levels of Mon2 in human inflammatory diseases (142).

Mon1 is characterized by high expression of CD14, Interleukin 6 (IL6) receptor, CD64, CCR2, CD163, with less dense expression of VCAM and CD204. The Intracellular adhesion molecule receptor (ICAM receptor), CXCR4, CD163, vascular endothelial growth factor (VEGF) receptor 1 have the highest expression on Mon2. Mon3 has maximal expression of CD16, VCAM 1 receptor, CD204, with much lower expression of CD14, IL6 receptor,

CD64, CCR2, and CD163 that Mon2 (143). **(Table 6)**

Table 6. Differential expression of surface markers based on monocyte subsets

Mon1	**Human**	**Mouse**
Proportion of total monocytes (%) (124)	85 (124)	40-45 (124)
Functional properties (124)	High phagocytic activity (124)	High phagocytic activity, pro-inflammatory (124)
Surface markers/receptors present		
CD14 (144), (134, 144)	High	High
CD16 (134, 144)	Low	Low
CCR2 (145), (146, 147)	High (Increased 26-fold)	High
CX3CR1 (134, 145, 146, 148)	Low	Low
CXCR1 (134, 141, 149)	High (Increased 5-fold)	Low
CXCR2 (134, 149)	High (Increased 7-fold)	-
CD11b (134, 147, 150-152)	Low	High
CD115(111) (111, 134)	-	High
CD62L (134, 144, 152)	High (Increased 3-fold)	High
CLEC4D (134, 153)	High (Increased 4-fold)	-
CLEC5A (109, 134, 153)	High (Increased 3-fold)	-
IL13Rα1 (134)	High (Increased 9-fold)	-
CD54 (134, 154)	Low (Decreased 2-fold)	-
CD40 (134, 152)	High (Increased 6-fold)	-
CD36 (109, 134, 155)	High (Increased 2-fold)	-
CD99 (134, 156)	High (Increased 2-fold)	-
CCR1(134, 157)	High (Increased 2-fold)	-
P2XR1 (134)	Low (4-fold)	-
HLA-ABC (134, 158)	Low (Decreased 1-fold)	-
CLEC10A (134)	Low (Decreased 6-fold)	-
GFRA2 (134)	Low (Decreased 6-fold)	-
HLA-DR (109, 134, 158, 159)	Low (Decreased 8-fold)	-
CD163 (134, 160, 161)	Low (Decreased 1-fold)	-
CD115 (112, 130, 134)	Low (Decreased 1-fold)	High
SLAN (134, 162, 163)	High (Increased 2-fold)	-
CD1d (134)	High (Increased 1-fold)	-
CCR5 (134, 164, 165)	Low (Decreased 1-fold)	-
CD294 (134)	Low (Decreased 1-fold)	-
Siglec10 (134, 166, 167)	Low (Decreased 7-fold)	-

Mon2	**Human**	**Mouse**
Proportion of total monocytes (%)	5 (124)	5-32 (124)
Functional Properties	High phagocytic activity, T cell proliferation and stimulation, angiogenesis, superior ROS production (124)	High phagocytic activity, pro inflammatory (124)
Surface markers/receptors present		
CD14(144) (109, 134)	High	High
CD16 (109, 134, 144)	Low	Low
CCR2 (146) (134, 168)	High (Increased 8-fold)	High
CX3CR1 (134, 141, 146)	Low	Low
CXCR1 (134, 141, 149)	High (Increased 4-fold)	Low
CXCR2 (131, 134, 149)	High (Increased 3-fold)	-
CD11b (134, 147, 152)	High	High
CD115 (109, 130, 134)	Low	High
CD62L (134, 152, 158)	High (Increased 1.3-fold)	-
CLEC4D (134)	High (Increased 18-fold)	-
CLEC5A (109, 134)	High (Increased 5-fold)	-
IL13Rα1 (134)	High (Increased 2-fold)	-
CD54 (134, 154)	High (Increased 1-fold)	-
CD40 (134, 152)	High (Increased 1-fold)	-
CD36 (134, 155)	High Increased 5-fold)	-
CD99 (134, 156)	High (Increased 5-fold)	-
P2XR1 (134)	Low (Decreased 5-fold)	-
HLA-ABC (134, 158)	High (Increased 1-fold)	-
CLEC10A (134)	High (Increased 4-fold)	-
GFRA2 (134)	High (Increased 3-fold)	-
HLA-DR(159) (134, 158)	High (Increased 2-fold)	-
CD163 (134, 161)	High (Increased 6-fold)	-
SLAN (134, 162, 163, 169)	Low (Decreased 3-fold)	-
CD1d (134)	Low (Decreased 5-fold)	-
CCR5 (134, 164, 165)	High (Increased 7-fold)	-
CD294 (134)	Low (Decreased 3-fold)	-
Siglec10 (134, 166, 167)	Low (Decreased 21-fold)	-

Mon3	Human	Mouse
Proportion of total monocytes (%)	10 (124)	26-50 (124)
Functional properties	Low phagocytic activity, high "patrolling" activity (in vivo), T- cell proliferation and stimulation (124)	Low phagocytic activity, patrolling function, tissue repair (124)
Surface markers/receptors present		
CD14 (134, 144)	Low	Low
CD16 (134, 144)	High	High
CCR2(146), (134, 147)	Low	Low/-
CX3CR1(130, 146), (134, 152)	High	High
CD11b (134, 147, 170)	High	High
CD62L(144, 152, 171)	Low	Low
P2XR1 (134)	High (Increased 1.2-fold)	-
HLA-ABC (134, 158)	Low (-)	-
CLEC10A (134)	Low (-)	-
GFRA2 (134)	Low (-)	-
HLA-DR (134, 158, 159)	Low (-)	-
CD163 (134, 160, 172)	High (Increased 7-fold)	-
CD115 (112, 130, 134)	Low (Decreased 2-fold)	High
SLAN (134, 162, 169, 173)	Low (Decreased 7-fold)	-
CD1d (134)	High (Increased 4-fold)	-
CCR5 (134, 164, 165)	High (Increased 8-fold)	-
CD294 (134)	Low (Decreased 2-fold)	-
Siglec10 (134, 166, 167)	Low (Decreased 3-fold)	-

Monocyte subpopulations differ in the range of cytokines they can produce in response to stimulation. Mon1 has been shown to preferentially express cytokines interleukin 1-β (IL-1β), IL-6, (Monocyte chemoattractant protein-1) MCP-1, inhibitor of nuclear factor kappa β kinase (IKKβ), whereas Mon2 produces anti-inflammatory IL-10. Interestingly, Mon3 stimulates cytokine production in response to viral rather than bacterial load, further emphasizing the functional differences between monocyte subsets (125). However, recent experiments showed specific release of IL-6 and IL-8 cytokines by Mon2 and Mon3 in response to bacterial endotoxaemia (174). **(Table 7)**

Table 7. Cytokine expression by monocyte subsets in response to LPS in human and mouse models' relative to Mon2.

Plasma cytokine	Mon1	Mon2	Mon3
G-CSF (134, 175, 176)	High	Low	Low
IL-10(125, 177) (134, 178)	High	Low	Low
CCL2 (95, 134)·(179)	High	Low	Low
RANTES (134, 180)	High	Low	Low
IL-6 (95, 134, 152)	High	Intermediate	Intermediate
IL-8 (125, 134, 176)	High	High	High
IL 1-β (95, 134, 152)	Intermediate	Intermediate	High
TNF-α (134, 152, 181)	Intermediate	Low	High

1.3 **Release Of Monocyte Subsets From The Bone Marrow And Spleen**

All three subsets are present in the bone marrow (111) (93). After maturation, Mon1 leaves the bone marrow, entering the peripheral circulation via CCR2 chemokine receptors (145). Previous studies had suggested the ability of Mon1 to differentiate further into Mon2 upon migration from the bone marrow, which enter the circulation (145). Most recently studies have shown the initial release of Mon1 in response to endotoxemia with the subsequent differentiation into Mon2 and Mon3 subsets (182). However, analysis of bone marrow samples indicates that cells with Mon2 phenotype are already present in human bone marrow (183). In fact, cells with the Mon2 phenotype were the dominant monocytic cells within the bone marrow (143).

Monocyte numbers have been found to follow a circadian rhythm controlled by Arnt-1, which is a key gene in regulating the molecular circadian clock (184). In contrast to the presence of CCR2 ligands present on monocytes during homeostasis the release of patrolling monocytes is dependent upon the G protein coupled receptor for sphingosine-1-phosphate, the deficiency of which leads to an inability of Mon3 to redistribute from the bone marrow (185).

Under certain inflammatory conditions (e.g. cancer, heart failure, myocardial infarction and stroke) mouse models have shown the spleen to be capable of extra medullary monopoiesis (186). Release of monocytes from the spleen is dependent upon angiotensin II as opposed to CCR2 in the bone marrow (187). Furthermore, in cardiovascular disease there is a dependency upon a separate chemokine, CXCL1, to direct the release of monocytes from both the splenic and bone marrow reservoirs (188).

1.4 **Roles Of Circulating Monocyte Subsets**

Monocyte studies involving gene analysis highlight the preferential expression of genes involved in angiogenesis, wound healing and coagulation (namely Mon3) (134). Alternatively, Mon1 have a higher capability to produce IL-1β and TNFα in response to bacterial lipopolysaccharides (125). During the inflammatory process both Mon1 and Mon2 bind to MCP-1 thus allowing monocytes to invade into human tissue and perpetuate the inflammatory cascade (189).

In contrast, Mon3 bind to CX3CR/CCL3 receptors via the leucocyte functional antigen 1, subsequently stimulating the release of IL-1β and TNFα. Such pathophysiology has been implicated in autoimmune conditions such as rheumatoid arthritis (125). Mon1 are capable of

changing their phenotype in response to a wide variety of stimuli. Under homeostatic conditions they maintain their function as patrolling monocytes in the Mon3 form. However, in inflammatory states there is a marked diversity in monocyte expression, driven by cytokines (namely GM-CSF, IFNγ, LPS, M-CSF, IL-4, IL-3) (190-192). There are 2 main opposing phenotypes released in such conditions, termed M1/M2 polarized monocytes/macrophages (193).

In vivo studies have found that polarisation of M1 (Mon1) monocytes is a result of pathogens that survive within phagocytes. Under such conditions NK cells, $CD8^+$ T cells or TH1 cells produce GM-CSF and/or IFN-γ (194-196) . The subsequent enhanced phagocytosis enables uptake and/or killing of pathogens but can also play a detrimental role in autoimmunity e.g., rheumatoid arthritis. In contrast M2 polarisation is driven by IL-4, IL-13, and TH2 cells, which inhibit excess inflammation and promote tissue repair (197, 198).

1.5 **Monocytosis**

Aside from the conventional inflammatory cascade, monocytes can also promote vascular inflammation through T cell signalling. By using the co-stimulatory receptor ligand pair CD40-CD40 (CD40L) binding to scavenger receptors such as the scavenger receptor A (found in foam cell formation) monocytes can trigger the inflammatory process found in atherosclerosis (199).

CD40 is present in monocytes, macrophages, and dendritic cells and is part of the TNF receptor superfamily. The CD40 ligand is found on $CD4^+$ T cells in addition to platelets and as such CD40-CD40L expression initiates platelet activation and thrombosis (200). Both

CD40 and CD40L are present on the endothelial cells of vascular endothelium and knock out mice studies have shown an up regulation of M2 macrophages (201).

Mon1 are essential in cardiovascular homeostasis, evidenced by regulation of LDL. Mon2 and Mon3 express higher levels of major histocompatibility complex class II (MHC-II) (202). Indeed, CD40 signalling enhances the expression of cell adhesion molecules, matrix metalloproteinase and pro inflammatory cytokines in macrophages (203).

1.6 **Functions Of Inflammatory And Patrolling Monocytes**

Multiple studies have found that Mon1 are recruited to the site of infection and differentiate to battle a wide spectrum of microorganisms (204). Most recently Mon1 have been found to be the mainstay in homeostatic conditions, even in the absence of inflammation. Monocytes up regulate MHC II expression, which in turn are able to control trafficking of cells to lymph nodes for presentation to antigen T cells (205).

In contrast Mon3 were thought to provide an anti-inflammatory role. Evidence is present to suggest the ability of Mon3 to express the appropriate cytokines to enable migration to the site of inflammation. Mon3 are able to initiate a transcription program that promotes tissue remodelling (206). In cardiovascular disease states, there is an initial recruitment of Mon1 with the expression of TNF, matrix metalloproteinase, and cathepsins. Mon3 are then recruited to the inflammatory site at a later time, expressing high levels of vascular endothelial growth factor, which propagates tissue healing. Current evidence supports the notion that Mon3 serve a “patrolling” function along the luminal surface of vascular endothelial cells during homeostasis (206). Whilst attached, Mon3 travel in close contact

along the vascular endothelium being able to detect sites of tissue injury of pathogens, undertaking phagocytosis where appropriate.

1.7 **Monocyte To Macrophage Transformation**

During early inflammation, there is a preferential differentiation of Mon1 into M1 inflammatory macrophages and Mon2 into M2 anti-inflammatory macrophages (115). Evidence points to the role of Mon1 in phagocytosis, proteolysis, and inflammation with the degradation of tissue. Mon2 dominate late in inflammation to potentiate angiogenesis, fibroblast formation, collagen deposition. Several studies have suggested that monocytes and macrophages can differentiate into different subsets in response to environmental triggers. For example, Mon1 in brain tissue exhibit phenotypic features of anti-inflammatory monocytes with the subsequent expression of M2 macrophages (207). Findings to date provide evidence for the ability of monocytes to exhibit multiple functional capabilities. They achieve this by preferentially differentiating into regulatory or inflammatory mature macrophages/dendritic cells.

1.8 **Monopoiesis And The Role Of Cytokines**

Once in the peripheral circulation, cytokines have been shown to influence the development of monocytes. Macrophage colony stimulating factor (MCSF) is the best recognised of such cytokines, with their importance highlighted with the presence of MCSF receptors on the surface of circulating monocytes (109). In vivo experiments have further shown that administration of exogenous MCSF promotes monocytosis (208). In contrast mouse models have shown that a deficiency in MCSF or their receptors leads to a reduction in monocyte

numbers both in the bone marrow and the peripheral circulation (209). The homeostatic control of monocyte numbers is linked to the modulation of MCSF production through the mononuclear phagocyte system (MPS) derived from the bone marrow leading to a feedback loop mechanism (210) . Stromal cells produce a steady state of MCSF within the blood. In contrast mature mononuclear phagocytes with MCSF receptor on their cell surface facilitate the clearance of MCSF.

In addition, specific cytokines have been implicated in monocytes maturation and differentiation during the inflammatory processes. For example, Granulocyte monocyte CSF is found to at low levels in homeostatic in vivo studies. However, during inflammation mouse models show an up regulation of this cytokine (211). This in turn has the potential to stimulate the production of monocytes with a predominantly inflammatory role. Furthermore, IL-4 has been shown to have a stimulatory effect upon the differentiation of monocytes to their inflammatory phenotype, demonstrated in IL-4 receptor deficient mice studies (212).

1.9 **Role Of Scavenger Receptors**

In order for the recognition and internalisation of foreign pathogens monocyte derived macrophages express scavenger receptors on their surface to complement the patrolling function of monocytes. 1st identified in 1979, scavenger receptors were associated with the ability to bind to and internalise oxidised low-density lipoproteins (oxLDL) (213). Scavenger receptors are able to recognise a diverse range of ligands, ranging from unmodified endogenous proteins and lipoproteins to microbial structures such as bacterial lipopolysaccharide and lipoteichoic acid (214).

The scavenger receptors are heterogeneous and thus divided into subclasses. However, their primary function remains the same, which is to identify and remove unwanted material through the recognition of modified self-molecules (e.g. apoptotic cells) or non-self-molecules (e.g. foreign particles or microorganisms). Removal is carried out by endocytosis, with more complex phagocytosis also being present.

In the physiological setting scavenger receptors have been found to play an important role in macrophage polarisation, which as detailed already is pivotal to the process of phagocytosis. The expression of several scavenger receptors such as CD163, SR A-1, and CD36 are found to be expressed on M2 macrophages which in turn have a role in tissue repair, remodelling and apoptotic cell death (215) (216) . An increase in scavenger receptor expression is indeed vital to the function of M2 cells (being more highly expressed than on M1). SRA-1 and CD36 are able to activate the apoptotic process where as CD163 has anti-inflammatory properties (215). However, it must be noted that although scavenger receptors are predominantly expressed on M2 macrophages they are not exclusive to this macrophage population and can contribute to the pro inflammatory (M1) response depending upon the external environment (217).

1.10 **Monocyte-Platelet Aggregation**

Activation of platelets results in the formation of monocyte-platelet aggregation complexes (MPA) that can be found in the peripheral circulation (218). As part of the immune system, infection or vascular injury stimulates the release of acute phase proteins in the form of CRP, serum amyloids A and P, complement proteins and fibrinogen by the liver. Acute phase

proteins inhibit or destroy microbes as well as stimulating a procoagulant effect. This can in itself limit pathogens by trapping microorganisms in blood clot. Circulating MPA complexes have been found at high levels in patients with underlying atherothrombotic risk factors such as hypertension and diabetes as well as in acute thrombotic cases such as myocardial infarction and stroke (219). MPA levels directly reflect the level of platelet hyperactivity thus providing a marker of blood thrombogenicity (218). More crucially, cross talk between platelets and monocytes is vital to the pro-inflammatory action of platelets with P selectin aiding in the mobilisation of monocytes to the site of tissue injury.

Pathophysiological mechanisms have been linked to the ability of platelets to enhance cytokine release by monocytes when MPA complexes are formed. It has been hypothesised that the presence of inflammatory stimuli triggers a change in monocyte phenotype such that a pro-inflammatory environment is achieved (220). The subsequent transformation to a pro-inflammatory monocyte phenotype gives rise to increased endothelial adhesiveness.
How exactly platelets alter the monocyte phenotype is not fully understood. One hypothesis is that there is a platelet COX 2 dependent up regulation in monocytes. Inhibition of this process with COX 2 inhibitors leads to a decline in CD 16 expression and a subsequent reduction in interaction between monocytes and the endothelium. This correlates with reduced P selectin activity (220).

1.11 **Role Of Monocytes In Cardiovascular Disease**

Inflammation plays a pivotal role in the pathogenesis cardiovascular disease. Up to this point the majority of research into the role of monocytes has been focused on heart failure and coronary artery disease, with little data available in relation to AF. Cytokines, such as IL-6

and TNF α are important markers of active disease and prognosis (221, 222). Compromise of the myocardium has multiple aetiologies, ranging from ischemic heart disease, hypertension, cardiac arrhythmias and metabolic diseases. Neopterin, which is a metabolite of guanosine triphosphate, has been found to be elevated in patients with HF and this is said to be a marker of monocyte activation (223).

The transmigration of cells to the site of tissue injury relies upon specific cell surface molecules namely monocytes and cell adhesion molecules that respond to signalling via cytokines released from the injured vessel wall (137). Once inside the vessel wall/myocardium, monocytes will differentiate into macrophages, which promote tissue repair. The complex interactions within injured myocardial cells leads to the formation of both pro and anti-inflammatory cells. In pathological conditions, there is an override of tissue homeostasis and uncontrolled inflammation leads to the exaggerated release of macrophages, which instead of healing tissue cause tissue damage with adverse remodelling. Therefore, trying to regulate the monocyte/macrophage balance is a logical therapeutic strategy.

As discussed, under specific stimuli monocytes will differentiate into macrophages. Macrophages play a vital role in the phagocytosis and removal of pathogens (224). Although inflammation aims to protect against infection, it can cause damage to the vascular endothelium, activation of tissue macrophage, activation of cytokine pathway migration of smooth muscle cells to the intima of the arterial wall thus accelerating the process of atherosclerosis (225).

Hypoxia and myocardial necrosis drive an inflammatory process in the injured myocardium, which involves activation of monocytes/macrophages. These cells in turn are capable of

producing cytokines, chemokines, and growth factors (226). In patients with diabetes who suffer an ST elevation myocardial infarction, Mon2 subsets were elevated and their high counts predict recurrent cardiovascular events and death (227).

In ischemic HF, Mon1 have similar counts to controls with coronary artery disease (CAD) without HF. In contrast Mon2 is the only subset increased in patients with stable HF and it shows a further sharp increase in acute HF (228) (227). Of interest, high Mon2 counts were associated with better survival in that study, using a combined outcome of death and re-hospitalization. This suggests a presence of potentially protective properties of this subset in patients with failing hearts. Analysis of their functional status was not analysed in the study, and it is difficult to be certain what drives possible benefits or dangers associated with the subset.

There is some controversy on the role of Mon3 in HF as both their depletion or no change were observed (228) (227). This may be due to differences in aetiology of the studied patients, for example an accelerated homing of Mon3 in patients with non-ischemic HF, as seen in the study with mixed HF aetiology. A notable limitation in some studies is the lack of control for comorbidities that may be responsible for the abnormal release of monocytes. Despite this potential limitation, one should still recognize the importance of monocytes in the inflammatory process that happens in HF with further research in human subjects, particularly focusing on Mon2 being justified (105).

A compromised myocardium provides multiple stimuli for monocyte recruitment in patients with HF. The presence of excessive left ventricular and atrial stretch in experimental studies of mice with heart failure with preserved ejection fraction on the background of hypertension has

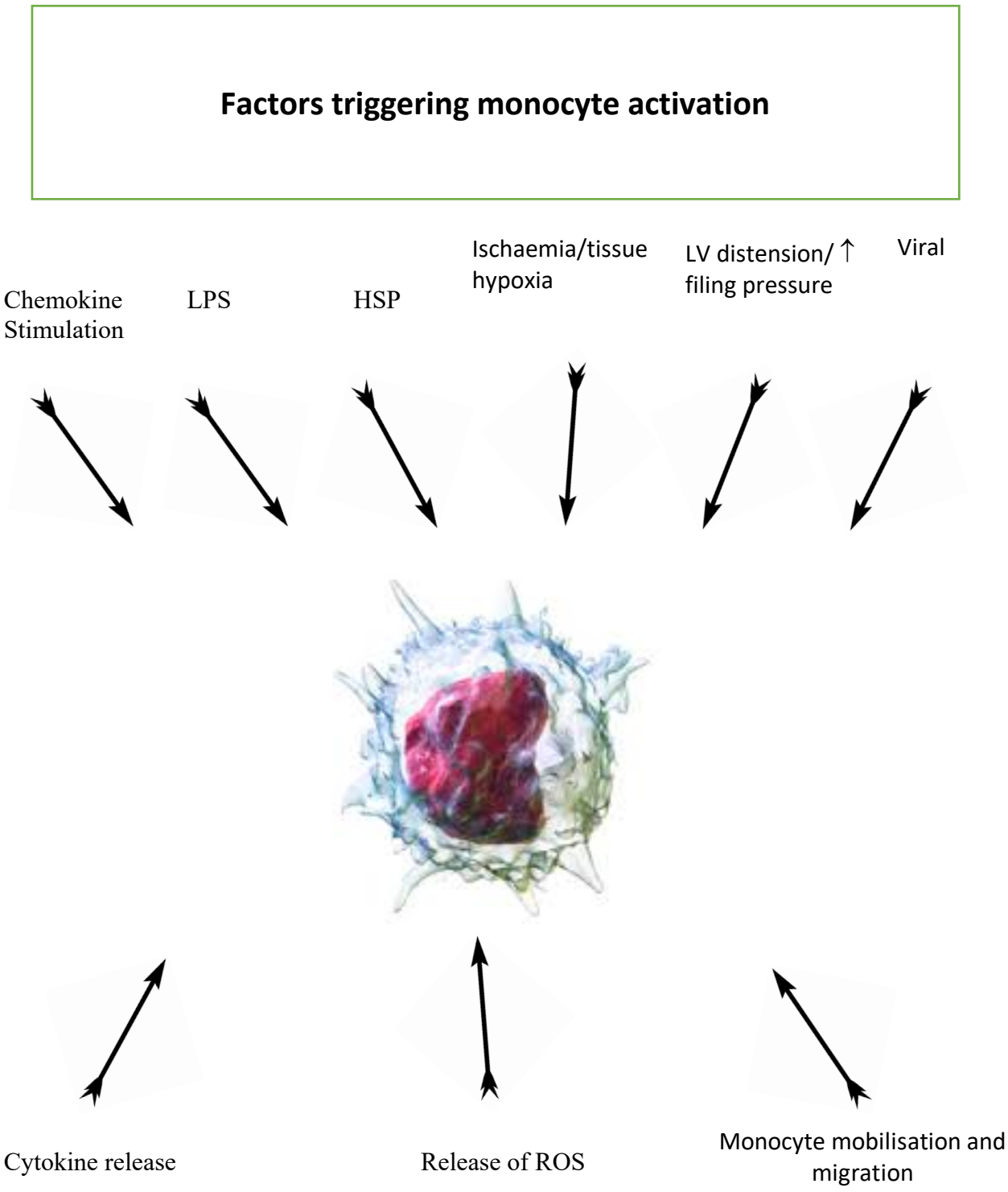
shown to stimulate myocardial resident macrophages to signal the release monocyte chemo-attractants (including MCP-1, interleukins) (229). Monocyte recruitment is further amplified by the presence of tissue hypoxia and ischemia(230) (231). **(Table** 8 highlights the involvement of Mon2 and Mon3 in cardiovascular diseases. Mon1 so far has not been firmly associated to particular cardiac states unlike its counterpart subsets.)

Table 8. Implications of Mon2 and Mon3 in common cardiovascular diseases

Condition	Mon1	Mon2	Mon3
Stable coronary artery disease (101, 232, 233)	No change vs healthy control	No change vs healthy control	No change vs health control
Acute myocardial infarction (234-236)	No change	2.5-fold increase, positively correlates with troponin T level	No change, no correlation with troponin T level
Unstable angina (237-239)	No change	Increased (in intermediate-high risk patients' vs low risk cohort)	No change (no difference with risk severity)
Acute heart failure (240) (241, 242)	No change	Increased, raised CD41 count relative to mon3	No change
Chronic heart failure (228, 243)	No change	Increased expression, correlates with NYHA class/LVEF/NT pro-BNP	Increased but no correlation to NYHA class/LVEF/NT pro-BNP
Chronic heart failure (244)	No change vs healthy control	No change vs healthy control No association with end diastolic dimension	Increased percentage vs health controls Inverse relationship with end diastolic dimension
Abdominal aortic Aneurysm (245)	No change vs healthy controls	Increased vs healthy controls	Increase count vs healthy controls

Excessive monocyte/macrophage cardiac recruitment leads to a vicious circle of myocardial damage and remodelling. This process involves apoptosis of cardiomyocytes(230) (231). It has been shown that monocyte TNFα triggers production of the inducible type of NO synthase, uncontrolled oxidative stress and consequently apoptosis and tissue necrosis (230). In the inflammatory process, cytokine release by stimulated monocytes attracts even more monocytes to the compromised myocardium, thus contributing to the vicious circle. **(Figure 4)**

Figure 4. Triggers for monocyte activation and subsequent function



1.12 **Monocyte Activation In Cardiac Fibrosis**

Cardiac fibrosis is the consequence of an activated monocyte/macrophage cascade. Both cellular and extra cellular processes are involved in cardiac fibrosis. Within the extracellular matrix (ECM), cardiac fibroblasts make up approximately 60% of all cells, in fact, outnumbering cardiomyocytes. They are relatively scarce in a healthy adult heart and a rise in the cell population occurs during a pathological process. This is suggested, for example, by evidence from mice models where tissue injury led to a rise in IL-β production and consequently to fibroblast expansion (246). This in turn propagates inflammatory cell infiltration and further cytokine production in the site of tissue injury (246). In such cases, there is an increase in rate of differentiation of precursor cells (e.g.monocytes, endothelial progenitors, pericytes and bone marrow circulating progenitor cells) into fibroblasts (247) (248).

The process by which monocytes can potentiate the cardiac inflammatory response leading to fibrosis is reliant on monocyte cell surface receptors. One such group of receptors is termed the Toll-like receptors (TLRs), which are members of pattern recognition system, but also able to respond to endogenic stimuli (249). Although TLR4 is present on different types of cells, its highest density has been noted on monocytes, reflective of their vital role in innate immunity. In addition, monocyte density of CD14 have also been found to be higher in patients with moderate-severe heart failure in comparison to normal or mild left ventricular impairment (250, 251).

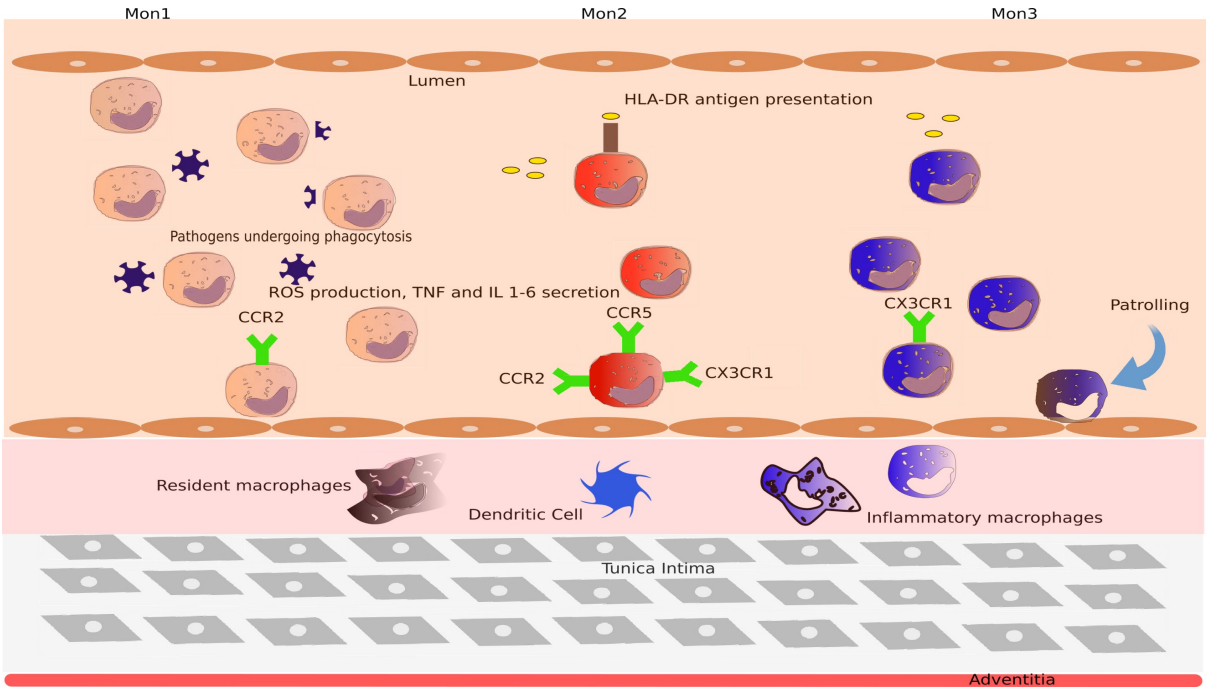
Expression of TLR4 on monocytes is linked to the degree of cardiac injury and remodelling (252). Evidence thus far points to the enhanced recruitment of TLR4 expressing monocytes into a compromised myocardium in both human and mouse studies (253, 254). The remodelled myocardium has a higher count of TLR4+ monocytes compared to a healthy myocardium (255), thus creating a proinflammatory environment. Indeed, TLR4 deficient mice have a lower inflammatory burden post-acute ischemia and reduced apoptosis of cardiomyocytes (256).

Further evidence has pointed to several mechanisms by which monocyte activation takes place, mostly studied in the heart failure population. With respect to the immune response found in cardiovascular disease, lipopolysaccharides that are found on gram-negative bacteria act as the ligand component for the activation of monocytes (e.g., binding to CD14 and TLR4 as previously mentioned) and trigger cytokine release. This endotoxin-cytokine hypothesis centres on bacterial transition into the circulation via entrance through a permeable bowel membrane (257). This is promoted by venous congestion developed through heart failure increasing membrane permeability. The over-expression of inflammatory cytokines amplifies the process leading to a pathological loop often culminating in symptomatic heart failure (251).

An alternative mechanism to the introduction of bacteria into the circulation in heart failure involves activation of the sympathetic system, a common feature of heart failure (258). The sympathetic activity is thought to redistribute blood flow away from the splanchnic circulation, which in turn leads to transient ischemia in the bowel. This causes an increase in endothelial permeability and entry of the pro inflammatory bowel contaminants into the circulation. This mobilises inflammatory cells from bone marrow (and the spleen depot) and numerous studies have found an increase in blood leucocytes in patients with advanced heart failure, thus supporting this hypothesis (259, 260).

It is likely that no one single hypothesis fully explains the process by which monocytes and their surface receptors are stimulated to propagate cardiac fibrosis. Both systemic and local cascades of inflammatory pathways exist to enhance the stimuli for monocyte triggered cardiac fibrosis. To date patients with AF are known to have evidence of cardiac fibrosis, namely in the left atrium. However, the pathophysiology behind the inflammatory cascade leading to fibrosis is unclear. My thesis aimed to delineate the role of monocytes in this pathway and to also provide a link between inflammation and fibrosis in this patient population with regards to their exercise capacity. (**Figure 5** represent a schematic diagram of the monocyte driven inflammatory process).

Figure 5. Role of monocyte subsets in inflammation and fibrosis.



Monocytes subsets in heart failure. Human monocytes are classified as Mon1, Mon2 and Mon3 respectively based on their level's expression of CD14 and CD 16. Mon2 are increased in patients with heart failure and are recruited to the myocardium in times of tissue injury, whereas Mon3 serve more of a patrolling function and are not so rapidly recruited. Monocyte subsets then differentiate into dendritic cells and inflammatory macrophages to further potentiate the fibrosis. process of inflammation and subsequent fibrosis.

1.13 **The Role Of Atrial Fibrosis In Patients With Atrial Fibrillation**

Atrial fibrosis is a hallmark of the structural cardiac remodelling that takes place in AF, causing an increase in the frequency of AF paroxysms, which in turn increase likelihood of progression to permanent AF (21) . Atrial fibrosis has been observed in biopsies from patients with AF (257) as well as in patients with specific risk factors predisposing to AF, such as valvular heart disease (261), dilated and hypertrophic cardiomyopathy (262), and advanced age (263).

Structural heart remodelling in ageing and heart disease is associated with fibrosis. With ageing, there is a progressive enlargement of the extracellular compartment in the atrial septum due to accumulation of connective tissue fibres (264). This process is even more prominent in a HF model (265), where larger areas of fibrosis were observed, similar to the "replacement fibrosis" seen after tissue damage and cell death. Atrial fibrosis may in itself be sufficient to increase susceptibility to AF, as shown in mice with atrial fibrosis due to overexpression of TGF-β1(266).

Although there are strong indications from animal models that atrial fibrosis can be proarrhythmic (267), some questions regarding the role of atrial fibrosis as a substrate of AF are still unresolved. Experimental data linking inflammation and atrial fibrosis have been conflicting. Most recent data point to the upregulation of profibrotic factors, such as TGF-β, and accumulation of collagen in the atrial interstitium (268). However, previous studies showed preserved interstitium despite changes in atrial architecture and myocyte characteristics (269). The discrepancy between the data can be partly explained by the findings that profibrotic factors may not accumulate over shorter time periods found in some studies and that increased gene expression of markers of fibrosis may be the first sign of later fibrosis (270). Another pro

inflammatory peptide, TNF-α has been shown to be elevated in patients with chronic AF. Released largely by monocytes and macrophages, TNF-α has been found at higher levels in patients with non-valvular AF than those in sinus rhythm (271). This correlates with a more significant leukocyte infiltration and more advance fibrotic changes in the atria.

Some, but not all, human studies have confirmed excessive atrial fibrosis in chronic AF patients compared to those in sinus rhythm (272, 273). The degree of atrial fibrosis and pro-fibrogenic status correlates with the persistence of AF (274). However, from these studies it is unclear whether the fibrosis is caused by underlying structural disease leading to AF or by AF itself. As the degree of the underlying heart disease is not well documented in every study, it is currently difficult to establish the magnitude of effects of particular conditions to the development of atrial fibrosis in AF patients. Some insights into pro-fibrotic effects of background cardiac pathology vs. AF comes from a comparison of structural heart disease patients with and without AF (261). In this study, AF itself has not been found to be associated with atrial fibrosis but is instead related to the severity of the structural heart disease. Given the significant differences in AF pathogenesis among patients with or without structural heart disease, studies dedicated to non-valvular AF would be essential to shed further light into the interactions between AF and connective tissue deposition within the atria.

The question therefore remains of how important atrial fibrosis is as a causative factor for AF in humans? Most animal models show that atrial dilation is accompanied by both atrial fibrosis and conduction disturbances, although conduction disturbances could also be seen in the absence of atrial fibrosis (275, 276). However, frequently used mice models of AF have significant limitations due to the fact that this species has a high physiological heart rate and thus AF induced in mice may not accurately reflect pathological processes in humans. In

patients undergoing open heart surgery, the degree of fibrosis does correlate with the occurrence of postoperative AF (277) and with the recurrence of AF (273).

Similar to AF, cardiac fibrosis is related to myocardial inflammation and oxidative stress secondary to infiltration of inflammatory cells, thus suggesting further pathophysiological links between the two (278). The oxidative stress seen in these conditions is further amplified by stimulation of the renin angiotensin-aldosterone system, which aids NADH oxidase release (279). IL-1, IL-6, TNF-α, MCP-1 are all up regulated in AF predisposing to fibrotic changes and the related electrical and structural remodeling, typical of AF. The role of inflammation in AF development is highlighted by the correlation with C-reactive protein (CRP) and has been found in postoperative patients to be a surrogate marker for predictor of new onset AF (280). Also, post ablation CRP levels can be used as a marker for risk of recurrence (281).

Further evidence on the role of cardiac fibrosis in AF comes from experimental and clinical studies demonstrating that prevention of atrial fibrosis can delay the development of AF. Several treatments (e.g., statins, Angiotensin converting enzyme inhibitors, AT_1-receptor blockers, fish oil, and glucocorticoids) have been proven to effectively delay the structural remodelling process and reduce AF burden in a variety of experimental models (282-287). Several post hoc analyses of clinical trials and small-scale proof-of-principle studies indicate utility of such approaches in human, but improvement of the patients' hemodynamics with normalisation of atrial pressures might also have contributed to the beneficial effects of these compounds (288).

The role of ventricular fibrosis in AF is less established. Patients with AF have more marked ventricular fibrosis than patients with sinus rhythm (289). Although atrial and ventricular fibrosis are likely to share a common mechanism there have much more limited findings of profibrotic gene expression in ventricular fibrosis in comparison to the atrium (290). TGF-β seems to play a major role for ventricular fibrosis in AF, but further data are needed to establish the mechanisms that trigger its expression in the myocardium and the role of monocytes and macrophages as source of TGF-β in the heart (291).

1.14 **Potential Biomarkers Of Cardiac Fibrosis (Collagen Turnover And Galectin 3)**

Plasma biomarkers of collagen turnover are easily accessible surrogate measures of systemic fibrotic processes. Procollagen secreted by fibroblasts undergoes enzymatic cleavage of its end-terminal sequences to enable collagen fibre formation. Serum levels of the pro-peptides, such as amino-terminal pro-peptide of pro-collagen type I (PINP) and type III (PIIINP) correlate with the amount of fibrillar collagen deposited (292, 293). For example, improved post-infarct LV remodelling in patients treated with spironolactone was associated with suppression of PIIINP levels (294). Galectin-3 has recently emerged as a marker of fibrosis implicated in pathogenesis of HF and AF (295, 296). Experimental data showed that galectin-3 is directly involved in myocardial fibrogenesis and mediates aldosterone-induced vascular fibrosis (297, 298). Clinical studies show that galectin-3 predicts the incident of HF in the community and it provides incremental prognostic information and predicts LV remodelling in chronic systolic HF (299, 300).

For my thesis, I will use PINP, PIIINP and Galectin 3 as surrogate markers of fibrosis to decipher a mode of mechanism by which monocytes propagate the inflammatory and fibrotic pathway in patients with AF and preserved left ventricular function. This will be further detailed in the methods section of my thesis.

1.15 **The Use Of Monocytes As Therapeutic Targets**

Human clinical trials using an anti-inflammatory approach has focused on patients with HF, but with limited success. Ever since the failure of broad-spectrum anti-inflammatory agents in HFrEF, more specific treatment has been tailored in HFpEF (301). Animal models of mice with hypertensive HFpEF have shown promising results with immunomodulators (302). Targets include inflammatory cytokines and chemokines, cytokine receptors, matrix modulating enzymes to name a few. Studies have focused on the use of anti-TNF agents to prevent binding to receptors on target cells and thus inhibit the cytokine cascade (303, 304).

Identifying specific pathways involved in the inflammatory process of HFpEF will allow the design of targeted immune-modulating agents. A recent small-scale study looked at the use of Anakinra, an IL1 receptor antagonist, used in rheumatoid arthritis, in patients with HFpEF (305). The hypothesis is that antagonism of this specific cytokine will have favourable effects on remodelling and pressure overload. Vitamin D and Sildenafil have also been proposed to be beneficial in HFpEF, having anti-inflammatory effects. However, studies showing no clinical benefits with the former 2 agents have also been published (306).

The monoclonal antibody Etanercept failed to show benefit in patients with regards to primary end points of death and hospitalisation from HF (303). Infliximab indeed showed harm with increase in deaths and hospitalisation compared to placebo (304). Most recently pentoxifylline has been found as a therapeutic source. It works by inhibiting the synthesis of TNF rather than inhibiting its action (307). However, not all studies have shown a reduction in circulating sources of TNF thus alluding to an alternative mechanism of action to cause beneficial effects.

Why targeting TNF has failed to show clear-cut beneficial effect is uncertain. One hypothesis is that the toxicity caused by drugs such as Infliximab may be harmful to cardiac muscle, as opposed to its use in Rheumatoid arthritis and Crohns Disease. Etanercept has been shown to exert a partial agonist effect and thus can induce toxicity by increasing the levels of cytokine TNF. This has been observed specifically in patients with HF (303). There seems to be a fine line between toxic and therapeutic levels of TNF and lower levels do in fact show protective effects on the myocardium and allow more favourable remodelling of the left ventricle. No large-scale randomised trials have been conducted to highlight this point and to date information from multiple small studies is only available.

Non-specifically angiotensin converting enzyme inhibitors (ACEI) have been shown to reduce circulating levels of monocytes and thus prevent adverse remodelling of the myocardium post MI (308). Similar findings are apparent for non-selective beta-blocker Carvedilol, which was found to reduce TNF medicated monocyte release (309). The mineralocorticoid receptor antagonist Eplerenone has also been shown to reduce monocyte levels by down regulating TNF enzyme. Work conducted by Kurrelmeyer et al acknowledged the role of the RAAS in the development of HFpEF. Chronic activation of the renin

angiotensinogen system increases ECM collagen causing left ventricular hypertrophy and increased myocardial stiffening, leading to both diastolic ad systolic dysfunction. In 48 women with echocardiographic diagnosis and symptomology of HFrEF randomisation to either Spironolactone or placebo showed a significant benefit in exercise tolerance, NYHA class, and 6MWT on follow up. Even though primary end points were not affected, rate of hospitalisations was reduced. Interestingly echocardiographic parameters were improved with Spironolactone (310). Spironolactone is thought to reduce levels of type III collagen, which are heavily involved in cardiac fibrosis and subsequent diastolic dysfunction.

1.16 **Aldosterone And Cardiac Fibrosis**

Cardiac expression of mineralocorticoid receptors is increased in AF, thus augmenting the genomic effects of aldosterone (311). Mechanisms of aldosterone-mediated cardiac fibrosis include myocardial inflammation, oxidative stress, and cardiomyocyte apoptosis and also direct stimulation of cardiac fibroblasts to produce collagen (312, 313). Clinical trials of aldosterone antagonists (RALES, EPHESUS, EMPHASIS-HF) uniformly showed their clinical benefits in systolic HF. Of note, according to a sub study of the RALES trial, the improved survival in participants treated by spironolactone was linked to its ability to reduce serum markers of ongoing fibrosis (type I and III collagen synthesis) (314). Additionally aldosterone leads to cardiac invasion by proinflammatory mononuclear cells (315).

Aldosterone antagonists (i.e., spironolactone or eplerenone) ameliorate LV fibrosis in animal models and reduce levels of serum markers of collagen turnover in humans with HFpEF (n=44) (316, 317). In a small, published pilot trial, spironolactone reduced LV fibrosis and improved diastolic function in participants with HFpEF (dilated cardiomyopathy, n=25) (318).

In a previous randomised clinical trial on 102 participants with chronic kidney disease with normal cardiac contractility cardiac diastolic function was significantly improved over 40 weeks of treatment by 25 mg daily of spironolactone vs. placebo (319, 320). Aldosterone inhibition with spironolactone significantly improves diastolic function and reduces cardiovascular stiffness in participants with chronic kidney disease (319, 320). Recently, the same dose of spironolactone within 1 year significantly improved diastolic function in participants with HFpEF (the ALDO-DHF trial, but only 5% of participants [n=22] had AF) (321). However, no data are available on effects of aldosterone antagonists on diastolic dysfunction and exercise tolerance in AF.

Spironolactone was recently tested in two clinical trials of HFpEF. The ALDO-DHF (321, 322) study was essentially a study of HFpEF in hypertensive subjects, which is another major cause of HFpEF as well as AF. Hypertension was present in 92% of participants in the ALDO-DHF study, with AF only present in 5% of the study participants at presentation (n=22). Thus, evidence in symptomatic permanent AF would be sparse, and ALDO-DHF would not address this clinical field. Further studies have consistently shown Mineralocorticoid receptor antagonists (MRA) to reduce the concentration of type III procollagen peptide (PIIINP) and thus myocardial fibrosis. Indeed, sub analysis of the RALES study showed that patients that most benefited from Spironolactone were those with a higher PIIINP levels (314).

1.16.1 **Other Potential Therapies**

The anti-inflammatory effects of statins have also been trailed and specifically Fluvastatin reduces TLR4 expression in vivo (323). Fenofibrate has been found to inhibit the action of multiple cytokines including IL1, IL6 and MCP1 (324).

Present and future studies are targeting the CCR2 receptor. Antagonistic action in mice studies has found a reduction in plasma levels of the monocyte subset Mon1. However, the atherosclerotic burden was not affected (325). Monoclonal antibodies against CCR2 have been used but again despite reductions in monocyte levels the clinical benefit has been disappointing (326). This suggests that CCR2 may exert inflammatory effects outside monocyte-mediated processes.

As highlighted previously certain monocyte subsets may have more of an impact on the fibrotic effects on the cardiac monocytes. Murine model studies have suggested monoclonal antibodies against Fractalkine, which binds to and stimulates the monocyte subset Mon3. This has shown promising results in patients with inflammatory arthritis (327). Most recently evidence has suggested antagonism of the CD14 receptor reduces the levels of TNF and IL6 in response to bacterial LPS (328). Inhibition by monoclonal antibodies has been shown beneficial in patients with pneumonia in minimising lung injury. Use in cardiovascular disease is yet to be undertaken.

1.21 **Summary**

Monocytes represent an essential component of the innate immune system and play a vital role in cardiovascular health. The beneficial effects of monocytes include their contribution to cardiac remodelling in response to physiological and pathologic changes in hemodynamics, elimination of pathogens, involvement in apoptosis and phagocytosis of necrotic tissues. However, excessive inflammatory response to cardiac insult can be harmful to the human body and can lead to cardiac fibrosis and heart failure. There is a fine balance between monocytes that predispose to beneficial or deleterious effects. Existence of several subsets of monocytes is likely to explain the diversity of the monocyte effects in health and disease. To date few studies have specifically looked at monocyte subsets and their key characteristics as contributors to cardiac fibrosis and AF.

Monocytes trigger an inflammatory cascade involving the release of cytokines. Such cytokines migrate to the myocardium and adhere to the endothelial wall. Infiltration into the myocardium is a complex process but one that ultimately leads to fibrosis and symptoms of heart failure. To better identify therapeutic targets, the role of monocytes and their individual subsets, in the pathophysiology of AF and its complications, such as HF must be determined.

1.30 **Hypotheses**

For my thesis, I hypothesised that patients with permanent AF and preserved left ventricular function would exhibit a comparative upregulation of Mon2 subset at baseline testing (in relation to Mon1 and Mon3), expression of surface markers and those of cardiac fibrosis which would be associated with reduced exercise capacity, quality of life and higher incidence of

hospitalisation. I further hypothesised that the heightened expression of Mon2 would be offset by the anti-fibrotic properties of Spironolactone when compared to placebo group. Specifically:

i) Monocyte subsets (namely Mon2) would act as a biomarker for outcomes with regards to Peak VO2 and 6-minute walk test at baseline and end of study

ii) Monocyte subsets at baseline would act as a biomarker for self-assessment score of quality of life at end of the study period

iii) Monocyte subsets (namely Mon2) at baseline would be associated with hospitalisations during the study period.

iv) Spironolactone would improve primary and secondary outcomes in the treatment group by providing an anti-fibrotic mechanism, seen by reduced markers of fibrosis (PINP, PIIINP and Galectin 3) and adverse monocyte subset biomarkers (i.e., reduction in the comparative population of Mon2)

1.40 **Aims And Objectives**

To test these hypotheses, my objectives for the thesis were:

i. To study monocyte subset numbers in patients with permanent AF and preserved left ventricular function and their association with reduced exercise capacity, impaired quality of life assessment and higher hospitalisation rates. Monocyte subsets would be measured using flow cytometry

ii. To measure cell surface markers of expression of activation/inflammation in patients with permanent AF and preserved left ventricular function and their

association with exercise capacity, quality of life assessment and hospitalisation. Cell surface markers would be measured using flow cytometry.

iii. To evaluate the interaction between monocytes and platelets in the form of monocyte platelet aggregation in patients with permanent AF and preserved left ventricular function and their association with exercise capacity, quality of life assessment and hospitalisation. Monocyte platelet aggregates would be measured using flow cytometry.

iv. To measure markers of fibrosis in the form of potential biomarkers PINP, PIIINP and Galectin 3 in patient with permanent AF and preserved left ventricular function and their association to exercise capacity, quality of life assessment and hospitalisation rates. Markers of cardiac fibrosis were measured using ELISA.

v. To compare monocyte subsets, surface markers of expression and markers of cardiac fibrosis in the Spironolactone vs placebo group over a 2-year study period and their association with changes in exercise capacity, quality of life assessment and hospitalisation rates.

In order to test this hypothesis patients underwent double blind randomisation to Spironolactone or Placebo once all baseline measures had been completed. A total of 250 patients participated in the study and were followed up regularly over a 2-year period.

Chapter 2

Methods

2.1. **Study Design**

For my thesis, I recruited patients from the IMPRESS AF trial for which I was the clinical research fellow. This was a double-blind randomised control trial evaluating the role of Spironolactone vs Placebo in the improvement of exercise tolerance in patients with AF and preserved left ventricular function (329). My thesis analysed the role of monocyte subsets and subsequent markers of inflammation in this population.

2.2. **Study Population**

The sample size was originally calculated to provide sufficient power to assess the impact of spironolactone treatment for the outcomes of the IMPRESS-AF trial and required recruitment of a total of 250 participants that were randomised 1:1 for treatment with spironolactone or placebo. This sample size provided statistical power sufficient to assess utility of the test fibrotic parameters in order to predict the study outcomes and efficacy of treatment with spironolactone (with more 80% power) using appropriate regression analysis. Further exploratory analyses incorporated interaction terms between intervention/control and each of the covariates, including parameters of fibrosis.

A total of 250 patients with permanent AF (PAF) and preserved ejection fraction were recruited from outpatient hospital settings via primary and secondary care centres. Primary care physicians were invited to identify patients within their practice that had been diagnosed with the criteria for this study (detailed below). Suitable patients were sent a formal invitation by the University of Birmingham to participate in the study. The study populations were chosen to represent the spectrum of disease within the population but also to enable precise

conclusions to be drawn with regards to therapeutic outcomes. For example, patients did not meet inclusion criteria if they had severe lungs disease as this would impact on performance during baseline testing, not representative of AF. All patients were recruited from the West Midlands area and underwent screening, baseline testing and regular follow up at the Institute of Cardiovascular Sciences City Hospital Birmingham. All research participants were recruited between the July 2015 to 31st June 2016.

2.2.1. **Subject Selection**

A set of inclusion and exclusion criteria were designed to address the study aims and hypotheses. This along with the randomised nature of the trial would minimise effect of potential confounders on the study results (e.g., monocyte count and exercise tolerance). All patients were required to have permanent AF rather than paroxysmal or persistent AF. This inclusion criterion reduced uncertainty surrounding impact of AF type on symptom patterns and exercise capacity and to reduce the number of potential confounders such as pharmacological treatment and need of electric cardioversions dependent on type of AF. I only included patients with preserved left ventricular function as benefits of spironolactone has already been proven in patients with impaired left ventricular systolic function (330).

2.2.1.2 **Inclusion Criteria**:

- Age 50 years old and over
- Permanent AF as defined by the European Society of Cardiology (ESC) criteria
- Ability to understand and complete questionnaires (with or without use of a translator/translated materials).

2.2.1.3 **Exclusion Criteria**:

- Severe systemic illness with life expectancy of less than 2 years from screening
- Left ventricular ejection fraction (LVEF) <50% (echocardiography)
- Severe *chronic obstructive pulmonary disease (COPD*) (e.g., requiring home oxygen or chronic oral steroid therapy)
- Severe mitral/aortal valve/tricuspid/pulmonary stenosis or regurgitation
- Significant renal dysfunction (serum creatinine 220 μmol/L or above)
- Increase in potassium level to >5mmol/L at baseline/screening
- Recent coronary artery bypass graft surgery (within 3 months)
- Use of aldosterone antagonist within 14 days before randomisation
- Use of or potassium sparing diuretic within 14 days before randomisation
- Systolic blood pressure >160 mm Hg

2.2.1.4 **Follow Up**

Patients were followed up for 2 years on a 3-monthly basis after an initial 1 month follow up post randomisation. Patients underwent a full blood count and renal function assessment during 3 monthly follow up in addition to a completion of a proforma to ensure patients were medically fit to continue in the study. Flow cytometry of monocytes was undertaken at 12 and 24 months follow up.

2.2.1.5 **Visit Schedule**

Baseline (participants who meet the inclusion criteria)

- Eligibility reviewed
- Medical history and medication review
- Standard clinical examination (including BP measurement)
- Cardiopulmonary exercise testing (CPET) with determination of peak VO_2
- 6-minute walking test
- EQ-5D and MLWHF quality of life questionnaires completed
- Randomisation to spironolactone or placebo

- Prescription and dispensing of blinded medication by qualified medical research personnel

Follow-up (months 1, 3, 6, 9, 12, 15, 18 and 21)

- Standard clinical examination (including BP measurement),
- Concomitant medication review
- Blood samples to test renal function, potassium, sodium and full blood count (including haematocrit).
- EQ-5D and MLWHF quality of life questionnaires completed at months 12 only.
- Information on outcomes such as hospitalisations and major adverse clinical events (MACE) collected, as well as safety outcomes (assessment of symptoms and possible side effects)
- Prescription and dispensing of blinded medication by qualified medical research personnel

Final Visit (24 months from randomisation)

- Standard clinical examination (including BP measurement),
- Medication review
- Repeat ECG
- Repeat echocardiogram
- Repeat Brain Natriuretic Peptide (BNP) level test using point of care device
- Repeat Cardiopulmonary exercise testing (CPET) with determination of peak VO_2
- Repeat 6-minute walk test
- Blood sample taken: potassium, sodium, full blood count (including hematocrit) and renal function

- EQ-5D and MLWHF quality of life questionnaires completed

  If the patient had an indication for spironolactone, the participants GP was informed. No further follow up arranged. The GP was informed about the trial completion.

Additional Visits (special conditions)

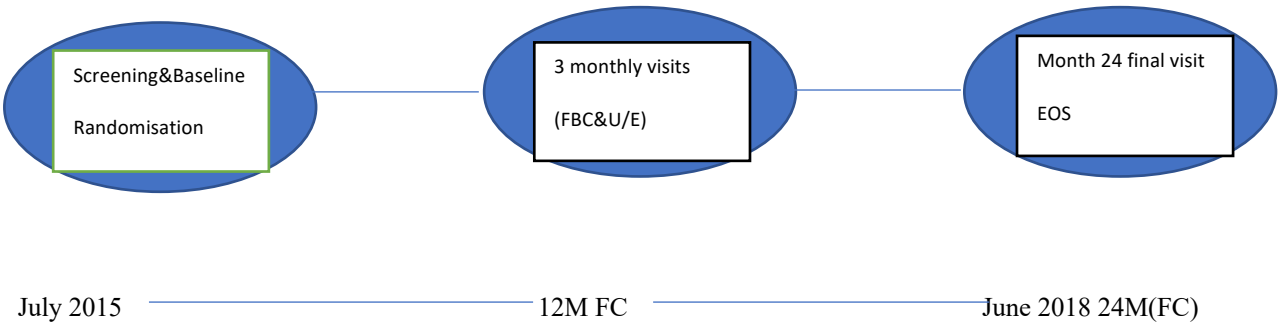
- Within 1 week of every dose adjustment a blood test was taken for analysis of renal function, potassium and sodium. The results were reviewed by the trial Research fellow. A fraction of patients would have an increase in potassium related to spironolactone. Based on the test results it may have been necessary to stop the drug or reduce its dose.

- A minority of participants voluntarily requested withdrawal from treatment. Those participants were requested to return any remaining medication. Where patients are only withdrawing from treatment but still wish to take part in the trial, patients were requested to complete the QoL questionnaire at months 12 and 24.

Participants were withdrawn from the trial medication in the following cases:

- Potassium level > 6 mmol/L.
- Severe renal impairment (e.g., acute renal failure, creatinine >220 μmol/L, creatinine clearance <30 mL/min)
- Significant breast pain or gynaecomastia despite reduction in the trial medication dose
- Allergy to the trial drug

Figure 6. Follow up timeline in study of the role of monocytes in patients with AF and HFpEF.



2.3. **Study Methods**

Patients underwent a baseline screening appointment upon which eligibility for participation into the trial was analysed.

2.3.1 **Assessment of Atrial Fibrillation**

The definition of AF was based upon the European Society of Cardiology guidelines (59). This was defined by the presence of a supraventricular tachyarrhythmia characterised by uncoordinated atrial activation with consequent deterioration of atrial mechanical function. All patients had an electrocardiogram (ECG) as part of the eligibility assessment. AF was described by the replacement of consistent P waves by rapid oscillations or fibrillatory waves that vary in size, shape, and timing, associated with an irregular, frequently rapid ventricular response when atrioventricular (AV) conduction is intact. Patients were recruited into the study only if AF was demonstrated on an ECG on the day of consent and initial screening and previous history was consistent with permanent AF. Patients with evidence of permanent AF on ECG underwent preliminary screening to ensure preserved ejection fraction in the absence of significant valve disease prior to undergoing further baseline investigations.

2.3.2. **Echocardiography**

Patients in whom permanent AF was confirmed underwent an echocardiogram. Patients with an ejection fraction of >55% with no severe valve disease were eligible for the study (i.e. preserved ejection fraction).

M-mode, 2D, Doppler and TDI transthoracic echocardiography were performed using Phillips iE33 ultrasound system (Bothel, WA, USA). Modern off-line QLAB software [Xcelera, Phillip (iE33) Ultrasound Quantification Module, USA] was used for quantification of LV systolic and diastolic function. E/e' (early mitral inflow velocity/TDI derived early septal mitral annular diastolic velocity) was used to assess diastolic function in AF. This parameter was strongly correlated with LV diastolic filling pressure in AF ($r=0.79$, $p<0.001$) and it is relatively independent of left atrial pressure (331) (332). An average from 10 consecutive cardiac cycles was calculated.

Quantification of the cardiac fibrosis was done using echocardiography via calibrated integrated backscatter. Calibrated integrated backscatter (cIB) is an echocardiographic parameter based on two-dimensional scans, which measures myocardial ultrasound reflectivity (integrated backscatter [IB]) and can be used to quantify myocardial fibrosis. IB intensity varies from low reflectivity of the blood in cardiac chambers, to medium reflectivity of the myocardium and high reflectivity of the pericardium. The reflectivity of the soft tissues is proportional to their content of the connective tissues. Accordingly, the pericardium, which is largely composed of the connective tissue, is conventionally used as a reference tissue for assessment of LV fibrosis. cIB is calculated as the difference between pericardial and myocardial IB and can be presented as negative or positive value (in this study, I choose to

present cIB as positive values). Lower cIB values indicate smaller differences in acoustic density between the pericardium and the myocardium, higher content of connective tissue and more advanced fibrosis. The utility of cIB as a measure myocardial fibrosis has been validated against myocardial biopsies and strongly correlates with myocardial collagen density (333).

Measurement of integrated backscatter and calculation of calibrated IB (cIB) was performed using acoustic densitometry software (part of QLAB package) according to the protocol established in my study department **(Appendix 1).** For quantification of LV fibrosis, I used two-dimensional images acquired from the parasternal long-axis view with frame rates between 80 and 120 frames/s. For measurement of LV cIB fixed size (5x5mm) regions of interest are positioned at the mid-myocardium of the interventricular septum, of the posterior wall, and on the posterior pericardium at LV level with as a control sample. cIB is calculated as a difference between myocardial and pericardial IB values, with LV cIB calculated as an average of septal and posterior wall cIB values. Previous intra observer coefficient of variability for LV cIB measurements in our department was 6.7%.

To further ensure minimal intra observer variability for LV cIB measurements assessment of cardiac fibrosis 10 echocardiography cases were independently analysed for consistency by a cardiology post doctorial research fellow. This showed variability of 2%.

2.3.3 **Clinical Assessment**

At baseline, all participants had a full medical history and clinical examination. This approach allowed the collection of detailed information on demographics (age, gender, smoking status and ethnicity), comorbidities (diabetes, hypertension, hyperlipidaemia etc), and medication use. The clinical examination yielded data on peripheral pulse rate, systolic and diastolic blood pressure (BP), height, weight, body mass index (BMI), evidence of valvular disease and co-morbid lung pathology. Blood pressure readings were taken using the Omron 705IT (HEM-759P-EZ) blood pressure machine. Systolic and diastolic blood pressure were recorded and interpreted in accordance with ESC guidelines on hypertension (334). The device was calibrated yearly. The results were not directly indicative of the treatment (although one could argue Spironolactone reduces BP) that the patient was receiving and were therefore recorded at each visit in the proforma.

2.3.4 **6 Minute Walk Test**

Participants undertook a 6-minute walk test as a simple measure of exercise tolerance (335). No specialist equipment was required for this procedure and instructions on how the test should be performed was in accordance with the trial protocol. For the trial, only the distance the patient walked needed to be recorded for analysis.

2.3.5 **Cardiopulmonary Exercise Testing**

Exercise capacity is a strong predictor of cardiovascular events and overall mortality (336). Cardiopulmonary exercise testing (CPET), with the determination of peak VO_2 is the 'gold-standard' assessment of exercise capacity, providing in depth information on cardiorespiratory system during intense, yet controlled periods of metabolic stress (337). This makes CPET an ideal non-invasive tool to establish the functional status of the heart, which provides a wealth of clinically relevant diagnostic and prognostic information.

CPET testing was performed to assess peak oxygen uptake (peak VO_2) and to define cardiovascular aetiology of the symptoms. Recently, CPET has been shown to be a highly accurate and reproducible measure of exercise tolerance in participants with preserved LV contractility (337). Exercise testing was performed with participants in the upright position on an electronically braked bicycle, with expired gas analysis under continuous electrocardiographic monitoring. Participants were encouraged to exercise to exhaustion. Peak VO_2 values were averaged from the final 30 seconds of the exercise test. Additionally, ventilator anaerobic threshold was evaluated by standardised methods using ventilator equivalents (338). The Statement of the American Thoracic Society and American College of Chest Physicians recommends that an increased VE/MVV ratio (e.g., > 85%) occurring at a relatively low work rate (e.g., 50 W) strongly suggests that ventilator factors are contributing to exercise limitation (339).

The CPET test was performed using the L COSMED CPET system based on cycle ergometer procedural instructions. Modern CPX systems contain rapidly responding O2 and CO2 sensors that allow for the calculation of oxygen uptake and carbon dioxide output at rest,

during exercise, and during recovery, as frequently as breath by breath. Although manufacturers' recommendations vary considerably regarding calibration, the CPET systems were calibrated immediately before each exercise test. This included calibration of airflow, volumes, and both the O2 and CO2 analysers. Because ambient conditions affect the concentration of O2 in the inspired air, temperature, barometric pressure, and humidity was taken into account. The CPET system automatically quantifies these conditions and make appropriate adjustments to calculate the inspired O2 concentration.

The CPET testing for my study was performed as per the RC-CSS SOP **(Appendix 2)**. Interpretation of the VO_2 maximum value result was used as a measure of exercise tolerance (i.e., higher value means better exercise tolerance).

2.4 **Randomisation**

Patients meeting all eligibility criteria and providing written informed consent underwent a baseline visit and the process of randomisation. A secure web-based randomisation system was used to randomise patients and allocate participants to receive a unique IMP number. After the patient was randomised, the system displayed the IMP number the patient had been assigned to but did not disclose the identity of the treatment that is associated with that particular IMP number.

Randomisation was stratified on the basis of baseline peak VO_2. Participants were stratified into two groups, those with baseline peak VO_2 below or above 16 ml/min/kg. Within each stratum a double-blinded blocked randomisation scheme was conducted, block size 4. Randomisation was implemented by the statistician initially producing two lists: peak VO_2

above and below 16 ml/min/kg. Within each of these lists, 126 A's and 126 B's were used to allocate participants to either a 2-year treatment with spironolactone or placebo. This was done by randomly selecting 21 blocks from the 6 different combinations of A's and B''s AABB, ABAB, ABBA, BBAA, BAAB and BABA. The two lists so produced therefore had an overall balance of an equal number of A's and B's and would also ensure that an imbalance of no more than one extra A or one extra B within any sequence of four randomisations. Thus, if participant recruitment was terminated prior to meeting the 252 goal there would be an imbalance of no more than one A or one B in the randomisation of participants.

In addition to the secure web-based randomisation system, a telephone back up was also incorporated into the randomisation methodology. This consisted of a paper-based randomisation system, available via the PC-CRTU randomisation line during, Monday to Friday during office opening hours.

2.5 **Blinding**

Trial participants, trial team in contact with the patient, care providers, outcome assessors, data analysts remained blinded to the treatment after assignment to interventions. To prevent accidental unblinding, code breaks were to be performed by the City Hospital pharmacy, who are independent to the study team.

Unblinding of the trial drug was rare, as the patients were treated symptomatically and the management was not usually altered by knowing the trial compound (i.e., spironolactone or placebo). Unblinding code breaks were avoided unless knowledge of the trial treatment was essential for the correct clinical care of the patient e.g. in the cases of acute liver injury, acute

renal failure, Stevens-Johnson syndrome or agranulocytosis, where Spironolactone may be the cause. Cases that were considered serious, unexpected and possible, probably or definitely related were unblinded.

For code breaks the City Hospital pharmacy were contacted to perform the unblinding. Code breaks had to be performed by following the instructions on the code break form and all fields completed before performing the code break. The PC-CRTU were informed as soon as possible if the code had been broken. This was done by sending a copy of the completed code break form to the PC-CRTU via email or fax. The codebreak envelope was resealed as soon as possible and the time and date of the code break documented on the front of the envelope along with the name of person performing the code break and specifying the reason for the code break.

Once the code was broken the patient was withdrawn from the trial treatment and became unblinded to their trial drug. However, where applicable these patients were asked to complete the QoL questionnaires at the 12 month and 24-month visits.

Blinding of the trial drug identity took place at the time of packaging and labelling. The trial drug was packaged by the Sharp Clinical Services, who labelled the drug with a unique IMP number. Two separate randomisation lists were made available to the Sharp Clinical Services by the trial statistician.

One list contained 126 A's and 126 B's and was used to allocate participants with a peak VO_2 above 16 ml/min/kg to receive either treatment A or treatment B. The second list was used to allocate participants with a peak VO_2 below 16 ml/min/kg to receive either treatment A or

treatment B. Upon receipt of the list, the Sharp Clinical Services determined the identity of A and B and documented the identity for the allocation for each patient. Each allocation was then assigned a unique IMP number. The finalised version of the list held by the Sharp Clinical Services, with treatment identities and IMP number documented became the code break list.

Using the code break list, the Sharp Clinical Services produced sealed code break envelopes which contained the unblinding information. This list was held by the Sharp Clinical Services. Code break envelopes were provided by the Sharp Clinical Services to the City Hospital pharmacy, which operated 24 hours day. Unblinding was restricted to emergency situations only!

2.6 **Dispensing of Treatment**

The Centre for cardiovascular Sciences was responsible for dispensing the trial medication to the participant on receipt of a prescription. The hospital NHS pharmacy had oversight and performed audits every 6 months.

2.7 **Treatment and Dosing Schedule**

Trial participants received either Spironolactone or matched placebo according to the following instructions:

- a single 25 milligram tablet
- to be taken orally once per day (typically to be taken during the morning but can be taken regularly at other time of day if preferable).
- to be taken with a drink to help swallowing of the drug

- for a 24-month period

In the case of an increase in potassium level to 5.1-6.0 mmol/L or in the presence of other non-life-threatening side effects (such as gynaecomastia) the trial drug (either placebo or Spironolactone) was down-titrated to 25 mg each second day (25mg being one capsule in either arm of the trial). In such cases, the investigators advised to re-up-titrate the trial medication if the reason for down-titration has resolved. Routine laboratory surveillance of serum potassium, sodium, full blood count with haematocrit, and renal function was done by protocol at each visit and within 1 week of any dose adjustment.

Drug toxicity was defined as an increase in potassium level to >6 mmol/L. In the case of toxicity or suspected toxicity, the trial medication (placebo or Spironolactone) was stopped for the duration of the trial, but the patient invited to attend the remaining follow up visits at 12 months and end of study visit at 24 months.

The participants were given bottles with the trial drug sufficient for 6 months of treatment. A pill count was performed at each visit. The remaining number of pills in the pack were checked and recorded in the patient CRF for that visit. At the visits at 6 months, 12 months and 18 months, the previous bottle and any remaining pills were returned, and the next supply dispensed. Bottles were returned to the nurse at the clinic visit and stored in the RC-CSS pharmacy until the end of the trial.

2.8 **Adverse Event Reporting**

Clinical aspects of the study were managed by the trials team at the University of Birmingham

2.9 **Blood Sampling**

All clinical laboratory procedures were undertaken by Pathology Department in City Hospital, Birmingham with regards to analysis for the trial. Routine biochemistry tests included urea, creatinine, sodium and potassium and were performed by the hospital laboratory as per standard protocols.

Prior to venepuncture, patients were rested in a chair but were not required to fast. The skin was cleaned with a sterile wipe and a tourniquet applied immediately before commencement of the procedure. The blood samples were obtained by inserting a 21-gauge needle into a vein located in the anterior cubital fossa of the patient's arm. Alternative anatomical sites were used if this was not feasible. In total, 18ml of non-fasting peripheral venous blood was collected into vacutainer tubes. This consisted of 5mls collected into an ethylene-diamine tetra-acetic acid (EDTA)-containing tube, 8mls into a citrated tube and 5mls into a serum tube with silicon coated interior. Routine haematological and biochemical tests were performed on the blood samples the same day, including BNP. In addition, 550µl of fresh EDTA sample was extracted and processed on the Becton Dickinson (BD) FACSCalibur flow cytometer within 60 minutes of collection to yield data on monocyte subsets and monocytes cell surface marker expression. Remaining blood samples were separated by centrifugation and the plasma stored at –70 ^{o}C for subsequent batched analysis.

2.9.1 **Analysis of monocyte subsets as makers of systemic inflammation**

To investigate correlations between monocyte subsets and response to mineralocorticoid receptor antagonists in this study population The BD FACS Calibur flow cytometer was used for data acquisition.

Flow cytometry (FCM - also known as FACS, stands for Fluorescence activated cell sorting) uses sophisticated technology that makes use of the principles of light scattering by particles crossing a beam of light, and excitation and fluorescence emission of fluorochromes attached to specific molecules or expressed by cells, to identify, analyse, and/or sort different populations of cells. The process begins with a population of single cells, or particles, suspended in a medium, injected into a stable stream that forces cells to travel one by one to be interrogated by the flow cytometer. Each particle passes through one or more beams of laser light. Scattered light and fluorescence emission provide information about the particle's properties. Information is gathered from the manner in which a particle scatters light or by the light emitted by fluorochromes attached to, or contained in, the particle.

Light scattered in the forward direction of a laser beam is focused by a confocal lens and detected by a light detector which converts it into an electrical signal that is digitalised to generate a parameter known as Forward Scatter (FSC). The FSC signal will give information about the size and shape of the cell, and information can also be gathered by a side confocal lens and detected by a detector reading side scattered light. The Side Scatter (SSC) signal gives information about the granularity of the cell. As FSC and SSC are unique for each type of particle, the combination of the two can help identify different types of cells.

Several optical detectors called photomultiplier tubes (PMT) are used in a flow cytometer to read fluorescence. These read the light emitted from the particle crossing the laser beams that excite the attached fluorochromes. Different fluorochromes will emit light at different wavelengths and these are split into specific colours by optical filters and sent to PMTs. PMTs convert light into an electrical pulse that is digitalised by other converters into signals readable by a computer as events, which are then used to generate histograms and dot plots.

All flow cytometers are able to interrogate cells and generate data, but only cell sorters can sort cells in a second stage where electrostatic cell sorting takes place. Once a cell type (or more than one cell type) has been identified, and after the machine is set with the sort criteria, the cell sorter will use electrostatic charge to separate the cells from the sample.

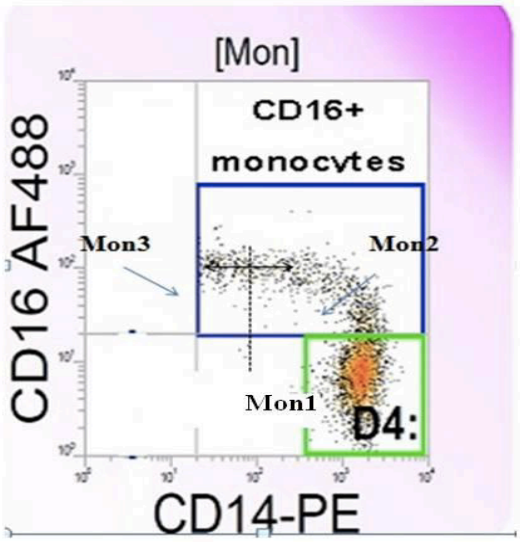
This electrostatic charge is applied to the stream when the particle matching criteria set on the machine reaches the 'break off point' (i.e. where the stream begins to form individual droplets that encapsulate single cells). So that the droplets do not break off at random distances from the nozzle the break off point is controlled as the nozzle through which it passes is vibrated at a high frequency. Once the charge is applied the droplet containing the cell of interest breaks away from the stream and is deflected left or right depending on the charge applied and can then be collected at the base for further study.

2.9.2 **Absolute Count of Monocytes and Monocyte Subsets**

Before publication of the revised nomenclature for monocyte subsets, the research group at the Institute of Cardiovascular Sciences had been involved in establishing a reliable flow cytometry protocol to allow the accurate enumeration of the 3 monocyte subsets and to

discriminate between Mon2 and Mon3, rather than relying on drawing an arbitrary line on the FC plot **(Appendix 3)**. (**Figure 7**).

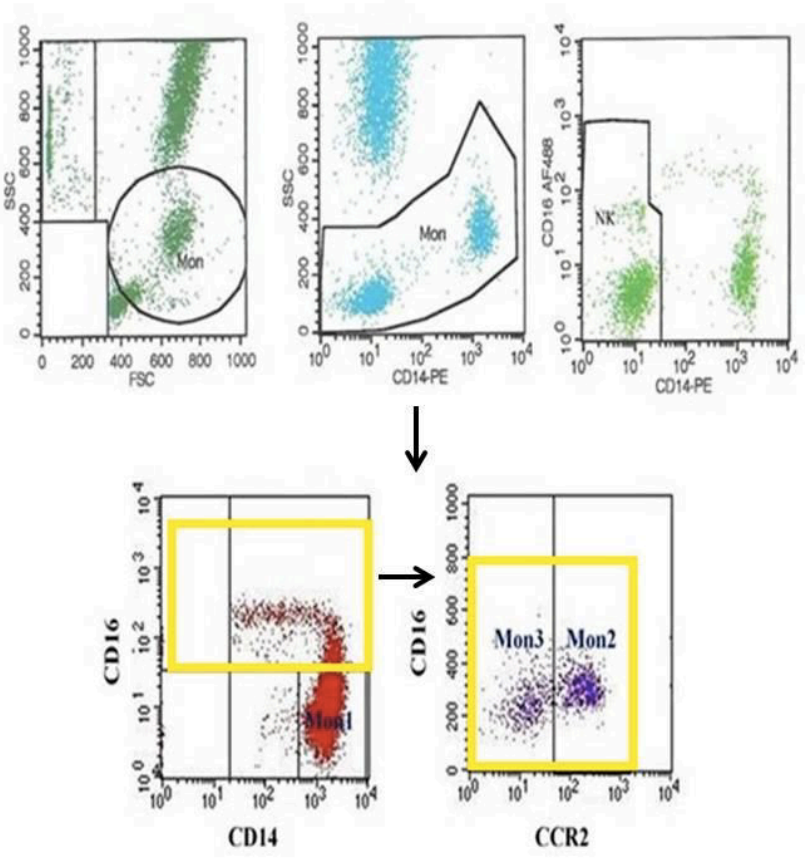
Figure 7. Ambiguity in drawing the boundary between Mon2 and Mon3 by using only CD14 and CD16 expression



Mon1: classical monocytes, Mon2: intermediate monocytes, Mon3: nonclassical monocytes

The additional use of CCR2 expression allows such discrimination, with Mon2 subset strongly expressing the marker whereas Mon3 subset does not. **(Figure 8)**

Figure 8. Accurate identification of monocyte subsets by flow cytometry using CCR2



Freshly obtained blood samples were kept at room temperature and processed immediately (ideally no later than 1 hour) as more prolonged storage can affect monocyte phenotype. For example, a 2 h delay in sample processing results in appearance of CD16 on Mon1, and numbers of CD16+ monocytes significantly increase after 4 h of storage at room temperature (340). Every attempt was made to take blood samples at the same time-of-day, typically in the morning since there is a diurnal variation in Mon2 subset. Before sampling, the patient was allowed to rest for at least 5 minutes to avoid increases of CD16+ monocytes due to any preceding exercise. Since excessive stress with catecholamine release may mobilise CD16+ monocytes, blood sampling was done under low stress conditions.

Gentle continuous rotation of samples during storage/staining was used to prevent possible adhesion of monocytes to the wall of sampling tubes and minimise their interaction with other blood cells such as platelets.

Mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MϕP9, BD) and anti-CCR2-APC (clone 48607, R&D systems, Oxford, UK), anti CD-42-PE (clone HIP1, BD) were mixed with 50µl of fresh EDTA anticoagulated whole blood in TruCount tubes (BD, Oxford, UK) containing a strictly defined number of fluorescent count beads. After incubation for 15 minutes, red blood cells were lysed by 450µl of lysing solution® (BD Oxford, UK) for 15 minutes, followed by dilution in 1.5 ml of phosphate buffer solution (PBS) and immediate flow cytometric analysis.

2.9.3 **Gating: Separation of Monocytes from Non-Monocytes**

The initial gating is used to separate monocytes from other leucocytes and debris. This was achieved using optical properties of monocytes and their expression of certain surface markers. Admittedly there is a certain scatter overlap between the three types of leucocytes and every care was taken to minimise loss of monocytes for further quantification, while avoiding contamination by other leucocyte populations.

Monocytes were selected by gating strategies based on forward and side scatter to select monocytes, side scatter versus CD14 expression to exclude granulocytes, and ungated CD14 versus CD16 expression to exclude natural killer lymphocytes.

2.9.4 **Identification of Monocyte Subsets**

Appropriate isotype controls were used, and subsets were defined as CD14++CD16-CCR2+ (‘classical’, Mon1), CD14++CD16+CCR2+ (‘intermediate’, Mon2) and CD14+CD16++CCR2- (‘non-classical’, Mon3) monocytes. Absolute counts of monocyte subsets (cells/µl) were obtained by calculating the number of monocytes proportional to the number of count beads in the TruCount tube according to the manufacturer’s recommendations.

2.9.5 **Other Considerations for Analysis**

Reproducibility of flow cytometry measurement depends on the number of events collected for analysis. It is recommended that a minimum of 400 events for the rarest subset is collected which will result into estimated coefficient of variability of measurement of 5 % providing all other standard quality control measures are ensured. Numbers of CD16+ monocytes have been shown to be lower in females and to increase with age (341). Therefore, clinical studies including analysis of monocyte subsets need age- and sex-matched control groups were needed (which is provided by the nature of a double-blind randomized control trial in this instance). Also, the effects of concurrent therapy had to be considered. For example, Glucocorticoids are most relevant, since they deplete Mon3 but may increase counts of Mon1 and Mon2 (172).

2.9.6 **Validation Studies**

Intra-assay reproducibility was assessed during development of the Standard Operating Procedure (SOP) previously for studying monocytes and MPAs in the preparatory stages for this project. An SOP is an absolute requirement for all laboratory investigations in the Atherosclerosis Thrombosis and Vascular Biology Unit of the University Of Birmingham Department Of Medicine at City Hospital, Birmingham. All SOPs must be evaluated and 'signed off' by the department's Consultant Clinical Scientist, Dr Andrew Blann, before they may be used in research projects. The SOP for this project is SOP 201 "Monocyte subsets, monocyte platelet aggregates by flow cytometry **(Appendix 3)**.

Validation for my study was undertaken on 2 separate healthy subjects. 4 EDTA bottles for flow cytometry analysis were taken from one male and one female (totalling 8 samples) and analysed to ensure consistent reproducibility of results.

Table 10. Mean intra assay coefficient of variation (%) for monocyte parameters

Volunteer	Subject A	Subject B	Mean
Total Mon	0.8	0.4	0.6
Mon1	1.0	1.6	1.3
Mon2	10.2	11.1	10.65
Mon3	3.5	4.2	3.85
Total MPA	3.8	4.6	4.2
MPA1	12.6	10.7	11.65
MPA2	14.4	10.4	12.4
MPA3	8.4	7.2	7.8

Total Mon : Total monocyte count, Mon1 : CD14++CD16-CCR2+ monocytes, Mon2 : CD14++CD16+CCR2- monocytes, Mon3 : CD14+CD16++CCR2- monocytes, MPA : Monocyte Platelet Aggregates, Total MPA : Total MPA count, MPA1 : MPAs associated with Mon1, MPA2 : MPAs associated with Mon2, MPA3 : MPAs associated with Mon3

2.9.7 **ELISA**

Activation of profibrotic pathways, increased production of myocardial collagen lead to increased pressure load in the heart, diastolic dysfunction and increased cardiac stiffness. This process is linked to an increased myocardial collagen turnover and shift in the balance between matrix metalloproteinases (MMP) and their inhibitors in favour of excessive myocardial fibrosis (342, 343). Published evidence from AF populations supports a central role of atrial fibrosis in electrical and structural atrial remodelling, and an independent predictive value for the high risk of cerebrovascular events (344). The research department has previously demonstrated that presence of AF and its progression from paroxysmal to chronic form are associated with incremental increase in left ventricular fibrosis, which significantly correlated with deterioration in parameters of diastolic function (28).

Aldosterone promotes cardiac fibrosis via myocardial inflammation, oxidative stress, and cardiomyocyte apoptosis and also direct stimulation of cardiac fibroblasts to express type I and III fibrillar collagen genes (312, 313). Cardiac expression of mineralocorticoid receptors is increased in AF, thus augmenting the genomic effects of aldosterone (311). Aldosterone antagonists (i.e., spironolactone or eplerenone) ameliorate LV fibrosis in animal models (316). Enzyme linked immunosorbent assay (ELISA) is a well-recognised method to quantify such markers of fibrosis and this is what was used for my thesis.

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate (stored serum) to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

ELISA was performed in 96-well polystyrene plate, which passively bind antibodies and proteins. Having the reactants of the ELISA immobilized to the microplate surface makes it possible to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes.

A detection enzyme or other tag is linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options.

In my thesis, I measured 3 specific markers: pro-peptide of pro- collagen type I (PINP), type III (PIIINP) and galectin 3. Approved protocols were designed and followed for each **(Appendix 4, 5 and 6 respectively)**. Plasma biomarkers of collagen turnover are easily accessible surrogate measures of systemic fibrotic processes. Procollagen secreted by fibroblasts undergoes enzymatic cleavage of its end-terminal sequences to enable collagen fibre formation. Serum levels of the pro-peptides, such as amino-terminal pro-peptide of PINP

and PIIINP correlate with the amount of fibrillar collagen deposited (292, 293). For example, improved post-infarct LV remodelling in patients treated with spironolactone was associated with suppression of PIIINP levels (294). Galectin-3 has recently emerged as a marker of fibrosis implicated in pathogenesis of HF. Experimental data showed that galectin-3 is directly involved in myocardial fibrogenesis and mediates aldosterone-induced vascular fibrosis (297, 298). Clinical studies show that galectin-3 predicts incident HF in the community and it provides incremental prognostic information and predicts LV remodelling in chronic systolic HF (299, 300).

2.9.8 **Brain Naturetic Peptide**

New-onset AF is associated with an elevation of BNP levels, with values that peak within 24–36 hours after AF onset. BNP concentrations gradually decrease in parallel to attainment of heart rate control, although many AF participants remain symptomatic long term (345). Various factors are implicated in BNP release in AF, including an increase in atrial load, tachycardia, and deterioration in systolic function.

These near patient tests were performed at screening and end of study visits. They were performed using the Roche Cobas H 232 BNP device. The BNP machines were used strictly per the manufacturer instructions. The device was calibrated yearly and certified. The results of this test would not be indicative of the treatment that the patient is receiving.

2.10 **Ethical Considerations**

The trial was performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Association General Assembly, Helsinki, Finland, June 1964, amended at the 48th World Medical Association General Assembly, Somerset West, Republic of South Africa, October 1996 (website: http://www.wma.net/en/30publications/10policies/b3/index.html).

The trial was conducted in accordance with the Research Governance Framework for Health and Social Care, The Medicines for Human Use (Clinical Trials) Regulations and subsequent amendments and the Data Protection Act 1998 *and Human Tissue Act 2008.* This trial was carried out under a Clinical Trial Authorisation. The protocol was submitted to and approved by the main Research Ethics Committee (REC) prior to circulation.

2.11 **Statistical Analyses**

All data was analysed using SPSS 23.0 for windows (SPSS Inc. Chicago, Illionis). The demographic data and clinical characteristics were presented as a median [interquartile range, IQR] where appropriate. Categorical data are presented as percentages. All analyses considered a p value of <0.05 as statistically significant.

1) Linear regression analysis was used to compare monocyte subsets, surface markers on inflammation measured using flow cytometry against primary and secondary outcomes. Linear regression was also used to analyse peak VO2 at two years between the intervention

and the control group. Covariates included baseline peak VO2, age, gender systolic/diastolic blood pressure and BMI measured at baseline.

2) Independent sample T test was used when undertaking inter group comparison where appropriate. Independent samples T test was used to decipher any statistically significant difference ($p<0.05$) in monocyte subsets, surface markers and those of cardiac fibrosis between the spironolactone and placebo group at baseline and at 24 months (end of study) visits.

3) Correlations between two continuous variables were assessed using Pearson's correlation test when comparing two normally distributed variables. Spearman's correlation test was used to compare two sets of not-normally distributed data or one normally distributed and one not normally distributed variable.

Chapter 3

Effects of Monocyte Subsets and Markers of Cardiac Fibrosis on Peak VO2 max

Abstract

Introduction: Monocytes play an important role in inflammation, angiogenesis and tissue repair and may contribute to the pathophysiology of heart failure (HF) and AF. I examined differences in monocyte subset numbers, subsequent markers of inflammation and cardiac fibrosis in patients with permanent AF and preserved left ventricular function with regards to peak exercise capacity. This was measured using Peak VO2 via cardiopulmonary exercise testing at the end of study 24-month visit.

Methods: Baseline monocyte subsets, surface markers and indicators of cardiac fibrosis and inflammation were analysed to ascertain any statistically significant relationship between peak VO2 and monocyte subsets over the course of the study period. Three monocyte subsets [CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3)] were analysed by flow cytometry in patients with permanent AF and preserved left ventricular function at baseline visit along with ELISA to quantify makers of fibrosis in the form of Galectin 3, PIIINP and PINP. SPSS software was used to carry out statistical analysis of all 250 patients in the study cohort. Baseline monocyte subsets and subsequent markers of inflammation and fibrosis were used to ascertain any relationship with the primary outcome using multivariant linear regression analysis. A total of 250 patients were analysed at baseline who were subsequently randomised in double blind fashion into either intervention or placebo arm of the study (125 in the Spironolactone treatment group and 125 in the placebo control group).

Results: The mean age of the study population was 72.5 years in this study cohort. The majority were males (76%) and of Caucasian ethnicity (94%). The average peak VO2 in the study population was 14 mL/kg/min. All patients were found to have an ejection fraction above 55% and measurements of clinical parameters measured physically on baseline visit were within normal limits. This included resting heart rate, peak heart rate, blood pressure

and body mass index. However, upon analysis of monocyte subsets CD16 Mon3 ($p=0.001$) and CD42 MPA3 ($p=0.026$) were found to be predictors of peak VO2 when measured at baseline and also when adjusted for treatment group intervention ($p=0.001$ and $p=0.030$ respectively).

Conclusion: My study shows that for the first-time monocyte subsets can be potential biomarkers, providing an association between peak exercise capacity in patients with permanent AF with preserved left ventricular function. Specifically, monocyte subset surface markers of CD16 Mon3 were significantly related to beneficial outcomes with regards to a peak exercise capacity in respect to VO2 max (higher CD16 Mon3 count associated with higher peak VO2 performance) when compared to 24-month study outcomes. Causality could not be established in his study.

3.1 **Introduction**

As described in the introduction chapter, monocytes play a pivotal role in inflammation and have functional characteristics that may be both detrimental and beneficial to the cardiovascular system, including phagocytosis, cytokine production, collagen synthesis and angiogenesis (93). Such functional diversity is likely to stem from the presence of distinct monocyte subsets. The mechanisms leading to reduced exercise capacity, related morbidity, and mortality in anticoagulated patients with permanent AF are likely related to an impairment in diastolic function, myocardial fibrosis and stiffening. Biomarkers of cardiac fibrosis have been shown to be elevated in patients with permanent AF (298) . The subsequent changes can lead to ventricular filling abnormalities, reduced cardiac output and decreasing exercise capacity (270).

Monocyte studies involving gene analysis highlight the preferential expression of genes involved in angiogenesis, wound healing and coagulation (namely Mon3). Alternatively, Mon1 have a higher capability to produce IL-1β and TNFα in response to bacterial lipopolysaccharides (125). During the inflammatory process both Mon1 and Mon2 bind to MCP-1 thus allowing monocytes to invade into human tissue and perpetuate the inflammatory cascade (346).

There is some controversy on the role of Mon3 in cardiovascular disease, namely heart failure, given that both their depletion and no change were observed (240). This may be attributed to differences in aetiology of the studied patients, for example, an accelerated homing of Mon3 in patients with nonischaemic HF, as observed in the study with mixed HF aetiology (347). A notable limitation in some studies is the lack of control for comorbidities

that may be responsible for the abnormal release of monocytes. With regards to AF the evidence is scarcer with regards to the beneficial and detrimental role of monocyte subsets in such patient cohorts. A recent study has pointed to the role of Mon2 in the remodelling of the left atrium in patients with AF. However, this involved a very small AF population (30 patients) and was done in the acute setting of catheter ablation (348).

In this chapter, I aimed to examine (i) the clinical characteristics of patients in the study group prior to performing cardiopulmonary exercise testing. (ii) correlations between monocyte subsets, their surface makers and those of cardiac fibrosis in relation to the primary end point of the study by analysing baseline monocyte subsets and their relationship with end of study peak VO2.

3.2 **Methods**

3.2.1 **Study Population**

The recruitment and data collection for patients with AF and preserved left ventricular function is detailed in chapter 2.

In order to explore the significance of monocyte subsets, surface markers and those of cardiac fibrosis upon peak VO2 max at the end of study visit I analysed patients at the following time points:

a. 12-month flow cytometry for monocyte subsets and surface makers.

b. baseline ELISA for cardiac biomarkers (Galectin 3, PIIINP and PINP).

c. 24 month visit cardiopulmonary exercise testing to record peak VO2

3.2.2 **Flow Cytometry and ELISA**

Flow cytometric and ELISA analysis was performed in all study patients as described in detail in chapter 2 (Appendix 3, 4, 5 and 6 contain the relevant SOP for further information).

3.2.3 **Cardiopulmonary Exercise Testing**

An in-depth description of the cardiopulmonary exercise testing procedure is detailed in chapter 2. In essence, exercise testing was performed with participants in the upright position on an electronically braked bicycle, with expired gas analysis under continuous electrocardiographic monitoring. Participants were encouraged to exercise to exhaustion. Peak VO_2 values were averaged from the final 30 seconds of the exercise test. Additionally, ventilator anaerobic threshold was evaluated by standardized methods using ventilator equivalents. (Appendix 2 contains the SOP for CPET).

3.2.4 **Statistical Analysis**

Details of statistical techniques have been described in chapter 2, but to summarise, any relationship between monocyte subsets at baseline and 24-month outcomes of peak VO_2 were analysed by a stepwise (forward) approach to multivariant linear regression analysis (prior linearity of data confirmed and suitability for regression analysis by way of Pearson method of correlation). A stepwise approach to identify the most significant variables impacting upon peak VO_2 was used to minimise collinearity and maintain the power of the study, which can be compromised by having a multitude of variables.

3.3 **Results**

3.3.1 **Subject Characteristics**

The study groups baseline demographic and clinical characteristics with regards to age, gender, ethnicity, smoking and alcohol consumption is detailed below, along with significant medication history **(Table 11)**. There were a higher proportion of males overall in the study (76%), as was the representation of Caucasian participants in the study (94%).

Table 11. Demographic and clinical characteristics of study at baseline.

	Study cohort n=250
Demographic characteristics	
Age (years)	73.5 (67-78)
Gender	
Female	59 (24%)
Male	191 (76%)
Ethnicity	
White	236 (94%)
Black	6 (3%)
Asian	5 (2%)
Mixed or other	3 (1%)
Smoker	
Current smoker	14 (6%)
Ex-smoker	66 (54%)
Never smoked	102 (40%)
Alcohol use (units/week)	9 (0-14)
Characteristics of the study outcomes	n=197
VO_{2peak} (mL/kg/min)	14.9 (11-18)
Clinical characteristics	n=250
Left ventricular ejection fraction (%)	58 (56-63)
E/E' ratio	9.8 (7.5-13.0)
Brain natriuretic peptide (pg/mL)	129 (73-241)
Body mass index (kg/m^2)	29.5 (26-34)
Systolic blood pressure (mmHg)	130 (117-142)
Diastolic blood pressure (mmHg)	75 (67-83)
Resting heart rate (bpm)	85 (74-99)
Peak heart rate (bpm)	130 (106-152)
Diabetes	45 (18%)
Echocardiographic Markers of fibrosis	

Echocardiographic backscatter	2.15 (1.14-3.3)
Medications	
Non-vitamin K oral anticoagulants	117 (47%)
Vitamin K antagonists	94 (38%)
Aspirin	19 (8%)
Clopidogrel	10 (4%)
Loop diuretic	49 (19%)
Thiazide diuretic	34 (14%)
Angiotensin converting enzyme inhibitor or angiotensin receptor blocker	147 (59%)
Calcium channel blocker	80 (32%)
Beta blocker	136 (54%)
Digoxin	49 (19%)
Amiodarone	1 (0.4%)
Statin	157 (63%)
Inhalers of asthma or COPD	37 (15%)

Baseline study outcomes showed that the marker of diastolic dysfunction on echocardiography in the patient population with permanent AF i.e., E/E' was 9.8. This is suggestive of the early signs of heart failure with preserved ejection fraction. This in itself is not uncommon in such a patient population and can be indicative of cardiac fibrosis in the left atrium and a marker for impaired exercise capacity.

3.3.2 **Cross Sectional Analysis of Baseline Monocyte Subsets, Surface Markers and Those of Cardiac Fibrosis in Relation to 24-Month Peak VO2**

3.3.2.1 **Monocyte Subsets**

The 3 monocytes subsets (Mon1, Mon2 and Mon3) were found to have no significant correlation with end of study peak VO2 max with p values of 0.63, 0.61 and 0.73 respectively **(Table 12)**. Based on my original hypothesis there was the potential for Mon 2 to be adversely associated with peak VO2 max performance with the subsequent decline of this subset in the spironolactone treated group, leading to improved exercise performance. Baseline data with regards to cardiopulmonary exercise testing did not suggest an association between these two continuous variables.

3.3.2.2 **Monocyte Platelet Aggregates and Monocyte Surface Markers**

As previously mentioned MPAs have been shown to have a pivotal role in the pathogenesis of coronary artery disease and acute heart failure, with little being known in the way of AF. In my study, analysis of monocyte subsets at baseline in relation to the primary study outcome of Peak VO2 max measure on cardiopulmonary bike exercise testing showed a significant relationship between the surface markers CD16 Mon3 (p=0.001),

Table 12. Analysis of 12 months monocyte data in relation to 24-month outcomes (adjustment made where appropriate for treatment groups) for peak VO2 max.

Analyses			
24-month VO2 max	Confidence Interval	β coefficient	p value
Monocyte Subset 1	-0.002-0.004	0.035	0.630
Monocyte Subset 2	-0.008-0.013	0.036	0.612
Monocyte Subset 3	-0.006-0.004	-0.025	0.725
MPA Subset 1	-0.034-0.018	-0.045	0.527
MPA Subset 2	-0.125-0.041	-0.071	0.321
MPA Subset 3	-0.080-0.022	-0.081	0.261
CD14 Mon1	-0.001-0.0001	-0.068	0.344
CD16 Mon 1	-0.160-0.124	-0.018	0.804
CD14 Mon2	-0.001-0.002	0.033	0.651
CD16 Mon 2	-0.010-0.018	0.042	0.557
CD14 Mon 3	-0.007-0.001	-0.092	0.201
CD16 Mon 3	0.004-0.016	0.228	0.001
CD42 MPA 1	-0.139-0.017	-0.110	0.124
CCR2 MON 1	-0.004-0.012	0.075	0.293
CCR2 MPA 1	-0.004-0.014	0.077	0.283
CD42 MPA 2	-0.026-0.007	-0.081	0.257
CCR2 MON 2	-0.009-0.016	0.041	0.571
CCR2 MPA 2	-0.005-0.012	0.059	0.412
CD42 MPA3	-0.085-(-)0.006	-0.159	0.026
CCR2 Mon 3	-0.199-0.272	0.022	0.761
CCR2 MPA 3	-0.356-0.089	-0.085	0.237
Galectin 3	-0.432-0.199	-0.048	0.766
PIIINP	-0.418-0.145	-0.299	0.132
PINP	-0.398-0.501	-0.803	0.876
Adjustment for treatment group			
CD16 Mon 3	0.004-0.016	0.229	0.001
CD42 MPA3	-0.082-(-)0.004	-0.151	0.030

Data presented as multivariant linear regression analysis. $p<0.05$ taken to statistically significant. Confidence interval is that in relation to the B coefficient

CD42 MPA (p=0.026) and subsequent performance on cardiopulmonary exercise testing when analysed using univariant linear regression analysis **(Table 12).**

Multiple linear regression analysis further showed a further significant correlation between CD16 Mon3 (p=0.001) and CD42 MPA3 (p= 0.03) suggesting that these biomarkers may be predictive of outcome in relation to peak exercise activity. The β coefficient would suggest that in this patient population a higher CD16 Mon3 count is associated with a better peak VO2 and vice versa for CD42 MPA3.

3.3.2.3 **Markers of Cardiac Fibrosis**

Baseline ELISA was carried out to assess the potential role of Galectin 3, PIIINP and PINP as a mechanism by which monocytes stimulate myofibroblast proliferation and procollagen-1 deposition, which eventually contributes to cardiac fibrosis, structural remodelling, and to further cardiomyocyte dysfunction, and thus forms an ideal substrate for ongoing cardiac fibrosis. In my study none of the 3 biomarkers tested were found at baseline to have a significant impact on VO2 outcome with p values of Galectin 3, PIIINP and PINP being 0.77, 0.13 and 0.88 respectively.

3.4 **Discussion**

In this chapter, I have shown for the first time that CD16 expression by Mon3 is a statistically significant predictor of peak exercise capacity in the form of peak VO2 max in patients with permanent AF and preserved left ventricular function. More specifically my results would suggest that as a biomarker CD16 Mon3 highlights a protective, beneficial effect in AF patients resulting in an improved exercise capacity.

Such observational associations are hypothesis generating and are in line with previous suggestions (albeit not shown before in this study setting) that there is a potential role for Mon3 and its key receptor CD16 in "tissue repair" (137). With Mon3 shown to have a role in limiting adverse remodelling in patients with cardiovascular disease one can hypothesise that the process of adverse left trial remodelling can be augmented depending on the differentiation of monocyte subsets in the bone marrow (349). Furthermore, the link with Mon3 as a protective subset has been shown in stroke survivors in in whom Mon3 counts were reduced (350). My study is the first to delineate the specific Mon3 surface marker, CD16 to be associated with better peak VO_2 in AF patients. The mechanism by which this is achieved is unclear from my study as the markers of fibrosis were not significantly associated with a change in peak VO_2. One may hypothesise that monocyte subsets play a key role in the process of inflammation and subsequent fibrosis that has been shown in non-AF patients with cardiovascular disease (234, 351).

Also, my study showed an association of higher CD42 expression on MPA3 with lower peak VO_2. It has been previously shown that circulating MPA were found at high levels in patients with underlying atherosclerotic disease (352). As stated earlier in my thesis MPAs are indicative of platelet activation and may have a role in induction of gene activation in

monocytes, such as genes for pro inflammatory cytokines. The findings from this chapter are again hypothesis generating in the associative link between MPA and a lower peak VO_2. Studies pertaining to the adverse role of MPA in cardiovascular disease have been highlighted in ischaemic heart disease both in angina and acute myocardial infarction (219, 353). More recently a higher percentage of platelets aggregated to Mon3 was found to be detrimental in patients with acute heart failure, with such a cohort having higher rates of hospitalisation and death (347). However, to date evidence has been lacking for their role in patients with AF with my study showing a possible association in this cohort with platelets aggregated to Mon3 via the surface ligand CD42 having a poorer outcome with regards to peak VO2.

My results have found an association with CD16 expression on Mon3 and an improved exercise performance in patients with permanent AF. The opposite was found for CD42 expression on MPA3. One could hypothesise a relationship that in this cohort of patients the process of favourable tissue repair and atrial remodelling is favoured by the presence of CD16 expression on Mon3. However, the presence of platelets aggregated to Mon3 via the surface ligand CD42 adds as a marker for heightened inflammation and platelet activity which is known to be an adverse maker of atherothrombotic events (219). A causal link cannot be justified from my findings but provides a justification for further research in order to delineate possible mechanisms by which monocyte subsets impact exercise capacity.

3.5 **Conclusion**

There is an association of CD16 Mon3 in patients with permanent AF and preserved left ventricular function with regards to outcomes of peak VO2. The same trend holds true for CD42 MPA3. There appears to be a role for CD16 Mon3 as a biomarker in this cohort of patients with the inverse holding true for CD42 MPA3. The function of Mon3 as opposed to the number could be a useful biomarker. Circulating monocyte numbers may not accurately reflect the overall monocyte production in chronic conditions. For example, if more monocytes are produced in bone marrow and more mobilised in tissues the numbers in circulation at a particular point may be similar.

There is therefore a potential therapeutic target in this patient population in both up regulation of CD16 Mon3 and possible down regulation of CD42 MPA3 in order to improve exercise capacity and possibly quality of life in patients with AF. However, such findings are hypothesis generating and will require further research in this field to further shine light on the mechanistic role by which monocytes are associated with both beneficial and detrimental effects on exercise capacity. The role of Spironolactone to provide a beneficial impact on primary and secondary outcomes will be explored in more detail in subsequent chapters.

Chapter 4

Effect of Monocyte Subsets and Markers of Cardiac Fibrosis on 6-Minute Walk Test, Quality of Life and Hospitalisation

Abstract

Introduction: Patients with AF are more likely to have impaired physical functioning, general health and subsequently compromised quality of life. Patients with AF often have confounding significant co morbidities leading to increased hospitalisation compared to those without AF. In this section I analyse the impact of monocyte subsets, their surface makers and biomarkers of cardiac fibrosis upon exercise capacity in the form of a 6-minute walk test, quality of life and hospitalisations

Methods: Three monocyte subsets, CD14++CD16-CCR2+ (Mon1), CD14++CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) were analysed by flow cytometry in patients with permanent AF and preserved left ventricular function at baseline, along with ELISA to quantify makers of fibrosis in the form of Galectin3, PIIINP and PINP. Statistical analysis involved SPSS software to carry out univariant and where appropriate multivariant linear regression analysis. A total of 197 patients were analysed in this part of my study. Analysis was made of 12-month monocyte subsets and end of study secondary outcomes. Quality of life was assessed using a well validated study questionnaire, with hospitalisations being recorded retrospectively by assessing electronic hospital records and patient's information on follow up visits regarding inpatient hospital admission.

Results: CD16 Mon3 (p=0.005) and CD42 MPA3 (0.043) were found to predict exercise capacity in the form of the 6-minute walk test when adjusted for treatment group intervention. Monocyte subsets, surface markers and those of cardiac fibrosis did not affect quality of life and hospitalisations.

Conclusions: Monocyte subsets surface markers of CD16 Mon3 and CD42 MPA3 were significantly related to outcomes with regards to a peak exercise capacity in respect to 6-minute walk test. Their role as potential biomarkers and therapeutic targets in patients with AF has been highlighted in this study.

4.1 **Introduction**

Patients with permanent AF have worse outcomes and reduced quality of life even despite optimal medical therapy. This holds true for patients in whom left ventricular function is preserved (354). There is conflicting data as to whether patients benefit prognostically from maintaining sinus rhythm (355). Targeting methods to improve quality of life and exercise capacity are likely to be more beneficial to this patient cohort rather than a rhythm control strategy.

The mechanisms leading to reduced exercise capacity, morbidity, and mortality in anticoagulated patients with permanent AF are likely related to an impairment in diastolic function, myocardial fibrosis and stiffening. Biomarkers of cardiac fibrosis have been shown to be elevated in patients with permanent AF (356). The subsequent changes can lead to ventricular filling abnormalities, reducing cardiac output and decreasing exercise capacity.

Monocytes have been implicated in fibrosis of different tissues. Along with (myo)fibroblasts, monocyte-derived macrophages are a source of TGFβ1, the key profibrotic factor (357). Activated monocytes represent a major MMP (matrix metalloprotease) source. Infact, monocytes/macrophages co-localise with myofibroblasts in areas of fibrosis during cardiac hypertrophy (358). The ability of an aldosterone inhibitor to reduce myocardial fibrosis, myocyte apoptosis, and MMP activity in rats was associated with reduced macrophage infiltration (359). Similar results have been obtained in animal models of diastolic dysfunction (360). Additionally, aldosterone leads to cardiac invasion of proinflammatory mononuclear cells.

In this chapter, I aimed to examine (i) monocyte subsets, surface markers and those of cardiac fibrosis at baseline in relation to patient performance in 6-minute walk test at 24 months. (ii) monocyte subsets, surface markers and those of cardiac fibrosis at baseline in relation to quality of life and hospitalisations.

4.2 **Methods**

4.2.1 **Study Population**

The recruitment and data collection for patients with AF and preserved left ventricular function is detailed in chapter 2.

In order to explore the significance of monocyte subsets, surface markers and those of cardiac fibrosis in relation to the set out secondary outcomes I analysed patients at the following time points:

a. 12-month flow cytometry for monocyte subsets and surface makers.

b. Baseline ELISA for cardiac biomarkers Galectin 3, PIIINP and PINP.

c. 24 months for 6-minute walk test, QOL and hospitalisations (ongoing prospective analysis)

The key outcome measures here were related to the impact/association of monocyte subsets and their surface markers, those of cardiac fibrosis, in relation to 6-minute walk test performance, quality of life and hospitalisation.

4.2.2 **Flow Cytometry and ELISA**

Flow cytometric and ELISA analysis was performed in study patients as described in detail in chapter 2.

4.2.3 **6-minute Walk Test and Quality of Life Questionnaire**

A brief summary of the 6-minute test is highlighted in chapter 2. To assess the quality of life of the patient cohort previously well validated health-related quality of life questionnaires were self-completed by patients (Minnesota Living with Heart Failure [MLWH and EuroQol EQ-5D [EQ-5D]).

4.2.4 **Statistical Analysis**

Details of statistical techniques have been described in chapter 2. Multivariant linear regression analysis in a forward stepwise method was undertaken as described in chapter 3.

4.3 **Results**

4.3.1 **Study Group Demographic and Clinical Characteristics**

The study groups baseline demographic and clinical characteristics have already been highlighted in chapter 3, Table 11.

4.3.2 **Relationship of Monocyte Subsets, Surface Markers and Markers of Cardiac Fibrosis with End of Study 6-Minute Walk Test.**

Analysis of monocyte subsets at baseline in relation to the secondary endpoint of the 6MWT further collaborates the association of CD16 Mon3 and CD42 MPA3 in relation to exercise performance **(Table 13).** This fits in line with the previous chapter outlining the relationship between these potential biomarkers and that of peak VO2.

A higher CD16 Mon3 count was associated with a significantly better outcome with regards to distance walked (p=0.0001) which was in contrast to CD42 MPA3 count which was associated with a negative effect on distance walked (p=0.021). This relationship held true upon multivariant linear regression analysis for both CD16 Mon3 (p=0.005) and CD42 MPA3 (P=0.043) respectively. **(Table 13).**

Table 13. Analysis of 12 months monocyte data in relation to 24-month outcomes (adjustment made where appropriate for treatment groups) for 6MWT.

Analyses (n=197)	Median (IQR)		
6-minute walk test (m)	269 (196-330)		
24-month 6MWT	Confidence Interval (B)	β coefficient	p value
Mon1	-0.059-0.064	0.006	0.934
Mon2	-0.109-0.308	0.066	0.348
Mon3	-0.137-0.065	-0.050	0.484
TMPA1	-0.651-0.410	-0.032	0.654
TMPA2	-2.386-0.993	-0.057	0.417
TMPA3	-1.778-0.322	-0.096	0.173
CD14 Mon1	-0.010-0.016	0.030	0.677
CD16 Mon 1	-3.146-2.469	-0.017	0.812
CD14 Mon2	-.010-.043	0.086	0.224
CD16 Mon 2	-0.285-0.296	0.003	0.969
CD14 Mon 3	-0.170-0.0001	-0.138	0.050
CD16 Mon 3	0.112-0.353	0.259	0.0001
CD42 MPA 1	-2.158-1.029	-0.049	0.486
CCR2 MON 1	.092-0.405	0.216	0.002
CCR2 MPA 1	0.109-0.462	0.220	0.002
CD42 MPA 2	-0.584-0.090	-0.102	0.150
CCR2 MON 2	-0.006-0.503	0.135	0.055
CCR2 MPA 2	-0.067-0.274	0.084	0.234
CD42 MPA3	-1.789-(-)0.146	-0.162	0.021
CCR2 Mon 3	-0.331-(-)0.010	0.128	0.068
CCR2 MPA 3	-5.194-3.604	-0.025	0.722
Galectin 3	-0.389-0.204	-0.040	0.701
PIIINP	-0.376-0.155	-0.214	0.122
PINP	-0.299-0.467	-0.789	0.813
Adjustment for treatment group			
CD16 Mon 3	0.059-.326	0.216	0.005
CD42 MPA3	-1.643-(-)0.026	-0.140	0.0043

Data presented as multivariant linear regression analysis. $p<0.05$ taken to statistically significant. Confidence interval is that in relation to the B coefficient.

4.3.3 **Relationship of Monocyte Subsets, Surface Markers and Markers of Cardiac Fibrosis with End of Study Quality of Life Scores and Hospitalisations.**

More subjective data in the form of the quality-of-life questionnaire showed CD16 Mon3 to be a predictor of improved scores in the initial analysis (p=0.009). CCR2 Mon1 (p=0.03), CCR2 MPA1 (0.012), CD42 MPA2 (p=0.03), CCR2 Mon3 (p=0.049) also were statistically significant with relation to QOL scores. Only CD42 MPA2 was associated with an inverse relationship (β coefficient -0.151) **(Table 14).**

However, upon multivariant analysis none of these predictors were shown to independently correlate with QOL outcomes **(Table 14).** There was no impact of monocyte subsets or biomarkers with respect to hospitalisations **(Table 15)**.

Table 14. Analysis of 12 months monocyte data in relation to 24-month outcomes (adjustment made where appropriate for treatment groups) for QOL.

Analyses			
24-month QOL	Confidence Interval	β coefficient	p value
Monocyte Subset 1	-0.007-0.015	0.054	0.449
Monocyte Subset 2	-0.024-0.049	0.049	0.494
Monocyte Subset 3	-0.020-0.015	-0.020	0.776
TMPA Subset 1	-0.073-0.115	0.032	0.657
TMPA Subset 2	-0.398-0.210	-0.043	0.542
TMPA Subset 3	-0.270-0.100	-0.065	0.364
CD14 Mon1	-0.002-0.003	0.033	0.643
CD16 Mon 1	-0.454-0.534	0.011	0.874
CD14 Mon2	-0.004-0.006	0.033	0.642
CD16 Mon 2	-0.096-0.005	-0.126	0.077
CD14 Mon 3	-0.030-0.0001	-0.138	0.051
CD16 Mon 3	0.007-0.050	0.184	0.009
CD42 MPA 1	-0.306-0.258	-0.012	0.867
CCR2 MON 1	0.003-0.059	0.153	0.030
CCR2 MPA 1	0.009-0.072	0.178	0.012
CD42 MPA 2	-0.122-(-)0.005	-0.151	0.033
CCR2 MON 2	-0.006-0.083	0.121	0.089
CCR2 MPA 2	-0.008-0.051	0.101	0.156
CD42 MPA3	-0.227-0.066	-0.076	0.282
CCR2 Mon 3	-0.004-1.639	0.139	0.049
CCR2 MPA 3	-0.250-1.299	0.095	0.183
Galectin 3	-0.299-0.180	-0.020	0.603
PIIINP	-0.201-0.130	-0.198	0.122
PINP	-0.276-0.400	-0.109	0.798
Adjustment for treatment group			
Nil Significant on adjustment			

Data presented multivariant linear regression analysis. $p<0.05$ taken to statistically significant. Confidence interval is that in relation to the B coefficient.

Table 15. Analysis of 12 months monocyte data in relation to 24-month outcomes (adjustment made where appropriate for treatment groups) hospitalisations.

Analyses			
Hospitalisations	Confidence Interval	β coefficient	p value
Monocyte Subset 1	0.0001-0.0001	0.024	0.723
Monocyte Subset 2	-0.001-0.001	0.022	0.739
Monocyte Subset 3	0.0001-0.0001	0.021	0.751
TMPA Subset 1	-0.003-0.001	-0.050	0.451
TMPA Subset 2	-0.008-0.006	-0.024	0.719
TMPA Subset 3	-0.005-0.003	-0.029	0.661
CD14 Mon1	0.0001-0.0001	-0.119	0.075
CD16 Mon 1	-0.015-0.007	-0.043	0.517
CD14 Mon2	0.0001-0.0001	0.057	0.396
CD16 Mon 2	-0.002-0.0001	-0.132	0.048
CD14 Mon 3	0.0001-0.0001	0.013	0.850
CD16 Mon 3	0.0001-0.001	0.023	0.726
CD42 MPA 1	-0.005-0.007	0.024	0.717
CCR2 MON 1	0.0001-0.001	0.077	0.248
CCR2 MPA 1	0.0001-0.001	0.080	0.230
CD42 MPA 2	-0.002-0.001	-0.036	0.585
CCR2 MON 2	-0.001-0.001	0.008	0.905
CCR2 MPA 2	-0.001-0.0001	-0.069	0.305
CD42 MPA3	-0.004-0.003	-0.022	0.746
CCR2 Mon 3	-0.011-0.027	0.058	0.381
CCR2 MPA 3	-0.006-0.029	0.084	0.207
Galectin 3	-0.178-0.118	-0.013	0.545
PIIINP	-0.132-0.172	-0.119	0.145
PINP	-0.202-0.276	-0.103	0.845
Adjustment for treatment group			
Nil Significant on adjustment			

Data presented multivariant linear regression analysis. $p<0.05$ taken to statistically significant. Confidence interval is that in relation to the B coefficient.

4.4 **Discussion**

In this chapter I have shown the potential biomarkers that can predict exercise capacity in the form of the well validated and used 6-minute walk test in patients with permanent AF and preserved left ventricular function. As mentioned in the introductory chapter exercise capacity is a strong predictor of cardiovascular events and overall mortality. Although CPET, with the determination of peak VO2 is the 'gold-standard' assessment of exercise capacity, not all patients can carry out such a physically challenging assessment due to co morbidities outside that of the cardio-respiratory system. For example, osteoarthritis can allow for reasonable daily functional status but limits more physically demanding tasks. To my knowledge this is the first study to highlight potential biomarkers that are able to predict outcomes in such regard.

I have highlighted the potential role of CD16 Mon3 and CD42 MPA3 in their association with exercise capacity which was highlighted in the previous chapter. CD42 MPA 3 is associated with reduced exercise capacity in this cohort of patients. My findings here are hypothesis generating and whether AF reflects a state of platelet hyperactivity and in turn is a potential cause of reduced exercise capacity cannot be concluded from my results. My results show there is an association between CD42 MPA3 and reduced exercise capacity, which to my knowledge has not been shown in this specific population. Previous research on the role of MPAs in ischaemic heart disease showed MPAs to be associated with a heightened risk of myocardial infarction in patients presenting with acute chest pain (odds ratio of 10.8, CI 3.6,32.0) when comparing lower and upper most quartiles (218). Evidence to date suggests that platelet activation induces gene activation in monocytes, which in turn propenoate an inflammatory cascade involving cytokine release (361, 362). Such studies have suggested a causative relationship between MPAs and cardiovascular disease (347). In my study the role of potential fibrotic factors in the form of galectin 3, PIIINP and PINP were not associated with outcomes

with regards to exercise capacity and thus an argument for causation could not be justified. Further research in this field should aim to build upon my findings to ascertain whether AF triggers a change in monocyte phenotype such that a pro-inflammatory environment is achieved. The subsequent transformation to a pro-inflammatory monocyte phenotype could give rise to increases endothelial adhesiveness and this can potentiate a continuum of fibrosis. Such a mechanism would help drive my hypothesis from that of association to more evidence for a causative relationship between MPAs and exercise capacity.

Conversely, I have shown once more that CD16 Mon3 is associated with more favourable exercise tolerance in this patient population. However, these findings are again hypothesis generating as, although an associated between CD16 Mon3 and exercise capacity was established a mechanism and thus causal link cannot yet be justified (markers of cardiac fibrosis were not found to be significantly correlated with exercise capacity). Previous data suggests that Mon3 exerts an anti-inflammatory and hence anti fibrotic role within the myocardial tissue (363). Some, but not all, human studies have confirmed excessive atrial fibrosis in chronic AF patients compared with those with sinus rhythm (364).One could hypothesise that regardless of the cause or effect mechanism of CD16 Mon3 in the AF population understanding more the inflammatory pathway by which this monocyte subset benefits exercise capacity could offer more insight into their role as a therapeutic target in the future.

Despite promising findings with such objective measures as cardiopulmonary exercise testing and 6MWT, the more subjective measure of assessing quality of life in patients with AF and preserved let ventricular function did not highlight any such promise. Although a well validated and established method of assessing patients physical and psychological well-being

the subjective nature of testing in this form may account for some of the lack of continuity in results when compared to more objective measurement such as peak VO2 max and 6MWT.

Hospitalisations did not show any correlation with monocyte subsets. However, my study population were cardiovascular stable and thus one could postulate that in this setting versus previous studies the inflammatory process is subdued thus explaining a muted impact of monocyte subsets in this patient cohort. Samples were not taken in the acute inpatient setting at the time of admission and thus the role of monocyte subsets at that time cannot be ascertained. Furthermore, only 6 patients were hospitalised over the study period, which in itself may not yield a true relationship in this regard. Longer follow up may highlight different results.

4.5 **Conclusion**

CD16 Mon3 and CD42 Mon3 were found to be associated with improved and reduced exercise capacity respectively in patients with permanent AF and preserved left ventricular function with regards to 6-minute walk test. These findings are hypothesis generating in the role for CD16 Mon3 and CD42 MPA3 as a biomarker in this cohort. Although reduced exercise capacity is in itself a marker of poor outcome the use of a biomarker can aid in the understanding of the underlying pathophysiology and at this point direct further research. However, causality cannot be derived from these findings but further studies in this field can build on such findings to provide a potential mechanism for the association provided. In addition, quality of life in this patient population is not impacted by monocyte subsets, neither is hospitalisation over the 24 months study period.

Chapter 5

Role of Spironolactone on Monocyte Subsets and Markers of Cardiac Fibrosis

Abstract

Introduction: Monocytes, and specifically monocyte subsets, have been shown to play a central role in the pathophysiology of cardiovascular disease. They play a role in the regulation of the inflammatory cascade and in turn cardiac fibrosis. In my study I set out to analyse the differences in monocyte subset numbers, surface makers and those of cardiac fibrosis in patients with permanent AF and preserved left ventricular function and the effects of spironolactone vs placebo on the quantitative measurement of these potential biomarkers. Furthermore, I analysed the clinical characteristics associated with this patient population prior to and at the end of the 24-month study period.

Methods: Three monocyte subsets Mon1, Mon2, and Mon3 were analysed by flow cytometry in patients with permanent AF and preserved left ventricular function at baseline, along with ELISA to quantify makers of fibrosis in the form of Galectin3, PIIINP and PINP. Statistical analysis involved SPSS software to carry out independent samples T test between spironolactone and placebo study groups. A total of 250 patients were analysed at baseline (125 in the Spironolactone treatment group and 125 in the placebo control group) and a total of 197 at the end of study (100 in the Spironolactone group and 97 in the placebo control group).

Results: Prior to treatment intervention there were no significant differences with regards to the demographic and clinical characteristics between the two patient groups. There were no significant differences between Mon1 ($p=0.17$), Mon2 (0.54). or Mon 3 levels ($p=0.09$) and their subsequent markers of inflammation and fibrosis. Comparison of monocyte subsets at end of study showed no differences between Mon1 ($p=0.86$), Mon2 ($p=0.66$) or Mon3 ($p=0.81$) subsets and their subsequent makers of inflammation and fibrosis between the treatment and placebo control group.

Conclusions: Monocyte subsets and their subsequent markers of inflammation and fibrosis were quantitively unchanged with the introduction of Spironolactone when compared to the placebo group over a 24-month period. Hence, the role of Spironolactone in providing potential anti fibrotic properties to enhance exercise capacity and quality of life in patient with permanent AF and preserved left ventricular function is limited from this study's findings.

5.1 **Introduction**

The mechanisms leading to reduced exercise capacity, related morbidity, and mortality in anticoagulated patients with permanent AF are likely related to disturbed diastolic ventricular function, myocardial fibrosis and stiffening. Activation of fibroblast signaling mechanisms are enhanced in patients with chronic AF (365). These changes can lead to ventricular filling abnormality, reducing cardiac output and decreasing exercise capacity. Aldosterone increases cardiac collagen deposition and left ventricular fibrosis. This involves direct stimulation of cardiac fibroblasts by aldosterone to produce collagen with chronification of oxidative stress and inflammation in the heart. Cardiac expression of mineralocorticoid receptors is increased in AF, thus augmenting the genomic effects of aldosterone (284). According to a sub study of the RALES trial, the improved survival in patients with heart failure with impaired cardiac contractility treated by spironolactone was linked to its ability to reduce serum markers of ongoing fibrosis (330). The RACE-3 study, reporting a complex intervention that included spironolactone (>75% difference in use between groups) suggested beneficial effects compared to usual care in patients with recent onset AF (366). Inhibition of monocyte accumulation in the myocardium has been found to supress myocardial fibrosis. The ability of an aldosterone inhibitor to reduce myocardial fibrosis, myocyte apoptosis, and MMP activity was associated with reduced macrophage infiltration (317).

In this chapter, I aimed to examine (i) the differences in monocyte subsets in patients receiving spironolactone vs placebo at baseline and at the end of the study. (ii) differences in monocyte surface markers and those of cardiac fibrosis between the spironolactone and placebo group at baseline and at the end of the study.

5.2 **Methods**

5.2.1 **Study Population**

The recruitment and data collection for patients with AF and preserved left ventricular function is detailed in chapter 2.

In order to explore the significance of spironolactone vs placebo on monocyte subsets, surface markers and those of cardiac fibrosis I analysed patients at the following time points:

a. 12 months and end of study flow cytometry for monocyte subsets and surface makers.

b. Baseline, 12 months and end of study for cardiac biomarkers Galectin 3, PIIINP and PINP).

In order to ascertain any significant correlation between monocyte subsets and primary endpoint of peak VO2 along with secondary endpoints of 6-minute walk test, QOL questionnaire and hospitalisation I analysed patients at:

a. 12 months for baseline monocyte subsets, surface markers and those of cardiac fibrosis
b. 24 months for peak VO2, 6-minute walk test, QOL and hospitalisations.

5.2.2 **Flow Cytometry and ELISA**

Flow cytometric analysis and ELSA was performed in all study patients as described in detail in chapter 2. (SOP can be found in Appendix 3,4 and 5 respectively)

5.2.3 **Statistical Analysis**

Details of statistical techniques have been described in chapter 2, but to summarise independent samples T test was used to decipher any statistically significant difference ($p<0.05$) in monocyte subsets, surface markers and those of cardiac fibrosis between the Spironolactone and placebo group at baseline and at 24 months end of study visits.

5.3 **Results**

5.3.1 **Subject Characteristics**

The study groups had similar baseline demographic and clinical characteristics with regards to age, gender, smoking and alcohol consumption. Medication history was also not significantly different between the Spironolactone and placebo groups apart from statin therapy ($p=0.04$) **(Table 16)**. There were a higher proportion of males overall in the study, but this was not statistically different between the two treatment groups as was the representation of Caucasian participants in the study.

Table 16. Demographic and clinical characteristics of study population in Spironolactone vs Placebo group at baseline.

	Spironolactone n=125	Placebo n=125
Demographic characteristics		
Age (years)	73 (68-77)	72 (67-78)
Gender		
Female	28 (22%)	31 (25%)
Male	97 (78%)	94 (75%)
Ethnicity		
White	118 (94%)	118 (94%)
Black	3 (2%)	3 (2%)
Asian	3 (2%)	2 (2%)
Mixed or other	1 (1%)	2 (2%)
Smoker		
Current smoker	6 (5%)	8 (6%)
Ex-smoker	66 (53%)	68 (54%)
Never smoked	53 (42%)	49 (39%)
Alcohol use (units/week)	3 (0-12)	6 (0- 14)
Characteristics of the study outcomes		
VO_{2peak} (mL/kg/min)	14 (11-18)	14 (11-18)
6-minute walk test (m)	266 (196-316)	271 (200-330)
E/E' ratio	9.8 (8.0-12.0)	9.7 (7.5-13.0)
EQ-5D-5L		
Score	0.84 (0.74-0.94)	0.88 (0.74-0.94)
Missing data	4 (3%)	5 (4%)
MLWHF score[1]		
Score	17.0 (6.3-35.8)	14.0 (5.8-30.0)
Missing data	8 (6%)	4 (3%)
Clinical characteristics		
Left ventricular ejection fraction (%)	58 (57-62)	58 (56-63)
Brain natriuretic peptide (pg/mL)	122 (73-230)	136 (82-241)
Body mass index (kg/m^2)	29 (26-33)	30 (26-34)

Systolic blood pressure (mmHg)	130 (117-140)	129 (118-142)
Diastolic blood pressure (mmHg)	75 (67-83)	74 (68-82)
Resting heart rate (bpm)	85 (74-99)	83 (74-97)
Peak heart rate (bpm)	129 (106-150)	130 (114-152)
Diabetes	24 (19%)	21 (17%)
Echocardiographic Markers of fibrosis		
Echocardiographic backscatter	2.36 (1.24-3.3))	1.93 (1.14-3.21)
Monocyte Count (per μl)	n=110	n=116
Total Monocyte Count	651 (511.9-801.3)	667 (539.9-824.9)
Mon1	536 (410.8-653.6)	541 (437.4-667.3)
Mon2	43.2 (25-80.1)	40.7 (23.7-65.2)
Mon3	49.8 (35.5-74)	59.6 (43.8-90.1)
Total MPA (per μl)	55.8 (34.4-77)	57.6 (40.9-79)
MPA 1	37.7 (23.3-53.2)	38.4 (27.3-52.6)
MPA 2	7.3 (4.4-10.7)	6.9 (4.4-11.8)
MPA 3	5.9 (3.5-9.7)	7.7 (4.8-11.2)
CD14 Mon1	1380.6 (1156.6-1600)	1477.1 (1240.7-1704.2)
CD16 Mon1	17.8 (14.8-22)	18.1 (15.3-21.3)
CD14 Mon2	1286 (914-1559.1)	1278.2 (916.2-1511.4)
CD16 Mon2	106.5 (95.8-121)	112 (97.1-129.4)
CD14 Mon3	184.6 (149.2-231.8)	179.4 (147.3-226.8)
CD16 Mon3	242.42 (192-311.9)	234.2 (177.2-292.6)
CD42 MPA1	40 (34.6-44)	40.9 (35.5-44)
CCR2 Mon1	159.1 (96.7-208)	149.6 (91-213.9)
CCR2 MPA1	145.5 (86-185.9)	137.4 (86.2-192.5)
CD42 MPA2	76.7(58.5-106.4)	80.5 (60.7-109.6)
CCR2 MON2	125.8 (91.4-162)	116.4 (90.4-148.9)
CCR2 MPA2	180.6 (139.3-234.6)	179.5 (139.3-227.6)
CD42 MPA3	51 (42.4-61.2)	50.8 (43.8-58.9)
CCR2 MON3	14.38 (12.56- 15.52)	13.8 (12.1-15.7)
CCR2MPA3	14.2 (12.1-15.9)	13.4 (11.8-15)
ELISA markers of cardiac fibrosis	n=110	n=116

Galectin 3 (Baseline)	1.38 (1.2-1.9)	1.42 (1.18-1.88)
Galectin 3 (12 months)	1.33 (1.18-2.1)	1.4 (1.2-1.48)
Galectin 3 (24 months)	1.29 (1.19-2.3)	1.31 (1.28-1.39)
PINP (Baseline)	149 (123.1-158.2)	157 (135.8-160.1)
PINP (12 months)	138 (110.1-138.9)	134 (121.4-140.4)
PINP (24 months)	129 (113.2-135.3)	131 (119.1-137.3)
PIIINP (Baseline)	360.4 (310.8-390.7)	376 (344.1-391.1)
PIIINP (12 months)	355.3 (301.2-370.9)	349.1 (340-360.1)
PIIINP (24 months)	352.1 (339.2-362.8)	347.1 (338.1-359)
Medications		
Non-vitamin K oral anticoagulants	60 (48%)	57 (46%)
Vitamin K antagonists	47 (38%)	47 (38%)
Aspirin	10 (8%)	9 (7%)
Clopidogrel	6 (5%)	4 (3%)
Loop diuretic	25 (20%)	24 (19%)
Thiazide diuretic	14 (11%)	20 (16%)
Angiotensin converting enzyme inhibitor or angiotensin receptor blocker	67 (54%)	80 (64%)
Calcium channel blocker	43 (34%)	37 (30%)
Beta blocker	66 (53%)	70 (56%)
Digoxin	26 (21%)	23 (18%)
Amiodarone	0 (0%)	1 (1%)
Statin	88 (70%)	69 (55%)
Inhalers of asthma or COPD	20 (16%)	17 (14%)

Continuous data presented as median (interquartile range)

To score MLWHF questionnaire, it was allowed that at most 20% of 21 responses missing which was equivalent to 4 data items. If there were less than or equal to 4 data items missing then we used mean substitution to impute the missing responses and then scored the questionnaire by summating the responses to all 21 questions; otherwise, the person's score was left missing

Baseline study outcomes showed that although there was no statistical difference between treatment group vs placebo the marker of diastolic dysfunction on echocardiography in the patient population with permanent AF, an E/E' of 9.8 and 9.7 respectively is suggestive of

the early signs of heart failure with preserved ejection fraction. This in itself is not uncommon in such a patient population and has been shown to be indicative of cardiac fibrosis in the left atrium and a marker for impaired exercise capacity in previous study cohorts. As expected in such a randomised control study monocyte subset, surface markers and EILSA markers of cardiac fibrosis were not significantly different at baseline between the 2 study groups.

5.3.2 **Cross-Sectional Analysis of 24-month (end of study) Characteristics of Cardiac Fibrosis**

5.3.2.1 **Monocyte Subsets**

Patients who received Spironolactone for 24 months were not found to have a significantly different monocyte subsets counts versus placebo (including the hypothesised Mon2 subset). Both Mon1 and Mon3 counts were found to be lower in the Spironolactone treated group (p= 0.86 and 0.66 respectively (**Table 17**). Mon3 count showed a trend towards being increased in the intervention arm.

5.3.2.2 **Monocyte Platelet Aggregates and Monocyte Surface Markers**

MPA has previously been shown to be have pivotal role in the pathogenesis of coronary artery disease and acute heart failure, with little being known in the way of AF (234, 347). In my analysis MPAs were not found to be affected by the intervention with spironolactone over a 24 months period when compared to the placebo group, p= 0.54, 0.80 and 0.31 respectively for MPA1, MPA2 and MPA3 **(Table 17)**.

However, CD42 MPA2 was found to be to be significantly reduced over the 2-year study period in the spironolactone treatment group vs placebo-controlled group (p=0.003) **(Table 17).** With evidence for non-significant variability in other continuous variables bar better control of systolic blood pressure this points to a likely treatment effect of spironolactone. This, however, did not translate into any significant benefit with regards to the set out primary and secondary outcomes.

Table 17. End of study characteristics for spironolactone vs placebo group

	Spironolactone n=97	Placebo n=100	Treatment effect (95% CI)	p value
Characteristics of the study outcomes	Mean (SD)	Mean (SD)		
VO_{2peak} (mL/kg/min)	14.69 (5.5)	15.1 (4.4)	0.63 (-0.63(-)-1.66)	0.52
6-minute walk test (m)	327.57(84.2)	341.72(98.3)	12.87 (-39.5- 11.2)	0.27
E/E' ratio	8.9 (3.1)	9.8 (3.6)	-0.8 (-1.71-.12)	0.87
EQ-5D-5L *Score*	0.81 (0.26)	0.84 (0.21)	-0.008 (-0.06-0.04)	0.74
MLWHF score[1]	17.4(22.7)	15.3 (20.4)	0.49 (-4.32-5.29)	0.84
Clinical characteristics				
Left ventricular ejection fraction (%)	56.9 (5.17)	57.4 (4.47)	-0.52 (0.67- (-)1.84)	0.44
Brain natriuretic peptide (pg/mL)	181.1 (170.7)	184.9 (109.7)	-3.77(-42.97-35.42)	0.85
Body mass index (kg/m^2)	30.7(5.4)	30.36 (5.82)	0.35 (-1.18-1.89)	0.65
Systolic blood pressure (mmHg)	128 (17.82)	135 (16.54)	-7.49(-12.2-(-)2.77)	0.02
Diastolic blood pressure (mmHg)	77 (10.51)	79 (11.13)	-2.26 (-5.23-0.71)	0.14
Resting heart rate (bpm)	80 (16)	80 (16.3)	0.6 (-3.94-5.13)	0.8
Peak heart rate (bpm)	125 (28.79)	124 (34.29)	0.88 (-8.14-9.91)	0.85
Echocardiographic Markers of fibrosis	n=101	n=106		
Echocardiographic backscatter	1.73(1.7)	1.47 (1.5)	.26 (-.18-0.7)	0.25

Monocyte Count (per μl)	n=101	n=106		
Total Monocyte Count	817.85 (282.5)	814.37 (245.2)	3.48 (-68.91-75.87)	0.93
Mon1	700.8 (256.35)	706.6 (209.72)	-5.85(-69.9-58.21)	0.86
Mon2	35.52 (33.42)	38.52 (60.25)	-3 (-16.45-10.44)	0.66
Mon3	81.24 (46.69)	79.63 (49.13)	1.61 (-11.54-14.76)	0.81
Total MPA (per μl)	75.96 (35.28)	72.93 (28.3)	3.03 (-5.72-11.77)	0.5
MPA 1	56.1 (30.47)	53.7 (22.71)	2.31 (-5.03-9.65)	0.54
MPA 2	8.4 (4.84)	8.6 (6.10)	-0.2 (-1.71-1.32)	0.80
MPA 3	11.51 (7.74)	10.49 (6.37)	1.01 (-0.93-2.95)	0.31
CD14 Mon1	1107.01(302.67)	1153.2(277.2)	-46.13(-125.6-33.3)	0.25
CD16 Mon1	14.14 (11.43)	12.47 (3.65)	1.67 (-0.63-3.97)	0.15
CD14 Mon2	739.42 (387.03)	704.7 (383.48)	34.74(-70.9-140.3)	0.52
CD16 Mon2	152.84 (79.21)	152.63 (96.76)	.21(-24.1-24.51	0.99
CD14 Mon3	148.12 (63.25)	138.77 (51.1)	9.35(-6.37-25.01)	0.24
CD16 Mon3	170.89 (74.87)	165.82 (73.57)	5.08 (-15.27-23.42)	0.62
CD42 MPA1	44.08 (7.32)	43.86 (6.2)	0.22 (-1.63-2.10)	0.81
CCR2 Mon1	110.38 (55.39)	110.11 (49.48)	0.26 (-14.12-14.63)	0.97
CCR2 MPA1	103.38 (51.73)	102.87 (46.07)	0.51 (-12.9-13.92)	0.94
CD42 MPA2	119.82(57.2)	104.85 (37.5)	14.98 (1.79-28.18)	0.03
CCR2 MON2	98.2 (34.37)	98.1 (26.61)	0.12 (-8.28-8.53)	0.98
CCR2 MPA2	165.24 (39.48)	160.68 (39.28)	4.55 (-6.24-15.35)	0.41

CD42 MPA3	55.78 (12.15)	54.19 (9.38)	1.59 (-1.37-4.56)	0.29
CCR2 MON3	13.51 (2.4)	13.27 (2.4)	0.24 (-.41-.90)	0.47
CCR2MPA3	14.67 (2.4)	14.44 (2.12)	0.24 (-.38-.86)	0.46
ELISA markers of cardiac fibrosis	n=101	n=106		
Galectin 3 (12 months)	1.29 (1.1)	1.26 (0.99)	0.12 (-.28-.92)	0.94
Galectin 3 (24 months)	1.48 (0.98)	1.52 (1.01)	0.16 (-.16-.64)	0.97
PINP (12 months)	138 (56.3)	133 (53.6)	7.28 (-2.8-9.4)	0.95
PINP (24 months)	139 (57.1)	128 (49.3)	6.23 (-1.36-8.4)	0.88
PIIINP (12 months)	298.3 (19.2))	282.1 (17.6)	2.44 (-7.1-5.1)	0.81
PIIINP (24 months)	279.4 (28.2)	268 (24.5)	3.1 (-1.29-4.26)	0.91
Continuous data presented as median (interquartile range) To score MLWHF questionnaire, it was allowed that at most 20% of 21 responses missing which was equivalent to 4 data items. If there were less than or equal to 4 data items missing then we used mean substitution to impute the missing responses and then scored the questionnaire by summating the responses to all 21 questions; otherwise, the person's score was left missing. Independent samples T test was used to compare the 2 study groups at 24 months.				

5.4 **Discussion**

The results from this chapter failed to prove my hypothesis that spironolactone would improve both the primary and secondary outcomes of my trial. Previous evidence pointed to the role of increased fibroblast activity and release of cytokines to induce a pro inflammatory cascade in patients with permanent AF (367). Subsequently, the changes in ventricular filling pressure, atrial fibrosis and cardiac output had been found to reduce exercise capacity in patients with AF (21). Patients with AF have an increased expression of mineralocorticoid receptors on their myocardial surface and thus aldosterone is thought to have greater fibrotic effects (318).

Despite evidence to support my hypothesis patients in the interventional arm of my study did not derive the anti-fibrotic properties initially predicted. From a clinical perspective one can argue that previous trials in this field have focused on patients with reduced rather than preserved left ventricular function (330, 368) and that the benefit in those preserved left ventricular function is lacking. Indeed, the ALDO-HF and TOPCAT studies were both trials that found spironolactone to have no beneficial role in exercise capacity, and those more promising results in the TOPCAT study were offset by potential quality concerns regarding the data collaboration from Eastern Europe (321, 369). Furthermore, any benefits seem in those patients with HFpEF were evident in those without AF (370).

In addition, there were no differences in quality-of-life assessment and hospitalisations between the 2 groups over the 24 months period. Quality life assessment, although a well-established tool for qualitive assessment can be deemed difficult to interpret in this context, especially in an elderly cohort of patients as was present in this study. Patients in this study had multiple co morbidities and their clinical health over the 2-year study period was subject

to change which itself would impact of quality life scores unrelated to their atrial fibrillation. Hospitalisations over the study period were minimal which is unsurprising as an outpatient, relatively stable population was chosen for the study in order to maximise the chances of study completion and compliance to the study protocol.

It was subsequently not surprising that the lack of benefit of Spironolactone translated into no significant changes in monocytes subsets, surface markers or those of fibrosis between the two trial arms. The finding of a higher MPA 3 count at baseline in the placebo group, reduction in CD42 MPA2 in the spironolactone group did not translate into significance with regards to the study's primary and secondary outcomes, and thus could be a chance finding. Furthermore, Spironolactone's role with regards to antifibrotic properties in this patient population failed to show any significance. Previous studies have focused on ischaemic heart disease and heart failure with preserved ejection fraction to suggest beneficial roles of spironolactone in this regard (370, 371). From my results one could hypothesise that patients AF and preserved ejection fraction have a different mechanism of inflammation and subsequent fibrosis which was not evident from my findings.

5.5 **Conclusion**

Treatment with the aldosterone antagonist, spironolactone in patients with permanent AF and preserved ejection fraction does not provide a beneficial impact on monocyte subsets, surface makers and those of cardiac fibrosis when compared to placebo. Furthermore, this translates into no statistically significant beneficial role with regards to exercise tolerance and quality of life. Although previous chapters have shown potential biomarkers in the form of monocyte surface markers and monocyte platelet aggregates to predict exercise capacity, the proposed anti fibrotic properties of Spironolactone did not provide any therapeutic gain.

Chapter 6

Discussion

6.1 **Summary Key Findings**

Much of the work presented in this final chapter has already been discussed in the individual results chapters (see chapters 3-5). The aim of this chapter is to bring all of these findings together in the context of the original hypotheses described in chapter 1 and to discuss how this work may lead on to future research.

The overall key findings in this thesis are:

i. In patients with permanent AF and preserved left ventricular function monocyte surface biomarkers, CD16 Mon3 and CD42 MPA3 that can predict peak exercise capacity. (Chapter 3 and 4)

ii. Quality of life in this patient population is not related to the counts of monocyte subsets, their surface markers and markers of cardiac fibrosis. (Chapter 4)

iii. Monocyte subsets, their surface makers and markers of cardiac fibrosis do not predict hospitalisations in patients with AF and preserved left ventricular function. (Chapter 4)

iv. Treatment with spironolactone vs placebo does not significantly change in monocyte subsets, surface markers and cardiac fibrosis in patients with AF and preserved left ventricular function. (Chapter 5)

v. Spironolactone has no statistically significant impact on primary and secondary outcomes over the 24 months study period when compared to the placebo cohort in patients with AF and preserved left ventricular function. (Chapter 5)

6.2 **General Discussion**

The specific findings from my study are that stable patients with permanent AF and preserved left ventricular function have inflammatory surface makers of CD16 associated with monocyte subset Mon3 and CD42 ligand on MPA associated with Mon3 have the ability to predict the peak exercise capacity and thus cardiovascular health in this patient population. In patients with AF a higher expression of CD16 Mon3 was observed to be associated with a higher peak VO2 and also better 6MWT performance. Conversely, a heightened expression of CD42 MPA3 was associated with a reduced peak exercise capacity in this cohort. Previous evidence ascertaining to the role of Mon3 and its surface marker expression in patient with AF is scarce. However, the results from my study are hypothesis generating and highlight a role for this monocyte subset that does not translate to the detrimental pro inflammatory process seen with Mon1 and Mon2. It must be emphasised that a causality link has not been found from my research, but the study has provided novel insight into potential for an association between Mon3 and exercise capacity in this specific patient population.

Mon3, similar to Mon1 have antigen processing capabilities, but are distinct by their association with wound healing (372). Furthermore, they have antagonizing functions to Mon1 and promote neutrophil adhesion at the endothelial interface via the secretion of TNF-α and do not reach the Mon1 production levels of pro-inflammatory cytokines (363). The observational association I have found between CD16 Mon3 and exercise capacity is likely due to their favourable role in inflammation and cardiac remodelling which is supported by previous finding in other cardiovascular conditions. Although no causal relationship can be established from my study, it is known that patients with AF have enhanced cardiac fibrosis

and this in turn has already been shown to lead to cardiovascular morbidity, including left ventricular dysfunction (373)

As expected, patients AF and preserved left ventricular function with reduced peak VO2 have higher all-cause mortality (374) . However, pathophysiology and mechanistic links have thus far been lacking in this population. My findings of the observed association between CD16 Mon3 and peak exercise capacity will help to guide future research as to whether there is a causal link and to what degree this may provide a potential therapeutic target by which augmenting the inflammatory response attenuates cardiac fibrosis in AF patients.

Conversely CD42 MPA3 in my study was associated with reduced exercise capacity. Previous research in non-AF patients has shown MPAs are associated with increased cardiovascular risk (375, 376). More recent evidence has actually pointed out to a more specific role of MPAs associated with Mon2 and presentation with acute decompensated HF (347). However, to date the role of MPAs in exercise capacity has not been studied in patients with permanent AF. From my results one could hypothesise that the noted observations likely fall in line with previous evidence (albeit in non-AF patients) that MPAs and the heightened state of thrombogenesis in this patient population is associated with reduced exercise capacity. Again, CD42 MPA3 is a marker of inflammation and in this case a detrimental observation related to exercise capacity has been seen. A causal link could not be derived from my results and indeed the markers of fibrosis chosen (galectin 3, PINP and PIIINP) did not provide further insight into a possible mechanism of inflammation leading to fibrosis.

Despite promising observational findings with regards to Mon3 and MPAs my original hypothesis was that Mon2 would be found to be detrimental with regards to peak exercise

capacity. This is on the background of the recent studies showing a pivotal role of Mon2 in the adverse atrial remodelling in patients with AF undergoing catheter ablation (348). Furthermore Mon2 has been well established as a pro inflammatory monocyte within cardiovascular disease (240). Patients with acute myocardial infarction (234), unstable angina (237), acute heart failure (241), abdominal aneurysm (245) and stroke all have elevated Mon2, which correlated with troponin T levels (350). In this patient population Mon3 showed no specific change. In fact, Mon3 was reduced in patients with stroke.

Why Mon2 was not associated with a reduced exercise capacity in this AF population cannot be established from my study. However, the patients recruited for my research were "stable" from a cardiovascular perspective. In the previous research highlighted above patients were often recruited in the setting of a "peak" pro inflammatory insult. In such cases Mon2 is a well-established marker of poor outcome (377). This context however was essential to allow patients in my study the best chance of completing a 2 year follow up and end of study exercise capacity testing. Furthermore, the role of monocyte subsets in the setting of cardiopulmonary exercise testing has not to my knowledge been investigated previously and thus my findings may allude to a different pathophysiological process for patients with AF that is separate from other cardiovascular disease states. Future research in this field will aid in deciphering this hypothesis.

Interestingly, despite objective evidence for the role of CD16 Mon3 and CD42 MPA 3 in predicting exercise capacity and thus to an extent functional status of patients with AF and preserved left ventricular function, such patients subjectively did not correlate their self-assessment score with either of these potential biomarkers. Furthermore, hospitalisations were unaffected by these inflammatory surface markers in this stable cohort of patients. One

could argue that cardiopulmonary exercise testing is a more reliable and effective measurement of cardiovascular and physical health vs a more subjective assessment. Indeed, quality of life assessment includes not only physical but social and psychological aspects of patient well-being and as such evidence in this field is lacking with regards to monocytes and inflammation.

Following on from this the second hypothesis of my study was that Spironolactone would be an ideal therapeutic agent to provide anti fibrotic properties which would result in improved exercise capacity, quality of life and potentially reduce hospitalisations in patients with AF and preserved left ventricular function. As already demonstrated in animal models, aldosterone promotes cardiac fibrosis via myocardial inflammation, oxidative stress, and cardiomyocyte apoptosis and also direct stimulation of cardiac fibroblasts to express type I and III fibrillar collagen genes (378). Cardiac expression of mineralocorticoid receptors is increased in AF, thus augmenting the genomic effects of aldosterone (311). Aldosterone antagonists (i.e., spironolactone or eplerenone) ameliorate LV fibrosis (379).

In my study Spironolactone did not cause any statistically significant change in monocyte subsets, inflammatory surface markers or those of cardiac fibrosis over the 24 months study period when compared to the placebo arm of the trial. Despite previous evidence in cardiovascular disease (albeit lacking in patients with AF) that attenuation of monocyte subsets, namely Mon2, can be beneficial to patients. I found that Spironolactone did not impact on this patient population with regards to the primary and secondary outcomes (Peak VO2, 6MWT, quality of life and hospitalisations). Furthermore, diastolic dysfunction on echocardiography and integrated backscatter were unchanged in the Spironolactone group in comparison to placebo over the 24 months period. A mechanism for which Spironolactone is

able to provide an anti-fibrotic pathway enabling reduced biomarkers of cardiac fibrosis to translate into enhanced exercise performance and functional capacity was not apparent in this 2-year study.

Therefore, my data suggest an observational association between CD16 Mon3 and higher exercise capacity measured by cardiopulmonary exercise testing and 6MWT. Furthermore, CD42 MPA3 was associated with a reduced exercise capacity. There was no association between monocyte subsets and markers of inflammation with neither measure of quality of life nor hospitalisation rates.

6.3 **Limitations**

My study has a number of limitations which need to be considered when interpreting the results. Although the statistical tests resulted in significant p-values at conventional levels with regards to surface makers as predictors of exercise capacity, it should be noted that numerous monocyte markers were analysed during the study which raises the argument around multiple comparisons. Adjusting for multiple comparisons would effectively just increase all of the p-values (e.g., by multiplying by the number of tests, in the case of Bonferroni-correction). As such, whilst this is effective in reducing false-positive rate, it does this by inflating the false-negative rate, meaning I would likely miss a "true" effect. My results are "hypothesis generating" and as such increasing the likelihood of false-negatives and missing a potentially important effect would be detrimental to such research. As a result, adjustment for multiple comparisons was not made.

Although cardiopulmonary exercise testing is a well validated method of assessing cardiovascular health and has been established in multiple studies to predict cardiovascular and all cause death, its utility in patients with permanent AF and preserved left ventricular dysfunction is less well documented. Thus, despite there being a clear correlation between specific surface markers and outcome with regards to Peak VO2 and 6MWT the extrapolation with regards to long term outcomes requires more long term follow up in a potentially larger cohort of patients.

Furthermore, whilst baseline characteristics were similar between patient groups (Spironolactone vs Placebo) more patients were taking statin therapy in the Spironolactone group. Previous evidence has linked anti-inflammatory properties of statin use as such may impact on inflammatory markers in the form of monocyte subsets (380). Going forward, had there been a role of monocytes in impacting primary and secondary outcomes one would have to have taken this into account as a potentially limiting factor. Due to the double-blind randomised nature of the trial design this would not have impacted on the initial results from baseline monocyte data and peak VO2. Furthermore, residual confounding from co-morbidities may still exist between groups as although all efforts were made to document those deemed relevant to the trial outcomes.

Finally, despite best efforts to ascertain functional mechanisms of monocyte action in patients with AF and preserved left ventricular function, the markers of cardiac fibrosis did not correlate with those of the monocyte surface markers. As such it remains unclear whether these monocyte surface markers are simply a reflection of the underlying disease process in AF or whether they directly contribute to its pathophysiology.

6.4 **Implications for Future Research**

This thesis has allowed me to systematically answer many questions relating to the role of monocytes in AF and preserved left ventricular function, as well as generating many more which will provide future direction for research in this field. Despite advances in the therapy of patients with AF and preserved left ventricular function this cohort of patients suffer drastically with regards to functional status and quality of life vs their non-AF counterparts (381).

Identifying potential 'markers' of poor exercise capacity and functional status may allow clinicians to plan the management for such patients more effectively, either in terms of utilising more aggressive therapies or acknowledging likely limitations in patient's future prognosis. Therefore, further larger scale studies are required to evaluate the prognostic role of monocytes in AF.

My work has provided an observational association which I hope will lead to future work to find causality associations between monocyte subsets and measures of exercise capacity in the future. I hypothesised that CD16 Mon3 and CD42 MPA 3 would provide their effects via a pathway mediated by changes in cardiac fibrosis. I hypothesised that plasma biomarkers of collagen turnover that are easily accessible surrogate measures of systemic fibrotic processes would provide a mechanistic answer to the role of monocyte subsets in determining exercise capacity in patients with AF. However, serum levels of the pro-peptides, such as PINP and type III PIIINP did not impact on this study outcome, as was the case for Galectin-3. Further work to identify more novel markers of cardiac fibrosis is warranted and will likely provide a

promising bridge to the mechanism by which monocyte surface markers influence exercise capacity in patients with AF and preserved left ventricular function.

Although monocyte subset numbers and phenotype addressed in this thesis may be important, assessing their functionality is likely to be crucial in furthering our understanding of their role in AF. For example, it would be interesting to utilise novel approaches of non-invasive imaging to track monocyte subsets in vivo. Furthermore, cardiac MRI would be an excellent tool to evaluate fibrosis in this patient cohort and to analyse the benefits of any potential therapeutic agents with anti-fibrotic properties. The role of MRI in highlighting ventricular and atrial fibrosis in patients with AF is well evidenced (382). However, whether pre-existing AF begets fibrosis or vice versa is not yet clear. Correlation between monocytes and evidence of fibrosis using MRI would provide a foundation to explore further the translational aspect of such cardiac inflammation. Furthermore, efficacy of therapeutic intervention could be correlated with changes in fibrosis seen on MRI.

The number of potentially interesting and exciting studies leading on from this thesis is clear. The brief outline above my no means encompasses the entirety of this extremely invigorating translational field of cardiovascular science. Although work in this field is progressing, it is very much in its infancy with regards to the AF population.

6.5 **Overall Conclusion**

The work described in this thesis has significantly added to our understanding of the role monocyte subsets in patients with AF and preserved left ventricular function. There are significant differences in cell surface receptor expression which may be important in the pathophysiology of this complex disease. Spironolactone intervention did not appear to be the answer to how patients with AF and preserved left ventricular function can attenuate monocyte subset surface marker expression in order to improve exercise capacity. At present the mainstay of treatment for patients with AF should be that of rate control and stroke prevention as highlighted through the most recent European guidelines in this field (37). This work also highlights several deficiencies in our understanding which need to be explored in future research.

APPENDIX 1: STANDARD OPERATING PROCEDURE

Echocardiography for Measurement of Cardiac Fibrosis

SOP written by Farhan Shahid and Eduard Shantsila

N.B. Use of the Echocardiography equipment is forbidden without having been officially trained

Contents

1. Introduction

Quantification of the cardiac fibrosis will be done using echocardiography via calibrated integrated backscatter. Calibrated integrated backscatter (cIB) is an echocardiographic parameter based on two-dimensional scans, which measures myocardial ultrasound reflectivity (integrated backscatter [IB]) and can be used to quantify myocardial fibrosis. IB intensity varies from low reflectivity of the blood in cardiac chambers, to medium reflectivity of the myocardium and high reflectivity of the pericardium. The reflectivity of the soft tissues is proportional to their content of the connective tissues. Accordingly, the pericardium, which is largely composed of the connective tissue, is conventionally used as a reference tissue for assessment of LV fibrosis. cIB is calculated as the difference between pericardial and myocardial IB and can be presented as negative or positive value (in this study, we choose to present cIB as positive values). Lower cIB values indicate smaller differences in acoustic

density between the pericardium and the myocardium, higher content of connective tissue and more advanced fibrosis. The utility of cIB as a measure myocardial fibrosis has been validated against myocardial biopsies and strongly correlates with myocardial collagen density.

M-mode, 2D, Doppler and TDI transthoracic echocardiography will be performed using Phillips iE33 ultrasound system (Bothel, WA, USA). Modern off-line QLAB software [Xcelera, Phillip (iE33) Ultrasound Quantification Module, USA] is used for quantification of LV systolic and diastolic function.[38] E/e' (early mitral inflow velocity/TDI derived early septal mitral annular diastolic velocity) will be used to assess diastolic function in AF. This parameter was strongly correlated with LV diastolic filling pressure in AF ($r=0.79$, $p<0.001$) and it is relatively independent of left atrial pressure.[39] An average value from 10 consecutive cardiac cycles will be calculated. Echocardiography was undertaken in line with British Society of Echocardiography standards.

Measurement of integrated backscatter and calculation of calibrated IB (cIB) is performed using acoustic densitometry software (part of QLAB package) according to the protocol established in the department. For quantification of LV fibrosis, we will use two-dimensional images acquired from the parasternal long-axis view with frame rates between 80 and 120 frames/s. At least 10 cardiac cycles are stored in cine-loop format for offline analysis. For measurement of LV cIB fixed size (5x5mm) regions of interest are positioned at the mid-myocardium of the interventricular septum, of the posterior wall, and on the posterior pericardium at LV level with as a control sample. cIB is calculated as a difference between myocardial and pericardial IB values, with LV cIB calculated as an average or septal and posterior wall cIB values. Previous intraobserver coefficient of variability for LV cIB measurements in our department was 6.7%.

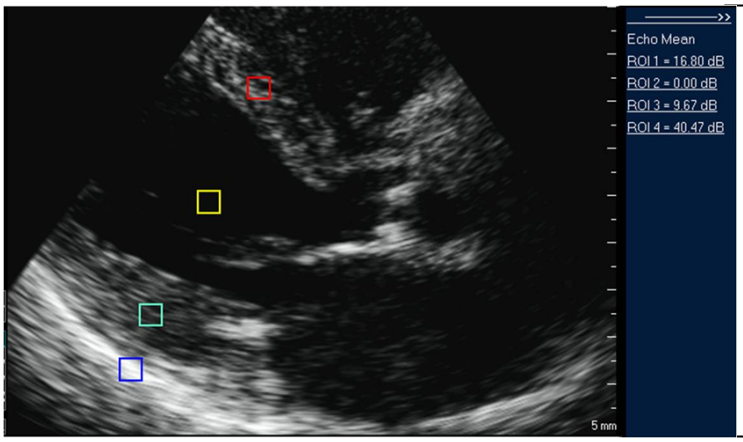


Figure 1. Echocardiographic assessment of cardiac fibrosis (integrated backscatter).
On the left, position of regions of interest [ROI] to measure integrated backscatters; ROI1, LV pericardium; ROI2, LV posterior wall; ROI3, LV lumen; ROI4, interventricular septum; On the right, correspondent values of integrated backscatters.

2. Equipment

The following equipment / supplies are required for Echocardiographic evaluation of cardiac fibrosis:

- Phillips iE33 ultrasound system (Bothel, WA, USA)
- Modern off-line QLAB software [Xcelera, Phillip (iE33) Ultrasound Quantification Module, USA]

3. Measuring cardiac fibrosis

- Images for analysis are saved and transferred to Xcelera.
- Identify patients for analysis and double click on name.
- Identify optimal parasternal long axis view for analysis and double click to view image.
- Click on double squared green Icon on bottom left of screen.
- Select ROI option from list at right hand side of screen. Then sub-select 5mm square icon.

- Drag square to area of intraventricular septum that is to be used for analysis. The software will then calculate the backscatter from area selected.
- Repeat this for a total of 3 sites on the intraventricular septum.
- Repeat the process for the posterior wall of the left ventricle followed by the pericardium
- Lastly click and drag one icon to the LV cavity for a baseline reference and record.
- A total of 10 measures should appear on the left-hand side of the screen

4. **Interpretation**

- An average of the 3 measures used for each the intraventricular septum, posterior wall and pericardium should be made and recorded
- This should then be compared to subsequent measurement to identify and change in cardiac fibrosis.

5. **Saving data**

- Data should be saved by clicking “save” and then the computer icon in the bottom right of the screen.
- It is recommended to name and save data to desktop and then storing inside a created file.

APPENDIX 2: STANDARD OPERATING PROCEDURE

Cardiopulmonary Exercise Testing (CPET)

SOP written by Farhan Shahid and Eduard Shantsila

N.B. Use of the CPET equipment is forbidden
without having been officially trained

Contents

Introduction

Exercise capacity is a strong predictor of cardiovascular events and overall mortality. Cardiopulmonary exercise testing (CPET), with the determination of peak VO_2 is the 'gold-standard' assessment of exercise capacity, providing in depth information on cardiorespiratory system during intense, yet controlled periods of metabolic stress. This makes CPET an ideal non-invasive tool to establish the functional status of the heart, which provides a wealth of clinically relevant diagnostic and prognostic information.

CPET testing with oximetry is performed to assess peak oxygen uptake (peak VO_2) and to define cardiovascular aetiology of the symptoms. Recently, CPET has been shown to be a highly accurate and reproducible measure of exercise tolerance in participants with preserved LV contractility. Exercise testing is performed with participants in the upright position on an electronically braked bicycle, with expired gas analysis under continuous electrocardiographic

and blood pressure monitoring. Participants are encouraged to exercise to exhaustion. Peak VO_2 values are averaged from the final 30 seconds of the exercise test. Additionally, ventilator anaerobic threshold is evaluated by standardized methods using ventilator equivalents.(338)

The Statement of the American Thoracic Society and American College of Chest Physicians recommends that an increased VE/MVV ratio (e.g., > 85%) occurring at a relatively low work rate (e.g., 50 W) strongly suggests that ventilator factors are contributing to exercise limitation (339).

Indications and Contraindications for CPET:

Evaluation of exercise tolerance and functional work capacity:

1. Determination of exercise-limiting factors and pathophysiologic mechanisms.

Evaluation of undiagnosed exercise intolerance:

1. Assessing the contribution of cardiac and pulmonary aetiology in coexisting disease
2. Symptoms disproportionate to resting pulmonary and cardiac tests
3. Unexplained dyspnea after initial other testing.

Evaluation of patients with respiratory and/or cardiovascular disease:

1. Functional evaluation and prognosis in patients with heart failure
2. Selection for cardiac transplantation.
3. Chronic obstructive pulmonary disease:

    - Establishing exercise limitation(s)
    - Determining the magnitude of hypoxaemia.

4. Interstitial lung diseases:
    - Detecting gas exchange abnormalities
    - Determining the magnitude of hypoxaemia
    - Determining potential exercise-limiting factors.

Pre-operative evaluation

Evaluation of response to treatment following surgery, rehabilitation, or pharmacological treatment Quantification of impairment for medico-legal purposes.

Contraindication:

Recent myocardial infarction (7 days) Unstable angina
Uncontrolled arrhythmias
Syncope

Active endocarditis
Acute myocarditis or pericarditis
Severe aortic stenosis
Uncontrolled heart failure
Acute pulmonary embolus or pulmonary infarct Thrombosis of the lower extremities
Suspected dissecting aneurysm
Uncontrolled asthma
Pulmonary oedema
Room air oxygen desaturation < 85%
Type I hypoxaemic respiratory failure
Severe mental impairment or any other inability to consent.

The CPET test is performed using the L COSMED CPET system based on cycle ergometer procedural instructions. Modern CPX systems contain rapidly responding O2 and CO2 sensors that allow for the calculation of oxygen uptake and carbon dioxide output at rest, during exercise, and during recovery, as frequently as breath by breath. Although manufacturers' recommendations vary considerably regarding calibration, the CPX systems

ware to be calibrated immediately before each exercise test. This includes calibration of airflow, volumes, and both the O2 and CO2 analysers.

Validation studies have been performed on many of the computerized systems. Because ambient conditions affect the concentration of O2 in the inspired air, temperature, barometric pressure, and humidity is taken into account. The CPX system automatically quantifies these conditions and make appropriate adjustments to calculate the inspired O2 concentration

Equipment

The following equipment / supplies are required for CPET: An integrated CPET module including the following:

- Cycle ergometer connected to 12-lead ECG monitoring system
- Pulse oximeter
- Flow sensor
- Oxygen and carbon dioxide gas analysers
- Software to operate the integrated components
- 3L calibration syringe (certified) for volume calibration
- Calibration gases for 2-point calibration of gas analysers
- Disposable nose clips, bacterial/viral filters and/or rubber flanged mouthpiece,
- Sphygmomanometer and stethoscope
- Resuscitation and other emergency equipment

N.B. Any equipment required should be requested through Sister Rebecca Brown or Manager Ronnie Haynes at the Institute of Cardiovascular Sciences.

Test Procedure

Patient preparation

- Suitable attire, including comfortable clothing and appropriate footwear should be worn.
- Normal medical regimes should be adhered to (i.e. use of prescription medication) unless instructed otherwise by the referring physician.
- Patients should abstain from smoking for at least 8 hours prior to testing
- The patient should not exercise heavily on the day of testing and be well rested before the test. Heavy meals are to be avoided, with a light meal eaten no less than 2 hours prior to the test

Start Up

1. Turn on CPET station and bicycle by turning on power from mains in wall behind CPET machine.
2. Turn on computer processor (DELL)
3. Double click on COSMED Omnia icon on windows screen
4. Password screen will appear (password: ascot (lowercase)

Calibration

5. Click calibration icon on screen followed by air calibration.
6. Detach cable from COSMED flowmeter and place in QUARK CPET port found just below power light of CPET machine.
7. Take the COSMED flowmeter and place it in holding tray where mask straps are found.

8. Click START on air calibration window and leave for approximately 5 minutes.
9. When air calibration is complete a data set will appear. NO RED areas should appear. If they do repeat calibration. Otherwise click ok and replace COSMED flowmeter in side holder.
10. Next click Flowmeters calibration and find flowmeter at bottom of CPET station. (It is a round cylinder)
11. Connect plastic tubing to funnel end.
12. Click START on flowmeter and then begin 6 cycles of steady strokes using the flowmeter handle.
13. Once complete programme will provide calibration information for this. No confirmation exit box exits so just back out of program once complete. *Pre-test Respiratory Function Testing*

Prior to the CPET each patient Is required to complete a flow volume loop:

Spirometry

14. Click Database option on screen
15. For new patient click on new subject icon on top left of screen and enter details
16. For existing patient find name in patient list and double click and then choose new test icon on left of screen
17. Once patient selected click new test and then Spirometry
18. Within this icon click FVC pre
19. Connect the Antibacterial Filter to the COSMED flowmeter

20. Instruct the patient that they will take a deep breath in on your command to fill their lungs. They are then to blow out hard and fast continuously for 6 seconds (one exhaled breath)
21. This will be repeated 3 times
22. Once patient understands click start on the top left of the screen and ask the patient to begin breathing normally in and out through the filter
23. Flow volume loops should appear on the screen. After 3-4 normal breaths ask the patients to breath in to fully expands their lungs and to blow out hard and fast for 6 seconds.
24. Click STOP on icon bar at end of test and repeat 3 times.
25. Click save and go back to spirometry menu window.
26. Now click MVV
27. Instruct the patient to breath rapidly and deeply in and out. This may cause light headedness so instruct the patient to sit down if they have the need to. This will be for 12 seconds and repeated on 2 occasions.
28. When patient is ready click START on the top left and end test after 12 seconds.

Save data and then exist

Performing CPET

The testing protocol should be briefly outlined to the patient (suggested below):

“This is an exercise test that requires a maximal effort. The more effort you put in, the more information we will obtain. We will be measuring your breathing through a mask. You will then go into the exercise task and be asked to pedal at approximately 60 rpm. The workload will increase with pedaling becoming more difficult until you won’t be able to continue”.

“We expect that the test will take 8-12 minutes. It is usually only hard during the last few minutes when we collect the most important data. During the test, we will be taking your blood pressure and monitoring your heart rhythm to make sure it is safe to keep exercising. When the test has finished, we want you to ride for another 2-4 minutes at a very low workload to help your body recover from the exercise”.

“If at any stage you feel any major discomfort such as strong chest pain, severe leg pain or nausea, let us know and we will stop the test. Otherwise, carry on pedaling until your legs or breathing prevents you from continuing.”

Exercise Bike

1. Next click METABOLIC ICON that is found next to spirometry
2. Within the next menu click Cardio pulmonary exercise testing
3. CPET- Breath by Breath should be clicked next.
4. Mover window to left screen, leaving right screen for ECG monitoring
5. At this point attach ECG to patient and click Stress ECG icon. The ECG should appear on screen.
6. Attach small/medium mask to patient (in most cases) and then fix COSMED flowmeter to mask.
7. Adjust height of bicycle seat to patient requirement
8. Once patient is on bicycle explain that they will pedal to maintain a speed of 60 RPH.
9. They should not talk throughout the test but we will use hand gestures to ensure all is well and for when the patient wishes to stop the test.
10. Once all is understood with patient press start on the top left of window and the 3-minute warm up phase will commence. Inform patient it will steadily get harder from

this point and they are strongly encouraged to try to reach their maximal effort during the test

11. Once the patient is nearing the end be ready to click the rest icon which will continue to monitor the patients but remove the resistance.
12. Once patient has completed CPET continue monitoring for approximately 3 minutes to the point where the patient HR is near baselines and ensure no abnormalities.
13. Detach all ECG electrodes and ask patient to rest.
14. Click on interpretation icon on screen and complete short report.
15. Save report by going to file-print-primo-pdf-then name report and save to IMPRESS AF reports.

Interpretation

- Key measures and variables must be identified (e.g. VO_2, HR, VE).
- It is also recommended that peak data for VO_2, HR, ventilation, and oxygen saturation, as well as peak work rate be presented in summary form report along with predicted values.

APPENDIX 3: STANDARD OPERATING PROCEDURE 201

MONOCYTE SUBSETS

Monocyte Platelet Aggregates by Flow Cytometry
SOP written by Eduard Shantsila and Andrew Blann

N.B. Use of the flow cytometry is forbidden
Without having been officially trained

Required pre-training

1. SOPs on venepuncture and on good clinical practice
2. SOP 195 – General operation of the flow cytometer

Contents

1. Introduction

Monocytes are large mononuclear cells (MNCs) derived from the bone marrow but on transit to the tissues where they seem likely to become semi-resident macrophages. Traditionally, they have been defined by glass-slide morphology, size, and scatter, but we now have the ability to define monocytes by cell surface molecules, using the FACS. For example, CD14 is a receptor for LPS present on monocytes, macrophages and neutrophils. CD16 is an antigen found on the Fc receptors and is present on natural killer cells, neutrophil polymorphonuclear leukocytes,

monocytes and macrophages. So, leukocytes populations can be further classified by the density of the expression of these markers, for example….

- M1 = CD14 strong CD16 negative
- M2 = CD14 strong CD16 strong
- M3 = CD14 weak CD16 strong

A further characteristic of monocytes in chemotaxis, such as to the chemokine monocyte chemoattractant protein-1 (MCP-1), a cytokine involved in monocyte infiltration in inflammatory diseases such as rheumatoid arthritis as well as in the inflammatory response against tumors. CCR2, short for chemokine (C-C motif) receptor 2, is a chemokine receptor for MCP-1 CCR2 has also recently been designated CD192.

Platelets are anucleate fragments of the cytoplasm of the megakaryocyte. They form thrombi when self-aggregating but more so in the presence of fibrin. However, platelets may also bind to monocytes. Cell surface markers of platelets include CD42a, also known as GpIX. It follows that dual labelling of blood with a monocyte marker (CD14/CD16/CCR2) and a platelet marker (CD42a) will identify monocyte-platelet aggregates (MPAs).

2. Materials and Supplier contact details:

Micro-reagents are kept in the fridge behind the door or on nearby shelves. Bulk fluids in boxes on other shelves and beneath the benches.

1) BD “FACS Flow” Running solution [Becton Dickinson (BD), Catalogue No. 342003]

10L containers.

2) 3 ml BD Falcon tubes [BD Catalogue No. 352054]

3) BD “FACS Clean” Cleaning Solution [BD Catalogue No. 340345]

4) BD Lysing solution [BD Catalogue No. 349202]

5) Sterile Phosphate Buffered Saline solution, 0.5L bottles [Invitrogen Ltd, Catalogue No 20012-068]

6) CD14 -PE conjugated monoclonal antibody - 100 tests [BD Catalogue No. 555398]

7) CD16 – Alex-flour 488 conjugated monoclonal antibody - 100 tests [ABD Serotec, Cambridge]

8) CD42a-PerCP conjugated monoclonal antibody [BD Catalogue No. 340537]

9) CCR2-APC conjugated monoclonal antibody [R&D Systems Europe Ltd, Cat No. FAB151A]

[n.b. this combination of antibodies constitutes a **Mastermix**: See ADB, ES]

10) Clear pipette tips [Alpha Laboratories Limited Catalogue No FR1250 1250ul Fastrak Refill NS]

11) Yellow pipette tips [[Alpha Laboratories Limited Catalogue No FR1200 200ul Fastrak Refill NS]

12) Count beads [BD (Trucount tubes)]. This is a crucial aspect as it will give us the number of monocytes/ml of venous blood. The product tube has a statement of the number of beads in each tube and so from this you can work out beads/mL.

Remember to dispose of all material thoughtfully.

3. Detailed method

3.1 General Preparation

3.1.1 Lysing solution.

Make from 50ml concentrate 10x FACS Lysing Solution (kept at room temperature). Dilute with 450ml distilled water in ½ litre bottle. This solution should not be used if it is older than a month (kept at room temperature).

3.2 Blood sample preparation

1. Add 12.5µL of Mastermix Absolute Monocyte Count (which includes CD14 2.5 µL, CD16 2.5µL, CD42a 5µL and CCR2 2.5 µL fluorochrome labelled antibodies) with

an electronic micropipette. Just place into the tube below a metal grid without touching the pellet.

2. Gently vortex the EDTA blood sample. Take 0.05 mL (=50 µL) of whole blood with electronic pipette and add to a Trucount tube.

3. Do not touch the pellet (this is critical!). Mix the tube gently with the vortex (3 sec). Incubate for 15 minutes in the dark, room temperature, shaking with horizontal shaker (set at 500 units). Add 0.45 ml (=450 µL) pre-diluted BD FACS Lyse solution (see 3.1.1) with a clear tip using the 1ml pipette. Incubate for 15 minutes on shaker as above.

4. Add 1.5 ml of PBS solution without touching the sample, followed by gentle vortex to ensure thoroughly mixed

3.3 Start up procedure

Part 1 – restoring reagents and preparation

1. Switch on Flow Cytometer by pressing the green switch on the right-hand side. The Apple Macintosh computer must also be switched on, but only 15 secs **after** the Flow Cytometer, or the link will not be recognized. Open the reagent panel on the left-hand side (LHS) by pulling the lid towards you. On the left is the sheath fluid reservoir, in the middle are switches and tubes, and on the right is the waste reservoir.

2. Carefully unscrew the top of the sheath fluid reservoir and fill with sheath fluid (in large box on shelf at head height – use plastic tube) to the level indicated on the top right-hand corner of the reservoir (little plastic bar).

3. Carefully disconnect/unscrew the waste container and empty contents down sink with plenty of water. Add approximately 40ml concentrated household bleach along with 360 ml of distilled water and reconnect container (plastic tubes available).

4. Pressurise the unit (takes about 20-30 sec) by moving the black toggle switch "Vent Valve" switch to the down/front position. It is located at the rear of the middle section between the sheath fluid tank and the waste container.

5. Air must be excluded from the tubing system by flushing it out. Any excess air trapped in the sheath filter can, if necessary, be cleared by venting through the bleed tube (the dead-ended rubber tube with a cap).

6. Close the drawer

Part 2 - Cleaning the machine

7. Ensure that a 3 ml Falcon tube (labelled 1) approximately 1/3 full of distilled water is positioned over the sample injection port (SIP) – a needle sheath - and that the swing arm is positioned under this tube. Press the prime button on the panel. When the system

enters “standby” with in 30 seconds then press the “prime” button again. When the standby and low buttons come on again then remove tube 1. We will re-use tube 1 in the shutdown procedure.

8. Prepare a second falcon tube (labelled 2) with FACS-clean (should contain about 2750 microlitres so that when inserted on to the sip it doesn’t touch the **O** ring). This is a smaller box on a shelf at above head height and above the bigger box of sheath flow fluid

9. Present tube 2 to the SIP and place support arm underneath it. Press the buttons “run” and “high” on the panel at the same time and run the FACS-clean in falcon tube 2 with supporting arm to left or right open for one minute. Then return the supporting arm to underneath the tube and “run-high” five minutes. This process ensures the machine is clean prior to running samples and helps minimise blockages.

10. Return to falcon tube 1 with distilled water. Repeat the above step 9 with this distilled water tube.

11. Press the ‘**STANDBY**’ and ‘**LOW**’ button on the system.

12. The machine is now ready to run samples.

3.4 Running blood samples.

Note. This must be learned from an experienced operator and you must seek scientific staff support to clear queries as the intricacies of the Cell Quest software are complex.

1. Open Cell Quest Pro software
2. Click 'File' – 'Open'
3. Click on the 'Monocyte Protocols' folder within 'Data 1' folder.
4. Click on the 'Monocyte Absolute Count'. This will open study protocol.

5. Click '**Connect to Cytometer**', located under the 'Acquire' menu.

6. Under the '**Cytometer**' menu, click '**Instrument Settings**'. The window appears displaying the compensations and threshold. Change settings by clicking on the **open** icon on the window which displays the folders select 'Monocyte Protocols' folder with in the 'Data 1' folder and click on the 'Monocyte Absolute Count' instrument settings in this folder. This will update the system settings to the preferred settings for the acquisition. Click '**Set'** on the window and by clicking '**Done'** the windows disappears. Make sure to click '**Set**' prior to clicking '**Done**'.

7. Click the '**Acquire'** menu once more and click '**Show browser**'.

8. Click directory- '**Change'** in order to specify the location folder.

9. Initial user must create new folder by clicking on '**New folder'** and by entering the title of the folder and choose that folder.

10. Change the custom suffix to the preferred title and number for data and click '**OK'**.

11. Untick the setup box (by clicking on it) in the browser Acquisition window. Now insert your sample and press "RUN" and "HIGH".

12. Open swing arm at bottom right of the cytometer and replace the Falcon tube with the sample to be run. Replace the swing arm under the Falcon tube.
13. Press the buttons '**Run**' and '**High**' on the control panel of the cytometer.

14. Click '**Acquire'** on the browser menu. The sample will now run for ~ 12 mins. Cell events will be displayed on the screen throughout the process (n.b. the higher the cell density, the more rapidly the cells will be acquired).

15. Click on '**Counters'** under the '**Acquire'** and observe the events per second which varies from 1000 to 8000 depending on various factors. The objective is to acquire 10,000 count beads for analysis.

16. Observe the acquisition closely since the system may get blocked (which happens very rarely) and the plots may not show any progress and the counters may not show any events per second.

17. Click pause on the acquisition window and replace the sample from the SIP with sterile PBS and run for 20/30 seconds minutes (clicking acquire wouldn't change the results) and then continue acquisition with your sample on the SIP. If the problem still persists please inform the senior scientific staff and seek assistance.

18. After attaining the target events the analysis stops and the file number changes automatically. Click on '**print'** under the '**files'**. Confirmation window appears again click on print.

19. Vortex your next sample gently. Re-programme the software with a new sample number and repeat the step 11.

20. If the cytometer is not ready message appears open the drawer and check the fluids level which may need refilling or emptying. The system may run out of Sheath fluid if there are more samples.

21. Be absolutely sure you have downloaded your results on to paper. Keep this paper safe. Do not assume the computer will keep the results safe, even if you have directed it to do so. Obtain all the raw data (cell numbers) and apply them into the specific spreadsheet you have designed for your project. The same spreadsheet should have the WBC and platelet count results from the Advia

3.5 Shut-down procedure

1. In this section we re-use tubes 1 and 2 with distilled water and FACS clean respectively. Install FACS Clean tube 2 over the SIP needle. Press button '**High'** and '**Run'** on the panel. Leave the support arm out at 90 degrees for approximately 1 minute. This cleans the outer portion of the aspiration sheath. The fluid will be rapidly aspirated, so ensure that the tube doesn't empty completely.

2. Now replace the side arm under the Falcon tube and allow it to run for approximately 5 minutes. This cleans the inner portion of the aspiration sheath and the FACS machine itself.

3. Repeat steps 2 and 3 with the distilled water tube 1. Once step 3 is complete, Leave the sheath in falcon tube 1 containing distilled water and press '**STANDBY'.**

4. Open the reservoir draw and depressurize the machine by moving the "Vent Valve" toggle switch to the up/rear position. The machine will hiss as it depressurizes.

5. Leave the machine on for a further 5 minutes to allow the laser lamp to cool. Turning the machine off prematurely will result in the lamp cracking.

6. Finally power down the FACScalibur (green button) and Apple Mac, and then clean up!

7. Note: * IF THE SYSTEM IS TO BE USED AGAIN ON THE SAME DAY LEAVE THE SYSTEM ON STANDBY and then DEPRESSURISE THE SYSTEM.

4. Interpretation of plots

For the first couple of analyses you will need to have all this explained to you by Dr Blann or Dr Shantsila. These numbers refer to the illustrative plot and nine individual plots…

TOP THREE PLOTS

1. The top left initial plots show the FSC/SSC plot (forward and side scatter, all in green). This is needed to gate the presumed monocytes. Be generous at this stage, include all monocytes. Contamination by granulocytes and lymphocytes will be removed during the next stage.

2. Immediately to the right (i.e. centre) is a plot of the cells stained with CD14 (light blue) which further gates the monocytes to separate them from granulocytes. Note a large residual proportion of granulocytes at the top of the SSC index.

3. Top right is plot of CD14/CD16 events (red/brown). Four gates have been drawn to define different populations of monocytes. M1 defines CD14strong/CD16-ve, whilst M4 defines cells expressing a lot of CD16.

CENTRE THREE PLOTS

4. Centre left is a plot of the Count beads (green), which are sampled at a concentration of, for example, 50,000 beads/tube. From this you will get monocytes/mL and thus MPAs/mL. The CD14-PE horizontal axis is irrelevant.

5. Centre middle is (green) plot of CD16 versus CD14, which allows you to gate and exclude lymphocytes from analysis. Note that pattern is a bit like the upper right box, but with CD14-ve/CD16-ve events present.

6. Centre right is a plot derived from Gate 4. It shows events (cells) that express high and low levels of CCR2 according to side scatter. There is a gating line down the middle of this plot to give cells staining high and low staining for CCR2. Gate 5 is cells staining weakly for CCR2 (=M3) whilst Gate 6 is cells staining strongly for CCR2 (=M2).

LOWER THREE PLOTS (all CD42a versus CCR2)

7. Lower left is a plot of CD42a versus CCR2 on population M1. MPAs are to the right of the line

8. Lower middle is a plot of CD42a versus CCR2 in M2. MPAs are to the right of the line

9. Lower right is a plot of CD42a versus CCR2 in M3. MPAs are to the right of the line

APPENDIX 4: STANDARD OPERATING PROCEDURE

PIIINP&PINP ELISA Protocol

1. Leave ELISA kit out and samples for 1 hour.

2. Prepare standard working solution: Centrifuge reference standard at 10,000g for 1 minute. Then add 1.0mL of reference standard & sample diluent. Leave for 10 minutes and invert several times. Then mix thoroughly with pipette. Take 8 tubes and add 500mL of reference standard & sample diluent to each tube. Then add 500mL from reference standard to 1st tube and mix and then so on to produce dilution series.

3. Add the standard working solution dilution series to columns 1 & 12. Add 100µL to remaining 80 wells. Cover plate with sealer and incubate at 37^{o}C for 90 mins

4. Prepare the Biotynlated Detection Ab working solution by adding 10.89mL of Biotinylated Ab Diluent to 110µL Biotinylated Detection Ab. Total volume 11mL. Centrifuge stock before use.

5. Remove liquid from each well but **DO NOT WASH.** Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Gently mix. Cover with plate sealer and incubate at 37^{o}C for 60 mins.

6. Prepare wash buffer by adding 30mL of Concentrated wash buffer to 720mL of distilled water to total volume of 750mL.

7. Prepare concentrated HRP Conjugate working solution by adding 10.89mL of Concentrated HRP Conjugate Diluent to 110µL Concentrated HRP Conjugate. Total volume 11mL. Centrifuge stock before use.

8. Aspirate each well and add 300mL wash buffer to each well. Soak for 1 minute and decant. Repeat for total of 4 washes.

9. Add 100µL of HRP Conjugate working solution to each well. Gently mix. Cover with plate sealer and incubate at 37ºC for 30 mins.

10. Aspirate each well and repeat wash process as per step 8 but for total of 5 washes.

11. Add 90µL substrate reagent to each well and cover with plate sealer. Incubate for 15 minutes at 37ºC (max 30 mins).

12. Add 50µL of stop solution to each well.

13. Place in microplate reader

APPENDIX 5: STANDARD OPERATING PROCEDURE

Galectin 3 ELISA Protocol (Dilution 1:2)

- Remove samples from freezer and ELISA kit and leave for half hour to reach room temperature
- Prepare samples worksheet (8x12) with patient ID
- Prepare Galectin 3 standard by adding 1 ml of Deionised water to vial and leave for 15 minutes.
- Prepare wash buffer by adding 20mls of wash buffer concentrate to total 500mls with deionised water.
- Prepare calibrator diluent RD6X (1:5) by adding 1ml of RD6X to 4ml deionised water (likely do to 4ml RD6X to 16ml deionised water as solution needed to dilute plasma samples)

Preparation of Standards (S1-S8)

- Add 900µL of 1:5 diluent to S1 along with 100µL of Human Galectin 3 standard
- Add 500µL of 1:5 diluent to S2-8
- Then pipette 500 µL of S1 to S2 and so on leaving S8 with only 1:5 diluent no transfer from S7.

Preparation of Plasma Samples

- Dilute samples prior to adding to assay plate by adding 50µL of sample to 50µL of RD6X (1:5). This forms a 1:2 dilution (confirmed with R&D)

Assay Procedure

- Add 100µL of Assay diluent RD1W to each well
- Add 50µL of standard to columns 1&12.
- Add 50µL of diluted sample to wells 2-11. (Remember the total volume in there is 100µL)
- Cover with adhesive strip and incubate for 2 hours at room temperature.
- Aspirate each well by firstly removing contents of columns 1-12 (changing pipette each time)
- Then add was buffer solution to each column and then empty into sink. Repeat process 4x and then blot to dry.
- Add 200µL of Human Galectin 3 conjugate to each well.
- Cover with adhesive strip and incubate for 2 hours.
- Repeat wash process
- Add 200µL of substrate solution (in the dark) and incubate for 30 minutes
- Turn on microplate reader and set up ready for analysis at 450 wavelengths.
- After 30 minutes add stop solution and ensure blue to yellow colour change.

Add to microplate reader

APPENDIX 6: Publications arising from thesis

Literature review

- The role of monocytes in heart failure and atrial fibrillation. Shahid F, Lip GY, Shantsila E. JAHA. 6 Feb. Volume 7. Issue 3. 2018.

Published abstracts

- Total monocyte count as a predictor of exercise capacity in patients with permanent atrial fibrillation and preserved left ventricular function. The West Birmingham AF Project. A Khan, F Shahid, G Y H Lip, E Shantsila. BCS 2018. Heart Jun 2018, 104 (Suppl 6) A79; DOI: 10.1136/heartjnl-2018-BCS.96 Abstract.

- Predictors of cardiac fibrosis in patients with permanent atrial fibrillation and preserved left ventricular function A Khan, F Shahid, G Y H Lip, E Shantsila. ESC congress. August 2018. Abstract.

- CHA2DS2VASc score predicts exercise capacity in patients with permanent atrial fibrillation and preserved left ventricular function. F Shahid, A Khan, G Y H Lip, E Shantsila. ESC congress August 2018. Abstract.

- High heart rate predicts cardiac fibrosis in patients with atrial fibrillation and preserved left ventricular function. Shahid F, Lip GYH, Shantsila E. Heart BMJ. 2018. Heart Jun 2018, 104 (Suppl 6) A76-A77; DOI: 10.1136/heartjnl-2018-BCS.9. Abstract.

Original manuscripts have been submitted to peer review journals for consideration of publication at the time of thesis submission.

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