Monocyte subpopulations in patients following ST-elevation myocardial infarction: implications for post-infarction left ventricular remodelling and clinical outcomes

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Summary of Thesis

Despite improvements in interventional and pharmacological therapy of atherosclerotic disease, it is still the leading cause of death in the developed world. Hence there is a need for further development of effective therapeutic approaches. This requires better understanding of the molecular mechanisms and pathophysiology of the disease. Atherosclerosis has long been identified as having an inflammatory component contributing to its pathogenesis, whilst the available therapy primarily targets hyperlipidaemia and prevention of thrombosis. Acknowledging a pleotropic anti-inflammatory effect to some therapies, such as acetyl salicylic acid and the statins, none of the currently approved medicines for management of either stable or complicated atherosclerosis has inflammation as a primary target. Monocytes, as representatives of the innate immune system, play a major role in the initiation, propagation and progression of atherosclerosis from a stable to an unstable state. Animal study data support a role of monocytes in acute coronary syndromes and in outcome post infarction; however, limited research has been done in humans.

In this thesis I describe for the first time in a large cohort of ST elevation myocardial infarction (STEMI) patients followed up for three years that total monocyte count, monocyte subset 1 (Mon 1), and monocyte subset 2 (Mon 2) are predictive of major adverse cardiac events (MACE) post STEMI (including death, new diagnosis of heart failure, recurrent acute coronary syndrome). Both the inflammatory function of monocyte subsets (via assessment and quantification of IKKβ as a surrogate for the NFκB inflammatory pathway activation) as well as the phagocytic activity of monocytes were studied in order to describe the mechanism through which monocytes affect their action. There was no significant difference in the NFκB pathway activity between those patients who developed an adverse event and those who did not. Also NFκB activity was not predictive of MACE. However the phagocytic activity of Mon 1 and Mon 2 were predictive of MACE suggesting that phagocytic activity of monocytes is the mechanism through which monocytes implement their action. Also this supports that the newly

described monocytes subset 2 (Mon 2) is predominantly an inflammatory monocyte subset, not reparative as Mon 3.

Major adverse cardiac events were driven mainly by heart failure diagnosis and echocardiographic findings. Hence the association between ventricular remodelling and phenotypic and functional characterisation of monocytes subsets was studied in this thesis. Total monocyte count, Mon 1 and Mon 2 were again predictive of negative ventricular remodelling with increase in end systolic indexed volume of >15% at 6 months follow up echocardiogram post infarction. Subclinical parameters of systolic dysfunction, namely global longitudinal strain and global circumferential strain were also significantly correlated with total monocyte count as well as Mon 1 levels.

Given the above, I studied the effect of incorporating total monocyte count in Thrombolysis in Myocardial Infarction (TIMI) STEMI score to predict patient outcome at 30 days post infraction. C- statistics indicated improved prognostication of the TIMI STEMI model after incorporation of the total monocyte count into the model with improved area under the curve from 0.67 (for TIMI STEMI score), to area under the curve of 0.77 (TIMIMon score). This allows individual tailoring of secondary preventative therapy in order to improve patient outcome post infarction. Having described a potential mechanism through which the innate immune system affects outcome in STEMI patients, namely Mon1 and Mon2 through their phagocytic activity, the results from this thesis could be a stepping stone into targeted anti-inflammatory therapy in management of myocardial infarction.

Dedication

To my wonderfully amasing mother and father for believing in me, for all their

support endless love and for always being there

To Anba Misael Omena Botamina Abona Nofeer

Abbreviations

ABC - pathway: ATP-binding cassette pathway

ACE - Angiotensin Converting Enzyme

ACS - Acute coronary syndrome

AHA/CDC - American Heart Association/ Centre for Disease control and prevention

BD – Becton Dickinson, Oxford, United Kingdom

CAD - Coronary artery disease

CRP – C-reactive protein

CRT – cardiac resynchronisation therapy

CV – coefficient of variability

DC - Dendritic cells

ECM - Extra cellular membrane

EDTA – ethylene-diamine tetra-acetic acid

EDV - end diastolic volume

EDVI- end diastolic volume indexed

EE' - early mitral inflow Doppler inflow

EF – ejection fraction

ELISA - enzyme linked immunosorbent assay

EPC – endothelial progenitor cells

ESVI- end systolic volume indexed

FACS - fluorescence activated cell sorting

FGF – fibroblast growth factor

FMD – flow-mediated dilation

GM-CSF – granulocyte-macrophage colony-stimulating factor

GP – glycoprotein

GTN – glycerol trinitrate

HRP - horseradish peroxidase

HSP - Heat shock protein

ICAM-1 - Intercellular adhesion molecule-1

IDCM – diopathic dilated cardiomyopathy

IGF-1- Insulin like growth factor-1

IgM - Immunoglobulin M.

IKK – inhibitory κB kinases

IL- Interleukin

IVRT- isovolumic relaxation time

LA- left atrium

LPS – lipopolysaccharide

LV – left ventricular

LVEDVi – left ventricular end diastolic volume indexed to body surface area

LVESVi - left ventricular end systolic volume indexed to body surface area

LVSD – left ventricular systolic dysfunction

MACE – major adverse cardiac events

MCP – monocyte chemo-attractant protein

M-CSF – macrophage colony-stimulating factor

MFI – median fluorescent intensity

MI – myocardial infarction

MMP – monocyte-derived microparticles

MNCs – mononuclear cells

Mon 1 – CD14++CD16–(CCR2+) monocytes

Mon 2 – CD14++CD16+(CCR2+) monocytes

Mon 3 – CD14+CD16+(CCR2–) monocytes

MPA – monocyte platelet aggregates

MRI: Magnetic resonance imaging

MV- mitral valve

NADPH – nicotinamide adenine dinucleotide phosphate

NFκB – nuclear factor κB

NO – nitric oxide

NOS – nitric oxide synthase

NYHA – New York Heart Association

PAI-1 – plasminogen activator inhibitor type 1

PBS - phosphate buffered saline

PCI – percutaneous coronary intervention

PWV – pulse wave velocity

ROS – reactive oxygen species

STEMI - ST elevation myocardial infarction

Tei index- myocardial performance index

TIMI- thrombolysis in myocardial infarction

TF – tissue factor

TLR – Toll-like receptor

TNF – tumour necrosis factor

VCAM – vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

WCC- white cell count

Acknowledgment

I would like to extend my deep thanks to Prof Lip and Dr Shantsila for supervising my work during this thesis and for their invaluable input throughout, without their support this project would have never seen the day of completion. Their guidance and direction led this project to completion and for that I am eternally grateful.

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As for me friends in Aston University and lab 358, especially Iru, Rita and Edyta, I can not thank them enough for making me feel so welcome and to decipher the realms of basic science techniques for me. They made me feel at home and kept up with my ignorance with extreme tolerance and admirable encouragement.

Declaration

All work presented in this thesis was carried out by myself, with the following exceptions. The idea and initial outline of the study was originally devised by Dr Shantsila, Prof Lip and Prof Griffiths with further development by myself. Application for approval by the research ethics committee was sought by Dr Shantsila and myself.

All recruited patients samples were collected and analysed solely and entirely by myself, this also included acquisition and analysis of echocardiograms of all recruited patients.

Assays for TNF-α, and MMP- 9 were also carried out by myself. Cell sorting experiments and cross over studies were also carried out by me.

Histogram in section 2.3.3.2 included in this thesis was created by Dr Shantsila and published elsewhere. He holds the intellectual authorship behind this histogram. I am grateful for his permission to include it for illustration in my thesis.

General Aims and hypothesis:

Three monocyte subsets have been recently characterised according to surface markers: CD14++CD16-CCR2+ (Mon1), CD14+CD16+CCR2+ (Mon2) and CD14+CD16++CCR2- (Mon3) subsets. Little is known of the function of these subsets in humans and especially in STEMI patients. Hence, this study aims to:

(i)   Characterise functional activity of different monocyte subsets (via NFκB activation and, phagocytosis) in patients with STEMI;

(ii)   Assess the role of monocyte parameters on cardiac function and remodelling at 6 months post infarction.

(iii)   Assess numbers and functional activity of different monocyte subsets on clinical outcome post myocardial recovery and assess their prognostic value.

In chapter one the role of monocytes in atherosclerotic disease and myocardial infarction is summarized. In chapter two the phenotype of monocyte subsets in recruited STEMI patients are summarized. The hypothesis is that total monocyte count and monocyte subsets are significantly increased in the 24 hours post infarction compared to healthy controls and patients with stable coronary artery disease. This is investigated in a cross sectional study. In a longitudinal study I hypothesise that the level of monocyte subset counts, after increasing acutely in the first 24 hours post STEMI, reduces after 14 days.

In chapter three, the methodology of measuring phagocytic activity of monocyte subsets is validated and the phagocytic function of the different subsets is described in a cross sectional study in STEMI patients, healthy controls and stable coronary artery disease subjects. The hypothesis is that the phagocytic activity will be significantly higher in the STEMI patients

compared to other health states and that phagocytic levels drop at follow up 14 days after infarction.

Chapter four describes the inflammatory effect of monocyte subsets, studying specifically NFκB pathway as well as the association with TNF-α and MMP- 9. I hypothesise that acutely, the inflammatory pathways are increased in STEMI patients and with inflammatory activity reduced at 14 days post infarction.

The effect of monocyte subsets, both in terms of counts as well as functional capacity, on ventricular remodelling is studied in chapter 5. Here we hypothesise, that total monocyte counts are predictive of negative remodelling. Functionally, NFκB levels and phagocytic activity are investigated as potential independent predictors of negative remodelling.

In chapter six, the effect of monocyte subsets and their functional parameters on predicting adverse cardiac events post myocardial infarction and after at least 2 years follow up is described. It is hypothesised that total monocyte count, and Mon 1 have a negative effect on patient outcome with a possible protective role for Mon 2.

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Preface

As this was a translational medicine study, the PhD was partly spent recruiting, analysing patient's blood samples and performing echocardiograms, and partly in the laboratory environment validating the basic science concepts and molecular theories behind the techniques used.

This is reflected in the writing of this thesis. Each chapter contains description of the molecular background driving the experiments with detailed account of the methodology used. The results section includes both laboratory based validation experiments followed by results in the recruited patient population with an aim to translate the molecular theories into patients' profiles. In order to avoid repetition, each chapter includes an aspect of the overall proposed hypothesis, e.g.: chapter 2 includes a description of monocyte subsets phenotype in STEMI patients with chapters 3 and 4 investigating monocyte subsets functional parameters, namely phagocytosis and inflammatory pathways (NFκB) respectively, in STEMI patients. Clinically, the implication of monocyte subsets and their function on cardiac performance and ventricular remodelling are discussed in chapter 5. Chapter 6 investigates the long term effect of monocyte subset in determining patient outcomes and prognosis.

Chapter 1

Introduction

1 Introduction

Atherosclerosis accounts for one fifth of all deaths in the world (Logue et al., 2011). It is the leading cause of death in the UK with more than 600,000 deaths annually due to its complications (Logue et al., 2011). Acute coronary syndrome (ACS) is an acute pathology associated with atherosclerotic plaque rupture and interruption of coronary blood supply to myocardial tissue. ACS carries a high mortality rate both *en route* to hospital and after receiving treatment. ACS is also associated with a high morbidity, especially when heart failure develops due to an extensive myocardial damage. Hence ACS forms an important field for research with an obvious need for improvement of current medical management, and introduction of new therapeutic targets. Better understanding of the underlying pathophysiological mechanisms leading to plaque development and rupture is essential to meet this need.

1.1 Definitions of Acute coronary syndromes

When myocardial blood flow is acutely impaired (ischaemia), and often not provoked by exertion, a person will commonly suffer prolonged pain; this is referred to as acute coronary syndrome (ACS). The underlying common pathophysiology of ACS involves the erosion or sudden rupture of an atherosclerotic plaque within the wall of a coronary artery. Exposure of the circulating blood to the cholesterol-rich material within the plaque stimulates blood clotting (thrombosis), which obstructs blood flow within the affected coronary artery. This coronary obstruction may be of short duration, and may not result in myocardial cell damage, in which case the clinical syndrome is termed unstable angina. Unstable angina may result in reversible changes on the electrocardiogram (ECG) but does not cause a rise in troponin, a protein released by infarcting myocardial cells. Ischaemia which causes myocardial necrosis (infarction) will result in elevated troponin. When the ischaemia-causing infarction is either short-lived or affects only a small territory of myocardium the ECG will often

show either no abnormality or subtle changes. This syndrome is termed non-ST-segment elevation myocardial infarction (NSTEMI) (Alpert et al., 2000).

STEMI is caused by complete and persistent occlusion of a coronary artery due to plaque rupture, with propagation of thrombus material downstream eventually completely occluding flow. As soon as the coronary blood supply is interrupted, myocardial damage ensues. The duration of deprivation of blood supply has great implications on recovery of heart muscle afterwards, hence in the modern era of cardiovascular medicine, percutaneous coronary intervention is the golden standard for restoring blood flow (re-vascularisation) of the infarcted myocardium.

For the purposes of this study, STEMI is diagnosed on the presence of typical chest pain symptoms (which may or may not be accompanied by haemodynamic compromise), as well as the typical ST elevation ECG changes. As prolonged ischaemia leads to death of cardiomyocyte, current guidelines thus recommend percutaneous revascularisation as the recommended treatment acutely for STEMI patients. This is in conjunction with a number of antiplatelet therapies as well as secondary prevention medications including, angiotensin receptor inhibits (to improve remodelling), and statin therapy for cholesterol management.

1.2 Inflammation in the atherosclerotic heart disease

Inflammation clearly contributes to the pathophysiology of atherosclerosis. Infectious agents, such as *Chlamydia pneumoniae,* have been detected in coronary atherosclerotic lesions (Kuo et al., 1993). Human atherosclerotic plaques contain viruses as well as numerous bacterial signatures, including nucleic acids, and peptidoglycan (Laman et al., 2002). In addition several studies suggest positive associations between oral bacterial colonization-levels and increased risk of atherosclerosis (e.g., coronary artery disease [CAD]) and cerebrovascular events (Zeituni et al., 2010, Ott et al., 2006), supporting the link between atherosclerosis and an inflammatory/infective pathophysiology (Ott et al., 2006, Wick et al., 2004, Epstein et al., 2000). The low grade inflammation associated with CAD

is acknowledged in day to day clinical practice, with a AHA/CDC consensus report (Pearson et al., 2003) recommending measurement of C-reactive protein (CRP) in asymptomatic subjects at intermediate risk for future coronary events (10-year risk of 10–20%) and in selected patients after an ACS (Pearson et al., 2003). In fact, the JUPITER trial indicated that statin therapy may reduce numbers of adverse cardiovascular events in subjects with normal LDL range, but high CRP concentrations (Ridker et al., 2001). Further evidence for the importance of inflammation in cardiovascular disease is seen from the ruptured plaque histology with abundance of macrophages, a thin fibrous cap and smooth muscle cell loss due to apoptosis (van der Wal et al., 1994).

The innate immune system plays a major role in the initiation and propagation of atherosclerosis, with monocytes/macrophages being the key players in this process (Oude Nijhuis et al., 2007). Monocyte involvement in the development of atherosclerotic plaques was reported in the 1970s with monocyte accumulation demonstrated in porcine atherosclerotic lesions (Kottke and Subbiah, 1978). Three indirectly related processes, which involve monocytes, have been identified in atherosclerosis (Figure 1). Monocytes have been shown to play a role:

(1) During the long-term process of initiation and formation of an atherosclerotic plaque, presumed to be accelerated by different risk factors, including smoking, hypertension, hyperglycaemia and critically hyperlipidaemia.

(2) During the acute inflammatory phase that follow destabilization, rupture of the atherosclerotic plaque and acute thrombus formation in ACS.

(3) During healing, where they reside in the myocardial tissue in the hypoxic phase during an acute coronary event and may promote myofibroblast accumulation, angiogenesis, and myocardial healing and remodelling, thus showing a protagonist or antagonist influence in post-ACS recovery.

In the present review, we critically examine these three roles of monocytes and evaluate data on the modulation of monocyte function indicating future direction of novel therapeutic interventions. Several characteristic features of monocytes are important in explaining this multitude of actions, namely, varying subpopulations; high plasticity and trafficking capacity; existence of multiple reservoirs and hematopoietic maturation sites.

1.2.1 *Monocyte diversity in animal models and humans*

Monocytes account for 3-8% of peripheral blood leukocytes. They are mononuclear cells often characterised by typical kidney-like shaped nuclei, but they are more accurately described by their expression of various surface receptors (Shalhoub et al., 2011). They are the main component of the innate immune system that is responsible for counteracting exogenous bacterial, viral and fungal infections mainly by phagocytosis (Libby, 2002). However, they are also involved in endogenous inflammatory processes. Monocytes contribute to atherogenesis through promoting leukocyte recruitment to plaques, and their roles are also mediated by activation of downstream signalling pathways, such as nuclear factor κB (NFκB) pathway (de Winther et al., 2005). Indeed, monocytes have been directly implicated in a number of chronic inflammatory conditions including glomerulonephritis, rheumatoid arthritis, lung fibrosis and atherosclerosis (Katschke et al., 2001, Duffield, 2010).

Cell surface receptor expression allows discrimination between monocyte subpopulations and was first described in murine models. Palframan et al. and Geissmann et al., using *CX3CR1 knockout* mice, demonstrated that peripheral blood monocytes differ in CX3CR1, CCR2, and CD62L expression (Palframan et al., 2001, Geissmann et al., 2003). Monocytes expressing CCR2, CD62L, and low levels of CX3CR1 appeared to be preferentially recruited to inflamed sites by virtue of their recognition of CCL2 (monocyte chemoattractant protein-1, MCP-1). Conversely, the CX3CR1[high] monocytes could migrate into non-inflamed sites (Geissmann et al., 2003) or migrate later during the recovery period after an acute inflammation (Nahrendorf et al., 2007).

In humans, "classical" monocytes, which represent 80-85% of the total population of circulating blood monocytes, can be identified by high expression of CD14 and lack of CD16 expression (also referred as Mon 1). They are considered inflammatory mediators and represent the predominant subpopulation identified in atherosclerotic plaques (Gerrity et al., 1979). These monocytes also

express CCR2, CD62L, and CD64 (Wong et al., 2011). The migration of this subpopulation depends strongly on MCP-1secreted by resident macrophages (Swirski et al., 2007).

Another human monocyte subset is defined as the CD14+CD16++ cells and it is referred to as 'non-classical' monocytes or Mon 3 population. They express high levels of CX3CR1 but do not express CCR2 or CD62L (Ancuta et al., 2003, Ancuta et al., 2006). This subtype depends on fractalkine (or CX3CL1, a soluble chemokine-like domain) for attraction and recruitment to endothelial surfaces. Fractalkine is expressed on activated endothelial cells as a transmembrane-anchored adhesion receptor thus attracting and arresting monocytes from the circulation into the atherosclerotic plaque. Indeed, CX3CR1 knockout mice, fed on a high fat diet showed a significant reduction in monocyte recruitment to the vascular wall and reduced atherosclerotic plaque formation. In fact genetic deletions of CCL2, CX3CL1, or their cognate receptors, CCR2 and CX3CR1, markedly reduced atherosclerotic lesion size in murine models of atherosclerosis (Saederup et al., 2008). When the three chemokine receptors CCR5, CCR2 and CX3CR1 were blocked the maximal reduction in the atherosclerotic plaque formation was evident, suggesting that all monocyte subpopulations are involved in atherogenesis (Combadiere et al., 2008). Table 1 summarises characteristics of different monocyte subsets in humans and mice.

Differences between monocyte subpopulation functions have been exemplified in an elegant in vivo mouse study by Auffray et al. who postulated that non-classical/resident' monocytes constantly patrol healthy tissues through long range crawling along the endothelium (Auffray et al., 2007). During acute inflammation these monocytes utilize CX3CR1 and the integrin lymphocyte function-associated antigen-1 (LFA-1) to "home" on the inflamed tissues on an "as required basis". In humans Cros et al. indicated that CD14low (i.e., CD14+CD16++) 'non-classical' monocytes also have similar patrolling properties and are involved in the innate local surveillance of tissues and the pathogenesis of autoimmune diseases (Cros et al., 2010).

More recently, a third human monocyte subpopulation has been identified as 'intermediate' CD14++CD16+ (also called Mon 2) cells (Shantsila et al., 2011). They are reported to be a predominant type of monocytes expressing Tie-2 (an angiopoietin receptor), which has been

implicated in angiogenesis. The presence of the three distinct monocyte subsets was recently confirmed by gene microarray analysis (Wong et al., 2011, Shantsila et al., 2011). The 'intermediate' subset has the highest expression of major histocompatibility complex class II molecules, whereas'non-classical'CD14+CD16++ monocytes are characterised by high expression of cytoskeletal rearrangement genes, inflammatory cytokines, and CD294 (Wong et al., 2011).

The varying nomenclature utilised to describe monocytes poses a problem in unified interpretation of animal and human data. As most of the current knowledge in the field originates from murine models, effort is directed towards establishment of parallels between monocyte subsets across species. Scarce information on their functions adds to the complexity of drawing reliable conclusions on the physiological and pathological roles of monocyte subsets in humans. For example, features of the so-called 'intermediate' subsets are increasingly demonstrated and recognised in a wide range of pathological conditions. An 'intermediate' pattern of the subset may be partly due to some overlap with other subsets (particularly with 'non-classical' monocytes), when their definition is solely based on their CD14 and CD16 expression. More accurate delineation of CD14++CD16+ and CD14+CD16++ monocytes can be achieved by additional marker CCR2 (Shantsila et al., 2011), with 'intermediate' monocytes being 'CD14++CD16+CCR2+' and 'non-classical' monocytes being 'CD14+CD16++CCR2–. Of note, CD14++CD16+ monocytes show the highest of all monocyte expression of many surface receptors, particularly those involved in reparative processes (e.g., CXCR4, Tie2, VEGF receptors type 1& 2). This together with the evidence of specific enrichment of this subset in bone marrow indicates that it is unlikely to just represent an 'intermediate' state between the other two subsets, but rather a unique and distinct monocyte subpopulation. Although it is not yet entirely clear whether specific monocyte subsets are predetermined to differentiate into particular types of tissue macrophages and dendritic cells, published data suggest existence of common features and links between CD14++CD16– (Mon 1) and CD14++CD16+ (Mon 2) monocytes and M1&M2 polarized macrophages, respectively. Also in vivo, Ly-6C^{lo} and Ly-6C^{hi} monocytes from mice differentiate more readily into M2-like cells and M1-like macrophages respectively, but macrophage development also depends on surrounding microenvironment and

interaction(s) with other cell types, such as lymphocytes (Geissmann et al., 2010, Gratchev et al., 2012). Accordingly, the assignment of numerical dominators (i.e., Mon 1, Mon 2, and Mon 3) for human monocyte subsets may be appropriate until more data are available on the-relationships between the subsets.

Table 1: Comparison of different monocyte subpopulations phenotype and function

Characteristic		Inflammatory/ Classical	Newly described intermediate population	Resident. /Non classical
Human		CD14++CD16-CCR2+ CX3CR1^{lo}; CD62L+ CD115+ (Mon 1)	CD14+CD16+CCR2+, CX3CR1^{hi}, CD62L-, CD115+ (Mon 2)	CD14+CD16++CCR2- VCAM^{hi} CCR2^{lo} CD64low (Mon 3)
Mice		Ly6-C^{hi}, CCR2+, CXCR1^{lo}, CD62L+, CD115+, CD11-, MHC class II -ve	No distinct model is available, however has characteristics of Ly6-C^{hi} models	Ly6Clow, CX3CR1hi, CD62L-, CD115, MHC- class II +ve
Recruitment		Early in acute inflammation	Early in Acute inflammation	Late in acute inflammation
Reservoirs		Spleen	None Known	Unknown
Function	Acute	Accumulate in injured myocardium and perform inflammatory and proteolytic function	? Inflammation	Assumed role in granulation tissue formation and angiogenesis, later mobilization
	Chronic	Accumulate in atherosclerotic plaque in response to activated endothelium.	Given high expression of CD163 and CD204 scavenger receptors then accumulation in atherosclerotic plaque is possible	Accumulate in atherosclerotic plaque via CCR5 and CX3CR1

MHC: Major histocompatibility complex; VCAM: vascular cell adhesion molecules.

1.2.2 *Monocyte trafficking and developmental plasticity*

High trafficking ability is another characteristic feature of monocytes. A hallmark of monocyte trafficking is their capacity to traverse from the circulation into areas of injury/inflammation, aiming for resolution of infection and contribution to the restoration of the tissue integrity via differentiation of different types of tissue macrophages and dendritic cells. However, presumably 'reparative' properties of the monocytes may fail and lead to a disease state, with atherosclerosis being an example. Indeed, monocytes are precursors of lipid-laden 'foam' cell macrophages, which are a critical component of atherosclerotic plaques. Even mature monocyte-derived macrophages do not lose their mobility entirely, under certain circumstances (which are not entirely understood) monocyte/macrophages, including 'foam cells' can migrate from the vascular wall back into the circulation (Gratchev et al., 2012). In several studies monocyte migration from atherosclerotic plaques led to plaque regression under experimental conditions (Feig et al., 2009, Llodra et al., 2004). In a mouse model, statin therapy augmented the egression of the plaque macrophages via removing the inhibitory effect on CCR7 and independently of lipid levels (Feig et al., 2011), possibly representing an additional pleiotropic effect of statins.

Monocytes are also characterised by an extremely high developmental plasticity, being able to differentiate under appropriate stimulation into different cell types ranging from epithelial, endothelial, cartilage cells to functional fibroblasts, cardiomyocytes and neuronal cells. Most of this work has been done under experimental conditions and the *in vivo* and (more importantly) clinical relevance is only beginning to emerge (as discussed below).

There has also been a recent shift from a long-term paradigm that the bone marrow is the sole source of circulating monocytes. At least in mouse models of myocardial infarction the spleen can serve as a reservoir of monocytes, which could be promptly released upon demand Swirski et al. have shown that Ly-6C^{hi} monocytes (the murine equivalent of 'classical' human monocytes) accumulated in the infarcted myocardium in numbers that exceeded their availability in the circulation, suggesting a different source of monocyte supply may exist, with the spleen identified as an important 'storage'

reservoir (Swirski et al., 2009). The group revealed that hematopoietic and progenitor cells can migrate from the bone marrow to the spleen, where they undergo maturation, followed by release of Ly-6C^{hi} monocytes that infiltrate atherosclerotic plaques producing inflammatory cytokines, and proteases, and leading to 'foam' cell formation (Robbins et al., 2012). These results indicate the existence of extramedullary monocyte sources in mice, whilst differential functional characteristic of bone marrow and plaque monocytes are still be investigated and also explored in humans (Robbins et al., 2012).

1.3 Endothelial activation and monocyte recruitment

Activation of the endothelium is the first step in the initiation of monocyte recruitment from peripheral blood. Multiple factors may lead to endothelial damage and activation, including oxidative stress, bacterial and viral infections, long-term hyperglycaemia, as well as increased plasma levels of both native and oxidised LDL (Quinn et al., 2011). The endothelium normally releases nitric oxide (which is vasodilatory); however, the presence of oxidised LDL hampers its activity (Vidal et al., 1998). Platelets are also involved in the activation of the endothelium, by producing platelet factor 4 and P-selectin which attract monocytes to the endothelium by forming monocyte-platelet aggregates (Vorchheimer and Becker, 2006).

Upon stimulation, endothelial cells over-express chemokines (e.g. CCL5 and CCL2), as well as toll like receptors (TLRs) and adhesion molecules (Crola Da Silva et al., 2009). Blood monocytes "roll" onto the endothelial surface and are recruited to the activated/damaged endothelium. The expression of adhesion molecules, such P- and E-selectins, VCAM-1 and ICAM-1 facilitates an initial adhesion followed by the firm attachment of activated monocytes expressing reciprocal ligands such as Mac-1 and VLA-4 onto endothelial cells (Huo and Ley, 2001). Monocytes from patients with hypercholesterolaemia are more prone to native LDL-mediated changes of adhesion molecule expression compared to monocytes from controls (Serrano et al., 2009). Simvastatin is capable of inhibiting these LDL mediated effects (Serrano et al., 2009).

The monocytes transmigrate across the endothelium via diapedesis to the subendothelial space. None the less junction adhesion molecules (JAM)-A and -C have been shown to be involved in the control of vascular permeability and thus leukocyte transmigration across endothelial cell surfaces (Weber et al., 2007). The process also relies on activation of the NFκB pathway to activate matrix metalloproteinases (MMPs) which assist monocytes in breaching the endothelium (Tobar et al., 2010).

1.3.1 *Monocytes in the stable atherosclerotic process*

Given monocyte plasticity, monocytes are able to cross from the blood into the subendothelial space where, monocytes differentiate to macrophages promoted by factors such as macrophage colony stimulating factor. The macrophages ingest oxidised LDL via scavenger receptors (SR), such as CD36, forming 'foam' cells (Collot-Teixeira et al., 2007). Lipid-laden macrophages undergo a process of apoptosis/necrosis which perpetuates the formation of further 'foam' cells. This, in conjunction with smooth muscle migration and proliferation across the intima leads to the formation of lipid-rich atherosclerotic core covered by a fibrous cap. The lipid laden macrophages also release cytokines, growth factors, MMP, reactive oxygen species (ROS) and tissue factor, all perpetuating the inflammatory response, and vascular remodelling (Badimon et al., 2011). This also activates and attracts platelets, thus increasing plaque susceptibility to thrombus formation.

Interestingly, lipid laden macrophages are also involved in the reverse transport pathway of lipid metabolism that circulates lipids to the liver via the ABCA1 transporters. In atherosclerosis the activity of this pathway is impaired especially in the presence of statin therapy, suggesting that these highly effective anti-atherosclerotic agents may also pose some (rather minor) pro-atherogenic properties (Wong et al., 2008). The cholesterol level and state of macrophage maturity appear to determine the effect of statin on the efficiency of the ABCA1 export pathway. Of note, supplementation of human macrophages with cholesterol reverses the statin-mediated down-regulation of the ABC transporter expression and vice-versa, indicating that statin beneficial effects

on plaque regression may be more prominent in subjects with more severe hypercholesterolaemia (Wong et al., 2008).

It is not only the monocyte-to-macrophage axis of monocyte differentiation that contributes to atherosclerosis formation, but monocyte-derived dendritic cells also playing a role. Dendritic cells are antigen presenting cells that prime the T cell response to "self" and "foreign" antigens, inducing self-tolerance (Bobryshev, 2010). These cells are also implicated into acute and chronic inflammatory responses, and contribute to memory T cell-mediated immune response. Hence DC are the sentinel of the adaptive immune system. DC are present within atherosclerotic plaques being selectively enriched in rupture prone areas of the atherosclerotic lesions (Bobryshev and Lord, 1995, Yilmaz et al., 2004). In an in vitro model, adhesion of DC to injured carotid arteries was mediated by platelets via Mac-1 and platelet JAM-C (Langer et al., 2007). Intraplaque accumulation of DC led to atherosclerosis progression due to DC induced lymphocyte proliferation.

In addition to presenting "atherosclerotic-relevant" antigens to lymphocytes, DC produce cytokines that create a local inflammatory microenvironment, which favours propagation of the atherosclerotic lesions. Indeed, in an in vitro model, plaque DC produced IL-12 which upregulated the expression of CCR5 on surface of T cells resulting in further attraction and activation of T cells (Zhang et al., 2006). DC may also have a role in foam cell formation, even though classically this was attributed to macrophages. In fact therapeutic manipulation of DC function by various clinically available agents (such as statins, ezetemibe, and diltiazem) resulted in atherosclerotic plaque regression (Feig and Feig, 2012).

1.3.2 *Monocyte activation and the role of toll like receptors*

Toll-like receptors (TLRs) are the most characterised members of the pattern recognition receptor family. At least 13 different TLRs have been identified in mammals. Activation of TLRs leads to downstream activation of NFκB-signalling pathway with pro-inflammatory consequences (Faure et al., 2001). TLR4 is required not only for the bacterial endotoxin-induced inflammatory responses,

but also for non-bacterial ligands, such as fatty acids and heat shock proteins (HSPs) (Lee et al., 2001, Vabulas et al., 2002, Asehnoune et al., 2004). The main TLRs implicated in the atherosclerotic process are TLR2 and TLR4 (Poltorak et al., 1998). Expression of both receptors is increased in LDL receptor deficient (LDLR−/−) and apolipoprotein E deficient (ApoE−/−) mice (Edfeldt et al., 2002). Whilst the biological role of TLR4 over-expression is likely to be protective against infection-related factors, chronic stimulation of the pathway by host ligands results into self-perpetuating inflammatory milieu inside the vascular wall, facilitating leukocyte accumulation and plaque formation and growth (Xu et al., 2001).

TLR4 signalling is involved in atherosclerotic plaque destabilization. Lipopolysaccharides (LPS) induce the macrophage expression of MMP- 9 via TLR4, whilst MMP- 9 has been shown to degrade collagen fibrous caps, thus predisposing to plaque rupture (Grenier and Grignon, 2006). TLR4 was also increased systematically in patients following myocardial infarction (MI) and locally at site of plaque rupture, yet suggesting that monocyte TLR4 has a role in plaque destabilization and rupture (Ishikawa et al., 2008). TLR4 was increased in both stable and unstable angina, with a congruent increase in TLR2 in the unstable state only (Ashida et al., 2005). This was recently echoed in the Atherosclerosis Risk in Communities (ARIC) study where monocyte TLR2 expression was associated with larger plaques, and TLR4 was associated with smaller ones (Matijevic et al., 2011). Recently our group demonstrated that TLR4 expression on surface of monocytes is unchanged in ACS patients, however with the increase in monocyte mobilization and attraction to areas of infarct led to a relative (but not true) increase in detection of TLR4 on surface of Mon 1 and Mon 2 (Tapp et al., 2013) .

Inhibition of TLR4 attenuates the inflammatory response in a mouse model of MI, as shown by a significant decrease in infarct size and expression of inflammatory mediators (Chong et al., 2004, Oyama et al., 2004, Kaczorowski et al., 2007). A new benzisothiazole derivative inhibitor of TLR4 signal transduction, suppressed LPS-induced up-regulation of cytokines, adhesion molecules and pro-coagulant activity in human vascular endothelial cells and peripheral mononuclear cells, suggesting a potential for management of atherosclerosis (Nakamura et al., 2007). Several agents

blocking TLR2 and TLR4 showed promising anti-inflammatory properties (Yang et al., 2009). They demonstrated that valsartan was able to inhibit TLR4 expression concomitant with a reduction in mouse myocardial injury, a smaller infarct size, and lower levels of myocardial enzymes post infarction.

1.3.3 *Activation of the NFκB pathway*

NFκB is one of the major transcription factors mediating inflammatory responses of monocytes and macrophages. Its activation leads to the production of proinflammatory and prothrombotic molecules, such as cytokines, MMPs, tissue factor (TF) and IκB, which exerts a negative feedback loop on NFκB activation (Palombella et al., 1994, De Rycke et al., 2005, D'Acquisto et al., 2002). Monocyte stimulation leads to activation of the NFκB which was linked to inflammation associated with atherosclerosis (D'Acquisto et al., 2002). Thus, measurement of NFκB levels could be used as a surrogate for the functional assessment of monocyte inflammatory activity (Monaco and Paleolog, 2004).

In non-stimulated monocytes, NFκB is sequestered within the cytosol by an inhibitory protein (i.e., IκB; inhibitor of NFκB) that prevents its translocation to the nuclei. Upon stimulation IκB is phosphorylated by IκB kinase (IKK), undergoes secondary ubiquitination and is degraded by the proteasome (Monaco and Paleolog, 2004). The IKK complex is a phosphorylating enzyme with several subunits, IKKα, IKKγ and IKKβ. The β subunit is the most active and is involved in transcriptional activation of the pathway by phosphorylating IκB. The degradation of IκB thus allows NFκB to translocate into the nuclei where it can act as a transcription factor (Monaco and Paleolog, 2004, Kollander et al., 2010). Several mechanisms lead to the activation of the NFκB pathway during the atherosclerotic process (Table 2).

Excessive NFκB activation is associated with negative ventricular remodelling (Hamid et al., 2011). Myocardial tissues from patients with heart failure had increased expression of NF*κ*B-regulated genes. This exacerbated ventricular remodelling with increased pro-inflammatory, pro-fibrotic and

pro-apoptotic effects (Hamid et al., 2011). Also, using gene transfer of IκBα Squadrito et al. (2003) have demonstrated that repression of NFκB transcription in models of ischaemia-reperfusion injury resulted in reduction in leukocyte recruitment to the myocardium, cardiomyocyte death and infarct size . Accordingly inhibition of NFκB may become an attractive pharmaceutical target and promising data on a well-tolerated pharmacological NFκB inhibitor, BAY 11-7082 have been recently presented (Kim et al., 2010). The latter reduces inflammation in a rat model of ischaemia-reperfusion injury leading to a significant reduction in the infarct size.

Table 2: NFκB activation in atherosclerosis

Stage of atherogenesis	NFκB role	Study	Results
Initiation of Atherogenesis	Contributing to the environment leading to LDL oxidation	Hajra et al. (2000) Ivandic et al. (1999), Han et al. (2001)	Mouse model LPS stimulation or high fat diet lead to NFκB activation and overexpression of NFκB genes Transgenic mice model
Role in monocyte migration across endothelium	NFκB controls transcription of MMPs (crucial enzymes in degradation of ECM)	Han et al. (2001) Bond et al. (1998)	In vitro model of fibroblast cells- activation of NFκB signalling in fibroblasts induces mt1-MMP gene expression. In vitro model- Activation of NFκB binding by inflammatory cytokines was therefore necessary but not sufficient for synergistic upregulation
Foam cell formation	Induction of monocyte to macrophage differentiation via MCSF Smooth muscle proliferation	Brach et al. (1991) Hoshi et al. (2000) Selzman et al. (1999)	In vitro- induction of MCSF (through TNF) is dependent on NFκB. In vitro- PD98059, an inhibitor of ERK, suppressed NFκB transcriptional activity and SMC proliferation In vitro- NFκB inhibited VSMC

| Zuckerbraun et al. (2003) | In vitro- Gene transfer of IkappaB alpha super-repressor inhibited development of intimal hyperplasia in vivo and SMC proliferation |

ERK: extracellular signal regulated kinase; LPS: lipopolysaccharide; LDL: low density lipoprotein; MMP: matrix metalloproteinases; MCSF: monocytes colony stimulating factor; SMC: smooth muscle cells; VSMC: vascular smooth muscle cells

1.4 Monocytes in the acute coronary syndromes

Acute coronary syndromes (ACS) refer to a clinical spectrum ranging from those for ST-segment elevation myocardial infarction (STEMI) to non–ST-segment elevation myocardial infarction (NSTEMI) or unstable angina (Alpert et al., 2000, Hamm et al., 2011, Underwood, 2009). ACS is almost always associated with rupture of an atherosclerotic plaque and partial or complete thrombosis of the infarct-related related artery. Most cases of ACS occur from disruption of a previously non occlusive but unstable (vulnerable) plaque (O'Connor et al., 2010, Chughtai et al., 2011). The characteristic features of a vulnerable plaque include a thin fibrous cap, a higher predominance of macrophages in the cap, smaller collagen content, and a large, lipid-rich necrotic core with overlying thrombus and platelet aggregates (Galis et al., 1994).

Monocytes promote destabilization of the fibrous cap leading to the plaque rupture. This is mainly orchestrated by MMPs (Newby et al., 2009, Medbury et al., 2008). Activated macrophages produce a wide range of lytic enzymes, including MMPs (e.g. MMP-1, -2, -3, -8, -9, and -14) (Newby, 2007). Increased expression and activity of MMPs has been noted in vulnerable plaque regions while elevated serum MMPs have been demonstrated in patients with ACS (Momiyama et al., 2010).

Plaque rupture could lead to downstream occlusion of coronary flow to the myocardium, the principle mechanism of STEMI. Monocytes have a role in thrombus propagation contributing to the coagulation cascade during the acute event. Patients with ACS show features of procoagulant monocyte activation with exposure of tissue factor (Altieri et al., 1988). Monocyte-platelet aggregates, and markers of monocyte and platelet activation involved in regulation of their function

are also increased in ACS patients, persisting even after one month of the acute event (Tapp et al., 2011). Also, microparticles derived from monocytes are abundant in ACS and support faster fibrin formation (Aleman et al., 2011).

Monocyte adherence to extracellular matrix and extravasation to the injured tissue induces the expression of a multitude of cytokines including TNF-α, IL1 and IL6 [potent inflammatory cytokines]; platelet derived endothelial cell growth factor [a potent chemoattractant and mitogen for fibroblasts]; TGFα and β [which contributes to fibrosis, by stimulating extracellular matrix release, primarily collagen, from myocardial fibroblasts]; M-CSF [cytokine necessary for macrophage survival],and insulin-like growth factor (Lambert et al., 2008, Frangogiannis et al., 2003).

Monocytes are also thought to participate in tissue injury. Downstream occlusion of blood supply to the myocardium (ischaemia) followed by pharmacological or interventional revascularization therapy (reperfusion) results in the ischaemia-reperfusion cascade. During hypoxia, ROS leakage from mitochondria is increased (Madamanchi and Runge, 2007). ROS-modified biomolecules formed during ischaemia stimulate infiltration of inflammatory cells, including monocytes, thus mediating an acute inflammatory response leading to cell injury and necrosis. Recently, a new model of 'innate autoimmunity' for ischaemia/reperfusion injury has been introduced, which integrates mechanisms of both intrinsic ischaemic cell injury and initiation of an extrinsic innate immune response (Zhang and Carroll, 2007). This hypothesis that the intrinsic changes associated with cell injury are augmented by a second wave of the innate immune system involvement largely represented by monocytes (e.g., mediated by the complement system, IgM and their receptors) helps to explain the continued loss of myocytes despite reperfusion and, in some in vitro studies, despite elimination of inflammatory cells (Haegert, 1979).

Within the subintimal space, monocytes mature into DC and macrophages, each with its separate polarity, and inflammatory functions that have further effect on tissue necrosis (Geissmann et al., 2010). Macrophages are currently considered to be comprised of two types: the classically activated (pro-inflammatory M1 type), and the alternatively activated M2 acting as anti-inflammatory cells (Lambert et al., 2008). M1 macrophages promote inflammation and extracellular matrix destruction.

IL-1β secretion from M1 induces MMP-9 and TGFβ secretion and stimulates fibroblast proliferation (Lambert et al., 2008). Macrophage phagocytosis of dying cells also triggers TGFβ production (Singer and Clark, 1999). Of interest, in murine models, monocyte tissue residence time was found only to be around 20 hours, with persistently high rates of recruitment to infarcted myocardium days after the acute event, and disproportionally slower rate of exiting from infarcted tissues at a maximum rate of 13%/day (Leuschner et al., 2012).

A mouse model of MI has demonstrated substantial functional differences between monocyte subsets in the course of the infarction. For instance, mice Ly-6C^{hi} monocytes (considered to be equivalent to CD14++CD16–CCR2+ human cells) are mobilised early after MI onset and show distinct phagocytic properties. In contrast Ly-6C^{lo} monocytes (considered to be the equivalent of human CD14+CD16++CCR2– cells) showed anti-inflammatory properties and were critical for myocardial healing and reverse remodelling in MI, promoting myofibroblast accumulation, angiogenesis and the deposition of collagen (Nahrendorf et al., 2007). The production of these subsets seemed to follow a biphasic mode, with an early release of Ly-6C^{hi} followed by a later production of Ly-6C^{lo} (Nahrendorf et al., 2007).

At the other end of the monocyte differentiation spectrum, levels of circulating DC (both plasmacytoid as well as myeloid DC) have been found to be significantly reduced in patients following an acute coronary syndrome, compared to healthy controls (Fukui et al., 2012). It is thought that this is due to increased recruitment of DC to the infarcted tissue. This hypothesis was supported with immunohistochemistry findings indicating an increase in the DC and T-cell infiltration of peri-infarct zone (Kretzschmar et al., 2012).

1.5 Cardiac Remodelling

An important structural event after myocardial infarction (MI) is left ventricular (LV) remodelling, which generally can be defined as changes within the cellular and extracellular constituents of the myocardial wall leading to changes in myocardial geometry subsequently leading to changes in LV

volumes. The rate and extent of this post-MI remodelling process have been established to be independent predictors of morbidity and mortality. Thus, identifying those patients at the greatest risk for developing post-MI remodelling and the basic mechanisms that contribute to post-MI remodelling holds great diagnostic/ therapeutic relevance. The factors that contribute to post-MI remodelling are multifactorial and include both cellular and extracellular processes. It is now becoming recognized that changes within the myocardial extracellular matrix (ECM) contribute to post-MI remodelling. Disruption within the fibrillar ECM network will cause a loss of normal structural support and continuity, resulting in myocyte fascicles being subjected to abnormal stress and strain patterns during the cardiac cycle, which in turn cause changes in myocardial geometry and function. Hence cardiac remodelling post-MI is defined by the degree of the residual impairment of LV contractility (e.g. ejection fraction) and geometry of LV after completion of the process of myocardial scar formation (e.g. LV end-systolic and end-diastolic volumes at 6 months after MI onset).

The matrix metalloproteinases (MMPs) constitute of a large family of proteolytic enzymes responsible for ECM degradation and remodelling under normal and pathological conditions. An important control point for MMP activation is binding to an endogenous family of inhibitors, the tissue inhibitors of MMPs (TIMPs). A clear cause-and-effect relationship between MMPs and the post-MI remodelling process has been demonstrated through the use of various animal models. In general, these preclinical studies have demonstrated that an imbalance between MMPs and TIMPs occurs in the post-MI myocardium and that increased MMP proteolytic activity facilitates post-MI remodelling and eventually LV dilation. Moreover, these preclinical studies have demonstrated that a specific temporal pattern of MMP/TIMP expression occurs after MI that is causally related to the degree of post-MI remodelling.

Illustration removed for copyright restrictions

Figure 1: LV remodelling on myocyte level (adapted from Konstam et al., 2011)

Cardiac remodelling plays a key role in the pathophysiology of LV dysfunction. The process of post-MI myocardial recovery depends on numerous factors, with multiple theories at the cellular and molecular level. Release of ROS, phagocytosis, fibroblast accumulation and angiogenesis have all been suggested to be involved and these relate to monocyte activity (Cavalera and Frangogiannis, 2014) . Inflammation plays an important role in cardiac healing (Frantz et al., 2009, Christia and Frangogiannis, 2013).

Cardiac remodelling can be assessed by transthoracic 2D and 3D echocardiography, using LV ejection fraction, volumes and mass as surrogate parameters. Using echocardiography, negative left ventricular remodelling is defined as an increase of 20% in the end diastolic volumes at 6 months follow up (Verma A, 2008, Konstam MA, 2011).

1.5.1 *Monocytes and cardiac remodelling*

Despite advances in medical and interventional therapy for ACS, many patients still develop heart failure. The process of post-MI myocardial recovery depends on numerous factors at the cellular and molecular levels. After the initial tissue damage induced by hypoxia, an acute inflammatory response ensues with recruitment of leukocytes to the infarcted areas (Nahrendorf et al., 2007). Subsequent release of ROS, phagocytosis, fibroblast accumulation, angiogenesis and, tissue formation occurs,

ultimately leading to cardiac remodelling and recovery modulated by the activity of these recruited cells. The role of inflammation is important. For example, an increase of the pro-inflammatory cytokine TNF-α with a corresponding decrease of anti-inflammatory cytokine IL10 is associated with adverse/reverse ventricular remodelling (Grieve et al., 2004). Together, leukocytes degrade extracellular matrix constituents and macromolecules released by the injured cells and aid clearance of dead cardiomyocytes and their debris.

Historically, monocytosis has been associated with left ventricular dysfunction post MI, with recruited monocytes releasing multiple cytokines, such as IL-1α and β, IL-6, TNF-α which are negatively associated with myocardial healing and development of heart failure (Jonsson et al., 2011). The balance between removing dead myocytes and prompt initiation of regeneration may determine patients' outcomes.

Monocyte injection and their cardiac recruitment through MCP-1, IGF-1 secretion, in enhanced remodelling with improved post-MI cardiac contractility in mice (Apostolakis et al., 2010). Monocytes are also involved in myocardial fibrosis and post-infarction scar formation, whilst their release of angiogenic factors (e.g., VEGF) promotes angiogenesis in and around the healing tissue.

Of note, prolonged Ly-6C^{hi} monocytosis early after MI onset could impair myocardial healing in a murine model (Bouchentouf et al., 2010, Panizzi et al., 2010). On the other hand, depletion of Ly6C^{hi} monocytes early post infarct led to increased areas of debris and necrotic tissue with impaired ventricular healing (Nahrendorf et al., 2007). This accords with results of previous murine study where post infarct macrophage depletion led to ventricular dilatation and myocardial wall thinning with concurrent decrease in neovascualisation, myofibroblast and collagen depositions (van Amerongen et al., 2007). Recently, depletion of DC led to a similar result, with sustained expression of inflammatory cytokines such as IL-1β, TNF-α, IL18, and MMP- 9, as well as a decrease in IL-10 within the infarcted area in a mouse model. Interestingly, there was an increase in Ly6C^{hi} monocytes in DC depleted mice (Anzai et al., 2012). It could be postulated that this is due to interruption of DC induced "negative feedback" on differentiation signalling that controls monocyte subpopulations/macrophage interaction. Hence depleting DC may lead to uncontrolled monocyte

differentiation and disruption of this phagocytic system thus illustrating the intricate balance and cross talk between it different components.

However our knowledge of the effect of monocytes on cardiac remodelling and clinical outcome in humans is still limited. Although similarities exist in MI pathophysiology between species, the inter-species differences between monocyte subsets are substantial (including the fact that monocytes account for 50% of murine leukocytes versus a much smaller proportion in humans). This may make extrapolation of animal data to humans challenging from a clinical and pharmaceutical perspective (Shantsila et al., 2010).

The effect of monocytes on ventricular healing in humans has been investigated in one small study, where numbers of CD14+CD16– monocytes were negatively associated with myocardial salvage following STEMI and poor clinical outcome (Tsujioka et al., 2009). CD16+ monocytes (analysed as a mixture or 'intermediate' and 'non-classical' monocytes) had no effect on ventricular remodelling, which contrasted with previous animal data (Nahrendorf et al., 2007). In a recent study where levels of the three human monocyte subsets in STEMI were analysed separately a prominent (over 2.5-fold) up-regulation of the CD14++CD16+CCR2+ (Mon 2) - described elsewhere in the literature as intermediate monocytes) subset in acute STEMI was observed with no changes in CD14+CD16++CCR2– (Mon 3) cells (Tapp et al., 2011). This was accompanied by a significant change in phenotype of the 'intermediate' subset (increase in CD14 and CCR2 expression, and a reduction in CD16 expression). These observations of distinctive changes related to this subset together with existing evidence of their pro-reparative and pro-angiogenic phenotype and anti-inflammatory properties are suggestive of their possible specific roles in cardiac recovery, but sufficiently powered data in this respect are lacking.

Interest in the 'intermediate' monocytes has been echoed into a number of clinical studies that indicated that their high levels have been associated with poor clinical outcome both in terms of future MI in stable coronary artery disease (the HOM SWEET HOMe study) (Rogacev et al., 2012); and lower left ventricular ejection fraction in patients post STEMI (Tapp et al., 2012); or as recurrence of coronary events in patients with chronic kidney disease and in stroke patients (Rogacev et al.,

2011, Heine et al., 2008). The biological roles of this subset are complex and expression of receptors with putative role in angiogenesis and repair (e.g., VEGF receptor 2, CD163, and CXCR4, which was found to be relevant to STEMI, are highest in this subset (Shantsila et al., 2013). One may speculate that the intermediate monocyte subset may play a role in myocardial reparation post MI. However the cause of such associations remains unclear and the exact function of the intermediate monocytes is still under investigation. Currently our knowledge only extends to very limited functional studies and a number of descriptive surface and genetic markers (to mention a few: CCR5, VEGF receptor 2, HLA-DR, ENG, CLEC10A, ACE, GFRA2) (Wong et al., 2011, Zawada et al., 2011). It is still unclear whether this subpopulation is a separate and independent entity from Mon 1 and Mon 2, and hence have their own differentiating pathway, or if they are an intermediate "stop" for classical monocytes as they shift towards a non-classical monocytes phenotype.

Of importance, 'classical' monocytes are featured by a distinct proinflammatory phenotype and their high counts are associated with poor myocardial recovery and worse outcome after MI (van der Laan et al., 2012). Although inflammatory stress associated with these cells is usually mentioned in the context of their potentially detrimental effects, the role of these cells is by far more complex and includes a number of potentially beneficial properties, including: phagocytic activity and regulation of extracellular matrix turnover. An appropriate balance in numbers and functional activity as well as in timing in relation to MI onset is probably the key in relation to the role of the cells in acute coronary catastrophes. However this perspective is based on numerical quantification and presence of monocytes as opposed to assessment of function, not to mention that the role of the 'intermediate' and 'non-classical' monocytes is still to be deciphered.

The role of progenitor cells in wound healing and cardiac remodelling is currently the object of intense scientific interest. Infusion of mesenchymal cells into the mouse myocardium 48 hours after MI induction reduced overall myocardial macrophage/monocyte levels. This included pro-inflammatory M1-type macrophages, whilst alternatively activated M2-type macrophages were significantly increased both in the circulation and the heart (Dayan et al., 2011). Delivery of the mesenchymal cells: reduced cardiac expression of IL-1b; reduced expression of IL-6; increased anti-

inflammatory IL-10 expression without changes in angiogenesis in the infarct area; and improved cardiac systolic function (Dayan et al., 2011). This could suggest that mesenchymal cells may regulate the switch in monocyte/macrophage phenotype towards M2 polarisation and favourable remodelling post infarction.

Of interest, Kuwana et al. described CD14+monocyte-derived mesenchymal progenitors, that can differentiate into a variety of mesenchymal cell types as well as having phagocytic function, thus providing a possible cellular source for tissue regeneration including wound healing and potentially heart remodelling (Kuwana et al., 2003). Also, monocyte-derived progenitor cells have been implicated in cardiac allograft vasculopathy likely due to their ability to differentiate into smooth muscle cells and promote intimal hyperplasia (Salama et al., 2011). However it is important to note that this group of patients routinely receive anti-rejection medication and it remains to be seen whether this has an effect on the abundance of monocyte-derived progenitor cell (Salama et al., 2011).

To address the potential role for inflammation in tissue damage post-MI, steroid therapy was used in the 70's and 80's in acute MI, but has not produced the anticipated beneficial effect, and failed to show an improvement in clinical outcome post STEMI (Bush et al., 1980, Peters et al., 1978). This may be partly due to creating an imbalance between the different monocyte subpopulations eradicating both the inflammatory as well as the reparative subtypes, altering the downstream differentiation of monocytes or potentially disturbing the cross talk between dendritic cells, monocytes and macrophages. Hence targeting specific subpopulations at appropriate phases of cardiac healing/remodelling is a theoretical alternative to improve outcome of inflammatory events post-MI (Figure 2).

Interestingly successful stem cell therapy in STEMI has been based on intracoronary administration of bone marrow mononuclear cells, which include a large proportion of monocytes (Table 3). However none of the studies used purified monocyte-derived progenitor cells but rather utilized a total pool of mononuclear progenitors.

Perhaps delivery or modulation of specific monocyte subpopulations, at different stages of healing will form the basis of future regenerative cell therapy after MI. Indeed Leor et al. have shown that

administration of activated human macrophages to the ischaemic myocardium in rats accelerated vascularisation and repair of the infarcted myocardium with improved cardiac remodelling and systolic function (Leor et al., 2006).

Table 3: Summary of clinical trials using bone marrow mononuclear cells in acute coronary syndrome patients with summary of trial findings

Study	Methodology	Study Period	Study Population (Intervention / control group)	Results
BALANCE study (Yousef et al., 2009)	BMNC in the culprit lesion/vessel	3, 12 and 60 months	60 (30/30)	Compared to control group, patients treated with BMNC exhibited: Improved contractility of infracted area; Reduced mortality; Improved exercise
REPAIR-AMI trial (Schachinger et al., 2006)	Bone marrow progenitor cells infusion into culprit vessel 3-7 days post AMI	12 months	204	In patients vs. placebo: BM PC administration was a significant predictor of a favourable outcome; Cumulative endpoints of death, recurrence of MI or revascularisation or hospitalization for heart failure significantly reduced.
BOOST study (Meyer et al., 2009)	BMNC intracoronary 4 days post AMI	6 and 8 months	60 (30/30)	Increased global EF in intervention group (6% at 6months). Effect between intervention group and control group lost at 18 months; However improved EF retained amongst transmural infarct group.
Janssens et al., (2006)	Bone marrow stem cells intracoronary 24 hours post AMI	4 months	67 (33/34)	No improvement in ejection fraction

ASTAMI (Beitnes et al., 2009)	BMNC intracoronary	3 years	100 (50/50)	The results indicate that intracoronary BMNC treatment in AMI is safe in the long term; A small improvement in exercise time in the BMNC group was found, but no effects of treatment on global LV systolic function.
Traverse et al., (2010)	Bone marrow MNC intracoronary at day 3-10	6 months	40 (30/10)	No difference in EF between groups; BMNC group had improved remodelling with significantly lower LVEDV.

BMNC- bone marrow mononuclear cells; LV- left ventricle; LVEDV- left ventricular end diastolic volume; AMI- acute myocardial infarction; EF- ejection fraction; PC- progenitor cells

Figure 2: The triple role of monocytes at different stages of the atherosclerotic process

The three panels depict the functions of monocytes). Monocytes patrolling in the circulation are activated by different factors. They traffic to the damaged/activated endothelium. The dysfunctional endothelium over-express monocyte chemotactic protein-1 ligand and adhesion molecules (VCAM-1, ICAM-1) on its surface. After rolling and attachment to the endothelium the monocytes cross the endothelial surface (diapedesis). In the subendothelial space monocytes differentiate to macrophages via macrophage colony stimulating factor. The macrophages ingest oxidised LDL via scavenger receptors, especially CD36, forming 'foam' cells. These undergo a process of apoptosis/necrosis which perpetuates the formation of further lipid laden macrophages. The middle panel depicts events during an ACS with plaque rupture, thinning of the fibrous cap on plaque surface and monocyte platelet aggregates. Panel three illustrates cardiac repair, acutely with attraction of monocytes CD14++CD16–CCR2+ (Mon 1) and later in the phase two of remodelling where CD14+CD16++CCR2– (Mon 3) (and potentially CD14++CD16+CCR2+ (Mon 2) cells) alters the extracellular matrix remodelling by myofibroblast deposition and angiogenesis, leading to thinning of the infarcted cells. [ICAM-1: Intercellular adhesion molecule-1; VCAM: Vascular cell adhesion molecule.

1.5.2 *Where are we now?*

No doubt monocytes play an important role in the pathophysiology of SCD and its complication. Blood monocytes are easier to detect and characterise, compared to tissue macrophages. As medical practice moves into the XXI century with more emphasis on the prediction and prophylaxis of future acute events monocyte numbers and functions may become a very attractive biomarker. However the key monocyte parameters with a potential to become prognostic markers and treatment targets are still to be defined and validated.

Indeed, there are still gaps in our knowledge of how monocytes differentiate into specific type of macrophages/dendritic cells in coronary plaques and later within infarcted tissue, and their role in regulating post-infarct reparative processes. The preferential differentiation of certain monocyte populations into particular macrophage types and other cells (e.g. myofibroblasts) has been suggested but needs further evidence.

The understanding of the function of intermediate monocytes is still in its early days. This venture will be blighted with practical difficulties: namely the isolation of the small amounts of intermediate monocytes from whole blood, then maintaining cell viability (without differentiation) to allow characterisation of their function and downstream inflammatory pathway activation. A multitude of murine or other animal models could be utilised but ultimately development of new high resolution molecular in-vivo imaging techniques to tag and track monocytes is needed. This will provide valuable information on mechanisms and magnitude of mobilisation of individual monocyte subsets to the myocardium, their differentiation and may shed further light on intimate aspects of monocyte activities which may prove to be new therapeutic targets. The role of the microenvironment and local factors in driving monocyte differentiation both in the sub-endothelial space and in the infarcted tissue also need to be further elucidated. Furthermore, detailed exploration of monocyte action via interaction and in coordination with other cells, such as lymphocytes and platelets, and in relation to different progenitor and stem cells is deserved.

The expression of chemokine receptors on the surface of infarcted cardiomyocytes is essential to be studied, even on animal models e.g. porcine hearts (the most closely related species

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anatomically and physiologically to human hearts). If therapeutic agents could modulate the expression of MCP-1, fractalkine or CCR5, then monocytes subpopulations could be attracted to infarcted tissues earlier leading to concurrent removal of debris, angiogenesis, fibroblast deposition and better myocardial recovery.

1.6 Unanswered questions in the field of monocyte research

Multiple essential roles are played by monocytes during the various stages of atherosclerosis, from its initiation to the progression and development of its complications - and later on, during myocardial healing and remodelling. These different roles can provide a fertile ground for pharmacological modulation of atherogenesis, stabilization of the atherosclerotic plaque or more importantly in myocardial healing and post infarction remodelling.

Monocyte-mediated pathways are not limited to the cardiovascular system and inhibition of any of the surrounding milieu of cellular mechanisms could actually alter this finely tuned balance in other inflammatory systems leading to deleterious side effects. Hence, direct targeting of specific monocyte subpopulations at different sites and stages of MI would seem to be the best option. In fact, stem cell therapy relying largely on monocyte subpopulations renders an attractive potential. Given that most of our current knowledge in the field comes from murine models, further clinical studies are clearly required to improve our understanding of monocyte pathophysiology in human cardiac damage post-ACS and the subsequent remodelling and recovery of the myocardium.

Whichever way we appraise our current knowledge about origin and progression of atherosclerosis, we are drawn to the same common origin: monocytes, their subpopulations, their function and their differentiation. With multiple therapeutic agents targeting "the clot" during ACS, an alternate and most attractive target for therapy lies in inflammation (Libby, 2013) and particularly specific monocytes subpopulations with their diverse phenotypes and sentinel role in both the innate and adaptive immune system.

PHASE I

CD14++CD16–CCR2+ and possibly CD14++CD16+CCR2+ monocytes: reduce or abrogate to reduce inflammatory effect and remove myocytes from infracted and peri-infarct areas.

PHASE II

CD14++CD16+CCR2+ monocytes and possibly CD14++CD16+CCR2+ monocytes: improve angiogenesis, myofibroblast deposition

Figure 3: Hypothesised principles of pharmaceutical targeting of monocyte subpopulations in the healing myocardium.

The exact role of CD14++CD16+CCR2+ (Mon2) cells remains unclear, more data is required. However as they are associated in humans with low ejection fraction after recovery from STEMI, it could be postulated that they have a role in debris removal early in the healing process. None the less their surface expression of Tie-2 is suggestive of a role in angiogenesis and reparation.

Chapter 2

Monocyte subsets in ST elevation myocardial infarction

2.1 Introduction

Ischaemic heart disease remains the most prevalent cause of death in the western world and the UK; coronary artery disease and heart failure are devastating pathologies for NHS resources and for patients' morbidity, mortality and quality of life. Over the last 30 years management of coronary artery disease saw the introduction of invasive percutaneous coronary intervention (PCI) for the management of acute myocardial infarction with reduction in the acute mortality rate following a myocardial infarction. Indeed PCI has now superseded thrombolysis therapy as the gold standard for managing myocardial infarction with reduction in infarct area, post infarct complications and mortality rates (Steg et al., 2012). PCI success is not only attributed to the mechanical restoration of coronary flow but also to concomitant use of antiplatelet therapy. However other avenues of clinical intervention, other than thrombus management, are now needed to continue improving patients' outcome on both clinical and subclinical measures.

Hence, the identification of an association between leucocytosis and development of adverse cardiac outcomes opened a new era in cardiovascular research (Horne et al., 2006). Thereafter there was rapid unravelling of subset-specific involvement of monocytes in murine models of atherosclerosis, their role in progression of coronary artery disease, finally leading to plaque rupture and myocardial infarction. Concurrently there was an adjunct enthusiasm in translating the findings from murine models to humans. This led to the characterisation of three different monocyte subsets in humans, in an attempt to translate murine model work.

This diversity of monocyte functions has been partly attributed to the existence of different monocyte subsets with specific phenotypic and functional properties (Geissmann et al., 2003, Auffray C, 2009, Cros et al., 2010, Zawada et al., 2011). Specific monocyte subsets have been shown to be differentially involved in the pathogenesis and outcomes of a range of diseases including heart failure (Wrigley et al., 2013), cerebrovascular accidents (Urra et al., 2009), and renal disease. Indicating the broad inflammatory effect of monocytes.

With the identification and reliable characterisation of three distinct monocyte subsets, the last few years were marked by a number of papers thoroughly describing the surface markers,

cytokine and integrin markers expression of the three monocytes subpopulations in humans (Zawada et al., 2011).

Mon 1 is characterised by high expression of CD14, IL6 receptor, CD64, CCR2 and CD163, but low expression of VCAM receptor and CD204 and absent CD16. Mon 2 is characterised by maximal expression of ICAM receptor, Tie2, CXCR4, CD163, VEGF receptor 1, KDR, ferritin, ApoB and CD115. Compared with Mon 1, Mon 2 have up-regulated CD16, VCAM1 receptor and CD204. In contrast, Mon 3 had maximal expression of CD16, VCAM1 receptor and CD204, but diminished levels of CD14, IL-6 receptor, CD64, CCR2 and CD163. On the basis of these findings, Mon1 appear to correspond to Ly-6Chi mouse monocytes and Mon 3 to Ly-6Clo monocytes (Shantsila et al., 2011). However, it is not clear whether any mouse monocyte subset may correspond to the human Mon 2.

It is now clearly described that there are two phases of monocyte subset recruitment to an infarcted myocardium post coronary artery ligation in murine models. The spleen is an important source of monocytes which can be mobilized in response to generalised inflammatory stimuli such as during myocardial infarction. These monocytes have a possible role in orchestrating post infarct healing by meeting the increased demand on macrophages, through supply of their progenitor monocytes, in order to aid "mopping up" of infarcted/ dead tissue.

The phenotypic and functional characteristics of monocyte subsets, especially that of Mon 2, remains undetermined.

2.2 Aims and Hypothesis

The aim of this study was to compare differences in frequency between the three human monocyte subsets in patients with CAD and healthy subjects defined according to contemporary nomenclature of the three monocyte subsets utilising CD16, CD14 and CCR2 surface markers.

The phenotypic and numerical comparison was performed in different health and clinical states (healthy controls, ST elevation myocardial infarction (STEMI), and stable coronary artery disease patients).

Total monocyte counts and their subset variations were also investigated longitudinally in prospectively recruited, consecutive STEMI patients to establish the characteristics of monocyte subpopulations in a large sample size. This chapter addresses the hypothesis that monocyte subpopulations are higher, acutely compared to the recovery phase 14 days post-STEMI.

2.3 Methods

2.3.1 Patient Recruitment

2.3.1.1 ST elevation myocardial infarction patients

Patients with STEMI admitted to Heartlands Hospital, Queen Elizabeth Hospital and Sandwell and West Birmingham Hospitals (SWBH) NHS Trust. Patients who met the study criteria were recruited between March 2011 and December 2012. Details of patients recruited are included in the appendix I.

2.3.1.2 Exclusion criteria

Exclusion criteria included: myocardial infarction in the preceding 6 months, sepsis on admission; pre-diagnosed haemodynamically significant valvular heart disease; ongoing malignant process with a prognosis of less than one year; as well as active inflammatory conditions requiring immune-suppressants (e.g. rheumatoid arthritis, psoriasis, systemic lupus erythematosus).

2.3.1.3 Control Subjects

Data from control subjects were utilised to compare counts in a cross sectional study with the main STEMI population patients. These included both patients with stable coronary artery disease as well as healthy individuals. More details on healthy control recruitment are included in appendix II

2.3.2 Statistical methods

2.3.2.1 Power Calculation

Power calculations were based on the results by Larose et al. who have shown that 20% of ACS patients have cardiovascular events during one year (Larose et al., 2010). This was

performed by resident statistician prior to the study commencing. Briefly, in order to achieve 80% statistical power to detect the difference in the MACE (with p<0.05) in count of the monocyte subsets, at least 35 MACE events were needed. MACE in this project includes: death, new diagnosis of heart failure and recurrent ACS. Thus, at least 175 patients were to be recruited. More details are available in appendix III.

2.3.2.2   Statistical analysis

Normality testing was undertaken using Shapori and Wilkes test with normality distribution curves. Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median with first and third quartiles (Q1-Q3) for non-normally distributed data. Statistical significance of differences between monocyte subsets in different health states was determined by one way analysis of variance (ANOVA) with Tukey's post-hoc test for normally distributed parameters and Kruskall and Wallis for non-parametric data with Dunns post-hoc test. A p-value (two tailed) of <0.05 was considered statistically significant. SPSS 21 (SPSS, Inc, Chicago, Illinois, USA) A value of p<0.05 and GraphPad Prism 4.0 software (La Jolla, CA, USA) statistical software was used to perform the statistical analyses. Monocytes counts were interrogated between day 1 and day 14 post infarct using paired t-test for parametric data and Wilcoxon rank test for non-parametric data.

2.3.3 Measuring monocyte subset counts

2.3.3.1   Sample collection:

Patients who consented for study after fulfilling inclusion criteria, phlebotomy was carried out via routine aseptic venesection at the point of recruitment into the study after attaining full consent. Venesection was attained at a single puncture with a 22 gauge wide bore sterile needle (to reduce risk of red blood cell haemolysis) into EDTA-containing tubes after the patients rested for 20 minutes (Chong et al., 2004). The venous samples collected were stored in the dark and transported from the recruitment site to the Centre for cardiovascular sciences

laboratory within an hour after which processing was initiated. Details for each analysis and sample preparation is included in the relevant chapters (Monocyte counts and phenotypic characterisation- chapter 2, section 2.3.3.2; Phagocytosis- chapter 3, section 3.3.3.; IKKβ- chapter 4, section 4.3.2). Patient serum was stored at -70 ^{O}C after centrifugation as described in chapter 4, section 4.3.3 and 4.3.4.

Coronary artery samples were aspirated from the guide catheter after the culprit vessel was stented and where applicable and aspiration catheter was utilised. Samples from the coronary arteries were stored and transferred in similar technique to venous samples.

In order to define monocyte subset counts in health, disease and over time, flow cytometric analysis of the fluorescence-labelled antibodies directed against specific cell surface receptors was undertaken. Flow cytometric analysis was performed using the BD FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK). VentuirOne, Version 3.1 software (Applied Cytometry, Sheffield, UK) was employed for data analysis. Flow cytometric analysis was performed by a single operator to minimize analytical error, (table 4 and 5 for reproducibility values) (Chughtai et al., 2011). The analyses were performed on whole blood samples obtained by Dr A Ghattas.

2.3.3.2 Immunophenotypic characterisation of monocyte subtypes and absolute count quantification

A mastermix, which includes mouse anti-human monoclonal fluorochrome-conjugated antibodies anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-PE (clone MφP9, BD), anti-CD42a-PerCP (clone Beb1, BD) and anti-CCR2-APC (clone 48607, R&D) CD14 2.5 μl, CD16 2.5 μl, CD42a 5 μl and CCR2 2.5 μl fluorochrome labelled antibodies, was prepared. Mastermix (12.5 μl) was mixed with 50μl of fresh EDTA anticoagulated whole blood in BD TruCount tubes (BD) containing 50,000 fluorescent count beads using an electronic micropipette. The TruCount tube was gently mixed on the vortex for 3 seconds. The tube was then incubated for 15 minutes in the dark at room temperature, shaking with a horizontal shaker (set at 500 units). Lysis was then performed by adding 450

µl pre-diluted BD FACS Lyse solution and the sample was then incubated for a further 15 minutes after being mixed gently on the vortex. Before analysing the sample through the flow cytometer, the sample was diluted with 1.5 ml of PBS solution, gently mixed again and then immediately analysed using the flow cytometer. Monocytes were selected using gating strategies and using forward and side scatter to exclude natural killer lymphocytes, and granulocytes. Granulocytes were excluded by CD14 expression versus side scatter (Figure 4, panel b). CD16+CD14- natural killer lymphocytes were excluded from CD16+ monocytes based on ungated CD14 versus CD16 expression (Figure 4, panel c). Mon 1 were identified as CD14++CD16- cells (Figure 4, panel e). The CD16+ cells (Figure 4, panel e) were separated into Mon 2 and Mon 3 based on their expression of CCR2. Absolute counts of monocyte subsets (cells/µl) were obtained by calculating the number of monocytes proportional to the number of count beads according to the manufacturer's recommendations. Definitions of CD14, CD16 and CCR2 positivity were made using appropriate isotype controls (Ziegler-Heitbrock et al., 2010).

The methodology developed differentiates between monocyte subpopulations according to surface expression of CD14, CD16 and CCR2. Three populations are recognized namely: a CD14++CD16-CCR2+ (Mon 1), CD14+CD16+CCR2+ (Mon 2) and CD14+CD16++CCR2- (Mon 3) subsets. Data was collected on 10,000 total cell events.

Aston University

Illustration removed for copyright restrictions

Figure 4: Absolute count gate from a recruited patient: A FSC/SSC plot (forward and side scatter plot) of a whole blood sample. All monocytes are gated within the circled area. Other leukocyte populations detected e.g granulocytes with higher side scatter are later removed. B, shows individual monocyte subpopulations to delineate different surface expression of CD14 and CD16 (X and Y axis respectively) to differentiate between Mon 1 and Mon 3. C, shows differentiation of the CD16+ subpopulations according to CCR2 expression, with CCR2 positive populations being Mon 2 (Shantsila et al., 2011)

This is a robust and reproducible protocol, for discriminating between different monocyte subpopulations based on surface expression of CD14 CD16 and CCR2 with good reproducibility has been developed (Shantsila et al., 2011). The coefficient of variation for the intra-assay reproducibility of the assay was: Mon 1: 5%, Mon 2: 6.5%, Mon 3: 6.6% Table 4). This was based on samples collected from healthy volunteers (N=2), each repeated six times. The mean and standard deviation are reported below. Inter-assay variability was from 2.5%-5.3 (table 5)

Table 4: Intra-assay variability of flow cytometry for measurement and quantification of monocyte

		Total monocyte count (cells/µl)	Mon 1 (cells/µl)	Mon 2 (cells/µl)	Mon 3 (cells/µl)
Subject 1	**Sample 1**	362.84	314.42	30.26	18.16
	Sample 2	338.35	304.25	34.17	20.48
	Sample 3	384.69	319.99	37.52	17.35
	Sample 4	313.41	317.63	33.52	19.14
	Sample 5	356.2	288.54	35.66	17.33
	Sample 6	345.25	297.51	36.24	18.65
	Mean ± SD	350 ± 24.1	307 ± 12	35 ± 2.6	18.5 ± 1.2
	CV %	6.9	4.1	7.4	6.4
Subject 2	**Sample 1**	455.4	317.18	106.67	26.69
	Sample 2	412.67	298.87	100.18	26.24
	Sample 3	468	338.96	116.78	28.25
	Sample 4	479.65	352.78	117.88	24.58
	Sample 5	438.67	299.21	118.62	28.62
	Sample 6	468.87	319.62	112.62	29.66
	Mean ± SD	454 ± 25	321 ± 22	112 ± 7.3	27 ± 1.8
	CV %	5.4	6.7	11.3	6.7
Intra-assay CV average (%)		*6.2*	*5.4*	*6.5*	*6.6*

Total monocyte count, Mon 1, Mon 2 and Mon 3 as well as total MPA (monocyte platelet aggregates). Mean and standard deviation are reported. CV: coefficient of variation (%).

Table 5: Inter-assay variability of flow cytometry for measurement and quantification of monocyte

	Total monocytes (per µl)	Mon 1 (per µl)	Mon 2 (per µl)	Mon 3 (per µl)	Total MPA (per µl)
Day 1	446	213	151	82.74	48
Day 2	470	236	167	89	51
Day 3	469	258	168	79	49
Day 4	457	247	152	76	52
Day 5	469	228	149	89	45
Day 6	468	239	163	85	50

Day 7	453	229	161	84	48
Day 8	479	239	169	80	49
Day 9	468	241	159	84	50
Day 10	478	221	151	82	48
Mean ± SD	466 ± 11	235.1 ± 13	159 ± 8	83 ± 4	49 ± 2
Inter-assay CV%	*2.3*	*5.5*	*4.9*	*4.9*	*4.0*

Total monocyte count, Mon 1, Mon 2 and Mon 3 as well as total MPA (monocyte platelet aggregates). Mean and standard deviation are reported. CV: coefficient of variation (%).

2.3.4 Ethical consideration

This study was performed in accordance with the Helsinki declaration. Ethical approval was granted by the Coventry Research Ethics Committee (reference number 09/H1210/11) and approval was obtained from the Research & Development departments at SWBH NHS Trust and Heart of England NHS Foundation Trust. All participants provided written informed consent.

2.4 Results

2.4.1 Absolute monocyte subpopulation count variation between peripheral versus coronary artery sampling

This investigation of monocytosis in STEMI, with its various subpopulations, on clinical outcome and ventricular remodelling, is based on systemic peripheral blood analysis. To demonstrate the relevance of this for the site of infarct, a study was undertaken to compare the absolute monocyte subpopulation count between the peripheral blood samples, and those obtained directly from affected coronary artery. Blood samples were thus attained directly from the occluded coronary artery in STEMI patients undergoing thrombus aspiration during percutaneous coronary intervention. These samples were obtained peri-intervention. The monocyte subpopulation counts in blood taken from the occluded artery were compared to counts obtained from samples taken peripheral from the antecubital fossa post-procedure.

There was no statistical difference between monocytes counts of the three subsets between the two sites. The results are illustrated in (Figure 5).
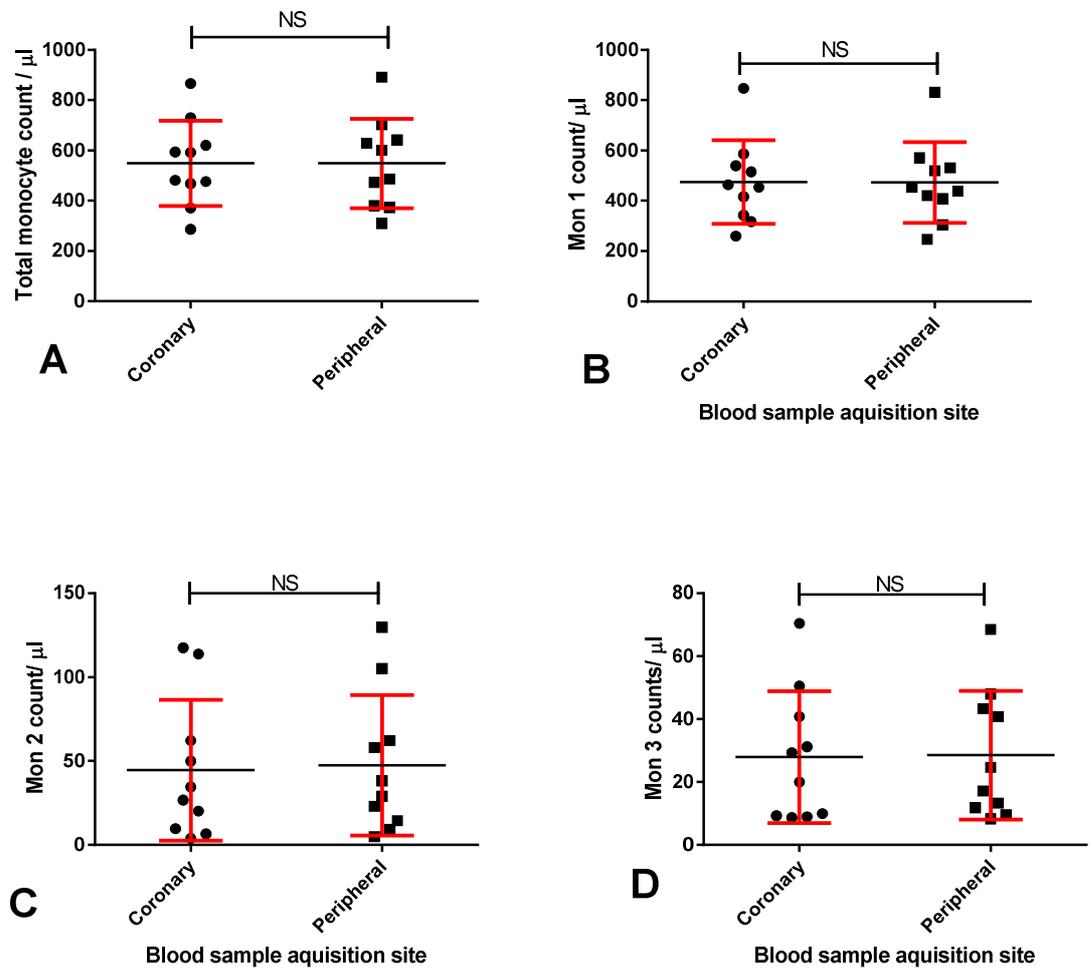
Figure 5: Monocyte subpopulation counts are not different between coronary artery circulation and peripheral venous samples: A total of ten STEMI patients were recruited with blood samples acquired simultaneously from the coronary artery post thrombus aspiration during percutaneous coronary intervention as well as peripherally from the brachial vein. The samples were analysed by flow cytometry for total monocyte counts, Mon 1, Mon 2, and Mon 3 counts. Statistical significance was tested using a paired t-test.

2.4.2 Time-delay of patient recruitment and effects on monocyte counts

It is logistically impossible to recruit patients at the same time with the same time gap between treatment and recruitment. Hence the effect of time delay between recruitment and sample analysis was investigated to direct time of recruitment, within the first 24 hours. Here blood samples were attained from seven consecutive STEMI patients. Blood samples were obtained

at 3 hours, 6 hours, 12 hours and 24 hours after the performance of coronary revascularisation.

Using paired t-test the differences between different time points were investigated.

Table 5: Effect of temporal variation on monocyte count

	3 hours post PCI	6 hours post PCI	12 hours post PCI	24 hours post PCI	pValue
Total monocytes, (cells/µl)	682 (592 – 769)	678 (624 – 752)	628 (618 – 736)	647 (575 – 694)	0.52
Mon 1 (cells/µl)	556 (468 – 639)	543 (469 – 617)	529 (479 – 592)	521 (438 – 585)	0.75
Mon 2 (cells/µl)	107 ± 40	103 ± 49	102 ± 43	96 ± 40	0.97
Mon 3 (cells/µl)	30 ± 23	39 ± 26	31 ± 19	32 ± 23	0.89

Samples were obtained and analysed for monocyte subset at 4 different time points after patients received percutaneous coronary intervention (PCI). Data are expressed as median with interquartile range in parenthesis for non-normally distributed data and as mean plus standard deviation for normally distributed data. Statistical significance was tested using paired t-test. There was no statistical difference in the different monocyte subsets between the four different sampling time points. Normally distributes data are described as mean ± SD, non-normally distributed data are presented as median (with range).

2.4.3 Patient Results

2.4.3.1 Monocyte subset counts in stable artery disease and healthy individuals

All three patient groups (i.e. STEMI, stable coronary artery disease and healthy individuals) were matched for age and gender (one way Anova with post Tukey post hoc, p= 0.42 and 0.84 respectively). Given the exclusion and recruitment criteria (chapter 2, section 2.3.1 and appendix I), there were no inherent differences between STEMI patients, or stable coronary artery disease patients.

Patients were recruited as described in (chapter 2, section 2.3.1 and appendix I). Total monocyte count, Mon 1 and Mon 2 monocytes were significantly increased in patients with STEMI compared with patients with stable coronary artery disease (SCD) and control groups

(table 7). There was no significant difference between stable coronary artery disease and healthy controls (p = 0.52) (Figure 6).

Table 6: Total monocyte count and monocyte subset counts differ according to health and disease

Monocyte subsets	STEMI (n=40)	Stable CAD (n=20)	Healthy controls (n=40)	p- Value
Total monocytes (cells/μl)	842 (595 – 1109)*†	439 ± 148	353 ± 92	<0.0001
Mon 1 (cells/μl)	646 ± 258*†	341 ± 102	281 (237 – 338)	<0.0001
Mon 2 (cells/μl)	191 ± 143 *†	66 ± 53	30 (16 – 75)	0.02
Mon 3 (cells/μl)	58 ± 38	46 (7.2 – 10.6)	9 (4 – 30)	<0.0001
Mon 1 proportion (%)	89 (72 – 93)	70 ± 17*	81 (67 – 86)	0.007
Mon 2 proportion (%)	21 ± 13*	13 ± 10	8 (5 – 18)	0.01
Mon 3 proportion (%)	7 ± 3	9 (7 – 11)	3 (1.2 – 8)	0.50

ST elevation myocardial infarction (STEMI), healthy controls and stable coronary artery disease (CAD) blood samples were analysed by flow cytometry for total monocyte, Mon 1, Mon 2, Mon3. Statistical significance was tested using ANOVA with Tukey post- hoc test for normally distributed parameters and Kruskall and Wallis for non-parametric data with Dunns post-hoc test, where (*) represents significant difference between STEMI and controls and (†) represents STEMI versus CAD. Normally distributed data are described as mean ± SD, non-normally distributed data are presented as median (with range).
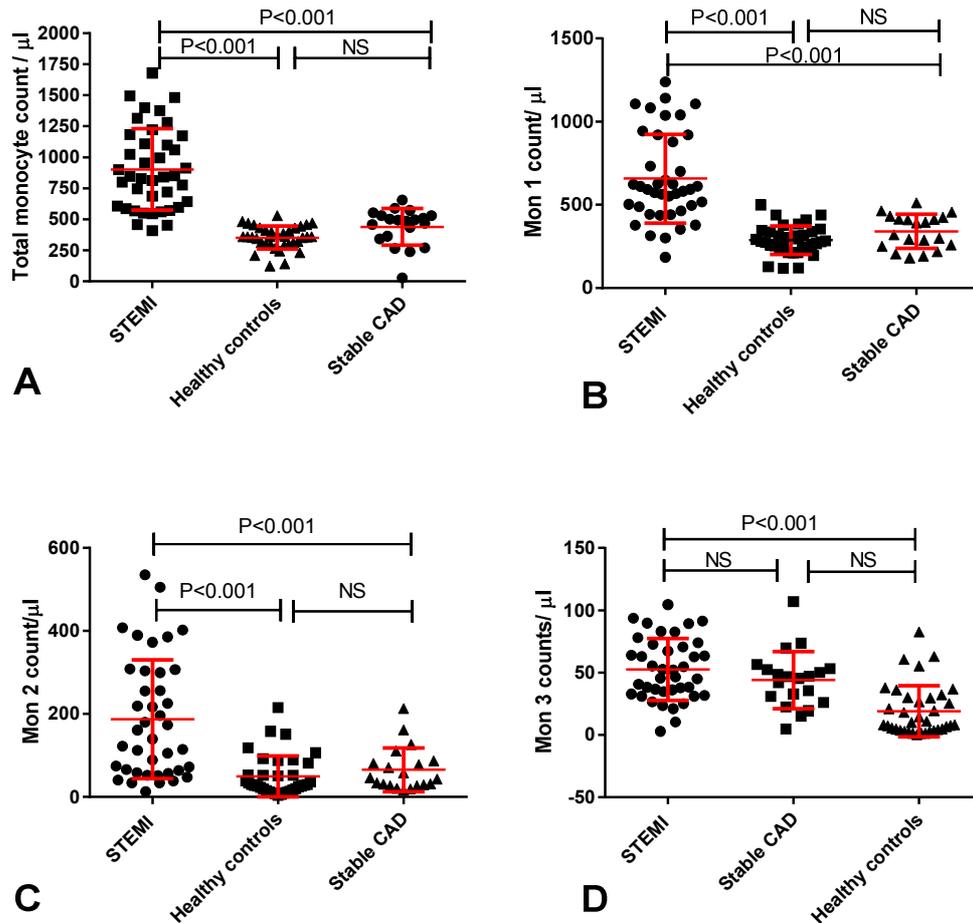
Figure 6: Monocyte subpopulations counts in different health states: A- Total monocyte counts were significantly higher in STEMI patients compared to stable coronary artery disease and healthy controls. B- Mon 1 and C- Mon 2 counts also had a similar pattern of phenotypic variation. Mon 3 counts were only higher in STEMI patients compared to healthy controls. Whole blood was obtained from 10 STEMI patient both directly from the coronary circulation during PCI and later peripherally form venous puncture.

2.4.3.2   Recruited STEMI patients' demographics

In total, 209 STEMI patients who met inclusion criteria were recruited from March 2011- Dec 2012. The average age of the recruited patients was 61 ± 11 (mean $\pm$SD) years with predominance of males being recruited accounting for 75% of subjects. This reflects the background epidemiology of ischaemic heart disease. There was a relatively short delay in patients presenting to hospital after the onset of symptoms with an average time of hospital admission post onset of symptoms of 4.7 ± 2.6h. In this patient population group there was a high prevalence of risk factors contributing to developing ischaemic heart disease (table 8)

including: smoking, hypertension, hypercholesterolaemia, history of previous myocardial infarction, as well as past medical history of chronic stable angina.

Table 7: Recruited patients demographics, past medical history and drug history

		N=209
Demographics	Age	61 ± 11
	Sex	75% males
	Time from symptoms onset to presentation to hospital (hours)	4.7 ± 2.6
	Time from hospital presentation to recruitment to study (hours)	16 ± 5
Biochemical profile at recruitment	Peak Troponin (ng/ml)	7065 (4521 – 14461)
	Peak Creatinine kinase (mg/ml)	1466 (894 – 1698)
	Total WCC (mmol/L)	11 (8.2 – 13.1)
	Estimated GFR (ml/min/1.73m^2)	74 ± 16
	Total Cholesterol (mmol/L)	4.9 (4.3 – 5.3)
	Body surface area	1.9 (1.8 – 2.0)
Past medical history at the point of recruitment (%)	Diabetes	23
	Hypertension	53
	Smoking	56
	Hypercholesterolaemia	43
	History of angina	5
	Previous myocardial infarction	15
	Obesity	15
	COPD	14
	CVA	8
	CABG	4
	Previous PCI	9
	LAD	41
	Inferior STEMI	38
Drug history at recruitment (%)	Aspirin	36
	Clopidogrel	3
	Statin therapy	34
	Calcium channel blocker	21
	Beta blocker	18
	Loop diuretic	15
	Thiazide diuretic	9
	ACE inhibitor	41
	Nitrates	7

A total of 209 patients were recruited. ACE inhibitor- angiotensin converting enzyme inhibitor; CABG: coronary artery bypass grafting; LAD: left anterior descending artery; COPD: chronic obstructive airway disease; CVA: cerebrovascular accident PCI: percutaneous coronary intervention; WCC: white cell count. Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median with (interquartile range) for non-normally distributed data

2.4.3.3   Monocyte subpopulation correlations with patients' demographics

Using Pearson's correlation analysis, total monocyte counts as well as Mon 1 correlated significantly with peak troponin (R = 0.19, p= 0.03). Both counts also correlated significantly with classical cardiovascular risk including myocardial infarction and history of PCI (table 9).

Table 8: Correlation of monocyte subpopulations with recruited patients' past medical history and biochemical markers

eGFR: estimated glomerular filtration rate; MI, myocardial infarction; PCI, percutaneous

	Total Monocyte count		Mon 1		Mon 2		Mon 3	
	R	p - Value	R	p - Value	R	p – Value	R	p - Value
Age	0.07	0.31	0.06	0.38	0.06	0.39	-0.02	0.78
Peak troponin	0.19*	0.03	0.17*	0.04	0.14	0.11	-0.03	0.74
CK troponin	0.13	0.07	0.08	0.26	0.16	0.02	0.03	0.73
Total WCC	0.04	0.56	0.07	0.35	-0.05	0.5	0.04	0.57
eGFR	0.06	0.40	0.04	0.54	0.04	0.56	0.07	0.34
Total cholesterol	-0.03	0.63	-0.04	0.59	0.02	0.74	-0.09	0.19
MI	0.18*	0.01	0.15*	0.03	0.12	0.08	0.10	0.14
PCI	0.15*	0.03	0.12	0.09	0.1	0.16	0.12	0.08
Hypertension	0.06	0.44	0.09	0.18	-0.07	0.33	0.03	0.64
Diabetes mellitus	0.07	0.33	0.08	0.28	-0.02	0.82	0.09	0.21
Smoking	0.00	0.95	-0.04	0.61	0.05	0.44	0.03	0.66

coronary intervention; WCC: white cell count' (Pearson's correlation was performed for all analytes).

2.4.3.4   Total monocyte and monocyte subset counts in recruited STEMI patients in a longitudinal study

There was a significant increase in the total monocyte counts as well as the three monocyte subset counts between the first 24 hours post infarction and at phase 2 (days 14- day 28 post index event). Interestingly there was no change in the proportion of Mon 1 and Mon 2 subpopulations between the two sampling time points. However, there was a significant increase in the proportion of Mon 3 at phase 2 with no significant increase in the total Mon 3 count. This is in keeping with the potential angiogenic and fibrotic reparative role of Mon 3 hence the increase in proportion during the recovery phase post infarction.

A concurrent significant decrease in the total number of monocyte platelet aggregates was also demonstrated at second sampling phase (day 14- day 28 post infarction) compared to recruitment during the index event. This may reflect the effect of therapeutic interventions that patients received on admission, including coronary intervention as well as antiplatelet therapy.

Table 9: Monocyte counts in STEMI patients at Day 1and second phase (day 14-28 days post infarct)

Monocytes	Median Florescence intensity at day 1 ± Standard deviation N= 208	Median Florescence intensity at phase 2 ± Standard deviation N= 151	Parametric analysis (paired t-test) pValue
Total monocyte count (cells/µl) **Mon 1 (cells/µl)** **Mon 2 (cells/µl)*** **Mon 3 (cells/µl)***	852 ± 294 634 ± 240 157 ± 113 61 ± 40	564 ± 288 399 ± 147 93 ± 72 51 ± 35	P< 0.01 P< 0.01 P< 0.01 0.005
Mon 1 proportion (%) **Mon 2 proportion (%)** **Mon 3 proportion (%)**	73 ± 14 18 ± 11 8 ± 6	78 ± 68 17 ± 12 10 ± 8	0.35 0.17 0.001

Normality testing was by Shapiro Wilk test and distribution curves. Dependent t-test was used as data were normally distributed. There was significant differences between total monocyte count and all three subsets. All cell counts were higher in the first 24 hours post infarction compared to day 14.

2.5 Discussion

This study was designed to understand the effect of STEMI on monocyte counts. Given the fact that ST elevation myocardial infarction is an acute occlusion of a coronary artery, there was a need to determine the difference, if any, in monocyte counts obtained centrally from a coronary circulation versus peripherally from a large calibre vein. In a small pilot study of 10 STEMI patients, no statistically significant differences were observed in monocyte counts analysed from blood obtained from the culprit coronary artery and the counts acquired from the peripheral circulation. This provides validity to the use of systemic blood samples for investigating monocyte counts in this work.

The rapid and persistent increase in monocyte counts acutely is in keeping with results by Dutta et al. where the increase in monocyte counts occurred within 24 hours of infarction with rapid transit time of macrophage and monocyte counts (Dutta et al., 2015). In another study there was a peak of monocyte and macrophage counts within 24 hours of infarction with a trough 2 days post infarction (Heidt et al., 2014). Its worthy of note that both these studies were using murine models. In contrast a small series in humans indicated that monocyte counts continue to rise up and reached a peak to day 3 post myocardial infarction (Tsujioka et al., 2009). Elsewhere, Tapp et al. reported an increase in Mon 1 and Mon 2 counts up to three days post ACS (Tapp et al., 2012). All together these findings could suggest the rapid production rate, transition time of monocytes through the arterial-venous system eventually reaching the coronary circulation and finally the rapid differentiation rate to macrophages and dendritic cells in the target tissue during the acute phase. The discrepancy in these time frames will ultimately require a study based on hourly monitoring of monocyte count in a number of subjects post myocardial infarction (not healthy individuals). In the early hours post infarction, it is difficult to exactly postulate the time frame during which monocyte counts start to increase, or indeed are at the highest. Such a study will ethically be difficult to implement as it is likely to impinge on patients' comfort during the peri-acute phase of the myocardial infarction. Also the time delay from symptoms onset, and hence acute coronary occlusion, to presentation to hospital will pose a time lag during which monocyte counts cannot be studied.

In recruiting STEMI patients it is logistically impossible to unify the time between having PCI and patients recruitment in this study. Hence, I investigated the effect of time delay between receiving treatment and recruitment time. This is of paramount importance given the dynamic differentiation and prompt physiological response to the acute inflammatory state posed by the myocardial infarction. Hence monocyte counts were measured at 3,6, 12, and 24 hours post PCI. The data shown here, in a group of 7 patients, confirmed that there was no significant change in monocyte counts acquired.

Our group has published previously on the effect of effect of exercise and diurnal variation on monocyte subpopulation. This suggested a diurnal variation in Mon 2 expression with highest Mon 2 counts expressed at 6:00 PM and lowest counts at 6:00 AM. There was no diurnal difference in total monocyte count or in Mon 1 and Mon 3 counts. We also demonstrated that a delay of up to 2 hours prior to sample analysis after venepuncture did not significantly affect counts of monocyte subsets. By 4 hours, the numbers of Mon 2 and Mon 3 significantly increased (p=0.005 and p=0.02, respectively). Studying the effect of these different physiological states and diurnal variation not only helps to unify the methodology utilized but also limits confounding factors that could significantly alter results.

Establishing the differences between monocyte counts in different health states in a cross sectional study, was essential in confirming the acute variation of monocyte and monocyte platelet aggregate counts in the STEMI study population. A significant increase in all monocyte subset counts in STEMI patients was observed compared to healthy individuals and stable coronary artery disease patients. These results concur with an earlier observational series by Tapp et al., who also described the dynamics of alterations of surface marker expression of the three monocyte subsets acutely after an infarction (Tapp et al., 2012). There was a prominent increase in Mon 2 but no changes in Mon 3 acutely post infarct. Significant increases in Mon 2 CD14 and CCR2 expression, and reduction in CD16 expression were also described by Shantsila et al. reported that the expression of IL-6R and VCAM-1 are reduced on circulating monocyte subsets in STEMI patients (Shantsila et al., 2013). These changes are hypothesized to represent a regulatory feed-back mechanism aimed at rebalancing the marked

inflammation which is typically present following acute MI or indeed to aid selective homing of monocytes with high receptor expression to damaged myocardium.

In stable coronary artery disease subjects, there were previous reports from small case series indicating an increase in CD16+ve percentages (likely to correspond to Mon 2 and Mon 3 here) in patients with symptomatic coronary artery disease compared to healthy controls even after adjusting for common risk factors including smoking and hypertension (Berg et al., 2012). In another study of stable coronary artery disease patients, the number of cardiovascular risk factors independently predicted elevated CD14++CD16+ monocytes (likely to represent Mon 1 and Mon 2) (Woollard and Geissmann, 2010). Interestingly inflammatory monocytes were not associated with stable atherosclerotic plaque assessed angiographically (Imanishi et al., 2010). However there was an association in patients with unstable angina, and thus unstable vulnerable atherosclerotic plaques (Imanishi et al., 2010).

This study is the largest study prospectively recruiting consecutive STEMI patients. The distribution of the three different monocyte subsets has been described in STEMI patients both acutely and 2 weeks after the index event of the myocardial infarction. Confirming previous findings, there was statistically significant higher counts of all three monocyte subsets at 24 hours post infarction when compared to counts 14 days post-STEMI. This acute increase in counts could be explained by the acute augmentation in expression of CCR2 on the surface of the myocytes (Hilgendorf et al., 2014, Dutta et al., 2015) as a response to the acute hypoxic state induced by the myocardial infarction as well as release of multiple inflammatory cytokines, e.g. TNF-α. The rapid mobilization of monocytes from the bone marrow as well as spleen is required acutely to replenish the supply of the rapidly consumed macrophages post-STEMI (Swirski et al., 2009). It is now recognized that there are resident macrophages within the myocardial tissue even in stable healthy state (Heidt et al., 2014). These are thought to be essential in maintaining healthy myocyte microenvironment. Acutely the increase in macrophage exit leads to an increase demand on monocytes mobilization and hence the higher counts measured in the hypoxic infarcted state (Heidt et al., 2014).

There is a multitude of observational studies describing surface makers and cytokine/chemokine associations of monocyte subpopulations in different pathologies

(Shantsila et al., 2011, Tapp et al., 2012, Zawada et al., 2011). However, there is a paucity of data describing the functional characteristics of these subsets. Indeed the effect of the above described increase in monocytes subpopulations, in the peripheral and central circulation, on patients' clinical outcome as well as the general function of the cardiac muscle post infarction, is yet to be determined. These unaddressed areas are the focus of the remaining studies of this thesis.

One limitation of my study design is that the glucose levels were not documented in STEMI patients at the point of admission. It is has been previously described that admission glucose levels in heart failure patients are an independent predictor of outcome, regardless of whether the patients have a history of pre-diagnosed diabetes mellitus or not. Monocyte counts association with glucose levels and clinical outcome remains to be an area requiring further investigation.

2.6 Conclusion

In the largest study recruiting STEMI patients and investigating numerical distribution of the newly characterised three monocyte subpopulation counts, there was a significant increase in inflammatory subset counts which were higher in the acute phase as well compared to counts 14 days post infarcts.

This work confirms with greater statistical power findings from pilot data and sets the path for the need to investigate the function of these subsets in the acute physiological state of STEMI.

Chapter 3

Monocyte subset chemotaxis and phagocytic activity

3.1 Introduction

Typically monocyte-derived macrophages have been associated with phagocytic function. With the avid interest in the role of macrophages and their precursors in the healing and recovery post infarction, it is hypothesised that the phagocytic activity of both monocytes and macrophages can account for their reparative process via removal of the infarcted hypoxic tissue damage hence influencing ventricular remodelling. A number of murine models have investigated the phagocytic activity of monocytes and macrophages. It was shown that the early influx of LyC6hi monocytes early post infarction are associated with poor outcome and adverse cardiac remodelling.

The behaviour of monocyte subpopulations is the centre of extensive research in a multitude of acute and chronic pathological conditions, including chronic kidney disease, cerebrovascular events, liver fibrosis, and patients who sustained an acute coronary syndrome or who have stable coronary artery disease. In fact peripheral monocytosis has been associated with poor outcome in atherosclerosis and coronary artery disease (Rogacev et al., 2012), as well as chronic kidney disease (Rogacev et al., 2011). The precise functions of these monocytes subsets remain ill defined, with limited gene-expression (Wong et al., 2011) and surface marker profiling (both angiogenic and scavenger) (Zawada et al., 2011, Shantsila et al., 2013). To date there has been no characterisation of the phagocytic activity of the different monocyte subpopulations and very few papers assess the functional characteristics of these subpopulations in healthy participants and under various normal physiological conditions (Shantsila et al., 2012).

Here we characterise the phagocytic capacity of the three defined monocyte-subpopulations in a novel flow cytometry assay. In doing this we assess further the phagocytic capabilities of monocytes in healthy individuals and investigate the effect of various physiological and disease states on phagocytic function of monocyte subpopulations.

3.2 Aims and Hypothesis

The aim of this chapter is to describe a flow cytometric method for measuring the phagocytic activity of the three different human monocyte subpopulations: Mon 1, Mon 2 and Mon 3. I will discuss the validation experiments carried out for the phagocytosis protocol; consider the effect of time delay in sample preparation on the phagocytic activity; investigate any effect of circadian rhythm variation on phagocytic activity of monocytes; and examine the effect of exercise. The beneficial effect of exercise in reducing mortality from cardiovascular disease in individuals with coronary artery disease, as well as reducing the generalised inflammatory state associated with ageing has been intensely studied. I hypothesis that exercise reduces the phagocytic activity of monocytes.

The difference in the phagocytic activity of the three monocyte subsets healthy controls, patients with stable coronary artery disease, STEMI and healthy controls will be investigated.

These experiments have been designed to test the hypothesis that the phagocytic activity of monocytes in STEMI patients is significantly higher compared to patients with stable coronary artery disease or healthy individuals.

3.3 Methods

3.3.1 Study subjects

Healthy controls, stable coronary artery disease patients, and STEMI patients were recruited as discussed in appendix I and section 2.3.1 of chapter 2. Briefly healthy individuals were consented for participation and recruited in City Hospital, Birmingham, UK. Stable coronary artery disease patients were recruited from City Hospital, Birmingham, UK or from Birmingham Heartlands Hospital. The inclusion criteria for STEMI patients are described in Chapter 2. All patients underwent primary coronary angioplasty as the default revascularisation strategy, and were initiated post-procedure on standard post-STEMI cardiovascular therapies as described in Chapter 2. Patients were recruited within 24 hours of admission. Further details on recruitment of study subjects are also included in appendix I.

The exercise studies were performed by 12 healthy subject (age 32±5, males, 60%). These subject had no significant past medical history and were not on any regular medication. They were recruited from Centre of Cardiovascular Sciences. Subjects were asked not to partake in aggressive physical activity in the 24 hours pre exercise experiment.

3.3.2 Flow cytometry

Flow cytometric analysis was performed using the BD FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK [BD]) as published previously (Shantsila et al., 2011).

3.3.3 Phagocytosis Assay

Whole blood (6ml) was collected into a lithium-heparinised bottle and stored on ice *en route* for sample preparation and analysis. All sample processing was undertaken within one hour of collection. The phagocytosis assay utilises pHrodo *E. coli* BioParticles (Invitrogen). These neutralized *E. coli* contain a fluorochrome that is inactive at neutral pH. However upon phagocytosis of the bacteria, within the acidified phagosome the acidic environment activates the fluorochrome which is detected by flow cytometry.

For each blood sample to be analysed, 100 μl of whole heparinised blood was mixed with 20 μl of pHrodo™ BioParticles®. A control, lacking bioparticles was prepared in parallel, with 20μL of PBS. The samples were mixed gently by vortexing and incubated at 37ºC for 10 minutes. Following incubation, monocyte sub-populations were labelled with an excess of fluorochrome-conjugated monoclonal antibodies: anti-CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK), anti-CD14-APC (clone MϕP9, BD) were added to both the sample and control. The tubes were mixed gently and placed in the incubator again for a further 10 minutes. Red blood cell lysis was then performed with a 100 μl of lysis buffer (Component A- enclosed with pHrodo *E.Coli* BioParticles Phagocytosis Kit for Flow Cytometry (Invitrogen, California, UK)). The tubes were incubated at room temperature for five minutes, thereafter 1 mL of buffer B was added to the tubes. These were mixed and incubated again at room temperature for 5 minutes. The samples were then centrifuged at 500 g for 5 minutes at room temperature. The supernatant was decanted and the cell pellet resuspended with 1mL of PBS. The samples were then washed again and the cell pellet finally resuspended in 300μls of PBS, gently mixed and analysed on the flow cytometer. In order to discriminate the three monocyte subpopulations (Mon 1-3), a multi-step flow cytometric method was developed previously (Figure 4; Shantsila et al., (2011)). Initially (Figure 4 a), light scatter characteristics were used to differentiate monocytes from other leukocytes (granulocytes and lymphocytes). This gated population was then analysed based on CD14 versus CD16 staining to identify Mon 1, 2 and 3 (Figure 4 b). Further sub-analysis was then undertaken to assess the phagocytic activity (pHrodo fluorescence) of these monocyte subpopulations (Figure 7).
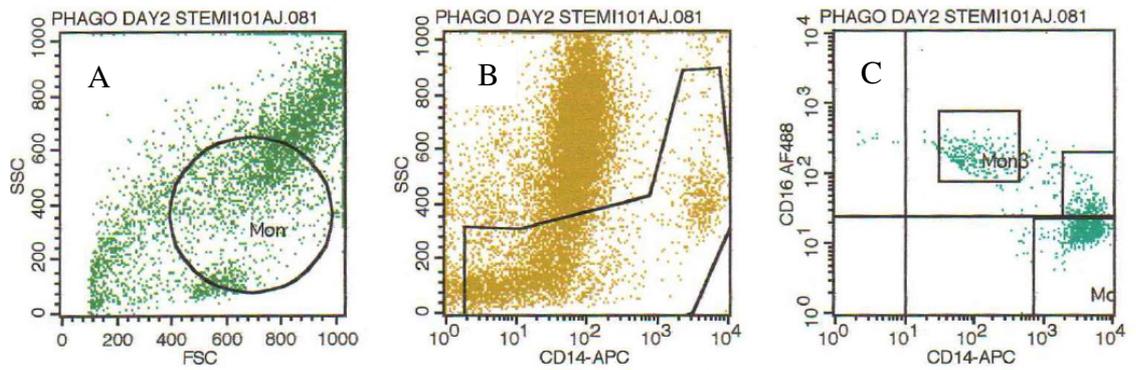
Figure 7: Flow cytometry gating for monocyte subset phagocytosis assay: Whole heparinised blood was incubated with *E. coli* pHrodo bioparticles and, after incubation, monocytes were stained with fluorescent anti-CD14 and anti-CD16 mAbs prior to lysis of red cells, fixation and flow cytometric analysis. A- Utilising forward scatter and side scatter to differentiate between monocytes, granulocytes and lymphocytes in whole blood. B- Discrimination between Mon 1 Mon 2 and Mon 3 monocyte subsets according CD14 and CD16 surface expression. C- Frequency histogram to show the relative phagocytic activity of the three monocyte subsets combined. Region M1 highlights positive fluorescence indicative of phagocytosis of *E. coli* pHrodo bioparticles.

The coefficient of variation of the phagocytosis assay was 5.4%, 7.1% and 1.4% for Mon 1, Mon 2 and Mon 3 respectively (table 11). These results were produced after analysing whole blood from three healthy individuals.

Table 10: Inter assay variability of the monocyte subset phagocytic assay

Parameter	Subject 1	Subject 2	Subject 3	CV%
Phagocytic activity of Mon 1 (MFI)	155.38	139.49	147.22	5.4
Phagocytic activity of Mon 2 (MFI)	155.38	137.00	138.24	7.1
Phagocytic activity of Mon 3 (MFI)	56.23	54.74	55.73	1.4

CV, coefficient of variation. Phagocytic activity of Mon 1, Mon 2, and Mon 3 were highly reproducible.

3.3.4 Analysis of monocyte CD14 and CD16 surface expression

For the analysis of cell surface antigen expression by the three monocyte subsets, 100μl of whole blood was incubated with mouse anti-human monoclonal fluorochrome-conjugated antibodies for 15 minutes in the dark. Subsequently red blood cells were lysed with 2ml of BD lysing solution® for 10 min, followed by washing in PBS and immediate analysis by flow cytometry. The three monocyte subsets were defined as CD14++CD16– monocytes (Mon 1), CD14++CD16+ monocytes (Mon 2) and CD14+CD16++ monocytes (Mon 3) in accordance with contemporary guidelines (Ziegler-Heitbrock et al., 2010) using anti-CD16-Alexa Fluor 488 (clone DJ130c, AbDSerotec, Oxford, UK) and anti-CD14-PerCP-Cy5.5 (clone M5E2, BD) antibodies.

3.3.5 Effects of physical exercise on phagocytic activity of monocyte subsets

Treadmill exercise was performed using Case 16 exercise testing system (Marquette Medical Systems Inc, Milwaukee, WI, USA) according to Bruce protocol with the volunteers exercising to exhaustion, as previously described (Watson et al., 2010). Venous blood samples were taken immediately pre-exercise, 15 minutes, 60 minutes and 24 hours post-exercise (four samples in total). The choice of time points for the post-exercise sampling was based on preliminary results from two volunteers with samples obtained at 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 24 hours. These time frames were determined from a previous paper from the laboratory investigating the effect of exercise on monocyte counts (Shantsila et al., 2012). All participants were asked to abstain from other strenuous physical activity for the period of the study.

3.3.6 Diurnal variation

Diurnal variations in monocyte subsets phagocytic activity were assessed in 6 healthy volunteers (age 36 ± 8.7 years, 5 male). Blood samples were taken at 6 hourly intervals, starting at 6 a.m. and finishing 6 a.m. on the following day (5 samples in total).

3.3.7 CD14+ monocyte chemotaxis

In a cross over study design, 100µl of patient plasma (5 patients having sustained STEMI, 5 had STEMI and developed a MACE event, 5 stable coronary artery disease) was added to a 2.5×10^5 of CD14+ cells isolated from healthy control subjects. These were placed in separate well inserts of an 8 well plate in triplicate. Control treatments were isolated monocytes with no patient serum, and isolated monocytes stimulated with 500ng/ml of LPS. The wells were placed in 8 well plate coated with 100µl of 100ng/ml of MCP1. The cell migration was then analysed using Cell-IQ® MLF (CM Technologies- Midland, ON, Canada). The samples were examined with 10x objective using a green filter cube and the number of migrating cells was recorded. The rate of migration was recorded hourly for a maximum of 9 hours.

3.3.8 Statistical analysis

Data distribution was assessed for normality by the Kolmogorov method (Wayne, 1990). Data are expressed as mean± SD for normally distributed parameters and median (interquartile range) for non-normally distributed parameters. Statistical significance of the difference in phagocytic activity between different monocyte subsets in health, disease and exercise was determined by repeated measures ANOVA with Tukey post-hoc analysis. The Friedman test was utilized for non-normally distributed parameters. A value of $p<0.05$ was considered statistically significant. Statistical analysis was performed with SPSS18.0 software and GraphPad Prism 4.0 software (La Jolla, CA, USA).

3.4 Results

3.4.1 Characteristics of recruited subjects

Healthy individuals recruited into the exercise programme were age 35.3±7.62 years (60% male). Healthy controls were recruited for the cross sectional study were age and gender matched to a group of STEMI and stable coronary artery disease patients. There were no differences between both groups in past medical history or medication at time of recruitment (Chapter 2, section 2.3.1 and appendix I).

3.4.2 Chemotaxis of isolated monocytes

In order to understand whether factors present in plasma may influence monocyte function, plasma from patients was incubated with primary monocytes from healthy donors and their migration to MCP1 was measured by cell IQ. There was no significant difference in monocyte migration to MCP1 between plasma from different patient groups (Figure8). The variance within group accounted for 15 %of the migrated cell number. There appeared to be a trend of increased rate of monocyte cells mobility in the MACE patients compared to SCD and STEMI patients who did not suffer from MACE with higher counts attained at the end of 9 hours. However, migration of monocyte subsets with no serum, and those stimulated with LPS also appeared to have a higher mobility rate compared to SCD and STEMI patients with no MACE. The total count of migrated CD14+ve cells incubated with MACE patients serum, isolated monocytes with no serum and isolated monocytes stimulated with LPS were higher at the end of 9 hours compared to counts from SCD and STEMI patients. It is very plausible that the serum of recruited patients had an inhibitory mechanism that controls the rate of monocytes chemotaxis and migration acutely. Isolated cells with LPS and those that are serum free lack this inhibitory effect and hence the rate of chemotaxis is higher. It appears that patients who suffered a MACE event loose this inhibitory effect and hence are able to migrate at a higher rate.

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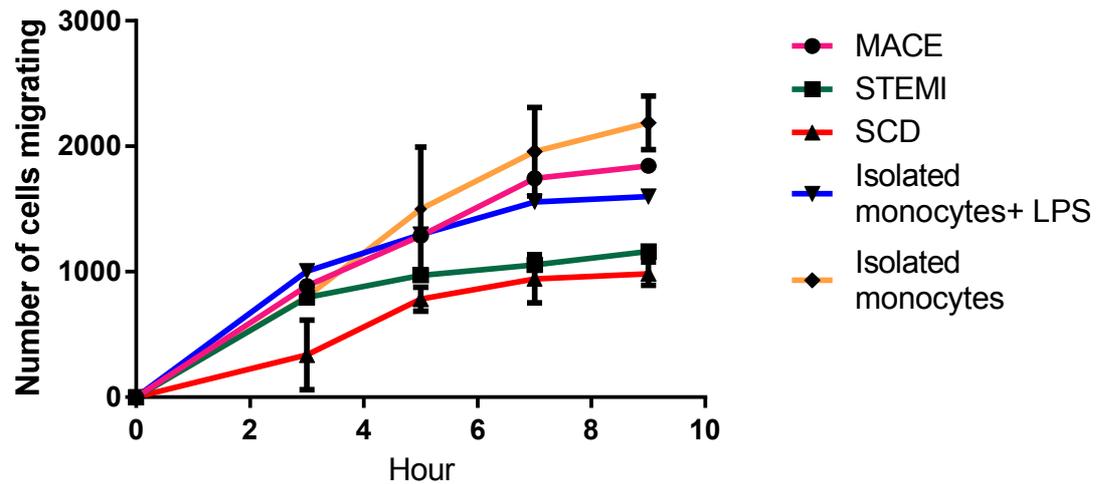
Figure 8: Monocyte migration to MCP-1 is affected by plasma from patients post-STEMI: CD14++ monocytes (2.5×10^5) were isolated from 30ml of venous blood from healthy individuals. Isolated cells were then incubated with 100µl of recruited patients' plasmas from MACE patients (n=5) STEMI patients (n=5, with uncomplicated recovery); stable coronary artery disease (n= 5, SCD). As controls each plate included isolated monocytes with no serum added, and isolated monocytes stimulated with LPS at 500ng/ml. The base of the well was coated with 100ng/ml of MCP-1 as the chemoattractant for subsets. The mobility of monocytes were measured using Cell-IQ® MLF. Data are expressed as the mean SD as error bars included in the SCD and isolated monocyte patients. One way Anova with Tukey post hoc analysis was utilised to test the differences between the different subject groups. There was a significant difference between MACE patients compared to stable coronary artery disease patients (SCD), and those who had STEMI but no MACE (p<0.001).

3.4.3 Surface CD14 expression and effect on phagocytosis

Given the high surface expression of CD14 (an LPS receptor) on both Mon 1 and Mon 2 and the phagocytosis *E-coli* bioparticles simulating a Gram negative organism (i.e. LPS-bearing), the requirement for CD14 in phagocytic activity was examined. CD14 expression was analysed versus *E. coli* uptake (i.e. MFI from phagocytosis assays). There was a negative correlation between the cell surface CD14 surface expression on Mon 1 cells and *E. coli* phagocytosis (r= -0.68, p=0.007), with a stronger negative correlation that was significant with Mon 2 (r= -0.64, p= 0.04). These data suggest that CD14 levels *per se* do not explain the different levels of phagocytosis noted within the subpopulations of monocytes. Interestingly there appeared to be a positive correlation between Mon 3 and *E. coli* (noting that Mon 3 are only weakly positive for CD14) (Figure 9).
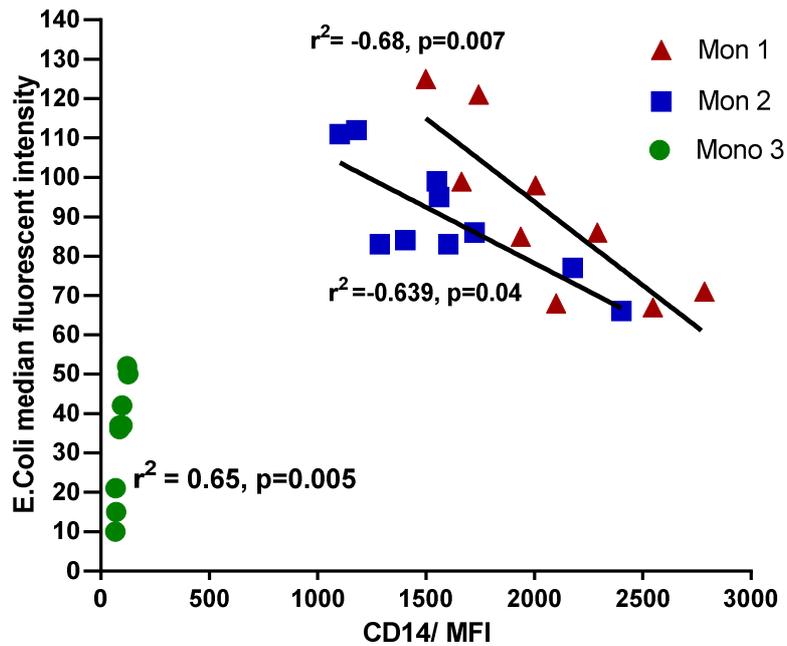
Figure 9: Mon 1 and Mon 2 subsets phagocytosis of *E. coli* correlates inversely with CD14 expression: CD14 surface expression levels against uptake of *E.coli* bioparticles as mean fluorescent intensity (MFI). Statistical analysis was performed using simple correlation analysis.

Given the inherent differences in monocyte subset functions assessed in the phagocytosis assay, it was of interest to identify the possible effect of physiology and pathology on this function.

3.4.4 Effect of exercise on phagocytic activity

The effects of exercise on the dynamics of phagocytic activity of monocytes was assessed in 12 healthy volunteers (age 35.3 ± 7.62 years, 8 male). To address the effect of exercise on monocyte subset function, healthy subjects were subjected to an incremental treadmill exercise for 14 minutes (Bruce protocol), equivalent to 12.9 mets (Shantsila et al., 2012, Hamm et al., 2011, Watson et al., 2010). At various time points post-exercise, blood samples were harvested and phagocytic activity assessed in comparison to baseline (pre-exercise) levels. The phagocytic activity of Mon 1 and Mon 3 was increased significantly at 24 hours post-exercise compared to baseline (pre-exercise) levels and 15 minutes post exercise (Table

11 and Figure) (p= 0.003 and p= 0.006 for Mon 1 and Mon 3 respectively). There was no significant change in the phagocytic activity of Mon 2 at any time point following exercise.

Table 11: Phagocytic activity of monocyte subsets is affected by exercise

	Baseline (pre-exercise)	15 minutes post	60 minutes post	24 hours post	p Value
Phagocytic activity of Mon 1 (MFI)	95 [65-115]	81 [61-118]	107 [73-139]	179 [73-195]	0.003
Phagocytic activity of Mon 2 (MFI)	84 [66-98]	78 [60-99]	85 [64-103]	65 [47-127]	0.71
Phagocytic activity of Mon 3 (MFI)	18 [12-28]	23 [12-32]	24 [16-44]	44 [22-50]	0.006

MFI, median fluorescence intensity**;** non-normally distributed data are presented as median [with interquartile range].
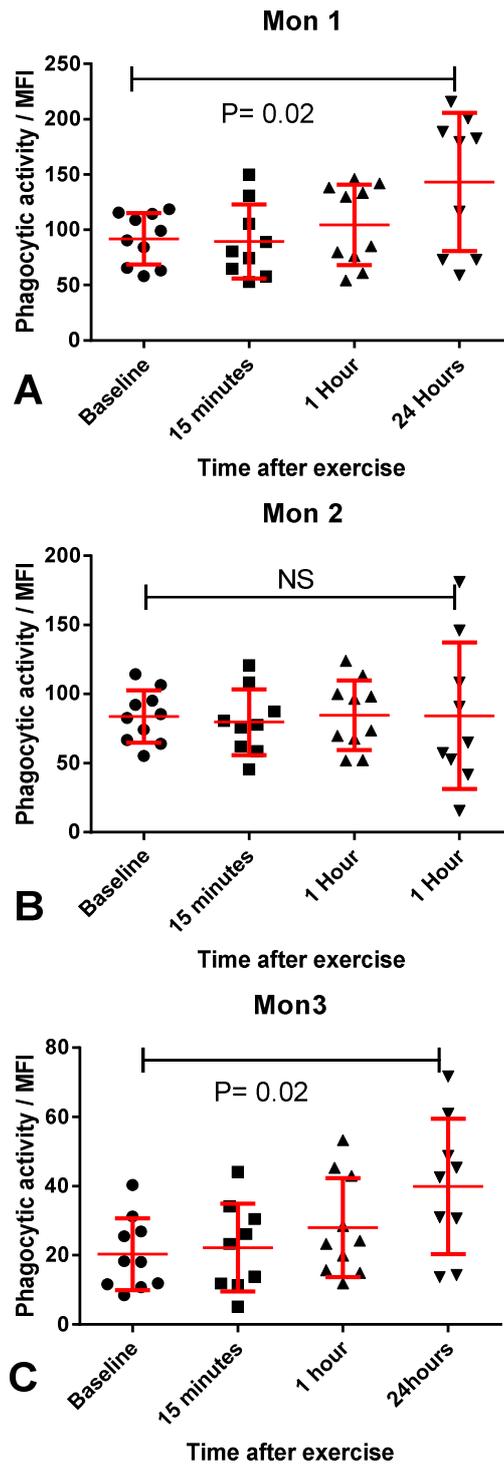
Figure 10: Phagocytic activity is increased in Mon 1 and Mon 3 after exercise: Healthy individuals (n=12) were exercised according to Bruce protocol until exhaustion. Phagocytic activity was measured as median fluorescence intensity (MFI) *E. coli* bioparticle uptake. Effect of time after exercise was determined by ANOVA.

3.4.5 Effect of circadian rhythm on phagocytic activity

This was important in delineating the logistics of patient recruitment during the longitudinal study of recruiting STEMI patients, six patients were recruited to investigate if diurnal variation had any effect on phagocytic activity. The maximum phagocytic activity of all the three monocyte subsets was at 12:00. This was not statistically different form phagocytic activity at 18:00. Even though it was logistically impossible to recruit patients at the same time of the day, most patients were recruited till early afternoon in order to reduce the effect of circadian rhythm on the results.

Table 12: Effect of diurnal variation on monocyte subsets phagocytic activity

	06:00	**12:00**	**18:00**	**00:00**	**06:00**	**p Value**
Phagocytic activity of Mon 1 (MFI)	126±24.4	161±10.9	137±16.5	94±17.9†	105±29.7	<0.005
Phagocytic activity of Mon 2 (MFI)	117±24.4	161±7.54	127±7.56	88±16.4†	113±9.73	<0.005
Phagocytic activity of Mon 3 (MFI)	38.7±8.29	58.4±13.6	57.5±5.95	33.6±6.80	52.5±36.8	0.21

MFI, median fluorescence intensity, Normally distributed data are described as mean ± SD

3.4.6 Phagocytic activity of monocyte subsets in different health states

In a cross sectional study, a total of 40 STEMI patients, 20 patients with stable coronary artery disease (SCD) and 40 healthy individuals were recruited to investigate the effect of disease on monocyte subset phagocytic activity. Patients were matched for age, gender, and clinical charactersitics (Chapter 2, section 2.3.1 and appendix I) Phagocytic uptake of *E. coli* bioparticles by the three different monocyte subsets were significantly higher in STEMI

patients compared to stable coronary artery disease patients and healthy controls (p values of <0.0001, 0.004 and <0.0001 for Mon 1, Mon 2, and Mon 3 respectively- Table 13 and Figure 11). There were no significant differences in phagocytic activity between stable coronary artery disease patients and healthy individuals (Table 13).

Table 13: Phagocytic activity of the three different monocyte subsets varies with health states

Monocyte subsets	STEMI (n=40)	Stable coronary disease (n=20)	Healthy controls (n=40)	pValue
Phagocytic activity of Mon 1 (MFI)	114 (97 – 129)	96 (73 – 114)	78 (55 – 102)	<0.0001
Phagocytic activity of Mon 2 (MFI)	106 (84 – 121)	85 (66 – 107)	66 (47 – 97)	0.004
Phagocytic activity of Mon 3 (MFI)	52 (29 – 78)	34 (18 – 49)	39 (26 – 56)	<0.0001

CAD, coronary artery disease; MFI, median fluorescence intensity. Phagocytic activity of monocytes in peripheral blood was determined as *E. coli* pHrodo uptake.
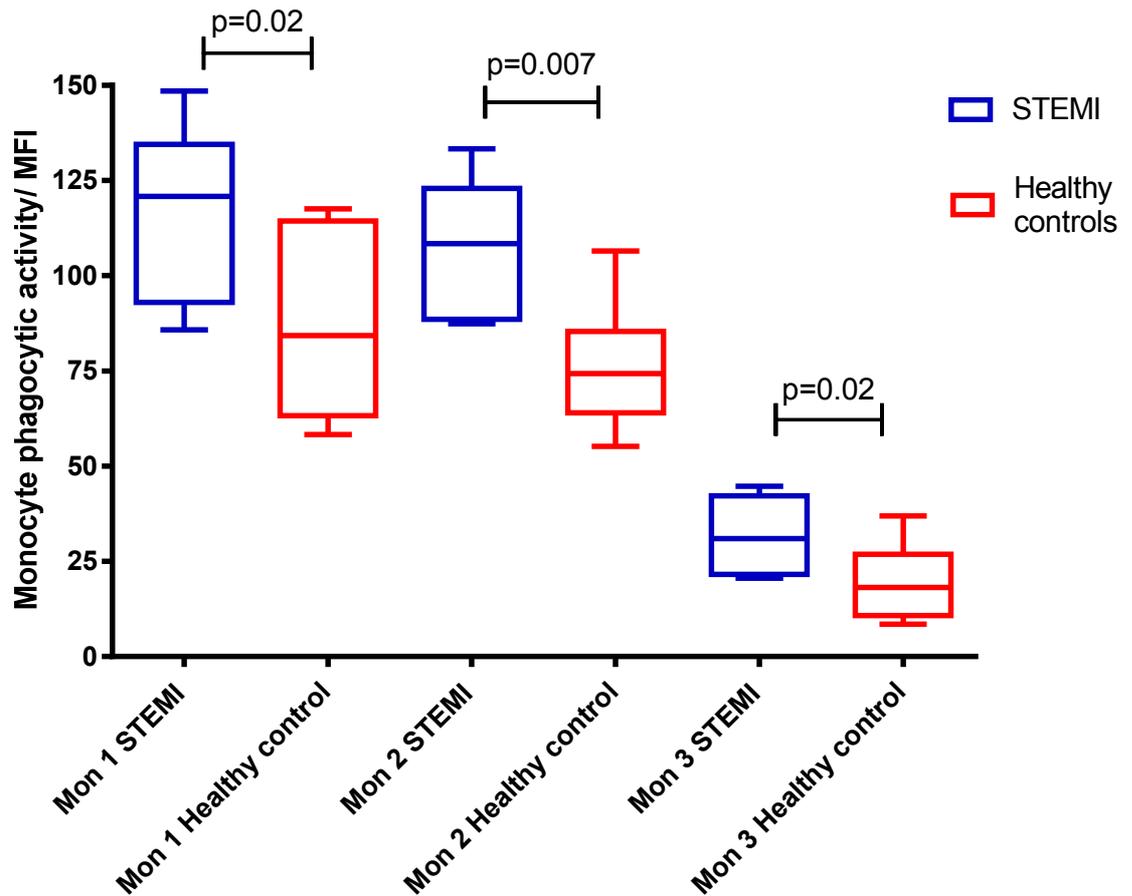
Figure 11: Phagocytic activity of the three monocyte subsets in in STEMI and healthy controls: Mon 1, Mon 2, and Mon 3 counts were assessed 24 hours post infarction (N=40) and compared with age- and gender-matched healthy controls (N=40). Phagocytic activity of Mon 1, Mon 2, and Mon 3 were significantly higher in STEMI subjects compared to healthy controls (Mann- Whitney U test was performed as data were non-parametric, p=0.02, p=0.007, p=0.02 for Mon 1, Mon 2 and Mon 3 respectively).

There was a significant increase in the phagocytic activity in all monocyte subpopulations from STEMI patients compared to those from healthy participants (Figure 11). Four-fold higher phagocytic activity was observed in Mon 1 compared to Mon 3 irrespective of health or disease.

3.3.7 Phagocytic activity in STEMI patients

The demographics of recruited STEMI patients are outlined in appendix I and Chapter 2, section 2.3.1.

The phagocytic activity of monocyte subsets in these STEMI patients (N= 209), did not change significantly when compared to the phagocytic activity at follow up 14 days post index event. This applied to all three monocyte subsets (p =0.36, 0.59, and 0.52 for Mon 1, Mon 2 and Mon 3 respectively), (table 15).

Table 14: Phagocytic activity of the three monocyte subsets is not affected over time after infarct :

Monocyte subsets	Within the first 24 hours post infarct (n=209)	At phase 2 post-infarct (n=149)	pValue
Phagocytic activity of Mon 1 (MFI)	119 (101 – 146)	115 (95 – 140)	0.36
Phagocytic activity of Mon 2 (MFI)	119 (92 – 145)	113 (91 – 143)	0.59
Phagocytic activity of Mon 3 (MFI)	41 (29 – 55)	42 (26 – 57)	0.52

Median fluorescence intensity, MFI. Data are expressed median with (interquartile range) for non-normally distributed data

3.5 Discussion

It is well described that during infarction there is an increase in surface expression of MCP-1 as a ligand for CCR2 hence leading to monocyte chemotaxis to the area of infarction (Tacke, 2007). Hence in order to investigate the function of monocyte after infarction the mobility of CD14+ monocyte subsets (Mon 1 and Mon 2) was investigated. Plasma from patients who had an adverse cardiac event on follow up (MACE) induced a higher rate of monocyte migration than from healthy controls, with higher cell numbers migrating to MCP-1 after 9 hours, compared to other patient population groups. There was also a trend for higher cell density and faster migration rates by isolated monocytes with no cross over plasma, and in those stimulated with LPS. It is possible that the plasma of patients who have sustained a STEMI, with no MACE, or those with stable coronary artery disease to have a suppressive chemokine/cytokine in the microenvironment that impairs the mobility of monocytes to infarcted tissue in an attempt to reduce monocyte chemotaxis acutely. Patients who sustain MACE might lose this inhibitory braking mechanism hence the higher count rates that are equivalent to LPS stimulated cells and control cells without patient's plasma. Here a reproducible, rapid flow cytometry-based technique was validated to assess phagocytic activity of monocyte subsets. As discussed in chapter 2, flow cytometry has been utilised to differentiate their identification based on cell surface expression of CD14 and CD16. The same immunophenotyping methodology was used to assess the phagocytic activity of these subpopulations (Figure 7). The methodology is highly reproducible (Table 11) and can be carried out on whole blood samples, processed within 2 hours of sample collection.

Mon 1 the classically described monocyte subset, is inflammatory in nature and here they exhibited a high phagocytic activity when compared to the 'non-classical' Mon 3 subset (table 14). This was evident in both the healthy subjects as well as patients following STEMI. However, for the first time, it has been shown that monocyte subset 2 (CD14++, CD16+) exhibits equivalent, high phagocytic activity to Mon 1 (Tables 13 and 14). With a high phagocytic activity, the likelihood of Mon 2 having reparative angiogenic role, comparable to

the well described Mon 3 subpopulation is debatable. Mon 3 subset has significantly lower phagocytic activity compared to Mon 1 and Mon 2 (Figure 11).

The beneficial effect of exercise in reducing mortality from cardiovascular disease in individuals with coronary artery disease, as well as reducing the generalised inflammatory state associated with ageing has been intensely studied (Woods et al., 2012). The increase in phagocytic activity of Mon 1 and Mon 3 24 hours post-exercise is reported for the first time. We have demonstrated previously that the total number of monocytes increased 15 minutes post-exercise but did not significantly change post exercise (Shantsila et al., 2012). Previously Woods et al., in an animal model, illustrated that moderate exercise had a stimulatory effect on the phagocytosis and chemotaxis of monocytes, whilst rats exposed to progressive training over 6 weeks showed a decrease in macrophage phagocytic activity (Woods et al., 2003). It might be that the increase in phagocytic activity is due to a generalised inflammatory response induced by the moderate exercise that our healthy individuals participated in during the study irrespective of monocyte numbers. This effect might be lost in persistent progressive training, partly explaining the overall beneficial effect of persistent (even mild) exercise observed and utilised in cardiac rehabilitation programmes worldwide (Timmerman et al., 2008). An increase in reactive oxygen species release post exercise, as well as other inflammatory markers has been previously reported (Nahrendorf and Swirski, 2015). In healthy individuals endurance exercise was associated with release of troponin but no detectable cardiac inflammatory increase in late gadolinium enhancement or fibrotic changes on cardiac magnetic resonance (Woods et al., 2003). Understanding the effect of phagocytosis on conventional cardiovascular risk factors could provide an additional venue for risk factor modification amongst STEMI patients (Walsh et al., 2011).

Within 24 hours of STEMI, the phagocytic activity of all three monocyte subsets was increased. Others have shown an increase in surface expression levels of scavenger receptors (e.g. CD163 and CXCR4) on Mon 2 with ejection lower fraction 6 weeks post STEMI (Shantsila et al., 2013). Having a high phagocytic activity would partly contribute to the effect of Mon 2 observed in predicting cardiovascular death and acute coronary syndromes in

patients with stable coronary artery disease (Rogacev et al., 2012). Studying this in conjunction with matrix metalloproteinase activity might shed might provide further understanding of the phagocytic effect of Mon 2. After STEMI cardiomyocytes die as a result of hypoxic injury. These dead apoptotic cells could attract monocytes and macrophages to phagocytose the dead cells to allow healing to initiate (Devitt and Marshall, 2011). Overzealous phagocytic activity early post infarct could account for the association of high counts of Mon 1 and a large size myocardial infarct and negative ventricular remodelling (Tsujioka et al., 2009, van der Laan et al., 2012). However this could excessive phagocytic activity could also be explained by reduced efferocytosis.

Enhancing clearance will likely decrease secondary necrosis and inflammation, leading to reduced macrophage content in infarcted tissue and potentially reducing the development of autoimmune T-cells and circulating auto-antibodies (Tang et al., 2012). As humans we preferentially enhance phagocytic clearance of apoptotic cells over blocking macrophage apoptosis, because as has been established recently, a resting steady state of macrophages are required for healthy myocyte structure. It might be likely that blocking apoptosis of the phagocytes may contribute to increased cellularity and autocrine and paracrine inflammation due to non-clearance of the phagosome. Oxidative stress and hypoxia have also been reported as in vitro inhibitors of clearance and may play a role in post MI repair (Zhang et al., 2014). Aberrant calcium signalling has also been recently implicated in efferocytosis. Both extracellular and intracellular sources of Ca^{2+} have long been implicated in phagocytic signalling, particularly during uptake of opsonized particles (Hackam et al., 1997). Changes in extracellular Ca^{2+} concentration, a feature of infarcted myocardium, can directly affect the conformation and therefore capacity of phagocytic receptors to bind targets for engulfment. Aberrant calcium signalling has been recently implicated in efferocytosis as well.

The data presented here suggest that the phagocytic activity of CD14+ monocytes are not CD14-dependent, despite these also being abundantly present on the surface of *E. coli*. Thus the detected results are reflective of the functional activity of monocytes and not merely a measurement of *E. coli* uptake. Phagocytosis of apoptotic polymorphonuclear cells was also

recently shown to be independent of CD14 with CD16+ve monocytes (both Mon 2 and Mon 3) having a higher phagocytic activity of apoptotic cells (Mikolajczyk et al., 2009). However Lingnau et al., describe that monocytes have both CD14-dependent and CD14-independent phagocytic activity either of which could be enhanced by the effect of surrounding cytokines including IL-10 and IFN-gamma (Lingnau et al., 2007).

With more data coming to light describing the transition of surface expression of CD16 on surface of monocytes and their function, it remains unclear whether Mon 2 is a separate entity of monocyte differentiation with their own differentiation end products (i.e. macrophages and dendritic cells); or whether they are a transient state observed between Mon 1 and Mon 3. Immunohistochemistry studies ought to be considered to address this question.

Recently the phagocytic activity of monocytes has been shown to be independent of their downstream differentiated macrophages (Lund et al., 2002). There is a need to investigate the effect of the phagocytic activity of monocytes both on ventricular remodelling and on clinical outcome. Such new information may support the potential for phagocytic activity to be utilised as therapeutic target in improving patient outcome post infarction.

3.6 Conclusion

For the first time a flow cytometry-based technique to assess the phagocytic activity of the three monocytes subpopulations in whole blood has been described. Mon 2 appears to have a high phagocytic activity that is not statistically different from Mon 1 in healthy individuals or in patients with STEMI. This suggests that they might have predominantly an inflammatory role. The phagocytic activity was not dependent on CD14 surface expression. Moderate interrupted exercise appears to cause an increase in natural phagocytic activity. This paper provides a stepping stone in describing phagocytic activity of the three monocyte subsets that could be used on wide scale in clinical studies and clinical practice.

Chapter 4

NFκB pathway activation in monocyte subsets

in relation to STEMI

4.1 Introduction

During myocardial infarction the acute hypoxic state associated with the infarction leads to overexpression of CCR2 and hence chemotaxis of monocytes.

The NFκB pathway is also thought to be activated in monocytes during hypoxia. NFκB is one of the major transcription factors mediating inflammatory responses of monocytes and macrophages. Its activation leads to the production of proinflammatory and prothrombotic molecules, such as cytokines, MMPs, TF and IkB. Excessive activation of NFκB has been linked to inflammation associated with atherosclerosis (Kutuk et al., 2003). Thus, measurement of NFκB levels could be used as a surrogate for the functional assessment of monocyte inflammatory activity.

In non-stimulated monocytes, NFκB is sequestered within the cytosol by an inhibitory protein (i.e., IκB; inhibitor of NFκB) that masks the nuclear localization signal present within the NFκB protein sequence. Upon stimulation IκB is phosphorylated by IKKβ, undergoes secondary ubiquitination and is degraded by the proteasome (Verma et al., 2004). The IκB kinase (IKK) complex is a phosphorylating enzyme with several subunits IKKα, IKKγ and IKKβ. The β subunit is the most active and is facilitates transcriptional activation by NFκB through phosphorylating IκB. The degradation of IκB thus allows NFκB to translocate into the nuclei where it can act as a transcription factor (Haden et al., 2004) (Figure 12).

Excessive NFκB activation has been associated with negative ventricular remodelling (Gupta et al., 2005). Myocardial tissues from patients with heart failure of various aetiologies exhibit activation of NF*κ*B and increased expression of NF*κ*B-regulated genes. Also, using gene transfer of I*κ*B-α, Squadrito et al. demonstrated that repression of NF*κ*B transcription in models of ischaemia-reperfusion injury reduced leukocyte recruitment to the myocardium, cardiomyocyte death and infarct size (Squadrito et al., 2003). A well-tolerated pharmacological NF*κ*B inhibitor, BAY 11-7082, reduced inflammation in a rat model of ischaemia-reperfusion injury leading to a significant reduction in the infarct size.

NFκB is activated in the heart during acute ischaemia and reperfusion and during unstable angina. Blocking NFκB activity reduces ischaemia–reperfusion injury in experimental studies

with short times of observation. Wong et al., 2008 showed that NFκB was activated in cardiomyocytes in failing hearts of patients with ischaemic heart disease, while similarly an increased NFκB expression was present in the fibrotic areas in patients with dilated cardiomyopathy. Later studies have confirmed that NFκB and its target genes are activated in cardiomyocytes of patients with failing hearts of various aetiologies and that the same is the case in mouse and rat models of heart failure (Gupta et al., 2005).

The outstanding technical problem with the measurement of NFκB is that the resting cytosolic form and the activated nuclear form cannot be distinguished by flow cytometry. Active nuclear NFκB is normally detected in cell lysates with an electrophoretic mobility shift assay, Western blot assays or by detecting its binding to a consensus sequence promoter oligonucleotides. There are no data on monocyte subset NFκB activity in STEMI. Therefore, here a method to measure the cytosolic IKKβ as a surrogate for NFκB activation has been optimized.

TNF-α is the most widely studied pro-inflammatory cytokine in cardiac pathophysiology. In murine models, TNF-α promotes progressive left ventricular dysfunction and remodelling in animal models. Serum TNF-α has been shown to be elevated in patients with heart failure and correlates with mortality. TNF-α stimulates NFκB as well as being a downstream product of the pathway stimulation. To further validate that IKKβ was a surrogate marker for NFκB activation, TNF-α secretion by monocytes was analysed after LPS activation. Furthermore, TNF-α, MMP- 9 and IKKβ activity were analysed in plasma from STEMI patients to investigate their interrelationship.
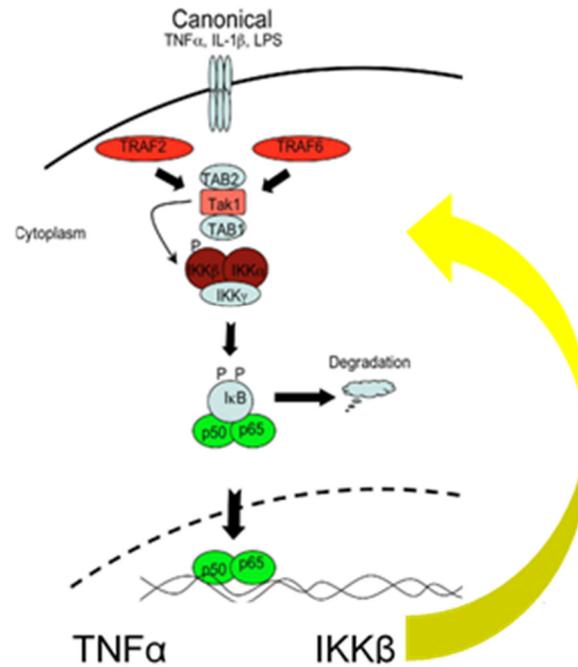
Figure 12: NFκB inflammatory pathway. Activation of the canonical NFκB pathway leads to downstream production of nuclearTNF-α and IKKβ**.** Its hypothesised that IKKβ is secreted back into the cytosol with a positive feedback loop on the NFκB pathway. Uprtream, activation of the NFκB pathway occurs by LPS and TNF-α. Upon stimulation IκB is phosphorylated by IKKβ, undergoes secondary ubiquitination and is degraded by the proteasome (Verma et al., 2004). The IκB kinase (IKK) complex is a phosphorylating enzyme with several subunits IKKα, IKKγ and IKKβ. The β subunit is the most active and is facilitates transcriptional activation by NFκB through phosphorylating IκB. The degradation of IκB thus allows NFκB to translocate into the nuclei where it can act as a transcription factor.

4.2 Aims and Hypothesis

In this study the aim to validate a flow cytometry based assay for the measurement of IKKβ activity as a surrogate for NFκB pathway activation. The aim is to utilize this method to describe IKKβ activity of different monocyte subsets in different health states in a cross-sectional study and also in STEMI patients over 6 months, as a longitudinal study.

The aim is to describe the relationship between monocyte subsets, TNF-α and MMP- 9 levels. The hypothesis is that IKKβ levels will be higher in STEMI patients compared to other health states with decrease in the level of IKKβ activity in STEMI patients during the follow up period at the second phase. As a consequence of NFκB activation, I hypothesis that there will be an increase in TNF-α and MMP-9 levels in STEMI patients.

4.3 Methods

4.3.1 Patient recruitment

STEMI patients, subjects with stable coronary artery disease and healthy individuals were recruited into the study as discussed in chapter 2, section 2.3.1

4.3.2 Assessment of NFκB pathway

A flow cytometry based technique for measuring NFκB activation indirectly was developed by determining intracellular IKKβ levels in monocyte subsets. Blood was obtained from two healthy individuals (age 30 ± 2).

4.3.2.1   Flow cytometry assessment of IKKβ levels

Whole blood in EDTA (100µl) was added to 5 µl of CD16-AF 488 and 2.5µl CD14 PerCP-CY5.5. The sample was then mixed gently on the vortex and incubated in the dark for 15 minutes. Red cell lysis was then carried out by adding 2.0 ml of 'PharmLyse' solution, gently mixed and then incubated for 10 minutes in the dark. The sample was centrifuged for 5 minutes at 500g and the supernatant was removed by a disposable pipette. To stain the cells, 2ml of 'Staining Buffer' was added to the tube, and the sample was centrifuged again for 5 minutes at 500g and the supernatant removed. After adding 500µl of 'Fixation and Permeation' solution [neutral pH-buffered saline (Dulbecco's Phosphate-Buffered Saline) that contains 4% w/v paraformaldehyde- BD], the sample was mixed and incubated for 20 minutes in the dark. The sample was spun again for 5 minutes at 500g and the supernatant removed. Washing was performed by adding 2 ml of 'Perm-Wash', incubating the cells afterwards for 10 minutes and then spinning for 5 minutes at 500g. Finally the supernatant was removed again and 10µl of anti-IKKβ-APC antibody was added to the sample and left to incubate for 30 minutes in the dark. A final washing step was repeated again with 2 ml of 'Perm-Wash' [BD Perm/Wash buffer: consists of 100 ml of concentrated stock solution (10X) containing both Fetal Bovine Serum (FBS) and saponin], spinning for 5 minutes at 500g. After removing the supernatant, 50µl of 4% PBS (Sigma E8008) was added, mixed with the sample and then run through the

flow cytometer. This is a very reproducible assay with my interassay variability of 6.3% and intra-assay of 4.9% (Figure 13).
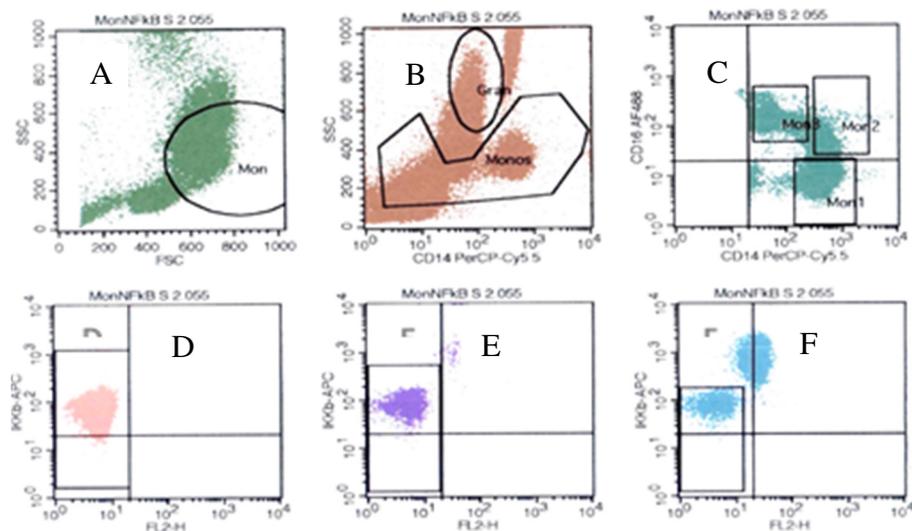
Figure 13: Flow cytometric discrimination between the IKKβ expression of three different monocyte subsets: Monocyte subsets are distinguished according to CD14, CD16 and CCR2 cell surface expression (panel C) after gating for total monocytes (panel A), and eliminating granulocytes (panel B). IKKβ expression of the different monocyte subsets (Mon 1, Mon 2 and Mon 3 in panels D-F respectively) is detected by APC binding.

4.3.2.2   Cell sorting and monocyte isolation

Whole blood (30ml) was collected into EDTA from a healthy volunteer. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation. Blood was diluted 1:1 with 0.5 mM EDTA in PBS pH 7.1-7.7 (2-8 °C) (Sigma, E8008), carefully and slowly layered over the Ficoll-Hypaque in a 15 ml conical tube, with 1 volume of Ficoll-Hypaque used for 2 volumes of diluted blood. The tubes were then centrifuged for 25 min at 1000 g with no break on deceleration (Hettich centrifuge). The upper layer is removed leaving the mononuclear cell layer undisturbed at the interphase. The interphase cells (consisting of lymphocytes and monocytes) are transferred to a new 15 ml conical tube. Ice cold PBS was added to form 15 ml total volume and then centrifuged for 10 min at 1900 at 4C using the Eppendorf centrifuge. The supernatant was removed carefully and cells resuspended to a total volume of 15 ml with ice cold PBS. The cells were washed twice with ice cold PBS at 4°C for 10 min at 430 g and repeated as necessary until the solution is clear.

Monocyte subsets were further isolated by magnetic sorting of the PBMC fraction using VarioMACS (Miltenyi Biotec, Düsseldorf, Germany) and direct labelling of CD14+ monocyte subpopulations using microbead-conjugated antibodies, using commercial monocyte Isolation Kit II (Miltenyi biotec 130-091-153). Per 3ml of collected whole blood, 10µl of FcR blocking reagent was added with 10µl of Biotin-Antibody Cocktail. The cells were mixed by pipetting and incubated for 10 minutes at 4-8 °C. Then 30µl of MACS buffer were added for every 3ml of blood along with 20µl of anti-biotin microbeads. The mixture was mixed well and incubated for 15 minutes at 4-8 °C. After washing the cells for 10 minutes at 270 g, the supernatant was removed and the cells resuspended in 500µl of MACS buffer. The LS column was in the meantime prepared by rinsing with 3ml of buffer and the cell suspension applied. This was washed 3 times with 3mls of buffer on each occasion. The eluent was collected in the same falcon tube containing the isolated CD14+ cells (Figure 14).
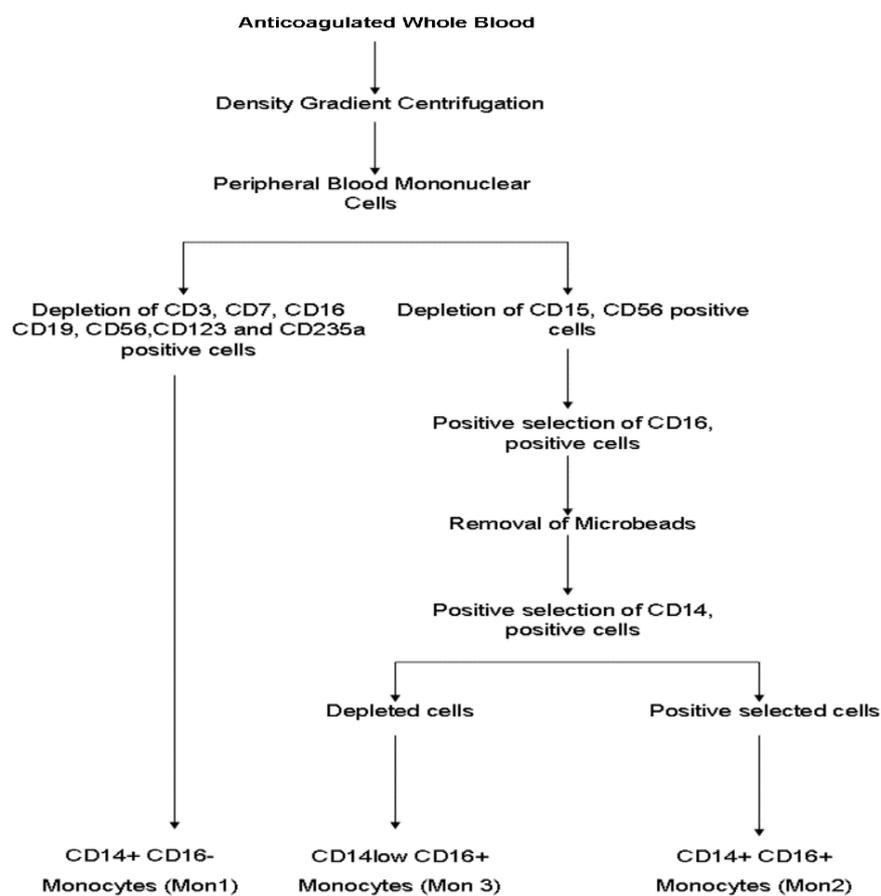
Figure 14: Cell sorting protocol for isolation of monocyte subsets from whole blood

Monocyte subsets obtained by cell sorting were suspended at a density of 1×10^6 cells/ml, in culture medium consisting of RPMI-1640, fetal bovine serum supplemented with 2 mM L-glutamine, 100µg/ml gentamycin and 100µg/ml penicillin. Cell suspensions were incubated, at 37°C, in a 5% CO_2 humidified atmosphere, in the presence or absence of 1µg/ml lipopolysaccharide (LPS) endotoxin (*Escherichia coli* 0127:B8). Cells were harvested and incubated CD16-Alexa Fluor 488 (clone DJ130c, AbD Serotec, Oxford, UK) and CD14-PerCP-Cy5.5 (clone M5E2, BD) for 15 min. After adding 500µl of 'Fixation and Permeation' solution [neutral pH-buffered saline (Dulbecco's Phosphate-Buffered Saline) that contains 4% w/v paraformaldehyde], the sample was mixed and incubated for 20 minutes in the dark. The sample was spun again for 5 minutes at 500g and the supernatant removed. Washing was performed by adding 2 ml of 'Perm-Wash' [BD Perm/Wash buffer: consists of 100 ml of concentrated stock solution (10X) containing both foetal bovine serum (FBS) and saponin]. The cells were incubated afterwards for 10 minutes and spun for 5 minutes at 500g. Finally the supernatant was removed again and 10µl of 'IKKbeta-APC' antibody added to the sample and incubated for 30 minutes in the dark. A final washing step was repeated again with 2 ml of 'Perm-Wash', spun for 5 minutes at 500g. After removing the supernatant, 50µl of 4% PFA/PBS (Sigma E8008) was added, mixed with the sample and analysed by flow cytometry.

4.3.2.3   Detection of NFκB protein after pathway activation

NFκB nuclear binding was assessed by using an ELISA based NFκB TransAM transactivation kit (Active Motif, Carlsbad, Calif) according to manufacturer's instructions. Nuclear extract was prepared from 2.5×10^5 isolated CD14+ that had been incubated with 50µl of STEMI patients' plasma. Nuclear protein (75µg/well) were loaded in 96-well plates pre-coated with consensus oligonucleotides, and the active- NFκB that bound to the oligonucleotide was detected HRP-conjugated secondary antibody. The absorbance was read using a plate reader at 450nm, and absorbance was expressed as the direct activity of NFκB.

4.3.2.4   Localisation of IKKβ in LPS stimulated CD14+ve monocytes

Isolated cells (2.5×10^5, 200 µl) in RPMi were added to microscope slides. The cells were left to adhere for one hour at room temperature in a dark, moist box. Then fixation permeabilisation agent was added for 15 minutes at room temperature. After wash with permeabilisation buffer twice by dipping slides in 50mls of buffer, the slides were left to dry prior to staining with IKKβ antibody. Slides were incubated for 30 minutes in a dark humid environment at room temperature. The slides were then washed once again with perm wash and allowed to dry for few seconds. Slides were mounted with a drop of mounting agent and fixed with a cover slip. A Leica Microsystems TCS SP5II system was used with a 63x objective lens (HCX PLAPO 63x/1.4-0.6 oil CS) and with 488nm laser to excite the anti-IKKβ fluorophore-conjugated antibody.

4.3.3 Measurement of MMP- 9 concentration using ELISA

MMP- 9 levels were measured in plasma using an enzyme-linked immunosorbent assay (ELISA) (R&D systems- USA). The capture antibody was diluted with PBS. One hundred µl of the diluted capture antibody was used to coat a 96 well microplate. The plate was then left at room temperature overnight. The plate was washed three time with washing buffer. The plates were then blocked with 300 µl of reagent diluent and the plate is incubated for 1 hour. The plates were washed afterwards three times and left to dry. 100µl of patient plasma is then added in triplicate to the plates and left to incubate in room temperature for two hours. The plate was washed and aspirated three times and then 100 µl of detection antibody was placed to each well and incubated for further two hours. After 10 minutes of incubation at room temperature in a light-protected environment the reaction was stopped using 0.5M sulphuric acid and the plate was read immediately using a microplate reader at an absorbance of 450 nm. Data were analysed using GraphPad Prism (GraphPad Software, California, USA) and unknown sample concentrations were calculated against the calibration curve generated from the plate standards. The intra-assay coefficient of variation was 4.1–6.9% and inter-assay coefficient of variation of 5.8–6.3%.

4.3.4 Measurement of TNF-α concentration using ELISA

TNF-α levels were measured using a quantitative enzyme immunoassay technique (Peprotech, Rocky Hill, NJ, USA). Respective monoclonal antibodies specific for TNF-α were pre-coated onto a microplate. Standards and samples were pipetted into the wells and any TNF-α present was bound by the immobilized antibody. A polyclonal antibody specific for TNF-α conjugated to peroxidase was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, ABTS substrate solution was added to the wells and left to develop for 20 minutes before the reaction was stopped with 0.5M sulphuric acid. The absorbance was read using a microplate reader at 430 nm. The concentration of TNF-α in plasma was analysed using GraphPad Prism (GraphPad Software, California, USA) against the calibration curve generated from the known standards.

4.4 Results

4.4.1 In vitro proof of concept studies of NFκB activation pathway

A novel flow cytometry based methodology was developed for the detection and quantification of IKKβ subunit activity upon NFkB pathway activation. A number of experiments were designed to validate the flow cytometry methodology.

4.4.1.1   IKKβ expression in whole blood after LPS stimulation

The first step was to confirm that primary monocyte activation elicited an increase in IKKβ expression. LPS was selected to activate monocytes as it elicits robust and reliable of NFkB activity in monocytes. Here whole blood was stimulated with different LPS concentrations for up to 3 hours. There was an increase in IKKβ level of CD14+ve cells stimulated cells both with prolonged incubation time and in response to higher LPS concentrations (Figure 15). However at 20µg/ml concentration of LPS the detectable IKKβ was reduced suggesting that such high concentrations of LPS lead to cell toxicity and death.
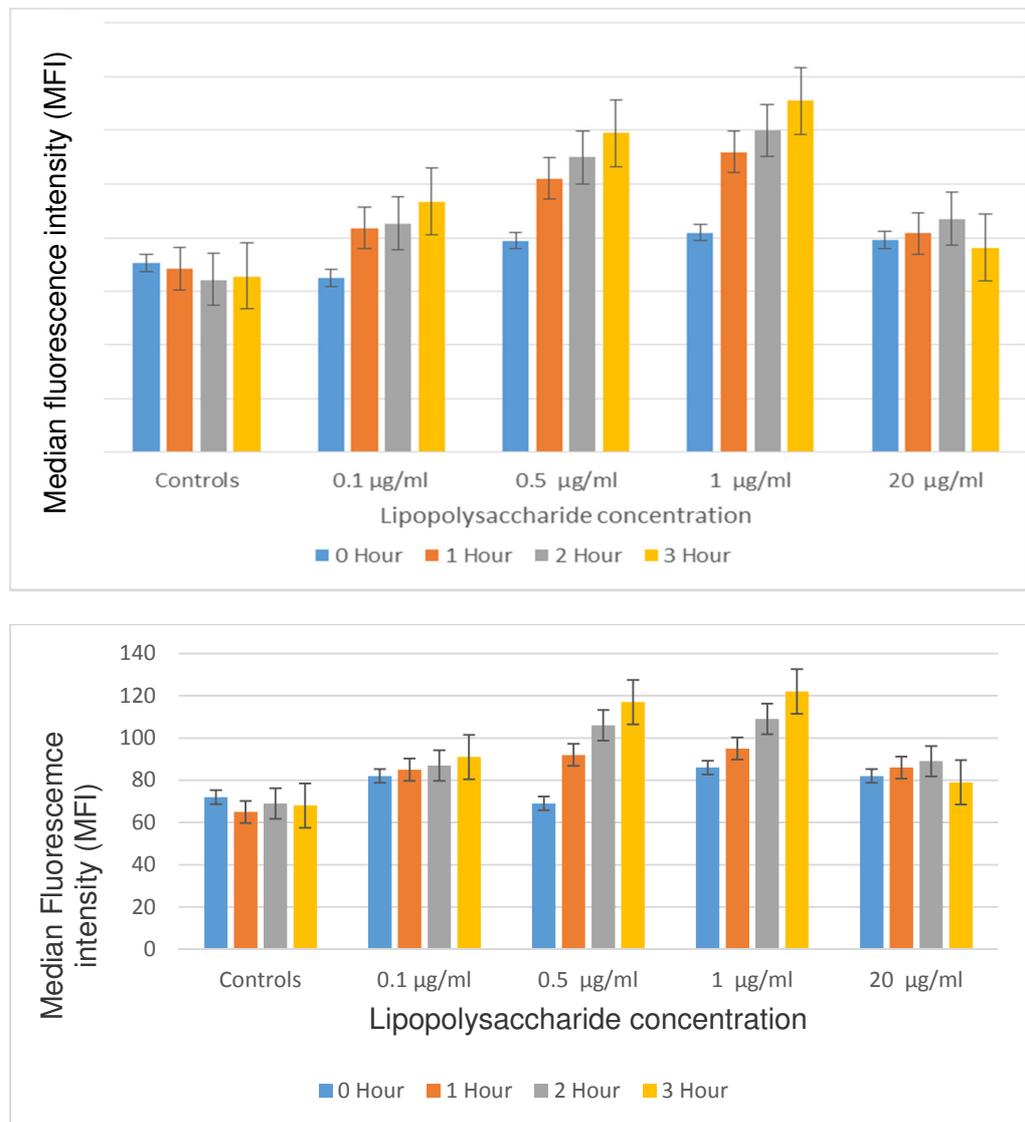
Figure 15: IKKβ levels increase in monocytes in whole blood stimulated with LPS: Blood was venesected from two healthy individuals (age: 28±3). Whole blood was venesected from two healthy volunteers. The blood was stimulated with increasing concentrations of LPS and allowed to incubate for different times. Red blood cells were lysed and stained IKKβ antibodies. Samples were run in duplicates. There was an increase in IKKβ levels in response to longer incubation with increasing concertation of LPS. A concentration of 20μg/ml led to a decrease in IKKβ levels probably due to cell toxicity at higher concentrations. Data are presented as mean with (SD- error bars).

4.4.1.2   IKKβ activity in isolated CD14+ cells after LPS stimulation

As a proof of concept, an in vitro study was carried out to determine the time frame for IKKβ transcription and detection in the cytosol. Whole blood was obtained from two healthy volunteers (one male and one female; age 28±3). Isolated monocytes were stimulated with four different concentrations of LPS and incubated for 24 hours. The supernatant from the cultured monocytes were removed and cells stained for IKKβ antibody and analysed by flow

cytometry at 0, 3, 12 and 24 hours after incubation with LPS. (Figure 16). The higher

concentrations of LPS (500ng/ml and 1μg/ml) led to highest levels of IKKβ levels at 12 hours

and 24 hours. Mean fluorescence intensity for IKKβ antibody was higher at 1μg/ml of LPS

stimulation compared to 500ng/ml. The maximum detected median fluorescent intensity of

IKKβ was higher at 24 hours than three hours post LPS stimulation.
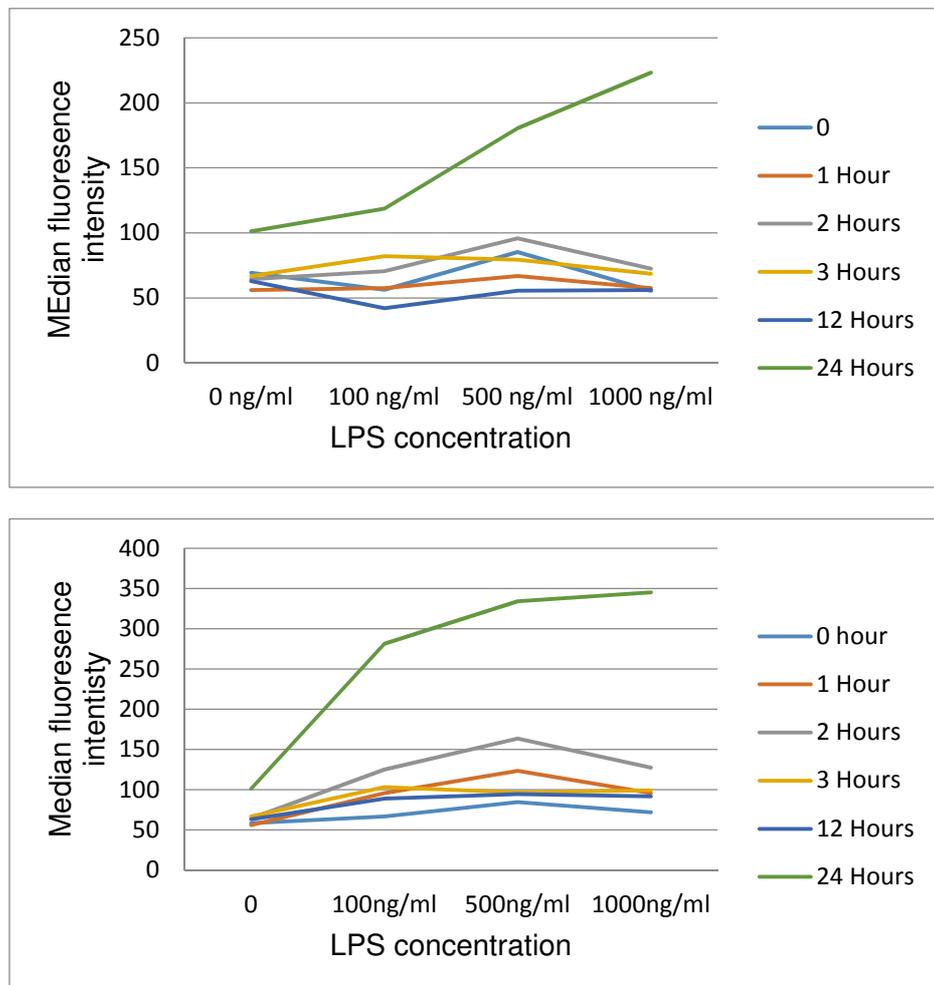
Figure 16: LPS increases IKKβ in monocyte populations after 24h: The response of (A) purified monocyte subpopulation 1 (CD14+veCD16-ve CCR2+ve) and (B) monocyte subpopulation 2 (CD14+veCD16+ve CCR2+ve) measured as the IKKβ mean fluorescence intensity (MFI) after stimulation with LPS (100ng/ml, 500ngml, 1ug/ml LPS) for 1, 2, 3, 12 and 24 hours (n-=1) At each time point there was a control sample with no LPS added. A group of samples (0 hour) was also analysed immediately after LPS stimulation.

4.4.1.3 IKKβ activity in isolated CD14+ cells after LPS stimulation with NFκB inhibition

To confirm that the increase in IKKβ level was due to NFκB activation, monocytes were isolated from the whole blood of a third healthy subject (age 36) with the addition of SN50 (a peptide that inhibits translocation of the NF-κB active complex into the nucleus). There was an increase in IKKβ levels with increasing concentrations of LPS and with prolonged incubation periods. However there was no change in IKKβ in the presence of the highest LPS concentration and SN50 (Figure 17).
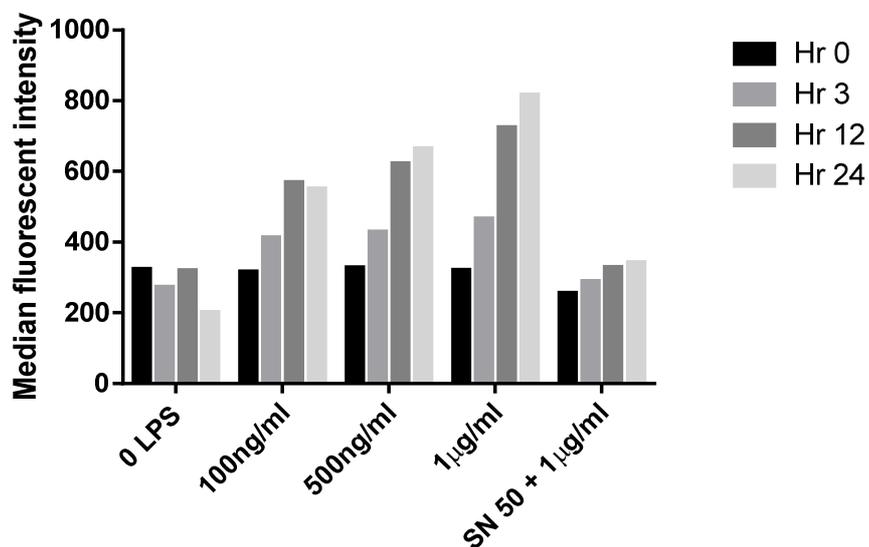
Figure 17: IKKβ levels upon LPS stimulation as well as incubation with SN50: Monocytes (2.5×10^5) isolated from one healthy individual were stimulated with different concentration of LPS (100ng/ml; 500ng/ml; 1ng/ml) as well as with SN50 (NFKB pathway inhibitor) all incubated with RPMI. 100 µl of supernatant was removed at different time points (Hr 0,3,12,24) these were then prepared and stained with IKKβ antibody and analysed on the flow cytometer. There was an increase in IKKβ levels with increasing concentrations of LPS and with prolonged incubation periods. However there was no change in IKKβ in the presence of SN50. Differences between groups was assessed by Anova statistical test.

4.4.1.4 TNF-α secretion by LPS stimulated CD14+ monocytes

NFκB pathway activation, and hence detection of IKKβ, leads to downstream production of various pro inflammatory cytokines, including TNF-α and IL-6. Thus, to confirm that the activation of monocytes with LPS was associated with downstream gene expression,

transcription and activation, an ELISA for TNF-α was performed on the supernatants from isolated monocytes after stimulation with LPS for different time periods (0, 3, 12, 24 hours). TNF-α secretion from primary monocytes of different healthy donors increased with longer incubation time and with increasing LPS concentrations studied here (Figure 18).
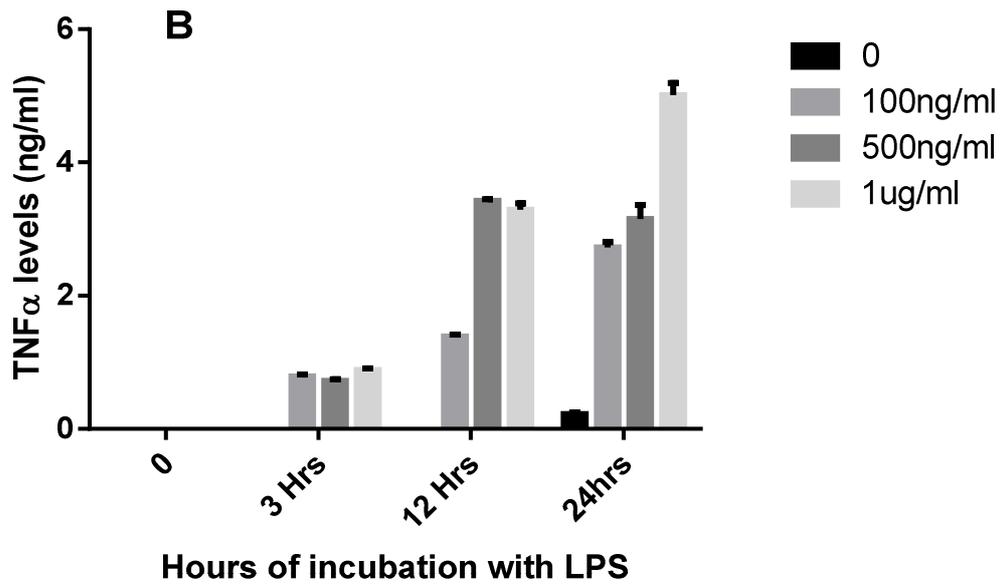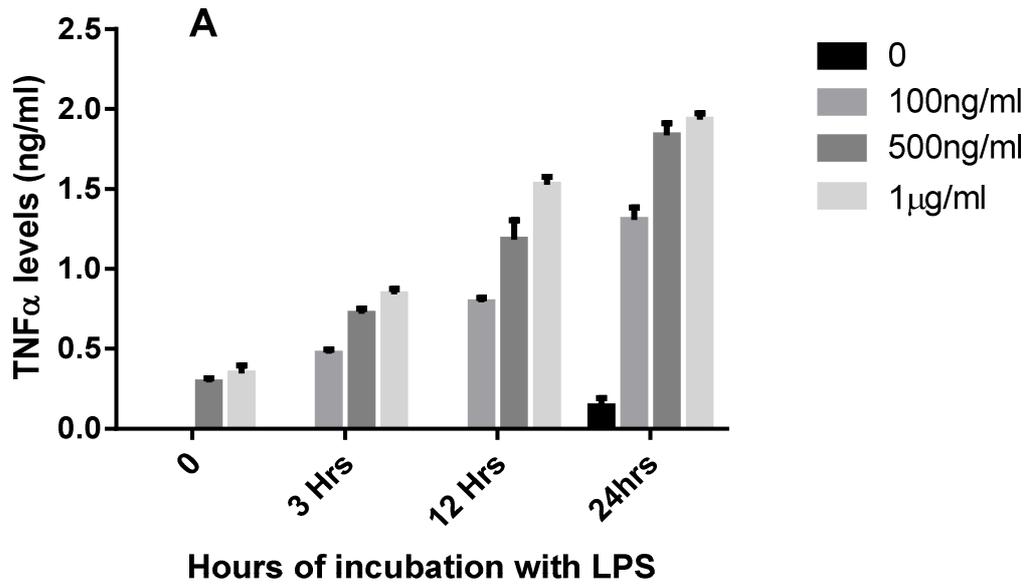
Figure 18: TNF-α secretion from LPS stimulated isolated monocytes: Isolated CD14+ve monocytes (2.5×10^5) from one healthy individual were stimulated in the presence and absence of LPS (100g/ml; 500ng/ml; 1μg/ml) in RPMI over a period of incubation. TNF-α secretion in the supernatant was determined using ELISA. Supernatant was collected at 0, 3, 12 and 24 hours. The experiment was repeated using monocytes isolated from two healthy individuals (A and B plots; age 28±3). Results are presented as means ± SD (as error bars).

4.4.1.5   NFκB protein in cross over study

We utilised the TransAm method, in order to detect NFκB protein as a confirmatory experiment using more standard techniques to detect activation. In a plasma cross over study 100µl of serum from different patient population was incubated with isolated monocyte cell (2.5×10^5).

There was a significant increase in NFκB protein after 12 hours of incubation compared to the first hour (p=0.02- paired t-test; Figure 19)

This corresponded to a change in CCR2 levels detected by flow cytometry

Figure 19: Nuclear NFκB is increased in CD14+ monocytes from healthy donors by plasma from STEMI patents: Monocytes isolated from whole blood of one healthy individual (female; age 33), were incubated with 20 µl plasma from STEMI patients for 12 hours. Nuclear NFκB was determined using TransAM ELISA. There was a significant increase in NFκB protein after 12 hours of incubation compared to the first hour (p= 0.02- paired t-test).

The level of the corresponding CCR2 levels on surface of isolated monocyte CD14+ cells were measured after 1 hour and 12 hours stimulation with LPS in vitro. CCR2 levels were significantly higher at one hour of stimulation compared to 12 hours (p <0.001).

The number of viable analysable cells measured at both time points was not statistically different (mean ± SD: 325± 37 at 1 hour compared to 297 ± 48 at 12 hours, p= 0.12) – (Figure 20).

Figure 20: Surface CCR2 expression is decreased after 12 hours of cross over study with patients' plasma: Monocytes isolated from whole blood of one healthy individual (female; age 33), were incubated with plasma from STEMI patients for 12 hours. CCR2 levels were significantly higher at one hour of incubation compared to 12 hours (paired t-test, p<0.001).

Having indicated an increase in NFκB levels are detected in the cross over studies after 12 hours of incubations, the different levels of NFκB expression were then investigated in different patient population groups to establish if there was a difference amongst different groups.

4.4.1.6   NFκB protein levels in different patient population groups

Isolated monocytes ($2.5x10^5$) were stimulated with the serum of different groups of recruited patients (5 stable coronary artery disease patients; 6 STEMI patients; 6 STEMI patients who sustained a MACE; 4 healthy controls all in duplicates. NFκB levels were significantly higher in STEMI and patients sustaining a MACE (p= 0.002, Figure 21).
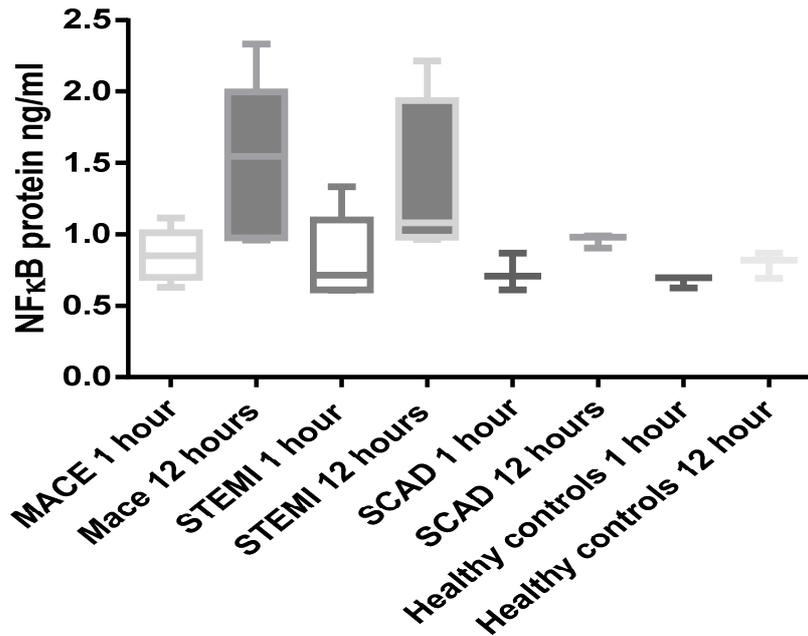
Figure 21: NFκB protein levels in different patient population groups after cross over study: Isolated CD14+ve cells (2.5×10^5) cells have been incubated for 12 hours with plasma from different patient population groups (6 STEMI patients, 6 MACE patient, 5 stable coronary artery disease (SCD), 4 healthy controls in duplicates). NFκB protein levels were significantly higher in STEMI patients and MACE patients at 12 hours compared to stable coronary artery disease.

4.4.1.7   Cytosolic IKKβ visualization

Finally, we attempted to visualize and localize the IKKβ protein detected using confocal microscopy. Monocytes were isolated and slides prepared, as described in methodology. The IKKβ protein was found to be cytoplasmic (Figure 22).

Having validated the methodology in vitro the NFκB activity pathways of different monocyte subpopulation were examined in STEMI and CAD.
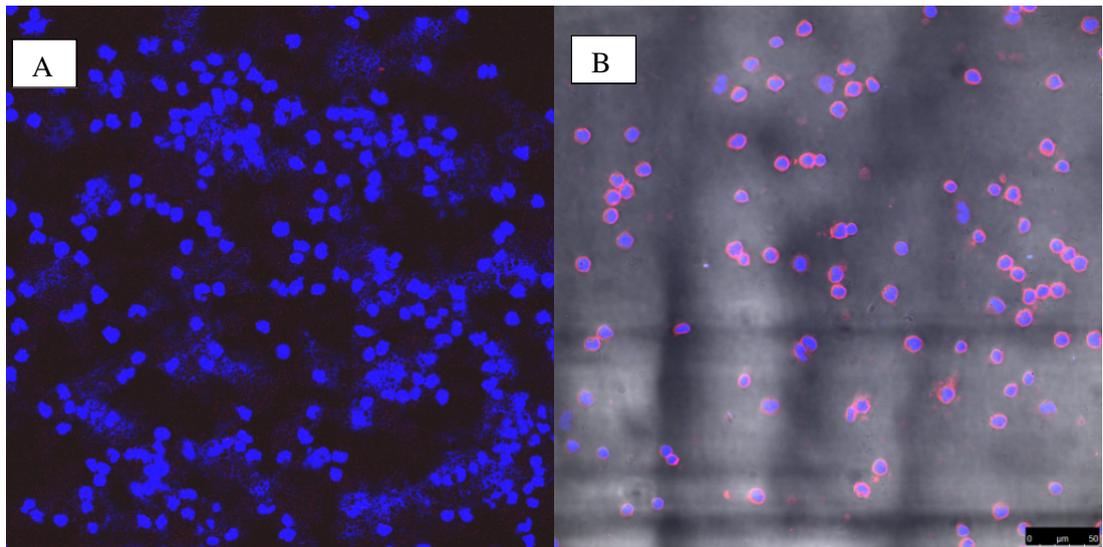
Figure 22: Cytoplasmic staining with IKKβ APC antibody in a proof of concept: This is demonstrated witth the pink halo around the nucleus (blue) of isolated cells (panel B). This is in comparison to control cell with no LPS stimulation or plasma crossover studies (panel A)

4.4.2 IKKβ activity in different health states

In order to investigate the ilammatory activity of monocyte subpopulation, IKKβ levels of the three different monocytes were measured in three different inflammatory vascular disease states represented by three different patient groups: ST elevation myocardial infarction patients within 24 hours of the infarct representing a high inflammatory state; patients with established stable coronary artery disease representing a chronic inflammatory state; and healthy individuals with quiescent inflammatory state.

The levels of IKKβ were significantly higher in all three monocyte subsets in STEMI patients within one day of infarct compared to those with stable coronary artery disease or healthy individuals. IKKβ levels were significantly higher in STEMI patients compared to subjects with stable coronary artery disease patients and healthy individuals (p=0.002) (Figure 23). IKKβ activity was not significantly different amongst stable coronary artery disease and healthy individuals (p=0.25) (Figure 23).
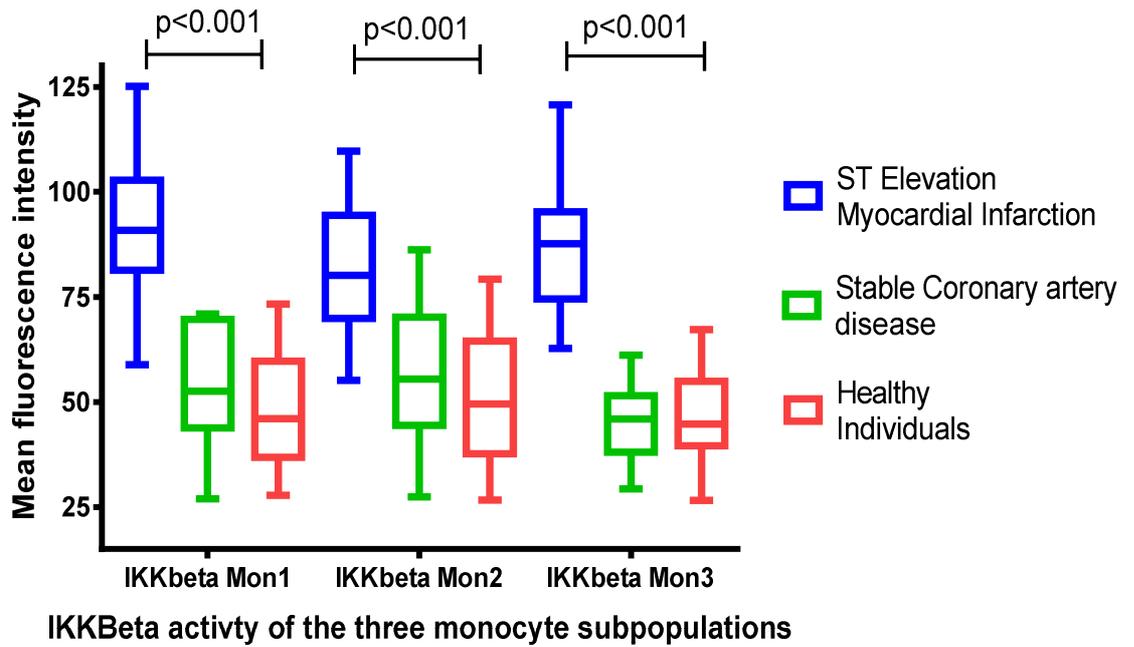
Figure 23: IKKβ expression is higher in monocyte subsets from STEMI patients: Monocyte subsets from 40 STEMI patient, 40 healthy participants and 20 stable coronary artery disease patients were compared for cellular IKKβ expression. Results are shown as mean ±SD, differences between groups have been analysed by one way anova with post hoc Tukey analysis.

4.4.3   NFκB pathway activity in STEMI patients

4.4.3.1   Patient demographics

209 STEMI patients were recruited to examine the effect of time on NFκB activation and outcome; the characteristic demographics are summarized in chapter 2, section 2.4.3

4.4.3.2   IKKβ level of activity in STEMI patients

There was a statistically significant decrease in the levels of IKKβ activity between day1 and follow up, which is consistent with the concept that inflammation reduces post-infarction (Table 16).

Table 15: IKKβ expression in monocytes from STEMI patients:

	Index Admission (N= 209)	Two weeks post MI (N= 140)	p Value
IKKβ Mon 1 (MFI)	71 ±26	64 ± 22	0.007
IKKβ Mon 2 (MFI)	67 ± 23	65 ± 24	0.396
IKKβ Mon 3 (MFI)	71 ± 23	66 ± 19	0.010

A paired t-test was used to investigate the difference between IKKβ activity at index event and two weeks afterwards. IKKβ levels of Mon 1 and Mon 3 were significantly higher at day 14 than day 1

4.4.4 Monocyte numbers correlate with circulating TNF-α and MMP- 9

To investigate the relationship between monocyte subset counts with other markers of inflammation, plasma TNF-α and MMP- 9 were examined, as critical factors in tissue remodelling and as markers of ongoing inflammatory activity regulated by NFκB pathway activation.

4.4.4.1 Patients' demographics

Of the 209 STEMI patients recruited, 162 plasma samples were available for measuring TNF-α and MMP- 9 levels.

The average age of patients that were recruited with STEMI was 61± 11 (mean ± SD) with 75% male. As reflected from general patients characteristic, chapter 2, section 2.4.3, there was abundance of classical cardiovascular risk factors amongst recruited patients; with 54% suffering from hypertension, 53% were smokers, 46% suffered from hypercholesterolaemia, and 17% had history of angina or previous MI. Eighty one percent of patients had either an anterior or an inferior infarct (43% and 38% respectively). Consistent with the existing vascular disease risk factors of the recruited patients, 38% were on aspirin, 25% were on calcium channel blockers for hypertension management, 19% were prescribed β- blocker

therapy, whilst 24% regularly took diuretic therapy (both thiazide and loop diuretics, 10% and

14% respectively (table 17).

Table 16: STEMI patient demographics

		N= 162
Demographics	Age	61 ± 11
	Gender	75% males
	Time from symptoms onset to presentation to hospital (hours)	4.7 ± 2.6
Biochemical profile at recruitment	Peak Troponin	2340 (843 – 6397)
	Peak Creatinine Kinase	959 (452 – 2189)
	Total WCC (mmol/L)	11 (8 – 13)
	Estimated GFR (ml/min/1.73m^2)	77 (63 – 90)
	Total Cholesterol (mmol/L)	4.9 (4.3 – 5.3)
	Body surface area	1.9 (1.8 – 2.0)
Past medical history at the point of recruitment [Number and (%)]	Diabetes	39 (24)
	Hypertension	94 (58)
	Smoking	86 (53)
	Hypercholestrolaemia	75 (46)
	History of angina	9 (6)
	Previous myocardial infarction	28 (17)
	Obesity	27 (17)
	COPD	21(13)
	CVA	14 (9)
	CABG	9 (6)
	Previous PCI	17 (10)
	Anterior infarct	69 (43)
	Inferior STEMI	62 (38)
Drug history at recruitment [Number and (%)]	Aspirin	62 (38)
	Clopidogrel	4 (2)
	Statin therapy	61 (4)
	Calcium channel blocker	41 (25)
	Beta blocker	31 (19)
	Loop diuretic	23 (14)
	Thiazide diuretic	15 (10)
	ACE inhibitor	70 (13)
	Nitrates	9 (6)

One hundred and sixty two patients had plasma available for measurement of TNF-α and MMP- 9. GFR: glomerular filtration rate; CVA: cerebrovascular accident; CABG: coronary artery bypass grafting; PCI; percutaneous coronary intervention; STEMI: ST elevation myocardial infarction. Normally distributes data are described as mean ± SD, non-normally distributed data are presented as median (with range).

4.4.4.2   Monocyte subset correlation with plasma MMP- 9

There was a significant correlation between MMP- 9 levels at day 1 post STEMI with total monocyte count, Mon 1 and Mon 2 (p<0.001). There was no correlation between MMP- 9 and functional studies as assessed by phagocytosis or IKKβ levels. At day 2 there were no correlations between monocyte numbers and MMP- 9 (Table 18).

Table 17: MMP- 9 correlation with monocyte subsets and functional parameters

	MMP- 9 day 1		MMP- 9 day 2	
	R^2	p Value	R^2	p Value
Total Monocyte count	0.43	<0.001	0.01	0.91
Mon 1 (cells/μl)	0.39	<0.001	0.03	0.77
Mon 2 (cells/μl)	0.28	<0.001	0.03	0.81
Mon 3 (cells/μl)	0.02	0.79	-0.19	0.09
IKKβ Mon 1 (MFI)	-0.01	0.87	0.22	0.06
IKKβ Mon 2 (MFI)	-0.01	0.87	0.17	0.15
IKKβ Mon 3 (MFI)	0.06	0.43	0.17	0.14
Phagocytosis Mon 1 (MFI)	-0.04 *	0.64	0.10	0.37
Phagocytosis Mon 2 (MFI)	0.02 *	0.82	0.17	0.14
Phagocytosis Mon 3 (MFI)	-0.01 *	0.94	0.01	0.96

Pearsons correlation was utilized to assess correlation of MMP- 9 with monocyte subsets and functional parameters.(* Phagocytosis parameters are non-parametric, hence Spearmans correlation was performed).

4.4.4.3   Monocyte subset correlations with TNF-α

There was a significant correlation between TNF-α at day 1 with total monocyte count, Mon 1, Mon 2 and Mon 3 (p<0.001). Functionally, IKKβ activity of Mon 1 and Mon 2 (p<0.001 and 0.006 respectively) were correlated with α. There was no correlation between TNF-α at day 14 with monocyte subsets (Table 19).

Table 18: TNF-α correlation with monocyte subsets and functional parameters

	TNF-α day 1		TNF-α day 14	
	R²	pValue	R²	pValue
Total Monocyte count	0.64	<0.001	-0.07	0.62
Mon 1 (cells/µl)	0.60	<0.001	0.03	0.83
Mon 2 (cells/µl)	0.26	<0.001	-0.17	0.25
Mon 3 (cells/µl)	0.27	<0.001	-0.14	0.36
IKKβ Mon 1 (MFI)	0.41	<0.001	-0.19	0.22
IKKβ Mon 2 (MFI)	0.27	0.006	-0.14	0.36
IKKβ Mon 3 (MFI)	0.06	0.53	-0.10	0.53
Phagocytosis Mon 1 (MFI)	0.05 *	0.58	-0.02	0.91
Phagocytosis Mon 2 (MFI)	0.17 *	0.07	-0.02	0.88
Phagocytosis Mon 3 (MFI)	0.16 *	0.09	0.22	0.13

Pearsons correlation was utilized to assess correlation of MMP- 9 with monocyte subsets and functional parameters. (*Phagocytosis parameters are non-parametric, hence Spearmans correlation).

4.5 Discussion

In this study a validated flow cytometric based methodology for the assessment of NFκB inflammatory pathway activation is presented. This approach uses the measurement of IKKβ as a surrogate marker in monocytes in whole blood after transcription and activation of the NFKB inflammatory pathway. This has been validated in both in-vitro and in-vivo experiments. There is a clear difference in detected IKKβ correlating with TNF-α, in STEMI patients compared to steady state of healthy individuals or patients with stable coronary artery disease.

Upon incubation of isolated monocytes with patients' plasma, there was an increase in the level of CCR2 expression in the supernatant of the isolated cells within 1 hour. NFKB protein was significantly increased in those samples 12 hours after incubation with patients' plasma. This suggests that an increase in CCR2 expression in response to a stimulant in STEMI patients' microenvironment led to activation of NFKB pathway 12 hours later. Chandrasekar and Freeman observed two waves of NF-κB activity in the heart after ischaemia–reperfusion. In their experiments, they observed NF-κB activity following 15 min of ischaemia and 15 min of reperfusion, which lasted until 1 h of reperfusion. The second wave began at 3 h of reperfusion and lasted until 6 h

In recruited STEMI patients there was a significant correlation between plasma TNF-α and total monocyte count, the three monocyte subsets and IKKβ levels of Mon 1 and Mon 2. This is in keeping with the in vitro studies that are summarized in figures 17 and 18 where TNF-α levels increased after stimulation with LPS and corresponded in an increase IKKβ levels. MMP- 9 on the other hand also correlated significantly with total monocyte count, Mon 1 and Mon 2, this could support the hypothesis that MMP- 9 is secreted by monocytes in response to hypoxia and reperfusion injury.

The interaction between NFκB pathway activation and TNF-α is complex and intricate: on the one hand NFκB pathway activation leads to downstream secretion and release, and on the other hand TNF-α is one the stimulants of the canonical NFκB pathway. Hence in the heart,

the interaction between TNF-α could lead to a vicious cycle of propagated inflammation and concurrently myocyte cell death or indeed uncontrolled apoptosis.

The literature illustrates that there are two varying roles for NFκB pathway activation. On the one hand, the cardioprotective role of NFκB signalling has been described in a number of studies. De Moissac et al. reported that Bcl-2 suppresses cell death of ventricular myocytes through a mechanism involving IKKβ mediated activation of NFκB pathway (de Moissac et al., 1998). Cardiac-specific expression of a similar IkB mutant increased the susceptibility of myocytes to apoptosis following acute coronary occlusion in vivo (de Moissac et al., 1998, Gordon et al., 2011), whereas p50 null mice displayed enhanced cardiac dysfunction following myocardial infarction. This finding is supported by recent evaluation of a natural occurring human polymorphism in the p50 gene that is associated with increased functional deterioration in patients with heart failure (Gordon et al., 2011).

The effect of monocyte stimulation of NFκB pathway stimulation on cardiac remodelling post infarct as well as clinical outcome in STEMI patients post infarction is discussed in chapters 5 and 6.

4.6 Conclusion

Here we describe a prompt, reproducible technique for measuring the activity of NFκB pathway. STEMI patients had an increased level of IKKβ at day one compared to phase 2. IKKβ was significantly higher in STEMI patients compared to patients with stable coronary artery

Chapter 5

Monocyte subsets and their functional assessment in determining ventricular remodelling and ventricular deformation post STEMI

5.1 Introduction

Post myocardial infarction the healing myocytes undergo complex geometrical changes as individual muscle cells and globally as an organ leading to alterations in the overall shape, size and function of the left ventricle, as the main pumping chamber of the heart, as well as overall of the heart as an organ.

The occurrence of left ventricular (LV) dilatation after acute myocardial infarction is not uncommon. Giannuzzi et al. noted severe LV remodelling 6 months after infarction in 16% of the patients (Giannuzzi et al., 2003). The clinical importance of LV remodelling was emphasized by White et al., who demonstrated that patients who died during follow-up after myocardial infarction had significantly larger LV volumes and lower left ventricular ejection fractions (LVEFs) than survivors (Frangogiannis, 2008, White et al., 2014). Furthermore, they indicated left ventricular end-systolic volume (LVESV) as the primary predictor of survival after myocardial infarction. As a consequence, early identification of patients with LV remodelling after acute myocardial infarction is of vital importance to initiate secondary prevention medication in an aim to reduce future risk profile.

Several mechanisms implicated in LV remodelling have been identified, including hypertrophy, fibrosis, apoptosis, and activation of proteolysis. These processes lead to changes both in the systolic and diastolic functions of the heart. Hence there has been a number of definitions for ventricular remodelling post infarction, with increasing in LV diastolic volume, reduction in systolic volumes or reduction in ejection fraction. However, over the last years LV remodelling post infarction was defined in a number of studies as an increase in left ventricular end diastolic volume of 20% as indexed to body surface area (LVEDVI). This is the definition utilised in assessing LV remodelling in his study.

5.1.1 Extracellular matrix and heart remodelling

Previous basic science studies have provided a cause-effect relation between MMP- 9 and adverse LV myocardial remodelling after MI. The results from these murine models suggest

that the robust increase in plasma MMP-9 levels observed early after MI likely reflects the initiation of an adverse myocardial structural remodelling process that is manifested as LV dilation in the later post-MI period.

A landmark paper described in murine models the sequential mobilisation and delivery of monocyte/macrophage phases in the immediate period after a myocardial infarction and later in the ensuing recovery period. In the initial phase post infarction, Ly-6c^{high} monocytes give rise to early inflammatory macrophages, both ultimately clearing damaged tissue by phagocytosis and secreting proteolytic enzymes. In the second phase, Ly-6c^{low} macrophages facilitate wound healing and regeneration by promoting myofibroblast accumulation, collagen deposition, and angiogenesis. Infiltrated monocytes may also interact with extracellular matrix in the damaged myocardium, leading to fibronectin release (Frantz and Nahrendorf, 2014). Once in the infarct, monocytes differentiate into macrophages in the presence of M-CSF. There are two macrophage subset, M1 and M2. It is yet unclear which monocyte subset differentiate into which macrophage subtype (van Amerongen et al., 2007). Macrophages promote angiogenesis, fibroblast proliferation, and extracellular matrix deposition. Macrophages also play a role in organ regeneration (Godwin et al., 2013). Though MI in adult mammals leads to scarring and diminished ventricular function, neonatal mouse hearts can regenerate after MI without scarring, but depleting cardiac macrophages impedes this repair process. A recent study by Pinto et al. showed that embryonic-derived cardiac macrophages promote angiogenesis and healing after myocardial damage (Pinto et al., 2012). Consistent with this, salamander limb regeneration also depends on macrophages (Godwin et al., 2013). Although inflammation is required for cellular debris removal and new tissue formation after ischaemic injury, exaggerated inflammation may impede the healing process, as shown in ApoE$^{-/-}$ mice with coronary ligation (Panizzi et al., 2010). Accordingly, blood monocyte count after MI positively correlates with left ventricular end-diastolic volume and negatively correlates with ejection fraction in patients.

5.1.2 Monocytes in heart remodelling

As the precursor of macrophages and dendritic cells monocytes have been previously reported to have an important role in timely "wound" healing post myocardial infarction with early phagocytosis of infarcted cells allowing for appropriate scar formation and hence containing and limiting the ensuing dilatation of cardiac chambers and volumes thus limiting the chance of adverse remodelling. In recent years however, peripheral monocytosis was demonstrated to be associated with worse clinical outcome with small series replicating these finding in STEMI patients.

Evidence supporting the hypothesis of monocyte functional diversity in MI in vivo is supported by the findings of sequential mobilisation of monocyte subsets, firstly in mouse (Nahrendorf et al., 2007) and more recently in humans (Tsujioka et al., 2009) . As discussed in chapter 1, sequential mobilisation of subsets is thought to be due to the orchestrated due to expression of subset- specific s. In the mouse model of MI, the Ly-6Chigh monocyte subset predominated within the myocardium during phase I after MI (peak level on day 3) and was found to exhibit phagocytic, proteolytic and inflammatory activity. In contrast, the Ly-6Clow monocyte subset predominated in phase II after MI (peak on day 7) and was implicated in myocardial healing and recovery processes, including anti-inflammatory properties, promotion of myofibroblast accumulation, angiogenesis and collagen deposition (Nahrendorf et al., 2007). In humans, the CD14+CD16- and CD14+CD16+ monocyte subsets peaked in the circulation on days 3 and 5 respectively after MI (Tsujioka et al., 2009). Tapp et al. indicated that classical monocytes and intermediate monocytes were associated with worse reduced EF 6 weeks post infarct (Tapp et al., 2012). Only two studies investigate the role of monocyte subsets

5.1.3 Inflammatory markers and heart remodelling

Tumor necrosis factor-α (TNF-α) is a master cytokine that is produced in significant quantities within the infarcted myocardium very soon after MI. TNF-α is secreted primarily by myocytes

and macrophages after injury (Kapadia et al., 1997). As discussed in chapter 4, there is an intricate relationship between TNF-α and the inflammatory pathway NF-κB. TNF-α binds to cell surface receptors and triggers intracellular signalling cascades that can result in activation of transcription factors such as NFκB (Mustapha et al., 2000). TNF-α also has direct effects on the matrix and collagen framework and is a potential major contributor to cardiac remodelling. In addition, TNF-α has direct effects on the matrix and collagen framework and is a potential major contributor to cardiac remodelling. TNF-α actives other cytokines such as IL-1 and IL-6 and orchestrates the host tissue response to acute injury Here we measure TNF-α, as an established marker of cardiac remodelling, and investigate its possible role with monocyte subsets and ventricular remodelling.

5.1.4 Echocardiogram in assessing heart remodelling

Transthoracic standard echocardiography (standard echo) is a widely available method; it is non-invasive and can be performed bedside. From a cost-benefit point of view, standard echo is the preferred method for evaluating left ventricular function. However, an echocardiographic examination is highly dependent on the examiners experience and has limitations in patients with obesity, obstructive pulmonary disorders or a poor acoustic window for other reasons, a limitation of three dimensional echocardiography as well. Given its high spatial resolution magnetic resonance imaging (MRI) is considered more sensitive in measuring ventricular volumes and ejection fraction.

Left ventricular (LV) remodelling following acute myocardial infarction (AMI) is a dynamic and complex process that develops in the response to myocardial damage. However, the exact determinants of this process are not completely understood. The assessment of LV remodelling parameters after AMI has therapeutic and prognostic implications as advanced LV re-modelling is associated with developing and progression of heart failure. Moreover, traditional echocardiographic parameters such as LV volumes and ejection fraction, wall motion score index, mitral regurgitation, and left atrial volume have been shown to provide prognostic information for LV remodelling.

Given the complexity and multidirectional cardiac fibres contractility as well as the multitude of molecular and histological changes associated with infarct healing and remodelling not all In order to assess of novel parameters, including strain (and strain rate) imaging based on speckle tracking imaging.

(STI) echocardiography or strain determining regional myocardial deformation measured by Doppler technique. Deformation studies, have already been correlated with degree of LV systolic dysfunction post infarction and with clinical outcome, with better sensitivity even than ejection fraction. In earlier technology strain assessment by Doppler is limited to the measurement of movement parallel to the ultrasound beam, whereas speckle strain may be measured independently of the angle, and measures regional deformation has thus speckle tracking has been found to predict the extent of myocardial injury post infarction as validated by CMR.

5.2 Aims and Hypothesis

The aim of this study was to prospectively identify that the associations of monocyte subsets and their functional parameters with systolic and diastolic function parameters. I also investigated the impact of monocytosis post STEMI on ventricular remodelling post STEMI and on the effect of monocyte subsets.

I hypothesised that the presence of higher counts of monocyte subsets 1 and 2 are associated with reduced ejection fraction 6 months after myocardial infarction as well as negative ventricular remodelling with a significant increase in end diastolic volumes at 6 months. In patients with normal or mildly impaired ejection fraction I hypothesised that high counts of monocyte subsets are predictive of reduced subclinical parameters of impaired systolic function namely, global and circumferential longitudinal strain.

5.3 Methods

5.3.1 Patient recruitment

All patients were recruited as summarized in appendix I and chapter 2, section 2.3.1.

5.3.2 Transthoracic echocardiography

Transthoracic echocardiography uses ultrasound waves to provide detailed imaging of the heart. It is non-invasive, portable and rapidly available making it the primary imaging modality for the assessment of cardiac anatomy and function.

5.3.2.1   Basic principles

Ultrasound waves are inaudible mechanical vibrations with a frequency greater than 20,000 cycles per second (20 KHz) that induces alternate refractions and compressions of the physical medium through which they pass. They are generated from the piezoelectric crystals within the transducer of the echo machine. An alternating current expands and compresses polarized particles within the crystal generating a burst or pulse of ultrasound wave which is transmitted (propagates) through tissue (medium) as a straight beam. The acoustic properties of the media determine how much of the beam is reflected, refracted (changes the direction of the wave) and attenuated (absorption of ultrasound energy) and are the basis of ultrasound imaging. The reflected ultrasound energy is received by the piezoelectric crystal in the transducer and converted to low amplitude voltage signals. The signal undergoes complex manipulation to form an image (Armstrong et al., 2010).

5.3.2.2   Imaging modalities

Advances in echo technology now allow multiple image displays. A summary of echo properties facilitating the study (A, 2009):

1. Motion (m) mode - allows a single dimension of anatomy to be graphically presented against time. It allows a high repetition frequency of the pulse transmission and receive phase of the transducer. The high sampling rate is valuable for evaluation of rapid intra-cardiac motion of continuously moving structures.

2. Two-dimensional imaging (2D) – generated by sweeping of the ultrasound beam made up of multiple scan lines across the tomographic plane. Reflected ultrasound signals from each scan line are received by the transducer to generate a cross-sectional image depicting structure and function in real time.

3. Doppler imaging- based on the change in frequency of the backscattered signal from small moving structures. It is frequently used to assess the velocity, direction and pattern of intra-cardiac blood flow.

5.3.2.3 Imaging Protocol

I performed all echocardiograms both at recruitment and at follow up 6 months post infarction. These were carried out at the Centre for Cardiovascular Sciences in City Hospital and at Birmingham Heartlands Hospital cardiology department depending on the patients' recruitment site. I used second harmonic imaging on a Phillips machine with multi-frequency phased-array transducer (1.5 MHz). Patients were positioned in a left decubitus positioning with ECG. Standard parameters for chamber quantification, left ventricular systolic and diastolic function are outlined in Table 20. All measurements were made in triplicate and averaged according to the recommendations of the American Society of Echocardiography (Lang et al., 2006). Studies were performed on the same model of the Phillips machine across all sites.

Mitral inflow velocities – measured using pulse wave Doppler with the sample volume at the tips of the mitral valve during diastole. Measurements include the peak early filling (E-wave) and late diastolic filling (A-wave) velocities, the E/A ratio and deceleration time (DT) of early filling velocity. Traditionally E/A has been used to assess diastolic function as a marker of the

LA-LV pressure gradient, however both are effected by increasing age (E velocity and E/A ratio decrease and DT and A velocity increase), heart rate and rhythm disturbance.

Colour m-mode propagation velocity (Vp) – measured in an A4C view using a colour m mode Doppler through the centre of the LV with measurement of the slope of the first aliasing velocity during early filling. It is a marker of LV diastolic function which measures the mitral-apical flow rate. In the normal ventricle there is rapid early filling of the LV (LV suction) driven by the LA-LV pressure gradient.

Isovolumic relaxation time – measured by continuous wave Doppler positioned in the LVOT outflow, simultaneously showing the onset of mitral inflow and end of aortic ejection. Impairment of myocardial relaxation reduces the LA-LV pressure gradient. The isovolumic relaxation period is therefore increased until LV pressure is < LA pressure and the mitral valve opens.

Myocardial performance Index (MPI) / Tei index – A Doppler-derived index of combined systolic and diastolic myocardial performance. It compares the total systolic time from mitral valve closure to mitral valve opening with systolic time involved in actual aortic flow

5.3.2.4   Myocardial deformation

The echocardiographic measurement of myocardial deformation provides a series of regional and global quantitative measures of myocardial function and contractility. Given the organization of the cardiac fibre and its complex anatomy. Assessment of this is done by deformation indices include strain, strain rate and torsion which provide a more direct assessment of intrinsic myocardial contractility than EF or wall motion analysis (Mor-Avi et al., 2011). Strain (S) is a measure of deformation (%) and refers to the change in myocardial length from an applied force. Strain rate (SR) is the time course of the deformation (s-1) and is derived from the difference of two velocities normalized to the distance between them. By

convention, ventricular contraction shortens longitudinal and circumferential fibres giving a negative strain and radial fibres lengthen and thicken giving positive strain. As such, normal longitudinal contraction is defined by negative systolic strain followed by biphasic positive diastolic strain corresponding to early and late diastolic filling.

Newer 2D speckle tracking (Doppler independent) semi-automated techniques for assessing deformation are accurate, well validated and not effected by 1D methodology limitations (Amundsen et al., 2006). Blocks of speckles in a discrete location are tracked from frame to frame (simultaneously in multiple regions) and tissue displacement data is derived from which strain and strain rates can be calculated. Two dimensional grey scale images from the apical four and two chamber views with sector depth and width optimised, were acquired at end-expiration at a frame rate ≥80 / second. The endocardial border was manually tracked at end-systole using commercially available software (Q-Lab 9- Philips). The software automatically defined the epicardial and mid myocardial line within the region of interest and calculates the frame to-frame displacements of the speckle-pattern throughout the cardiac cycle (Figure 24). The advantage of speckle tracking include: quantification of deformation indices in any direction within the imaging plane and in simultaneous ROIs, less angle dependence and less time consuming semi-automated off-line analysis packages (BD, 2011).

All analysed images were recorded with a frame rate of at least 40 fps for reliable analysis by the software. First, the LV end-systolic frame was defined by determining the closure of the aortic valve in the apical long-axis view. Then the time interval between R-wave and aortic valve was automatically measured and used as a reference for the four- and two-chamber views. After defining the mitral annulus and LV apex with three index points in all three apical views, the LV endocardial border was automatically traced at end-systole and the created region of interest was manually adjusted to the thickness of the myocardium. Segments which failed to track by the software were manually adjusted by the operator. Any segments which subsequently failed to track were automatically discarded by the software for the calculation of global strain (Phillips Q lab 9 manual). Analysis was feasible in 90% of the segments. Global longitudinal peak systolic strain for the complete LV was provided by the software

using a 17-segment model in a 'bull's eye' plot calculated as the average of longitudinal or circumferential peak systolic strain of each view.

Figure 24: An example of speckle tracking of Q-lab and cardiac delineation- A. B Bulls-eye illustrating areas of reduced deformation (blue coloured segments: The echocardiogram is a highly reproducible assessment of cardiac volumes. My inter observer coefficient of variation ranged from 4.9-8.8% (Table 19).

Table 19: Echocardiogram inter-observer variability

	Chamber volumes			Systolic function				Diastolic function				RV function	
	ESV	EDV	LA volume	EF	TIE index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
Subject 1													
Day 1	32	80	18	63	0.1	70	35	1.1	269	8	10	17	25
Day 2	33	82	20	59	0.1	68	33	1.3	251	7	9	18	26
Day 3	28	75	21	62	0.1	65	32	1.4	268	7	9	18	26
Day 4	30	86	22	64	0.1	72	30	1.3	267	7	10	17	27
Day 5	33	72	19	60	0.1	76	35	1.2	250	6	10	20	26
Standard deviation	2.17	5.57	1.58	2.7	0	4.14	2.12	0.11	9.61	0.71	0.55	1.22	0.71
Coefficient of Variation (%)	6.95	7.04	7.95	3.37	0	5.9	6.4	9.05	3.68	10.1	5.70	6.8	2.72
Subject 2													
Day 1	48	60	25	51	0.2	65	40	0.9	216	6	9	21	23
Day 2	40	66	26	48	0.3	72	37	0.9	189	6	9	22	23
Day 3	45	68	28	49	0.3	84	36	0.8	254	6	10	23	26
Day 4	49	69	24	47	0.2	78	40	0.8	268	7	9	23	26
Day 5	44	75	26	51	0.1	80	39	0.8	248	7	8	24	27
Standard deviation	3.6	5.4	1.5	1.8	0.0	7.4	1.8	0.05	24	0.55	0.71	1.14	1.87
Coefficient of variation (%)	7.9	8.1	5.7	3.6	0	9.8	4.7	6.5	9.5	8.6	7.9	5	7.5
Subject 3													
Day 1	60	100	25	56	0.3	66	28	1	199	6	10	20	29
Day 2	58	95	22	60	0.3	79	28	1.1	210	6	11	20	29
Day 3	54	90	25	58	0.3	75	30	1.2	222	6	9	19	29
Day 4	59	99	26	51	0.25	73	31	1	187	6	10	18	26
Day 5	54	95	28	55	0.3	70	29	1.1	224	5	11	18	26
Standard deviation	2.8	3.9	2.2	3.4	0.02	4.9	1.3	0.08	15.6	0.44	0.84	1	1.64
Coefficient of variation (%)	4.9	4.1	8.6	6.1	7.7	6.8	4.5	7.7	7.5	9.7	13	5.3	5.9

Subject 4													
Day 1	55	90	26	63	0.1	80	35	1.4	170	4	6	21	31
Day 2	45	75	28	65	0.1	85	36	1.3	201	4	8	21	29
Day 3	49	88	29	61	0.1	76	37	1.3	188	5	7	18	32
Day 4	52	86	30	59	0.3	79	34	1.2	166	4	4	18	32
Day 5	58	84	27	63	0.3	80	38	1.2	205	4	9	18	31
Standard deviation	5.1	5.8	1.6	2.3	0	3.2	1.6	0.08	17.6	0.44	0.84	1.64	1.22
Coefficient of variation (%)	9.8	6.9	5.6	3.7	0	4.1	4.4	6.5	9.4	10.6	11.6	8.6	4
Subject 5													
Day 1	45	60	27	62	0.2	76	40	1.1	167	8	5	19	32
Day 2	46	65	25	66	0.3	66	42	1.3	175	7	6	19	32
Day 3	44	66	24	59	0.3	67	45	1.4	184	8	6	17	29
Day 4	39	75	26	61	0.2	79	39	1.5	168	5	6	16	30
Day 5	40	80	28	68	0.1	70	42	1.4	187	7	6	16	31
Standard deviation	3.1	8.1	1.6	3.7	0.08	5.7	2.3	0.15	9.1	0.54	0.44	1.51	1.30
Coefficient of variation (%)	7.3	11	6.1	5.9	0	7.9	5.5	11	5.2	7.2	7.7	8.7	4.2
Average coefficient of variation (%)	7.4	7.6	6.8	4.5	1.5	6.9	5.1	8.2	7.1	8.8	8.2	6.9	4.9

Interobserver variability of the echocardiogram study performed on 5 different subjects on 5 consecutive times. I was blinded to patient's recruitment status or past medical history. Average of coefficient of variation was between 1.5 – 8.8. Subjects recruited were healthy individuals with no prior cardiac history

5.3.3 TNF-alpha and MMP- 9

These were performed by ELISA on recruited patient's plasma as described in chapter 4 section 4.3.3 and 4.3.4.

5.3.4 Statistical analysis

All data in this work were analysed using SPSS version 21 (SPSS Inc., Chicago, Illinois, USA) as well as GraphPad Prism version 6. Normality of data distribution for each variable was determined using the Kolmogorov-Smirnov test and normality plots. Normally distributed variables are presented as mean ± standard deviation and analysed using parametric tests. Any non-normally distributed variables were analysed as non-parametric data and were quoted as median (interquartile range). Inter-group comparisons were performed using independent t-tests (two groups) or a one-way analysis of variance (more than two groups). Non parametric data were analysed using a Wilcoxon t-test for independent data and within group analysis was performed using Kruskal-Wallis. Within-group comparisons across different time points between recruitment and follow up were assessed using paired samples t-test. In case of non-parametric measures between-group comparisons across different time points were performed using a repeated measures analysis of variance, with the time point. Linear regression analysis was performed to determine the relationship between continuous outcome variables. Correlation parametric variables were assessed using Pearson's correlation and any non-parametric variables assessed using Spearman's. Categorical variables were presented as frequency (percentage) and analysed using the Pearson and two tailed significance was reported. A Type I error rate below 5% ($p < 0.05$) was considered statistically significance. In correlation statistics between echocardiographic parameters and monocyte counts as well as functional assessment of monocytes, the statistical significance is established at $p < 0.01$, in order to reduce the possibility of random associations given the number of studied parameters.

5.4 Results

5.4.1 Parameters of echocardiographic study

A total of 172 patients went on to have the first echocardiogram at recruitment, of those 121 attended their second echocardiogram at 6 months post infarction with remaining subjects having either died or did not attend the invitation or follow up echocardiogram. Using dependent t-test for parametric data, or Wilcoxon test for non-parametric data, the echocardiographic parameters between the two sampling point were compared (Table 20).

End systolic indexed volume (ESVI) and end diastolic indexed volume (EDVI) were both significantly increased in the second echocardiogram compared to first echocardiographic study, p= 0.01 and p <0.001, respectively.

LV Ejection fraction by Simpson's biplane was not statistically different between the first and second echocardiogram study. The Tei index, fractional shortening, and strove volume with higher in the first 24 hours compared to six months post infarction (p = 0.03, p <0.001, and p <0.001 respectively).

As for the diastolic parameters of cardiac function, the deceleration rate and isovolumic relaxation time (p=0.62) (IVRT), as well as E/A ratio were prolonged at follow up compared to the post infarct study, p <0.001 and 0.02 respectively.

In deformation studies the global longitudinal strain (GLS) were significantly lower in the first 24 hours compared to 6 months after infarction, p <0.001.

Table 20: Echocardiographic study parameters of recruited patients undergoing both echo studies

	Parameter	Within the first 24 hours post infarct (121)	Six months post infarct (N= 121)	p Value
Chamber volumes	End systolic volume /ml (ESV)	41 (32-54)	42 (33-55)	0.17
	End systolic volume index ml/m^2 (ESVI)	21 (16-27)	18 (15-23)	0.01
	End diastolic volume /ml (EDV)	88 (74-102)	84 (65- 110)	0.59
	End diastolic volume index ml/m^2 (EDVI)	37 (31-50)	45 (39-53)	<0.001
	LA volume /ml	43 (32-53)	46 (36-51)	0.02
LV systolic function	EF SIMPSON'S biplane	50 (43-56)	50 (43-56)	0.66
	Tie index	0.28 ± 0.14	0.3± 0.16	0.03
	Stroke volume	51 (35-69)	17 (11-56)	<0.001
LV diastolic function	MV E/A	1.2 (0.9-1.5)	0.9 (0.8-1.2)	0.02
	MV deceleration time	210 (164-246)	204 (120-248)	<0.001
	IVRT	98 (69-121)	84 (58-120)	0.02
	EE' lateral	8.4 (6.6-11.4)	9 (7-11)	0.72
	EE' septal	11 (8-13)	9 (6-16)	0.08
RV function	FAC of RV	33 (27-38)	31 (28-38)	0.02
	TAPSE	19 (17-22)	19 (14-22)	0.47
Deformation study parameter	GLS	-14 (-16 - -11)	-16 (-18 - -13)	<0.001
	GCS	-15 (-20- -11)	-16 (-21 - -11)	0.31

Dependent t-test was used for analysing parametric variables. Wilcoxon t-test was used to interrogate non-parametric data. The following parameters were significantly higher in the first 48 hours post recruitment compared to the 6 months follow up study: ESVI, EDVI, LA volume, Tei index, stroke volume, fractional shortening, MV deceleration time, IVRT, FAC (Fractional area change) of the right ventricle (RV). GLS, global longitudinal strain; GCS global circumferential strain. Data are expressed as mean ± SD for normally distributed data or as median with (interquartile range) for nonparametric data.

5.4.2 *Monocytes correlation with echocardiographic study*

Using linear regression analysis all monocyte subpopulations were negatively associated with EF within the first 7 days post admission (R^2 = 0.6, 0.7 and 0.3 for Mon 1, Mon 2 and Mon 3 respectively; p). At 14 days only Mon 2 remained correlated with R of 0.6, p =0.02).

At day 1 total monocyte count, Mon 1 and Mon 2 were negatively associated with TEI index as another determinant of systolic function (p <0.0001).

The fractional area change (FAC) of the right ventricle was also associated with total monocyte count and Mon 1. IKKβ Mon 1, Mon 2 and Mon 3 were associated with SV. Phagocytic activity of Mon 2 was associated with ejection fraction (EF).

When associating monocyte subset counts and their functional assessment parameters with the second echocardiogram 6 months post infarction, there remained to be a negative association between total monocyte count, Mon 1, and Mon 2 with ejection fraction (Table25). Higher total monocyte count, Mon 1 and were associated with reduced EF (R= -0.42, p<0.001; R= -0.33, p<0.001), with a very week correlation and for Mon2.

Table 21: Association of monocyte subset counts with different parameters measured on echocardiography at the point of recruitment

	Chamber Volumes					Systolic function				Diastolic function				Right heart	
	ESV	ESVI	EDV	EDVI	LA volume	EF	TEI index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
Total monocyte count/ µl															
R	0.181	0.190	0.189	0.200	0.178	-0.429	0.345	-0.191	0.063	0.025	0.048	0.089	0.101	0.032	0.233
p Value	0.018	0.019	0.013	0.015	0.019	**<0.0001**	**<0.0001**	0.043	0.613	0.789	0538	0.248	0.303	0.730	**0.003**
Mon 1/ µl															
R	0.177	0.179	0.216	0.207	0.151	-0.343	0.304	0.152	0.099	0.002	0.007	0.050	0.055	-0.029	0.212
p Value	0.021	0.028	0.005	0.012	0.040	**<0.0001**	**<0.0001**	0.068	0.247	0.981	0.927	0.517	0.473	0.698	**0.006**
Mon 2/ µl															
R	0.048	0.112	0.014	0.071	0.202	-0.288	0.205	0.148	0.252	0.079	0.154	0.025	0.074	-0.176	0.071
p Value	0.533	0.173	0.854	0.391	0.008	**0.0001**	0.007	0.046	0.003	0.303	0.046	0.749	0.336	0.022	0.364
Mon 3/ µl															
R	0.109	0.130	0.043	0.021	-0.006	0.159	0.094	-0.051	0.093	0.087	0.182	0.062	-0.874	0.141	0.113
p Value	0.157	0.114	0.576	0.802	0.942	0.038	0.221	0.541	0.278	0.257	0.018	0.420	0.257	0.065	0.147

Total monocyte count, Mon 1 and Mon 2 had significant negative association with systolic function parameters including EF and TIE index. The former two were also associated with fractional area change (FAC) of the right ventricle. ESV- end systolic volume; ESVI- end systolic volume indexed to body surface area; EDV- end diastolic volume; EDVI- end diastolic volume indexed to body surface area; LA- left atrium; EF- ejection fraction; SV- stroke volume; FS- fractional shortening

Table 22: Association of monocyte subset functional parameters with echocardiographic findings

	Chamber volumes					Systolic function				Diastolic function				Right heart	
	ESV	ESVI	EDV	EDVI	LA volume	EF	TIE index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
IKKß 1															
R	-0.089	-0.117	-0.119	-0.157	0.04	0.099	-0.222	0.38	0.15	-0.09	-0.47	0.06	-0.08	0.12	-0.12
p Value	0.252	0.159	0.123	0.059	0.61	0.196	**0.004**	**<0.0001**	0.10	0.22	0.55	0.47	0.30	0.11	0.15
IKKß 2															
R	-0.112	-0.160	-0.121	-0.18	0.05	0.068	-0.238	0.30	0.17	-0.07	-0.08	0.07	-0.09	0.15	-0.15
p Value	0.149	0.053	0.118	0.03	0.52	0.377	**0.002**	**0.0003**	0.06	0.39	0.29	0.35	0.23	0.06	0.05
IKKß 3															
R	0.007	-0.064	0.025	-0.04	0.06	-0.065	-0.118	0.29	0.07	-0.04	0.04	-0.01	-0.08	0.002	-0.15
p Value	0.931	0.443	0.751	0.63	0.47	0.404	0.128	**0.0006**	0.45	0.574	0.63	0.86	0.33	0.98	0.06
Phagocytosis 1															
R	0.082	0.106	0.048	0.05	-0.10	-0.102	0.100	-0.03	0.03	0.01	-0.01	0.15	0.04	-0.07	-0.12
p Value	0.291	0.206	0.539	0.55	0.22	0.189	0.196	0.73	0.72	0.93	0.94	0.06	0.63	0.40	0.13
Phagocytosis 2															
R	0.154	0.161	0.131	0.13	-0.06	-0.154	0.07	0.06	0.01	0.03	-0.05	0.09	0.08	-0.03	-0.13
p Value	0.046	0.054	0.091	0.12	0.42	**0.007**	0.34	0.49	0.90	0.66	0.52	0.27	0.32	0.70	0.10
Phagocytosis 3															
R	-0.042	-0.115	-0.041	-0.14	0.03	-0.027	-0.017	-0.001	-0.02	-0.01	-0.07	0.17	0.004	-0.04	0.04
p Value	0.59	0.170	0.575	0.099	0.75	0.727	0.826	0.99	0.79	0.93	0.36	0.03	0.99	0.62	0.62

At day 1: IKKβ 1 and IKKβ 2 were negatively associated with TIE index and stoke volume. With IKKβ 3 also positively associated with SV (stroke volume). ESV- end systolic volume; ESVI- end systolic volume indexed to body surface area; EDV- end diastolic volume; EDVI- end diastolic volume indexed to body surface area; LA- left atrium; EF- ejection fraction; SV- stroke volume; FS- fractional shortening

Table 23: Relationship between matrix metaloproteinases and TNF-α with echocardiographic study parameters at day 1 after recruitment

	Chamber volumes					Systolic function				Diastolic function				Right heart	
	ESV	ESVI	EDV	EDVI	LA volume	EF	TIE index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
MMP- 9															
R	0.15	0.09	0.03	-0.01	0.09	-0.24	.03	0.08	-0.17	0.09	-0.11	0.05	0.05	-0.13	0.11
p Value	0.14	0.35	0.71	0.91	0.31	**0.004**	0.73	0.39	0.09	0.32	0.21	0.59	0.57	0.13	0.20
TNF-α															
R	0.27	0.25	0.27	0.20	0.08	-0.40	0.32	-0.14	-0.16	-0.03	-0.03	0.02	0.04	-0.06	0.12
p Value	**0.005**	0.02	**0.005**	0.06	0.41	**<0.0001**	**0.001**	0.18	0.14	0.78	0.79	0.85	0.58	0.52	0.24

There was a week but highly significant negative association between MMP- 9 and TNF-α with EF. ESV- end systolic volume; ESVI- end systolic volume indexed to body surface area; EDV- end diastolic volume; EDVI- end diastolic volume indexed to body surface area; LA- left atrium; EF- ejection fraction; SV- stroke volume; FS- fractional shortening

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Table 24: Relationship of monocyte subset counts with different parameters measured on echocardiography at phase two of sampling

	Chamber volumes				Systolic function				Diastolic function				Right heart	
	ESVI	EDV	EDVI	LA volume	EF	TIE index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
Total monocyte count														
R	0.18	0.17	0.11	0.04	-0.42	0.39	-0.19	0.06	-0.03	0.12	-0.10	0.10	0.03	-0.02
p Value	0.03	0.06	0.17	0.66	**<0.001**	**<0.001**	0.04	0.63	0.79	0.27	0.26	0.30	0.73	0.87
Mon 1														
R	0.15	0.22	0.09	0.09	-0.33	0.31	-0.16	0.02	-0.05	0.21	-0.06	0.07	0.03	-0.04
p Value	0.08	0.02	0.26	0.39	**<0.001**	**<0.001**	0.09	0.87	0.63	0.06	0.51	0.51	0.78	0.69
Mon 2														
R	0.21	0.00	0.12	0.02	0.06	0.42	-0.10	-0.08	-0.04	-0.10	-0.16	0.13	-0.07	-0.13
p Value	0.01	0.97	0.14	0.84	**<0.001**	**<0.001**	0.32	0.53	0.69	0.36	0.09	0.20	0.43	0.21
Mon 3														
R	0.02	0.06	0.01	-0.02	0.04	-0.02	0.03	0.12	-0.01	0.05	-0.06	-0.09	0.13	0.01
p Value	0.84	0.54	0.87	0.86	0.62	0.80	0.73	0.32	0.90	0.68	0.53	0.38	0.16	0.32

Total monocyte count, Mon 1 and Mon 2 had significant negative association with systolic function parameters including EF and TIE index. ESV- end systolic volume; ESVI- end systolic volume indexed to body surface area; EDV- end diastolic volume; EDVI- end diastolic volume indexed to body surface area; LA- left atrium; EF- ejection fraction; SV- stroke volume; FS- fractional shortening

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Table 25: Association of monocyte subset functional parameters with echocardiographic findings at phase 2 of sampling

	Chamber volumes					Systolic function				Diastolic function				Right heart	
	ESV	ESVI	EDV	EDVI	LA volume	EF	TIE index	SV	FS	E/A	MV Dec	E/E' septal	E/E' lateral	TAPSE	FAC
IKKβ 1															
R	-0.08	0.09	-0.12	0.03	-0.10	0.16	-0.27	-0.42	0.20	0.09	0.24	-0.09	0.10	0.21	-0.07
p Value	0.37	0.31	0.19	0.72	0.31	0.09	0.004	**<0.001**	0.87	0.33	0.03	0.29	0.31	0.03	0.50
IKKβ 2															
R	-0.12	0.04	-0.17	-0.02	-0.16	0.21	-0.27	-0.36	-0.02	0.11	0.17	-0.14	0.06	0.26	-0.09
p Value	0.22	0.67	0.07	0.82	0.12	0.02	0.004	**<0.001**	0.87	0.23	0.14	0.14	0.58	0.005	0.37
IKKβ 3															
R	-0.05	0.06	-0.13	-0.02	-0.18	0.09	-0.14	-0.44	-0.07	0.05	0.17	-0.21	0.15	0.11	-0.17
p Value	0.58	0.52	0.17	0.81	0.08	0.31	0.14	**<0.001**	0.59	0.58	0.12	0.02	0.15	0.22	0.91
Phago 1															
R	0.15	0.16	0.07	0.12	-0.14	-0.27	0.04	0.02	0.12	-0.11	-0.10	-0.02	-0.16	0.19	-0.11
p Value	0.10	0.07	0.48	0.20	0.16	**0.004**	0.67	0.817	0.36	0.23	0.39	0.58	0.12	0.04	0.27
Phago 2															
R	0.25	0.25	0.19	0.23	-0.19	-0.27	0.09	-0.09	0.05	-0.03	-0.05	-0.06	-0.11	0.10	-0.09
p Value	0.007	0.006	0.04	0.009	0.06	**0.003**	0.35	0.35	0.72	0.78	0.69	0.52	0.27	0.29	0.36
Phago 3															
R	0.07	0.04	0.04	0.02	-0.02	-0.5	0.08	-0.14	-0.08	-0.05	0.00	-0.01	-0.20	0.17	0.07
p Value	0.43	0.67	0.67	0.80	0.85	0.57	0.38	0.14	0.54	0.57	0.97	0.89	0.04	0.06	0.51

Phagocytic activity of Mon 1 and Mon 2 were negatively associated with EF. IKKβ levels of Mon 1, Mon 2 and Mon 3 were negatively associated with SV. ESV- end systolic volume; ESVI- end systolic volume indexed to body surface area; EDV- end diastolic volume; EDVI- end diastolic volume indexed to body surface area; LA- left atrium; EF- ejection fraction; SV- stroke volume; FS- fractional shortening

5.4.3 Monocyte quartiles associations with ejection fraction:

Higher monocyte count quartiles correlated with lower ejection fraction. Total monocyte count, Mon 1, and Mon 2 higher count quartiles were associated negatively with lower EF. Using one-way Anova testing with

Tukey post hoc analysis, the number of echocardiograms in each monocyte count quartile were statistically different amongst the different quartiles (Figure 25).
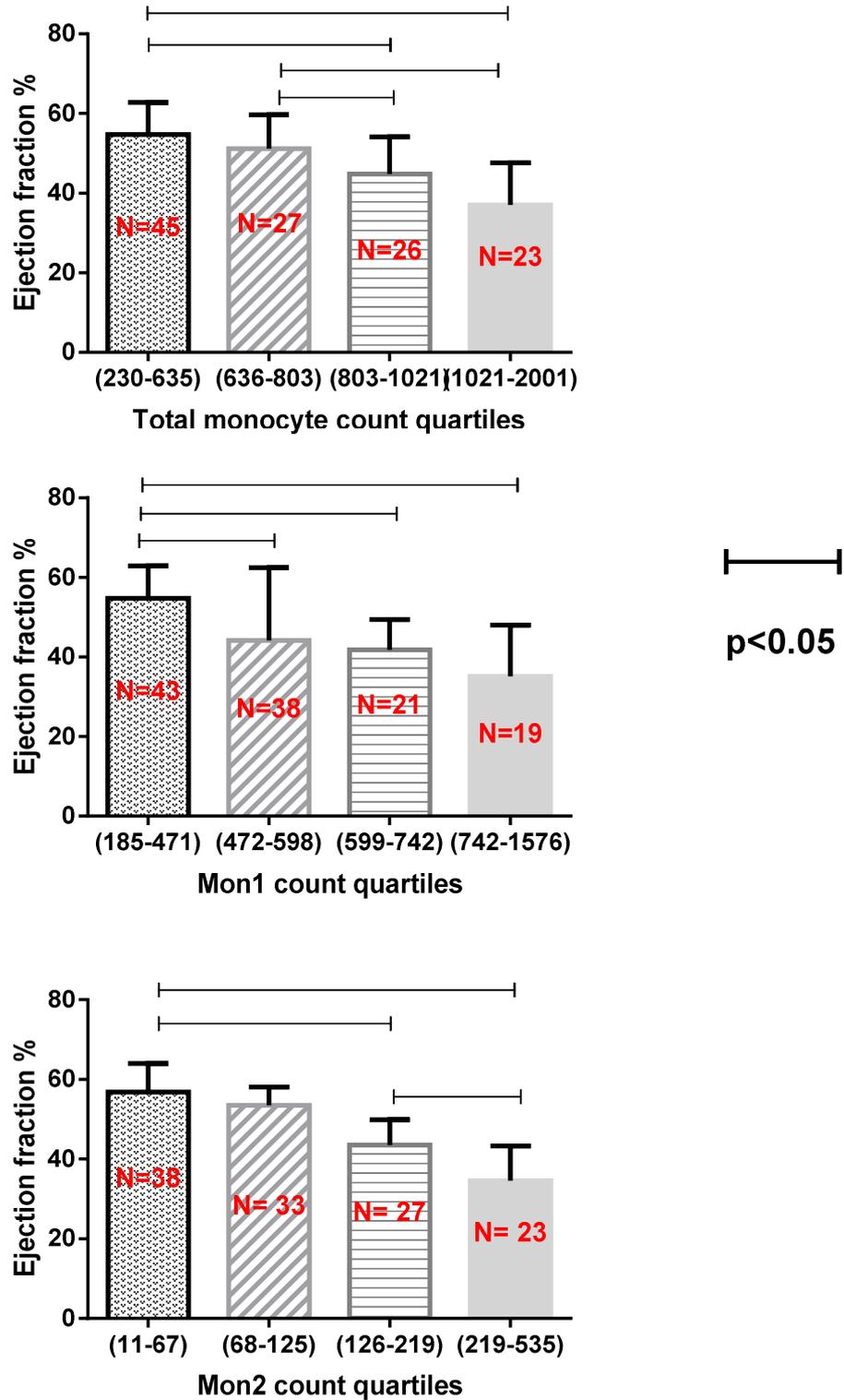
Figure 25: Monocytes quartiles association with ejection fraction: Higher quartiles of total monocyte count, Mon 1 and Mon 2 were associated with lower ejection fraction. (one way anova with Tukey post hoc analysis). Differences between groups was postivie at levels of p< 0.05.

5.4.4 Monocytes and negative ventricular remodelling

Negative ventricular remodelling is defined as an increase in end diastolic volume indexed to body surface area of ≥20% on the six months echocardiogram compared to first echocardiographic study performed 24 hours post infarction.

5.4.4.1   Demographics of patients with negative ventricular remodelling

In total, 121 patients from the total recruited 209 underwent both echocardiogram studies and hence were assessed for ventricular remodelling. Thirty-four patients suffered from negative remodelling at the completion of the healing process, i.e. 6 months post infarction. Table 26 summarizes the clinical characteristics of patients who sustained negative ventricular remodelling. Patients who have 'negative' LV remodelling were generally well matched to those who had a non-remodelled outcome. However, patients who sustained negative remodelling were more likely to be on aspirin and ACE inhibitors (p = 0.01, and p = 0.01 respectively). There was also a higher percentage of a history of previous myocardial infarction amongst those who suffered negative remodelling (p= 0.01). These demographics are summarized in Table 26.

Table 26: Patient demographics of those sustaining negative remodelling compared to patients with no remodelling

	Parameter	No remodelling (N= 87)	Negative remodelling (N=34)	p Value
Patient's Demographics **(%)**	Age	61 ± 11	64 ± 11	0.10
	Sex	62/85	25/35	0.99
	Peak Troponin (ng/l)	7520 ± 13665	5627 ± 9511	0.55
	Peak Creatinine Kinase	1494 ± 1427	1524 ± 1527	0.92
	Total WCC (mmol/L)	12 (8.6 – 13)	12 (9 – 13)	0.64
	Estimated GFR (ml/min/1.72m^2)	73 ± 16	74 ± 15	0.91
	Total Cholesterol (mmol/L)	4.8 (4.3 – 5.2)	5 (4.7 – 5.3)	0.65
	Time from symptoms onset to presentation to hospital (hours)	4.7 ± 2.4	5.2 ± 2.7	0.40
	Inferior infarct	33 (39)	12 (34)	0.68
	Anterior infarct	41 (48)	11 (31)	0.11
Past Medical History [n (%)]	Diabetes (%)	19 (22)	10 (32)	0.24
	Hypertension (%)	44 (50)	20 (65)	0.21
	Smoking (%)	47 (53)	20 (65)	0.30
	Hypercholestrolaemia (%)	39 (44)	15 (48)	0.83
	Heart failure (%)	11 (13)	3 (8.6)	0.76
	History of angina (%)	2 (2.3)	1 (2.9)	0.99
	Previous myocardial infarction (%)	6 (7.1)	10 (29)	0.01
	Obesity (%)	11 (13)	7 (19)	0.24
	Cerebrovascular events (%)	5 (5.7)	3 (6.5)	0.89
	Previous CABG (%)	5 (5.7)	3 (10)	0.43
	Previous PCI (%)	4 (4.7)	4 (12)	0.23
	COPD (%)	8 (9.5)	14	0.52
Drug history at Recruitment (%)	Aspirin	31 (36)	20 (65)	0.01
	Clopidogrel	2 (2.3)	1 (3.2)	0.99
	Statin therapy	29 (33)	14 (45)	0.28
	Calcium channel blocker	22 (25)	8 (26)	0.99
	Beta blocker	13 (15)	9 (29)	0.11
	Loop diuretic	10 (11)	6 (19)	0.36
	Thiazide diuretic	8 (9.2)	4 (13)	0.51
	ACE inhibitor	31 (36)	20 (65)	0.01
	Nitrates	6 (6.9)	1 (3.2)	0.67

Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median with (interquartile range) for non-normally distributed data

5.4.4.2 Monocyte counts and function in patients with negative remodelling

At day Patients who suffered from negative remodelling had higher counts of total monocyte count, Mon 1 (p= 0.004) and Mon 2 (0.007). Their phagocytic activity were also significantly higher for both Mon 1 (p= 0.008) and Mon 2 (p= 0.005) (Table 28). There was no difference in the IKKβ activity.

Table 27: Monocyte count and functional activity in negatively remodeled patients

	Negative remodelling (n= 34)	No remodelling (n= 87)	p Value
Total Monocyte count (cells/ μl)	1004 ± 385	821 ± 263	0.004
Mon 1 (cells/ μl)	752 ± 282	613 ± 225	0.007
Mon 2 (cells/ μl)	188 ± 134	144 ± 103	0.07
Mon 3 (cells/ μl)	64 ± 43	63 ± 31	0.87
IKKβ Mon 1 (MFI)	69 ± 26	73 ± 25	0.39
IKKβ Mon 2 (MFI)	65 ± 25	68 ± 22	0.57
IKKβ Mon 3 (MFI)	68 ± 32	73 ± 21	0.36
Phagocytosis Mon 1 (MFI)	139 ± 30	121 ± 32	0.008
Phagocytosis Mon 2 (MFI)	140 ± 49	115 ± 38	0.005
Phagocytosis Mon 3 (MFI)	44 ± 19	42 ± 20	0.73

Data are expressed as mean ± standard deviation (SD) for normally distributed data

At 14 days (phase 2), there was no significant difference between monocyte counts and any of their functional parameters (table 29).

Table 28: Monocyte count and functional activity in negatively remodeled patient during phase 2 (day 14)

	Negative remodelling (n= 34)	No remodelling (n= 87)	p Value
Total Monocyte count (cells/ μl)	578 ±	573 ± 362	0.
Mon 1 (cells/ μl)	419 ± 99	404 ± 103	1.0
Mon 2 (cells/ μl)	101± 44	82 ± 35	0.69
Mon 3 (cells/ μl)	53 ± 34	49 ± 12	0.26

IKKβ Mon 1 (MFI)	58 ± 20	64 ± 23	0.67
IKKβ Mon 2 (MFI)	57 ± 20	64 ± 22	0.27
IKKβ Mon 3 (MFI)	65 ± 19	66 ± 19	0.69
Phagocytosis Mon 1 (MFI)	123 ± 29	118 ± 34	0.56
Phagocytosis Mon 2 (MFI)	112 ± 36	120 ± 38	0.32
Phagocytosis Mon 3 (MFI)	43 ± 16	46 ± 12	0..4

Data are expressed as mean ± standard deviation (SD) for normally distributed data

5.4.4.3   Predictors of negative remodelling

A univariate and multivariate linear regression model for factors predicting LV remodelling was built to determine factors predicting LV remodelling.

5.4.4.3.1   *Univariate predictors of negative remodelling*

A univariate regression model indicated that amongst patients with negative remodelling having a previous history of a myocardial infarction or being a current smoker were both predictive of negative remodelling (OR= 1.2, p= 0.03 and OR= 1.41, p=0.04 respectively). MMP- 9 and TNF-α were also predictive of negative remodelling (OR= 3.58, p=0.02; OR=4.11, p= 0.02 respectively) (Table 30).

Table 29: Logistic regression model for predictors of negative ventricular remodelling

Patient Demographics	Logistic regression model		
	OR	**95% CI**	**p Value**
Age	1.01	0.88 – 1.16	0.88
Diabetes Mellitus	1.39	0.57 – 3.39	0.47
Hypertension	0.51	0.23 – 1.15	0.11
Previous myocardial infarction	1.20	0.06 - .058	0.03
Smoking	1.41	0.18 – 0.95	0.04
Time to Presentation	1.07	0.92 – 1.25	0.40

Time to recruitment	1.00	0.93 – 1.08	0.99
Obesity	0.59	0.20 – 1.66	0.33
Hypercholestrolaemia	0.62	0.28 – 1.37	0.24
LAD infarct	2.03	0.89 – 4.67	0.09
Peak Troponin	1.00	1.00 – 1.00	0.55
Peak CK	1.00	1.00 – 1.00	0.92
Total WCC	1.032	0.97 – 1.09	0.30
MMP- 9 at recruitment	3.58	1.21 – 10.57	0.02
TNF-α at recruitment	4.11	1.24 – 13.64	0.02
MMP- 9 at 14 days	0.85	0.014 – 52.47	0.94
TNF-α at 14 days	30	0.008 – 11.7	0.42

Logistic regression model for factors predicting negative ventricular remodelling.

5.4.4.3.2   Multivariate predictors of negative remodelling

Using the univariate model results a forward multivariate regression model was built adjusting for: age, sex, hypertension, diabetes, smoking, hypercholesterolaemia, peak troponin, previous myocardial infarction and history of angina as well as MMP- 9. This indicated that total monocyte count, Mon 1 and phagocytic activity of Mon 2 were independent predictors of LV remodelling (p= 0.001, 0.003, 0.03 respectively). Phagocytic activity of Mon 1 and Mon 2 were also increased the likelihood for developing negative ventricular remodelling (p= 0.04 and 0.007 respectively (Table 31).

Table 30: Multivariate regression model for prediction of negative ventricular remodelling

	Phenotypic and functional characteristics of monocyte subpopulations			
	Total Monocyte count/ 1 cell	1.002	0.001 – 0.004	0.001
	Mon 1, per 1 cell	1.003	0.001 – 0.004	0.003
	Mon 2 /1 cell	1.004	0.002 – 0.007	0.03
Day 1	Mon 3/ 1 cell	1.003	0.994 – 1.012	0.54
	IKKβ Mon 1 (MFI)	0.991	0.974 – 1.008	0.29
	IKKβ Mon 2 (MFI)	0.992	0.974 – 1.010	0.37
	IKKβ Mon 3 (MFI)	0.990	0.972 – 1.008	0.26

	Phagocytosis Mon 1 (MFI)	1.013	1.001 – 1.026	0.04
	Phagocytosis Mon 2 (MFI)	1.015	1.004 – 1.26	0.007
	Phagocytosis Mon 3 (MFI)	1.001	0.982 – 1.001	0.945

The model adjust for adjusting for: age, sex, hypertension, diabetes, smoking, hypercholesterolaemia, peak troponin, previous myocardial infarction, history of angina, TNF-α and MMP- 9

5.4.5 Monocytes correlation with deformation study:

Subclinical changes of the cardiac function measured by deformation assessment of the global longitudinal and circumferential strain were correlated with total monocyte count, Mon 1, Mon 2 and Mon 3. At day 1 impaired global longitudinal and circumferential strain were associated with higher counts of total monocyte count (R^2=0.37, p <0.0001; R^2=0.24, p <0.0001 respectively). The association was weaker at 14 days post infarction albeit it being significant (R^2=0.17, p <0.0001; R^2=0.06, p= 0.02) (Figure 26).

Total monocyte was equally strongly associated with global longitudinal strain at day 1 (R^2=0.36, p <0.0001). The correlation with global circumferential strain was stronger compared to total monocyte count with R^2=0.26 (p <0.0001). When compared with day 2, Mon 1 was also weekly negatively associated with global longitudinal and circumferential strain (R^2=0.14, p= 0.0003; R^2=0.07, p= 0.01 respectively) (Figure 27).

With significance level determined to be <0.01, Mon 2 was not associated with global longitudinal and circumferential strain at day 1 post infarction (R^2=0.06, p =0.01; R^2=0.02; p=0.13). There was also no association at day 14 between Mon 2 and strain studies (R^2 =0.08, p=0.01; R^2 =0.02, p=0.24) (Figure 28).

Mon 3 conversely were very weekly, but significantly, associated with global longitudinal strain and global circumferential strain at day 1. There was no association when Mon 3 were correlated with deformation studies at day 14 (R^2= 0.01, P=0.01- for global longitudinal strain; and R^2= <0.000, P= 0.93) (Figure 29).

130
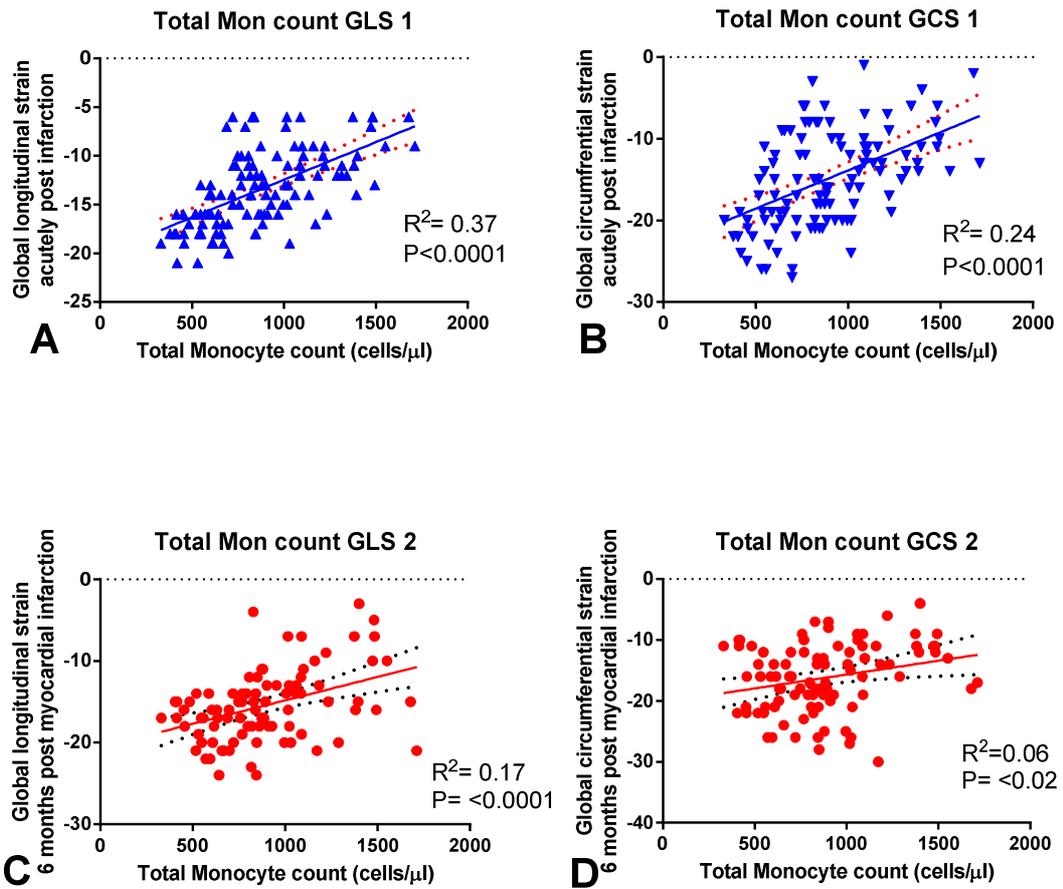
Figure 26: Total monocyte count association with deformation study: A- Mon association with global longitudinal strain (GLS) and B- global circumferential strain (GCS) at day one. There was a week correlation that was not statistically significant (P>0.01). C- Mon association with day 14 GLS and D- GCS, there remained to statistically significant association was found between Mon and global longitudinal deformation at day 14.
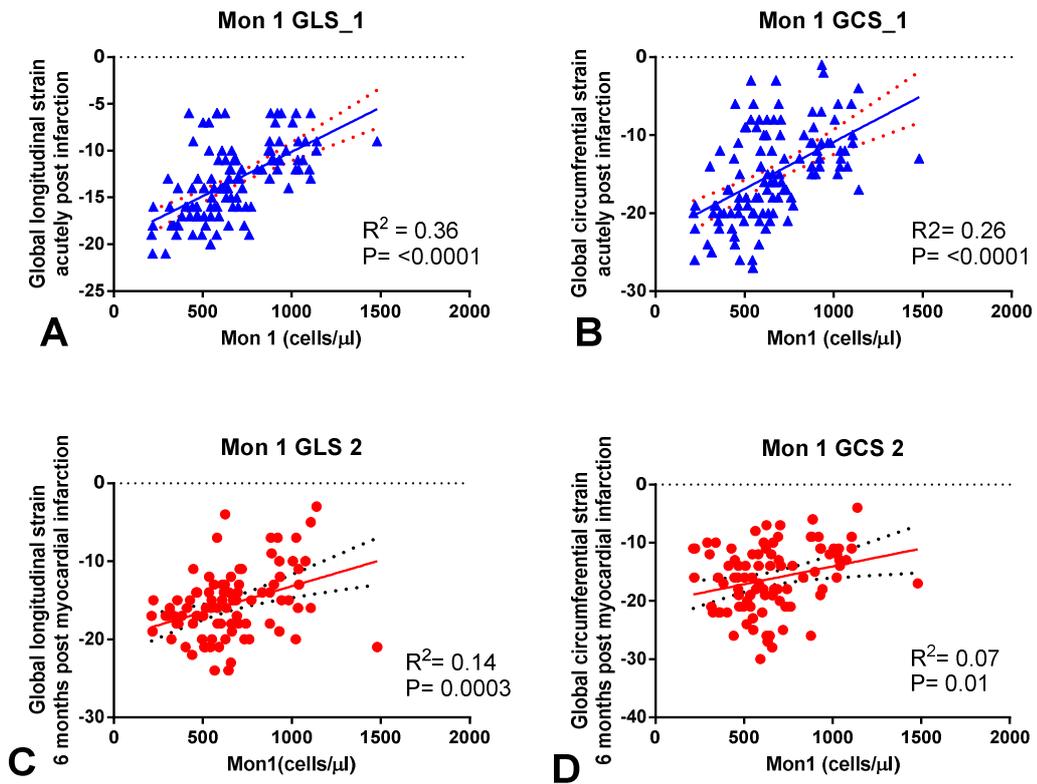
Figure 27: Mon 1 association with deformation study: A- Mon 1 association with global longitudinal strain (GLS) and B- global circumferential strain (GCS) at day one. There was a week correlation that was not statistically significant (p >0.01). C- Mon 1 association with day 14 GLS and D- GCS, there remained to statistically significant association was found between Mon 1 and global longitudinal deformation at day 14.
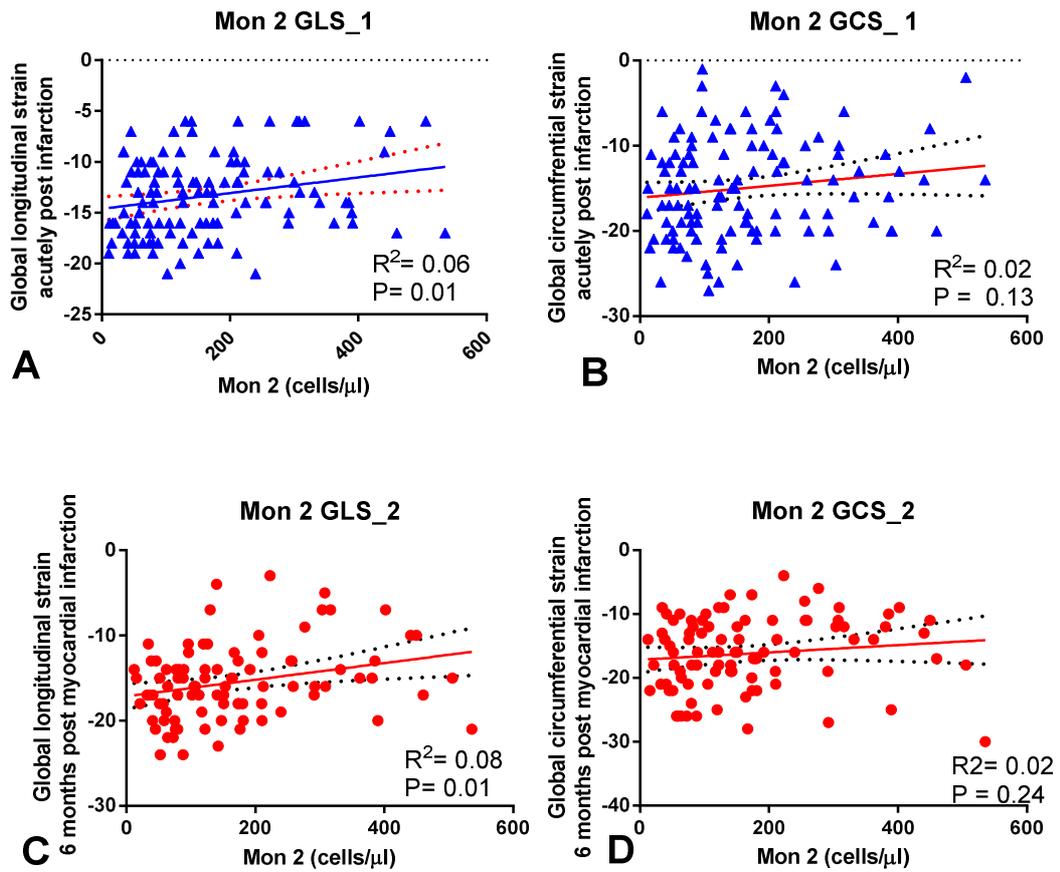
Figure 28: Mon 2 association with deformation study A- Mon 2 association with global longitudinal strain (GLS) and B- global circumferential strain (GCS) at day one. There was a week correlation that was not statistically significant (p >0.01). C- Mon 2 association with day 14 GLS and D- GCS at day 2, no statistically significant association was found between Mon 2 and deformation studies at day 2.
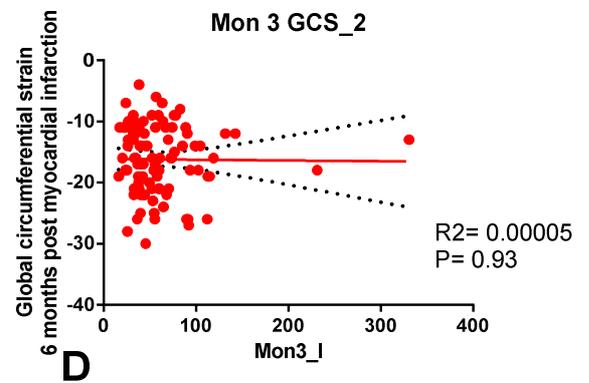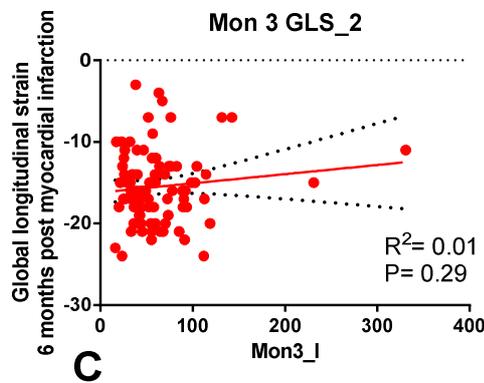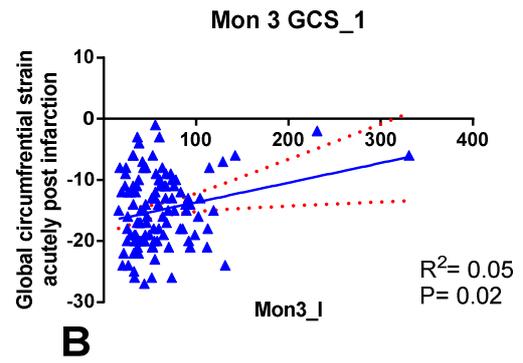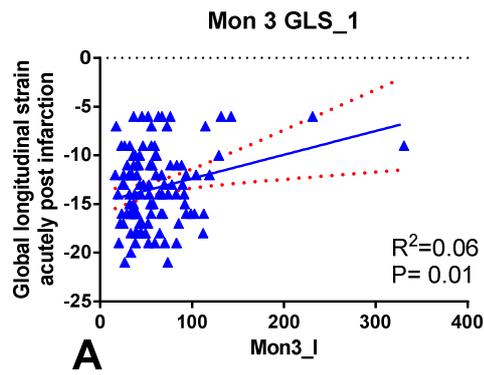
Figure 29: Mon 3 relationship with GLS and GCS at day 1 and 14: No meaningful significant association was found between Mon 3 and GLS or GCS at any time point.

5.5 Discussion

My study is the largest study that has investigated the effect of monocytes on ventricular remodelling post STEMI. It is also the study that has allowed for the longest recovery period post STEMI, with follow up echocardiograms being performed after six months from the index STEMI, other studies followed patients for 4, 6 or 12 weeks month (Maekawa et al., 2002, van der Laan et al., 2002). My study used echocardiogram and body surface area indexed volumes to assess ventricular remodelling as an easily accessible, highly reproducible least intrusive imaging modality for investigating ventricular remodelling.

I opted to utilise echocardiography for the assessment of LV volumes and measurement of EF. Compared to CMR, echocardiography is the most readily available clinical modality with least intrusion to patient care acutely within the first 24 hours post myocardial infarction. Even though end systolic and end diastolic volumes have been shown to be systematically underestimated on echocardiogram compared to cardiac magnetic resonance studies, however as I performed all the echocardiogram studies myself with a good inter-observer variability (table 20), the uniformity of the data would have been attained despite underestimated volumes.

Echocardiography also provided robust information on subclinical changes to LV systolic function i.e. deformation studies. At the time of patient recruitment CMR obtained deformation studies were still being validated as a research utility.

As this was an echocardiographic based study, LV mass was not measured in this given the inherent changes in the left ventricular wall geometry following STEMI: with the likelihood of thinning of ventricular wall in the infarcted zone with segmental hypertrophy in non-infarcted segments as a compensatory mechanism. This non uniform myocardial response to infarction undermines the assumptions of homogeneity of contractility assumed by both mathematical equations utilised in calculating LV mass using echocardiography (Lang et al., 2006).

I illustrate that high counts of total monocytes as well as monocyte populations 1 and 2 measured in the first 24 hours post infarction correlated with ejection fraction and tie index, both as measu1res of LV systolic function. The tie index is not dependent on volumes changes and hence is not affected by preload and afterload alteration. This correlation remained significant at 6

months with the final remodelled ejection fraction being negatively associated with total monocyte count, Mon 1, Mon 2 as well as the phagocytic activity of Mon 1 and Mon 2. Higher quartiles of total monocyte count, Mon 1 and Mon 2 were all associated with lower ejection fractions. This reflects the possible inflammatory function of the Mon 1, as well as the newly described Mon 2.

As illustrated by other groups, MMP- 9 was predictive of negative ventricular remodelling due to its well established effect on increasing LV geometry eventually leading to an increase in ventricular volumes. MMP- 9 is secreted by monocytes on stimulation by acute insult (Brunner et al., 2010) . For the first time we illustrate the monocyte total monocyte count and Mon 1 as well as Mon 2 are predictive of negative ventricular remodelling in a multivariate model that is adjusted for MMP- 9.

I describe for the first time that phagocytic effect of Mon 1 and Mon 2 are independent predictor factors of negative remodelling in STEMI patients. I've discussed the monocyte role in phagocytosis in Chapter 3. Here phagocytosis is illustrated to be the mechanism through which Mon 1 and Mon 2 exert their deleterious effects on reduced systolic function. This is in keeping with findings from Panizi et al. in murine models (Panizzi et al., 2010). None of the human studies in the area of monocytes so far has described a functional parameter to account for the effect of monocytes on myocardial salvage, microvascular obstruction or reduced EF. The role of impaired phagocytome clearance, i.e. delayed apoptosis, could explain the discrepancy in the literature.

Tumour necrosis factor- α (TNF-α) is a pro-inflammatory cytokine secreted by activated macrophages and T cells, and is instrumental in the mediation of vascular inflammation. TNF-α production is in increased concentrations in patients with acute myocardial infarction (AMI). Furthermore, plasma TNF-α concentrations are predictive of death and recurrent ischaemic events following AMI (Satoh et al., 2008, Schlitt et al., 2004). This study indicates that in a multi variate model, including the effect of TNF-α, Total monocyte count, Mon 1 and Mon 2 remained independent predictors of ventricular remodelling.

There was no effect on IKKβ in predicting ventricular remodelling. There was also no difference in IKKβ levels in patients who developed negative ventricular remodelling. Even though NFκB pathway has been associated with developing heart failure, this study does not reflect this finding.

No doubt NFκB pathway has an inflammatory role, however as discussed in chapter 4, there are two presumed functions of NFκB pathway, one cardiotoxic and the other cardioprotective (Han et al., 2001).

Given the complexity of cardiac fibre alignment and dynamic contractility of the cardiac fibres in different planes during contraction, subclinical cardiac contractility dysfunction could arise. Global longitudinal and circumferential strain provide measurement of subclinical impairment in the contractility of myocytes prior to patients frankly developing signs or symptoms of heart failure. In the era of rapid revascularisation with PCI, and thus reduction in the number of patients overtly presenting with heart failure complication initially, the importance of assessment of subclinical parameters of heart muscle dysfunction is paramount. Global longitudinal and circumferential strain have been shown predictive of negative outcome in the future as well as developing overt heart failure on prolonged follow up (Lyseggen et al., 2005, Skulstad et al., 2006, Vartdal et al., 2007). This is the first study that investigates the effect of monocyte subset counts on monocyte longitudinal and circumferential strain rate. There was a significant relationship between total monocyte, Mon 1 and Mon 2 both in global longitudinal and circumferential strains. Indicating the monocyte effect not just on the global function of the heart but also on subclinical parameters.

5.6 Conclusion

Mon 1 and Mon 2 are predictive of negative ventricular remodelling with phagocytosis appearing to be the functional parameter through which this is implemented. Total monocyte count, Mon 1 and Mon 2 correlate well with deformation studies.

Chapter 6

Monocytes counts and function in depicting major adverse cardiac events (MACE) in patients post STEMI

6.1 Introduction

As described earlier in chapter 1 a plateau has been reached in optimizing management of patients sustaining acute coronary syndrome with an urgent need arising to investigate alternative pathophysiological parameters for therapeutic intervention during management of coronary occlusion. A plethora of literature identified an association of inflammation with progression of stable coronary artery disease with patients with a leukocytosis, and specifically monocytosis being at higher risk for progression of stable coronary artery disease and developing an adverse cardiovascular event, as well as the involvement of monocytes in the progression of the atherosclerotic plaque formation and fibrous cap rupture, as discussed in chapter one.

It has long been recognized that an increase in the peripheral white cell count correlates with the outcome of MI. A large-scale study with 153,213 patients aged 65 years or older with MI demonstrated that a higher WBC count within 24 h of admission was associated with higher mortality within 30 days. However, leukocytosis immediately after MI is caused by the release of neutrophils pooled in blood vessels, spleen and liver, and in that case, the peripheral WBC count changes within hours. Horne et al. in a large retrospective study of 3227 patients, confirmed that leukocytosis is an independent predictor of future myocardial infarction or death (Horne et al., 2005). Maekawa et al. also described monocytosis as a predictor of LV aneurysm formation and LV rupture post infarction (Maekawa Y et al., 2002). During acute MI, the number of circulating classical and intermediate monocytes increased acutely over 3 days and were associated with impaired left ventricular function and larger infarct size. Furthermore, preferential accumulation of CD14++CD16– cells in the infarct border zone has been observed in the myocardium of STEMI patients (Libby et al., 2008). Interesting, compared with cells in patients with stable coronary artery disease, circulating classical monocytes (likely to present Mon 1 and Mon 2 in this study) are more adhesive and increase in number in patients with heart failure.

A number of small observational series described an increase in Mon 1 and Mon 2 subpopulations counts in STEMI patients up to three days post infarction with a negative correlation to ejection fraction. A small scale cardiac magnetic resonance based study recruiting 50 patients, with a short

follow up period of six months, indicated that a negative correlation between classical monocytes and ejection fraction as well as microvascular obstruction. Elsewhere, assessing non-culprit coronary plaque size in 90 acute MI patients during the acute phase and 7-month later found that peak monocyte counts in the blood independently predicted plaque progression after ST-elevation MI.

In humans very few studies have investigated the effect of the newly described intermediate monocyte (this is represented as Mon 2 in this study) in clinical conditions. In a large prospective cohort of 951 patients undergoing elective coronary angiography, increased numbers of intermediate CD14++CD16+ monocytes (similar to Mon 2 in this study) independently predicted cardiovascular death, myocardial infarction (MI) and stroke over a period of 2 and a half years (Rogacev et al., 2012). On the other hand, recently a small study of 60 diabetic patients who sustained a STEMI were matched to 60 non-diabetic STEMI patients and followed up for 6 months. Here intermediate monocytes were found to be positively correlated with ventricular remodelling, suggestive of a probable reparative role for Mon 2.

Hence, given the paucity and conflicting data from small studies with short follow up, it became essential to device a study with longer follow up and adequate statistical power to investigate the effect of monocyte subsets, especially the intermediate subset Mon 2, on clinical outcome post STEMI. Furthermore, the need for investigating the translational and functional pathways to explain the above observations strongly presents itself as an important unanswered question.

6.1.1 Risk stratification of STEMI patients

Physicians sometimes rely on clinically derived scoring symptoms to risk stratify patients' mortality risk and thus aid the decision making process of balancing risk versus benefit on making process to ensure better outcome for the patient. A number of scores are currently available for the assessment of risks in STEMI patients, including the GRACE score, TIMI score, and SKOWELL scores (Morrow et al., 2000). However due to the inherent medical emergency of STEMI, the current clinical pathways in patient management demand intervention/thrombolysis

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to restore flow to the infarcted cardiac myocardium within 30 minutes of presentation to hospital. Hence these scores are mainly for retrospective assessment of mortality risks and do not all apply to STEMI patients, but include other parameters of the acute coronary syndrome, i.e. NSTEMI (Yan et al., 2005).

The TIMI STEMI scores accounts for clinical parameters including blood pressure at presentation, anterior ST elevation, heart rate at presentation, as well as time delay in presentation and body mass index (Table 31). Amongst the above mentioned clinical risk stratification scores, no score accounts for inflammation at the time of STEMI. Investigating the efficacy of these scores in predicting outcome with the inclusion of inflammatory markers could provide a new venue for refining prediction of mortality in STEMI patients.

Table 31: TIMI score criteria and calculated 30 day mortality

TIMI score criteria	Points awarded	Calculated Score and 30 day mortality risk after an myocardial infarction	
		Total Score	**30 day mortality (%)**
Diabetes mellitus, hypertension, or angina	1		
Systolic blood pressure less than 100 mmHg	3	0	0.8
Heart rate greater than 100 BPM	2	1	1.6
Killip class II-IV	2	2	2.2
Body weight less than 67 kg	1	3	4.4
Anterior lead ST elevation or left BBB	1	4	7.3
Time to treat more than 4 hours	1	5	12
Age		6	16
>= 75 years old	3	7	23
65-74 years old	2	8	27
Less than 65	0	9-14	36

Incorporating inflammatory markers, e.g. monocyte count, in the risk stratification score would add another parameter that could possibly improve the sensitivity of these scoring mechanisms in predicting patient mortality. This could also determine which patients could potentially benefit from targeted anti-inflammatory medication post infarction. The efficacy of such a model was never investigated before.

The effect of monocyte counts, Mon 1, Mon 2 and their functional parameters on predicting outcome on prolonged follow up period is not clearly described. The effect of the newly described Mon 2 subpopulation is particularly unclear. There is no data in humans describing a cause and effect relationship of monocytes subsets in STEMI patients.

6.2 Aims and Hypothesis

In this study the aim is to assess the predictive value of monocytes and their functional parameters in predicting clinical outcome of STEMI patients post infarction. We also aim to investigate the effect of adding monocytes into existing risk stratification score to improve their predictive performance.

The hypothesis is that total monocyte count, Mon 1, and Mon 2 are independent predictors of patients' clinical outcomes. Functionally, IKKβ activity of Mon 1 and Mon 2 as well as their phagocytic activity are likely to be predictive of poor clinical outcome.

6.3 Methods

6.3.1 Patient recruitment

Patient population were recruited as described in appendix I and chapter 2, section 2.3.1

6.3.2 Flow cytometry

Flow cytometry studies to measure monocytes, phagocytic activity and IKKβ and were carried out as described in chapters 2, 3 and 4 respectively: sections 2.3.3; 3.3.2; 4.3.2.1

6.3.3 TNF-α and MMP- 9

ELISA measurements of MMP-9 and TNF-α were carried out on stored plasma as described in chapter 4, sections 4.3.3 and 4.3.4 respectively.

6.3.4 Defining MACE

Primary endpoints of adverse cardiac events (MACE) was defined as death, recurrence of acute coronary syndrome and revascularisation, new clinical diagnosis of heart failure based on patient symptoms in conjunction with echocardiographic features suggestive of left ventricular dysfunction. Patients were followed up using electronic hospital records from each recruitment site. I contacted GP surgeries for patients who were not reviewed in hospital post STEMI. All events were adjudicated by myself and I was blinded to monocyte data.

6.3.5 *Statistical analysis*

Data management and statistical analysis were performed using SPSS 21 (SPSS, Inc., Chicago, Illinois). Two-sided p values 0.05 were considered significant. Categorical variables are presented as percentage of patients and were compared using chi-square tests.

Normally distributed data are expressed as mean ± standard deviation. Whilst non-parametric data are expressed as median and quoting the interquartile range. Differences between patient groups were compared using independent t-test for normally distributed data or Mann-Whitney *U* tests for non-parametric data. Long term MACE events after STEMI were assessed by a Cox proportional-hazards model. This was initially carried out as a univariate model, and then a multivariate model was built to analyse the relationship between monocyte subsets, IKKβ and phagocytic activity with event free survival after adjusting for risk factors for cardiovascular disease, biochemical profile. I also adjusted independently for TNF-α as a marker for inflammation and MMP- 9 extracellular matrix remodelling factor.

Subjects were divided into 4 groups (quartiles) according to their total monocyte counts and monocyte subset counts. Kaplan-Meier survival curves were used to compare event-free survival (i.e., time until first occurrence for an adverse cardiac event) between groups. The log-rank test was used to test the hypothesis that at least 1 of the survival curves depicted by the different quartiles differs from the others and then a pairwise comparison between strata was performed to interrogate the relationship between different quartiles. Statistical significance was determined at 5%. ROC curves were calculated for total monocyte count, and individual monocyte subsets with the area under the curve being compared for each subset as well as for troponin and the TIMI score. The monocyte count reaching the maximum sensitivity and specificity (at least 75% and 25% respectively) was then used as a cut off level to convert the continuous data in the Cox regression model into dichotomous data. A multivariate Cox regression model was built again using the dichotomous variables.

Comparison of differences between area under the curve was carried using Z statistics (DeLonge et al., 1998) (Analyse-it®, Excel 2013).

6.4 Results

6.4.1 Recruited Patients

Between March 2011 and Dec 2012 I solely recruited 209 patients from the three different sites. Patients were followed up for a mean 33 ± 17 months during which there were 75 MACE events recorded. MACE cases were recorded in total 75 events mainly driven by heart failure diagnosis (n= 36); need for repeat revascularisation (n=27); death (n=12).

Patients who suffered an adverse cardiac event were significantly older (64 ± 11 years vs 59 ± 11) than those who had an uncomplicated post MI recovery and recuperation. There was also a predominance of male patients (75% males, p=0.02) amongst the MACE group. This is in keeping with already established epidemiology of ischaemic heart disease population. Interestingly there was a higher WCC amongst patients who developed MACE (p=0.03), this is in agreement with previously reports (Table 33).

Table 32: Demographics of patients developing MACE

		MACE N = 75	No MACE N = 134	p Value
Patient's demographics	**Age**	64±11	59 ±11	0.01
	Gender (male/ %)	72	59	0.02
	Peak Troponin	11431 ± 20817	4497 ± 7915	0.03
	Peak Creatinine Kinase	1578 ± 1491	1405 ± 1393	0.43
	Total WCC (mmol/L)	12 (9 – 14)	11 (7.6 – 12)	0.03
	Estimated GFR	71 ± 17	75 ± 16	0.06
	Total Cholesterol (mmol/L)	5 (4.5 – 5.3)	4.8 (4.3 – 5.3)	0.99
	Time from symptoms onset to presentation to hospital (hours)	5.2 ± 2.6	4.5 ± 2.6	0.08
	Time from hospital presentation to recruitment to study (hours)	20 (16 – 25)	19 (17 – 22)	0.27
Past medical history (%)	**Diabetes**	20	27	0.40
	Hypertension	56	51	0.56
	Smoking	56	57	0.99
	Hypercholesterolaemia	39	49	0.19
	Heart failure	11	11	1.00
	History of angina	2	9	0.04
	Previous myocardial infarction	12	21	0.11
	Obesity	15	16	0.84
	Cerebrovascular events	8	9	0.80
	Previous CABG	4	5	0.73
	Previous PCI	8	11	0.45
	COPD	16	11	0.41
	Anterior infarct	46	38	0.24
	Inferior infarct	29	43	0.06
Drug history at recruitment (%)	**Aspirin**	40	35	0.54
	Clopidogrel	2.7	2.3	0.98
	Statin therapy	37	29	0.29
	Calcium channel blocker	19	24	0.37
	Beta blocker	15	21	0.34
	Loop diuretic	14	16	0.69
	Thiazide diuretic	10	8.1	0.80
	ACE inhibitor	36	50	0.54
	Nitrates	7.8	5.4	0.77

Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median with (interquartile range) for non-normally distributed data

6.4.2 Monocyte counts and functional parameters of patients developing MACE

Patients who sustained a MACE event had higher total monocyte counts, Mon 1 and Mon 2 as well as Mon 3 counts compare to patients who have not developed MACE (p <0.0001 for total monocyte count, Mon 1 and Mon 2; p= 0.03 for Mon 3). The phagocytic activity of Mon 1 was significantly higher in day 1 in patients who developed MACE (p= 0.004). This is in conjunction with a significantly higher phagocytic activity for Mon 2 at day 14 (p= 0.005). There was no difference in the IKKβ levels, and hence NFκB pathway activity, between patients who developed MACE and those who did not have MACE events (Table 34)

At day 14, there remained to be a significantly higher count of Mon 1 and Mon 2 amongst patients who developed MACE events (p= 0.03 and p=0.002 respectively) (Table 34)

Table 33: Difference in monocyte subset counts and functional assessment in patients who developed MACE vs no MACE

	MACE (n= 75)	No MACE (n= 134)	p Value
Total Monocyte count	1064 ± 272	736 ± 239	<0.001
Mon 1 (cells/µl)	794 ± 221	546 ± 203	<0.001
Mon 2 (cells/µl)	200 ± 122	134 ± 104	<0.001
Mon 3 (cells/µl)	70 ± 46	56 ± 34	0.03
IKKβ Mon 1 (MFI)	70 ± 24	72 ± 27	0.48
IKKβ Mon 2 (MFI)	65 ± 22	68 ± 23	0.41
IKKβ Mon 3 (MFI)	71 ±23	72 ± 23	0.75
Phagocytosis Mon 1 (MFI)	129 (110 – 152)	114 (95 – 146)	0.004
Phagocytosis Mon 2 (MFI)	111 (84 – 141)	105 (105 – 149)	0.005
Phagocytosis Mon 3 (MFI)	27 (31 – 60)	38 (27 – 52)	0.47
Day 14 results (phase 2) at follow up			
Total Monocyte count	608 ± 206	537 ± 327	0.14

Mon 1 (cells/μl)	433 ± 177	379 ± 121	0.03
Mon 2 (cells/μl)	117± 80	78 ± 43	0.002
Mon 3 (cells/μl)	58 ± 41	47 ± 21	0.08
IKKβ Mon 1 (MFI)	64 ± 20	64 ± 24	0.87
IKKβ Mon 2 (MFI)	67 ± 26	63 ± 23	0.45
IKKβ Mon 3 (MFI)	69 ± 16	65 ± 20	0.29
Phagocytosis Mon 1 (MFI)	115 (96 – 139)	114 (95 – 141)	0.93
Phagocytosis Mon 2 (MFI)	114 (93 – 149)	113 (90 – 141)	0.89
Phagocytosis Mon 3 (MFI)	43 (23 – 70)	42 (28 – 55)	0.63

Data are expressed as mean ± standard deviation (SD) for normally distributed data; or median with (interquartile range) for non-normally distributed data

6.4.3 Univariate Cox regression model

A univariate Cox regression model indicated that independent predictors of MACE amongst recruited patients were age and gender from the patient demographics (HR of 1.03 and 1.89, p= 0.008 and 0.03 respectively) (table 34). Male gender nearly doubled the risk of MACE, with higher age and impaired kidney function marginally increasing risk of adverse cardiac event. CVA, cerebrovascular accident; CABG, previous coronary artery bypass grafting were not predictive of MACE in our patient population.

Biochemically having a high troponin level and impaired renal function also predicted MACE in recruited patient population risk, (HR 1.002, p=0.003; HR 0.99, p=0.03). This is in keeping withtraditional cardiovascular disease risk factors,

Table 35: Univariate Cox regression of factors predicting MACE

Patient Demographics		Unadjusted Cox regression		
		Hazard Ratio	CI	p Value
Age		1.03	1.007-1.050	0.008
Gender		1.89	1.04 – 3.47	0.03
Diabetes Mellitus		1.22	0.75 – 1.99	0.42
Hypertension		1.18	0.75 – 1.87	0.47
Hypercholesterolaemia		1.32	0.84 – 2.08	0.23
CVA		1.41	0.65 – 3.07	0.39
Smoking		1.04	0.66 – 1.64	0.88
Obesity		1.07	0.56 – 1.99	0.83
History of Ischaemic Heart disease				
Previous myocardial infarction		1.71	0.98 – 2.98	0.06
Previous CABG		1.31	0.48 – 3.60	0.59
Previous PCI		1.39	0.67 – 2.90	0.38
Angina		2.55	1.17 – 5.56	0.02
Time to Presentation/hour		1.07	0.99 – 1.08	0.08
Time to recruitment / hour		1.02	0.97 – 1.07	0.40
LAD infarct		1.33	0.85 – 2.10	0.21
Inferior STEMI		1.63	0.99 – 2.68	0.05
Peak Troponin (ng/ml)		1.002	1.001 - 1.002	0.003
Peak CK		1.000	1.00 – 1.00	0.49
Total WCC		1.003	0.99 – 1.007	0.12
eGFR ml/min/1.73m^2		0.99	0.97 – 0.99	0.03
Inflammatory markers				
MMP- 9		2.50	1.45 – 4.32	0.001
TNF-α levels at day 1		3.15	1.72 – 5.76	<0.001
Phenotypic and functional characteristics of monocyte subpopulations				
Day 1	Total Monocyte count/ 1 cell	1.002	1.002 -1.003	‹ 0.0001
	Mon 1 / 1 cell	1.003	1.002 - 1.004	‹ 0.0001
	Mon 2 /1 cell	1.004	1.002 - 1.005	‹ 0.0001
	Mon 3/ 1 cell	1.005	1.000 - 1.005	0.056
	Total Monocyte count/ 100 cell	1.277	1.202 - 1.356	‹ 0.0001
	Mon 1 / 100 cell	1.329	1.232 - 1.433	‹ 0.0001
	Mon 2 / 50 cell	1.197	1.100 - 1.302	‹ 0.0001
	Mon 3 / 50 cell	1.053	0.99 – 1.528	0.061
	IKKβ Mon 1 / MFI	0.997	0.99 - 1.007	0.56
	IKKβ Mon 2 / MFI	0.996	0.986 - 1.007	0.47
	IKKβ Mon 3 / MFI	1.000	0.990 -1.01	0.98
	Phagocytosis Mon 1/ MFI	1.012	1.005 - 1.019	0.001
	Phagocytosis Mon 2/ MFI	1.008	1.003 - 1.014	0.001
	Phagocytosis Mon 3/ MFI	1.010	1.002 - 1.019	0.020
Day 2	Total Monocyte count/ 1 cell	1.002	1.001 - 1.003	<0.0001
	Mon / 1cell	1.002	1.000 - 1.003	0.03
	Mon 2 /1 cell	1.005	1.002 - 1.008	0.001
	Mon 3/ 1 cell	1.005	0.999 - 1.011	0.12
	IKKβ Mon 1 / MFI	1.001	0.989 – 1.013	0.89
	IKKβ Mon 2/ MFI	1.005	0.995 - 1.016	0.33
	IKKβ Mon 3/ MFI	1.010	0.996 - 1.023	0.16
	Phagocytosis Mon 1/ MFI	0.999	0.991 - 1.006	0.72
	Phagocytosis Mon 2/MFI	1.000	0.994 - 1.007	0.97
	Phagocytosis Mon 3/MFI	1.008	0.997 - 1.018	0.16

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6.4.4 Multivariate Cox regression model:

Using this data a multivariate model was built including: age, sex, peak troponin, peak CK, total WCC, renal function (as demonstrated by eGFR levels), the patient sustaining an anterior infarct, and the time delay of patient presenting to hospital. The model also included previous history of myocardial infarction, angina, hypertension, diabetes, hypercholesterolaemia, and being a current smoker. Also levels of MMP- 9 and TNF-α at recruitment point. This multivariate model indicated total counts of monocytes, monocyte 1, and monocyte 2 as well as the phagocytic activity of Mon 1 and Mon2 measured at the index event as well as Mon 2 counts measured in the recovery phase 2 weeks post recruitment, were predictive of MACE (Figure 30).

Table 36: Multivaraite Cox regression model

	Hazard ratio	CI	p Value
Total Monocyte count (/ 100 cells)	1.528	1.288 - 1.791	<0.0001
Mon 1 (/100 cells)	1.639	1.186 – 2.266	0.003
Mon 2 (/100 cells)	1.280	1.049 - 1.563	0.015
Phagocytosis Mon 1 (MFI)	1.022	1.005 - 1.039	0.011
Phagocytosis Mon 2 (MFI)	1.017	1.002 - 1.032	0.022
Phagocytosis Mon 3 (MFI)	1.010	0.996 - 1.024	0.167

Multivariate model demonstrates that total counts of monocytes, Mon 1, and Mon 2 as well as the phagocytic activity of Mon 1 and Mon2 measured at the index event are positively predictive of MACE (the hazard ratio and p Values are summarised in table 36).
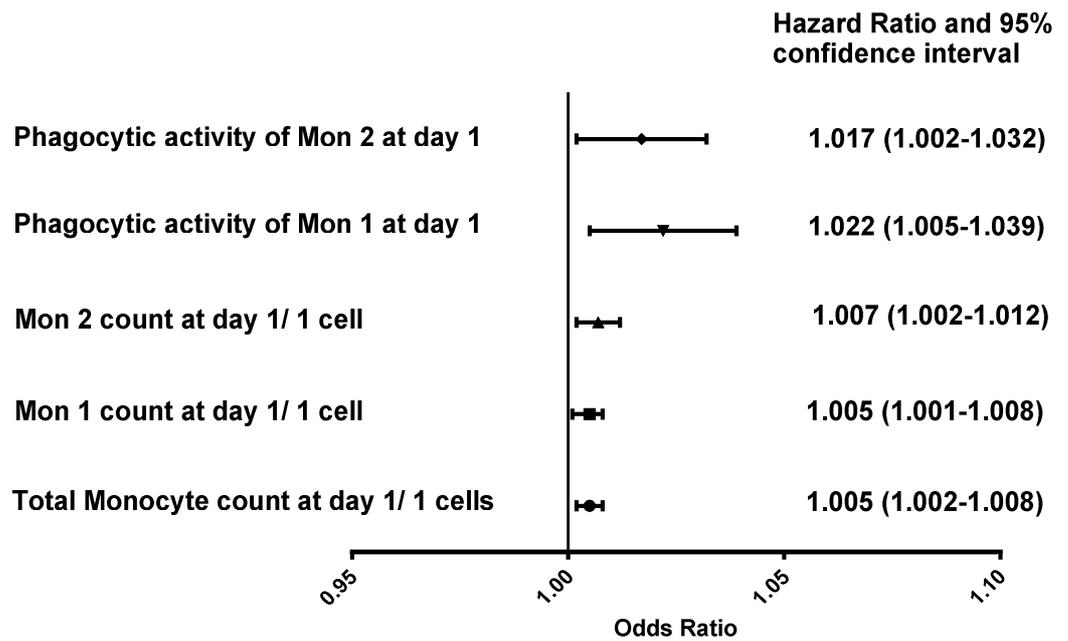
Figure 30: Forest plot of independent factors predicting MACE on multivariate analysis: Total Mon1 and Mon 2 count at day 1 as well as the phagocytic activity of both subsets were independent predictors of MACE with significant hazard ratios.

6.4.5 Monocyte count quartiles and clinical outcome

6.4.5.1 Higher counts of Total monocyte counts predict worse clinical outcome

After stratifying the study cohort by monocyte subsets cell counts into quartiles, higher counts of total monocytes, Mon 1 and Mon 2, but not Mon 3 were univariately associated with any MACE events as illustrated by Kaplan Meier survival curves (Figures 31, 32, 33 and 34 respectively). Significant higher counts for the monocyte subsets were presented in the third and fourth quartiles of each subpopulations.

The effect of higher total monocyte count in predicting MACE continued for 400 days post index event (Figure 31)
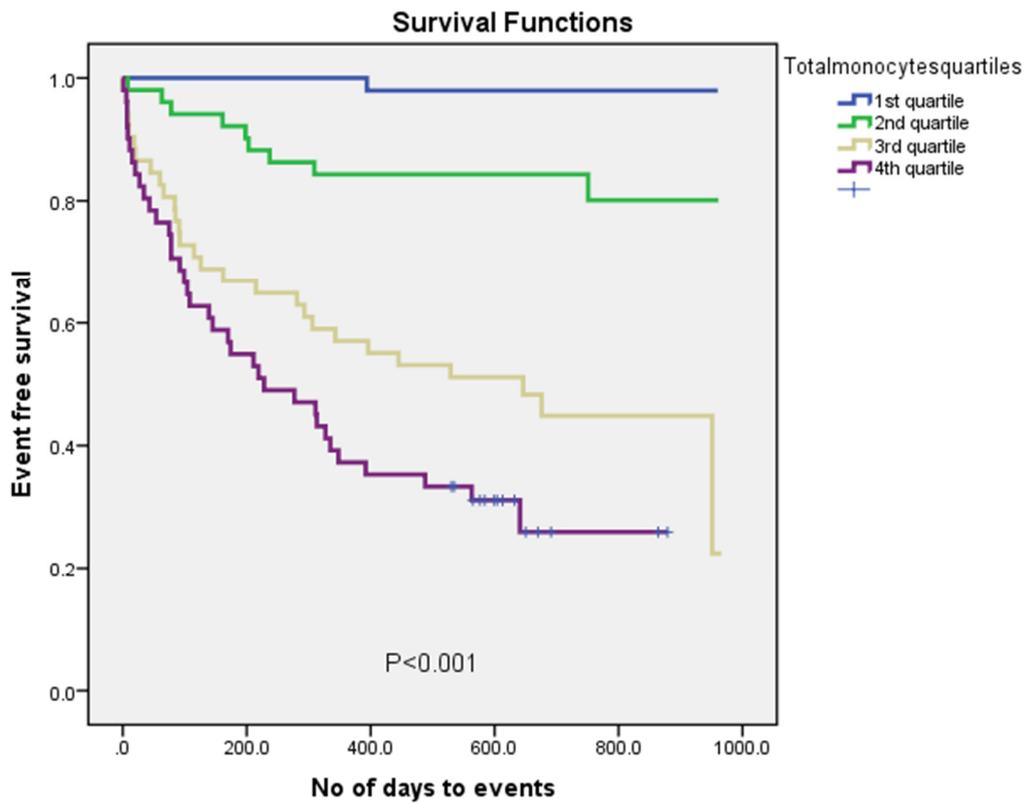
Figure 31: Total monocyte count quartiles and ability to predict MACE: There was a significant increase in mortality in patients who had higher total monocyte counts presented in the third and fourth quartiles compared to counts in the first two quartiles. Eighty per cent of outcomes occurred in patients whose total monocyte counts were in the third and fourth quartiles.

6.4.5.2   Higher Mon 1 counts predict worse clinical outcome

Patients who had Mon 1 counts in the higher third and fourth quartiles were strongly predictive of MACE events an effect that was maintained for more than 600 days (Figure 32).
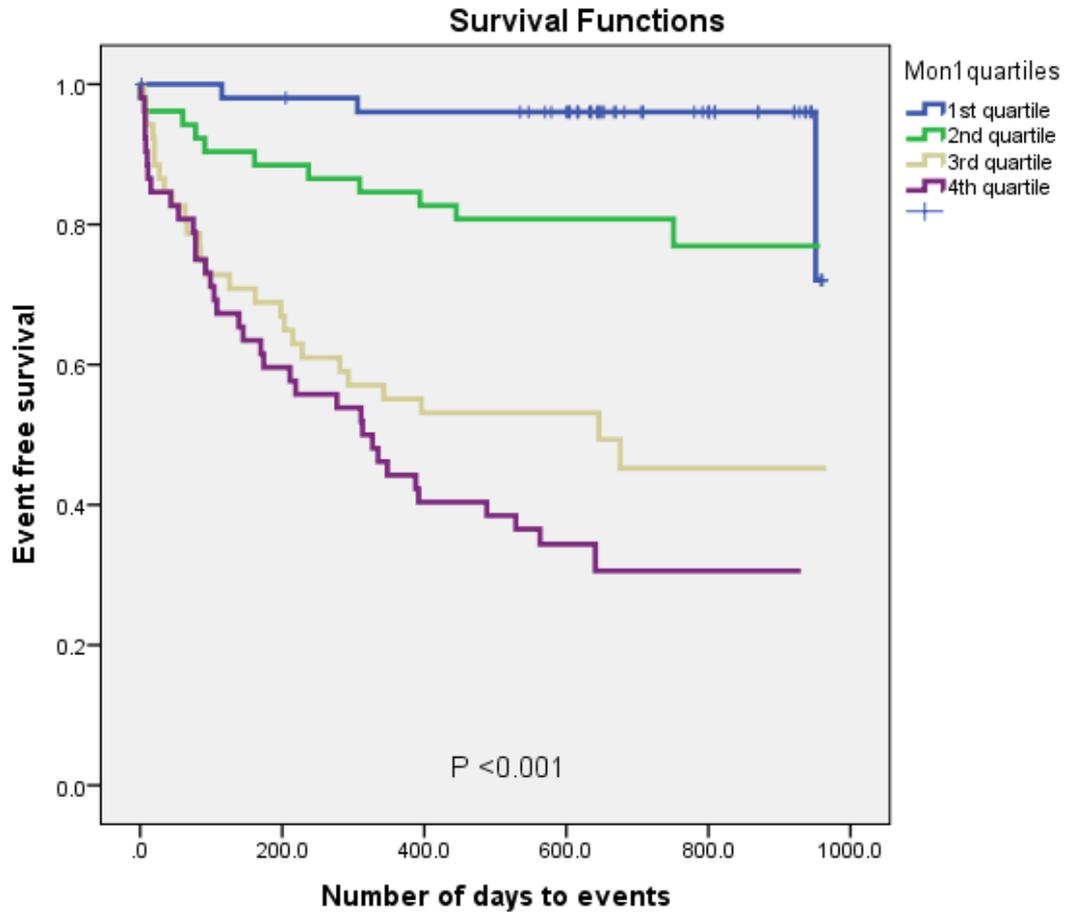
Figure 32: Mon 1 count in Kaplan Meier survival curve: There was a significant increase in mortality in patients who had Mon 1 in the third and fourth quartiles compared to the first two quartiles. Eighty per cent of outcomes occurred in patients whose total monocyte count were in the third and fourth quartile**.**

6.4.5.3   Higher Mon 2 counts predict worse clinical outcome

There was a significant increase in MACE events in patients who had monocyte counts in the higher third and fourth Mon 2 counts compared to the first and second quartile counts. This effect persisted 333 days post index event.
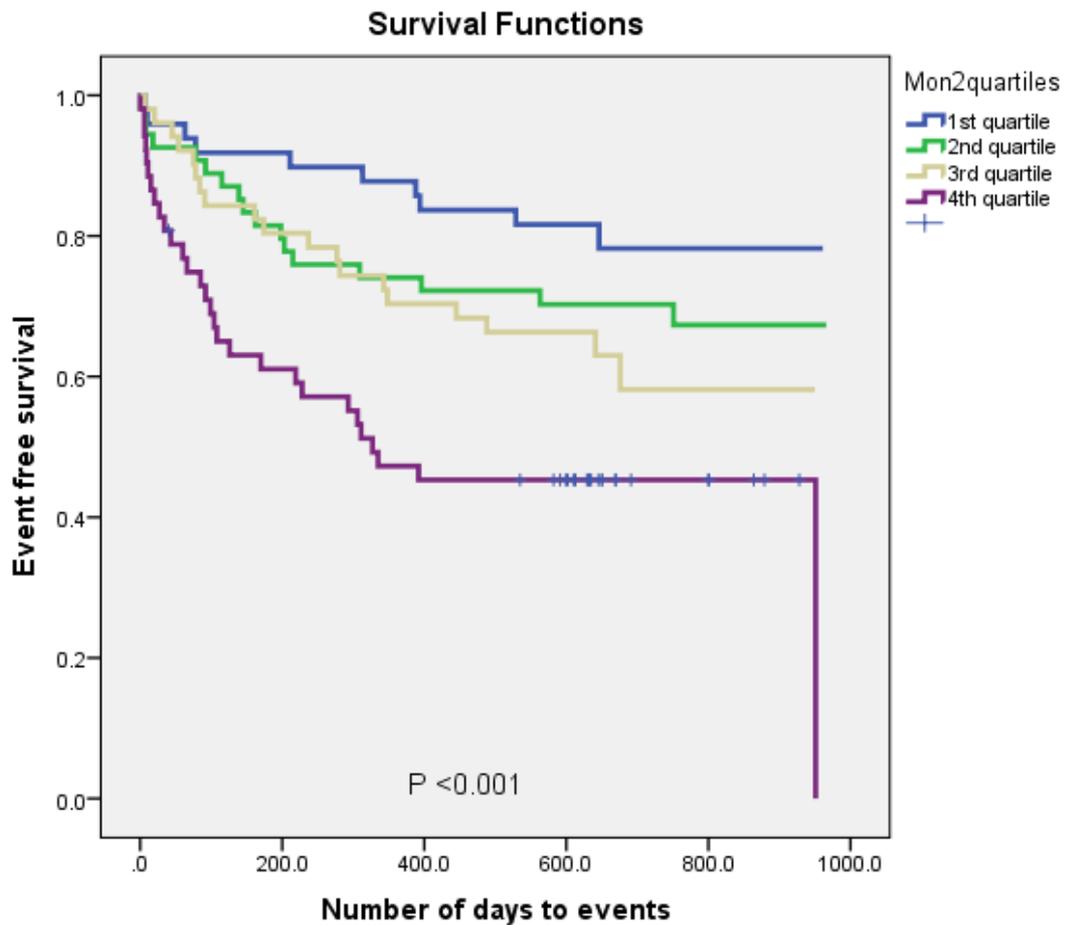
Figure 33: Mon 2 count in Kaplan Meier curve: There was a significant increase in mortality in patients who had higher Mon2 in the third and fourth quartiles compared to the first two quartiles.

6.4.5.4   Higher Mon 3 counts do not predict outcome in STEMI patients

Higher quartiles of Mon 3 did not increase risk for MACE events. There was no difference in the

effect of the four quartiles of Mon 3 on clinical outcome at follow up.
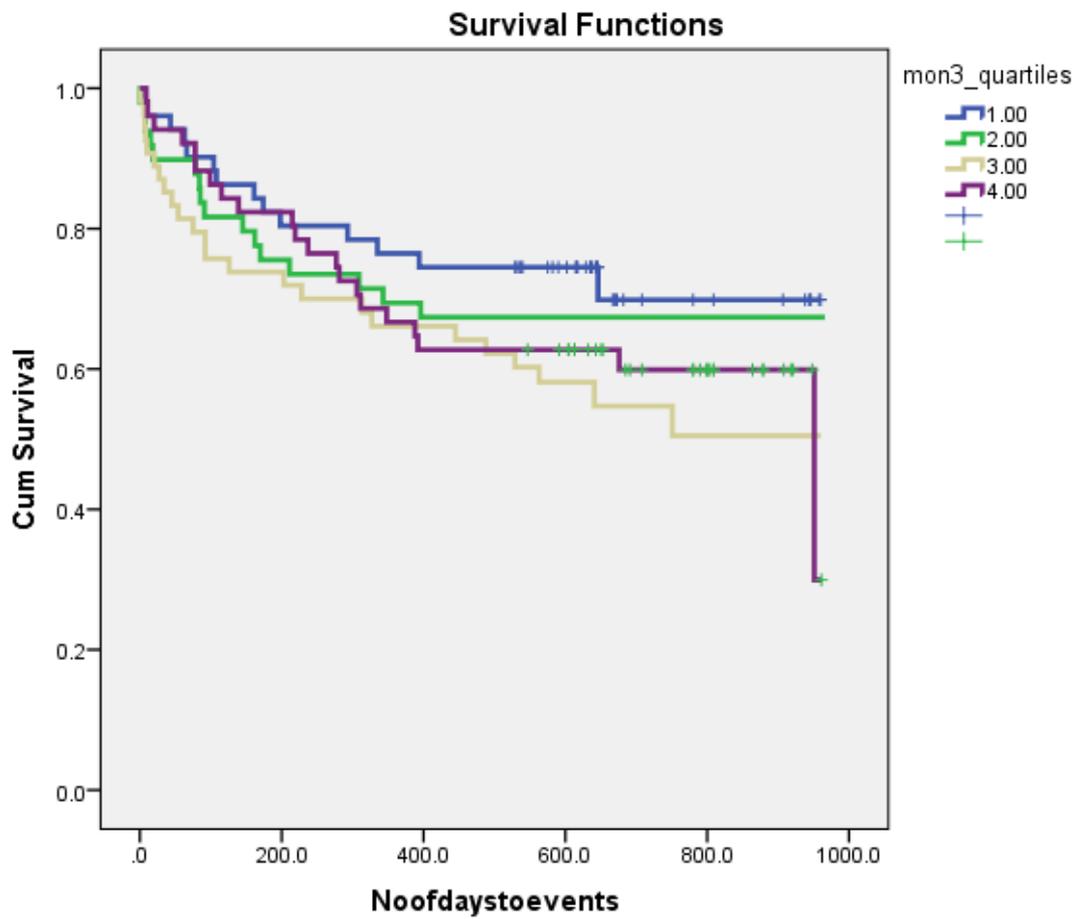
Figure 34: Mon 3 quartiles does not increase risk for developing MACE in STEMI patients: There was no statistically significant difference between the four quartiles of Mon 3 counts.

6.4.6 C statistics for total monocyte count, Mon 1 and Mon 2

C statistics analysis was performed to assess the performance of monocytes in addition to the already clinically established TIMI STEMI score in risk stratifying patients' risk of mortality at 30 days. Receiver operator curve (ROC) analysis showed that total monocytes and Mon1 had the best predictive performance for MACE (area under the curve of 0.85, p<0.0001; and 0.81 p<0.0001 for total monocyte count and Mon 1 respectively) (Table 37). In our cohort the TIMI score had poor discriminatory capacity for MACE as reflected by the c-indexes in Figure 35. Area under the curve for the TIMI scoring system is 0.64
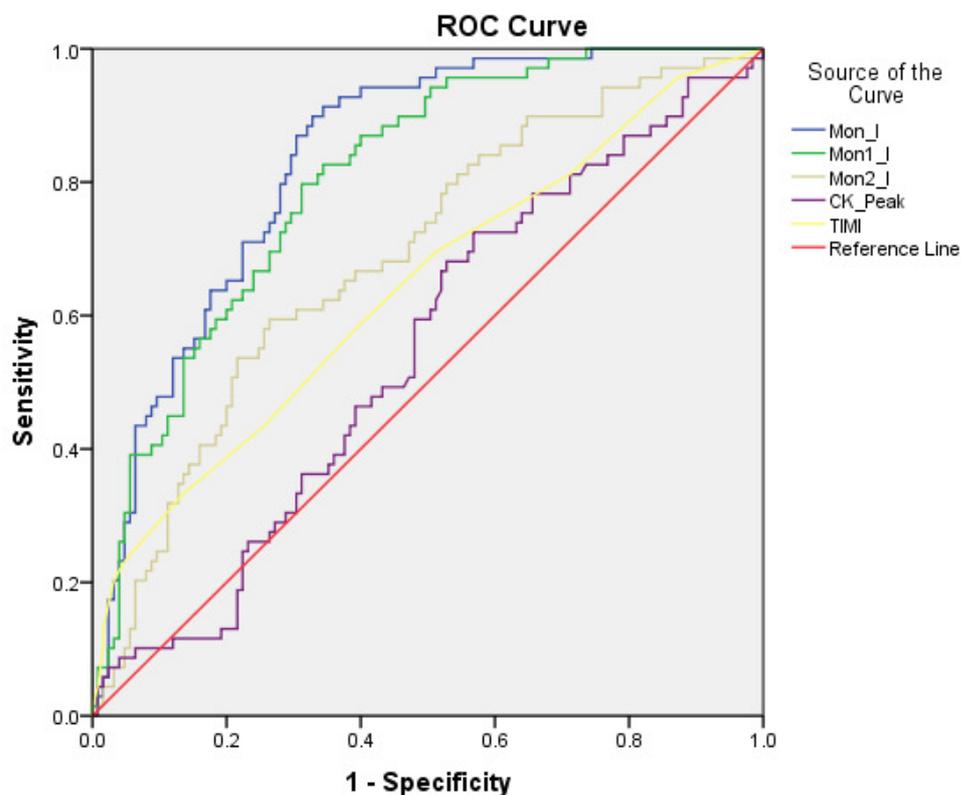
Figure 35: Receiver operator curves (ROC) comparing the discriminatory power of total monocyte counts, Mon 1, Mon 2, TIMI score and peak CK levels. The paramter of Mon 1 shows the highest discrimnatory power (area under the curve of 0.85, p<0.0001) and the CK parameter showing no discrimnitaory effect. The legend gives the indices in order of increasing area under the curve.

Table 34: Area under the curve of the TIMI Score

Parameter	Area under curve	95% CI	p Value
Total Mon count (cells/ µl)	0.85	0.78 – 0.89	<0.0001
Mon 1 (cells/ µl)	0.81	0.74 – 0.87	<0.0001
Mon 2 (cells/ µl)	0.69	0.61 – 0.77	<0.0001
Creatine Kinase	0.55	0.46 – 0.63	0.31
TIMI Score	0.64	0.56 – 0.72	0.001

6.4.6.1 Total monocyte count

Nevertheless, after incorporating total monocyte count to the TIMI STEMI model, the sensitivity of model in predicting poor outcome improved (Figure 36) with an area under the curve of 0.77 (TIMIMon score in Table 37)
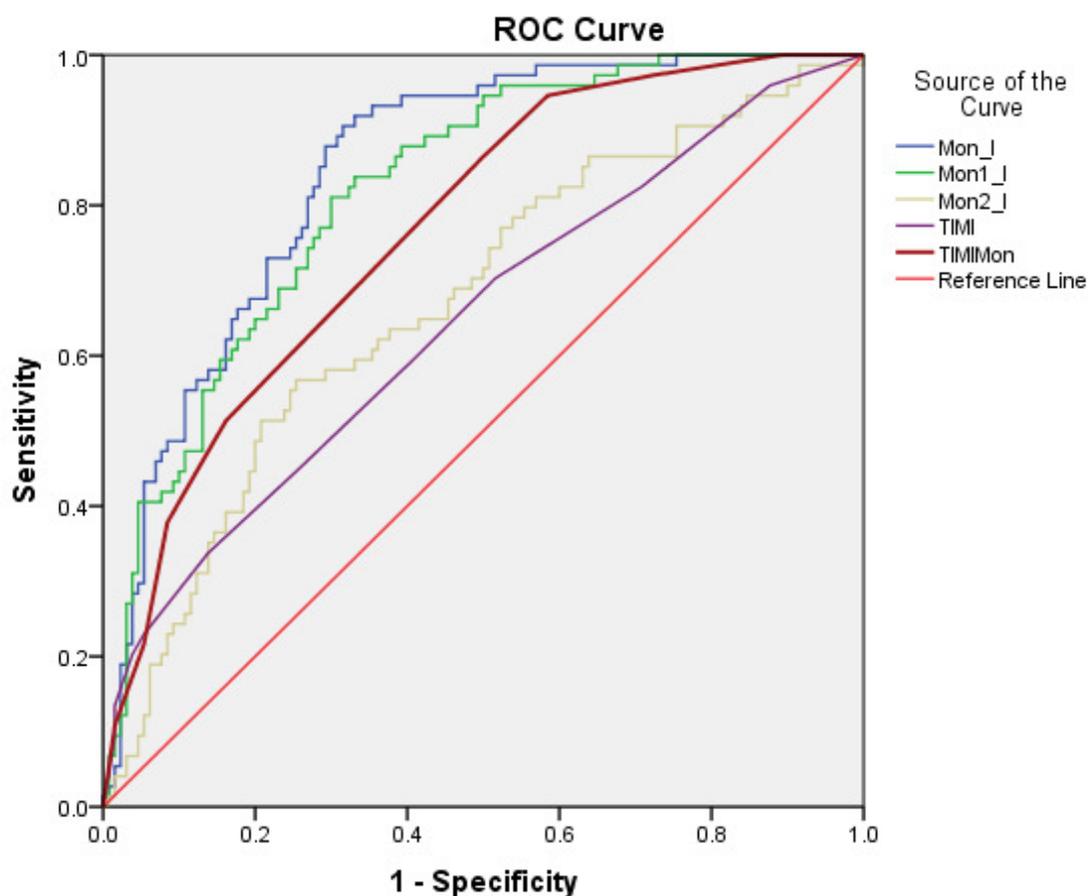
Figure 36: Receiver operator curves (ROC) comparing the discriminatory power of total monocyte counts, Mon 1, Mon 2, TIMI score and TIMIMon score. The paramter of Mon 1 shows the highest discrimnatory power. The combined TIMIMON paramter has higher discriminatory power (Area under the curve of 0.77) than TIMI score and Mon2 as separate

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paramters (area under the curve of 0.65 and 0.67 respectively). The legend gives the indices in order of increasing area under the curve.

Table 35: Area under the curve for monocyte counts

Parameter	Area under curve	95% CI	pValue
Total Mon count (cells/ μl)	0.85	0.80 – 0.90	<0.000
Mon 1 (cells/ μl)	0.82	0.76 – 0.80	<0.000
Mon 2 (cells/ μl)	0.67	0.59 - .75	<0.000
TIMI	0.65	0.57 – 0.73	0.001
TIMIMon Score	0.77	0.70 – 0.83	<0.001

Finally comparing the difference between the areas under the curves for each of ROC curves was carried out. It indicated that the differences between total monocyte count vs Mon 1, Mon 2 and TIMI score were significantly different (0.03, 0.2, 0.2 respectively, p<0.0001 respectively) (Table 36).

Table 36: Comparison of area under the curve

ROC comparisons of area under curve	Difference of ROC	95% confidence Interval	Z Test	pValue
Total Monocyte vs TIMI	0.20	0.11 – 0.29	-6.50	<0.0001
Total monocyte vs Mon 1	0.03	0.01 – 0.06	-24.9	<0.0001
Total Monocytes vs Mon 2	0.20	0.11 – 0.25	-8.95	<0.0001
Mon 1 Vs TIMI	0.17	0.08 – 0.27	-6.79	<0.0001
Mon 1 vs Mon 2	0.15	0.06 – 0.25	-7.19	<0.0001
Mon 2 vs TIMI	0.02	0.09 – 0.12	-8.97	<0.0001

Comparison of area under curve between different comparison models for predicting MACE. Total monocyte count vs TIMI, Total monocyte count Cs Mon 1; Total monocyte count vs Mon 2; Mon 1 vs TIMI; Mon 1 vs Mon 2; and Mon2 vs TIMI. Statistical analysis for difference between areas under the curve was done via Z- statistics (Analyse-it ®- Excel 2013).

6.4.7 Dichotomous data in Cox regression model

Using the ROC analysis curve, I was able to determine the total monocyte count, Mon 1 and Mon 2 counts that provided the best combination of sensitivity and specificity in predicting adverse clinical outcome. These counts were 818 cells/ml; 620 cells/ml; 117 cells/ml for total monocyte count, Mon 1, and Mon 2 respectively. As discussed in statistics section, I created dichotomous variable for total monocyte count, Mon 1, and Mon 2 and included that in a multivariate Cox regression model. This indicated that higher total Monocyte count, Mon 1, and Mon 3 carry a higher hazard ratio (HR) in predicting MACE events if higher monocyte subset counts are studied (HR 5.2, $p < 0.0001$; HR 4.6, $p<0.0001$; HR 2.6, $p=0.005$).

6.5 Discussion

In this section of the study the results of follow up of the 209 recruited patients and the MACE events incurred are described. The predominant adverse cardiac event was developing heart failure, based on echocardiographic findings and clinical symptoms. My study is the largest prospective study with the longest follow up of consecutive STEMI patients treated with percutaneous coronary intervention (PCI) and receiving adequate antiplatelet therapy during PCI according to ESC guidelines for revascularisation for STEMI.

A landmark paper investigating the effect of intermediate monocyte on the outcome of 900 patients undergoing coronary angiography, indicated that solely Mon 2 was associated with cardiovascular outcome at three years follow up. My study now confirms the negative predictive effect of the CD14++CD16+CCR2+ Mon 2 subset, equivalent to intermediate monocyte, in STEMI patients both acutely and at the beginning of recovery phase. The fact that total monocyte count and Mon1 at the index point were predictive, in contrast to the Rogacev et al. paper, could be explained by the differences in the populations studied (Rogacev et al., 2011) whilst I studied acute myocardial infarction they followed up patients with stable coronary artery disease undergoing coronary angiography.

The phagocytic activity of monocytes appeared to be the monocyte functional mechanism affecting patients' outcome with no role from NFκB activation pathway. This is in keeping with presumed increase in phagocytic activity of monocytes in the acute phase post infarction. As MACE events were driven by development of clinical and echocardiographic features of heart failure, an increase in the number of local monocyte and their phagocytic activity can lead to adverse cardiac remodelling hence increased rate of MACE.

The results of this section of my study provide more evidence on the function of the recently identified monocyte subset 2, suggesting an inflammatory role for Mon 2. My study also suggests that the phagocytic activity of monocytes is the predominant function of subset 1 and subset 2. These results suggest that monocytes subsets especially subset 1 and 2 pose important information

for future patient prognostication as well as forming a possible focus for new medical therapies in improving patient outcome.

There was no effect of measuring IKKβ and clinical outcome. Despite the well proven association of the NFκB pathway and developing heart failure, it is important to acknowledge that most of these studies were done in murine models. Human based studies were not performed with long follow up period, until my study was carried out.

ROC analysis and C- statistics of total monocyte counts, Mon 1 and Mon 2 indicate a very good area under the curve indicating that patients with higher monocyte counts, Mon 1 and Mon 2 are more likely to develop an adverse cardiac event compared to patients with lower counts. The area under the curve was better for monocyte subsets compared to Troponin levels and TIMI score independent. This provides an immediate avenue for the utilisation of monocyte counts measurement in clinical practice. The ability to risk stratify patients likely to develop adverse cardiac events depending on their monocyte subset counts, would be able to tailor secondary preventative medical therapy to those patients who are at risk. This is likely to improve both the cost effectiveness of secondary preventative therapy as well as scientifically individualising patient care.

6.6 Conclusion

This study is the largest study with the longest follow up period investigating the impact of three different monocyte subsets on long term outcomes. It investigated the impact of monocytes both at the time of infarct as well as 2 weeks into recovery. It indicates that higher total count of monocytes, as well as Mon 1 and Mon 2 are associated with worse outcome This was associated with an area under the curve of 0.85 and 0.6 for Mon1 and Mon 2 respectively. The phagocytic activity of monocytes was the functional mechanism that significantly and negatively impacted on patients' outcome. There was no statistically significant effect from the NFKB activation pathway suggesting that it is the overzealous activity of the phagocytic of monocytes.

Chapter 7

Conclusions

7.1 Final Conclusion

This thesis was the largest study investigating the phenotypic and functional characteristics of the three monocytes subpopulations, as defined of recent years [Mon 1 (CD14++CD16-CCR2+); Mon 2 (CD14+CD16+CCR2+); Mon 3 (CD14-CD16++CCR2-)], in STEMI patients. After a plethora of descriptive data on surface markers of monocyte subsets to try to establish their functional characteristics, in this study I investigate two of the functional pathways through which monocytes implement their function: namely inflammation and phagocytosis.

In chapter two for the first time, we describe that the monocyte subset distribution counts in peripheral and coronary circulation are not statistically different. This finding supports the concept of utilising peripheral sampling to study monocyte subset in whole venous blood. In this chapter the effect of STEMI on monocyte counts is characterised. All three monocyte subsets were significantly increased in STEMI patients compared to healthy individuals and patients with underlying stable coronary artery disease.

I studied the phagocytic activity of monocyte subsets in chapter 3. A robust, reproducible, rapid flow cytometry based assay for the assessment of monocyte subset phagocytic activity in whole blood is described. The method was validated in multiple physiological states. I demonstrated that 24 hours post exercise there was an increase in phagocytic activity of Mon1 and Mon3 in response to extreme exertion. I also illustrated that the phagocytic activity of monocytes were higher for all monocyte subsets in STEMI patients compared to healthy controls and patients with stable coronary artery disease. There was also a no significant change in Mon 1 and Mon 3 phagocytic action at the second phase post STEMI. However there was no reduction in phagocytic activity of Mon 2 at 14 days. This persistent activity of Mon 2 could explain the effect of Mon 1 and Mon 2 in ventricular remodelling and clinical outcome. Indeed in chapter 5, I describe for the first time that the phagocytic activity of Mon 1 and Mon 2 were independent predictors of negative ventricular remodelling. Later in chapter 6, I describe that the phagocytic activity of Mon 1 and Mon 2 were independent predictor of major adverse cardiac events and increased the risk of mortality.

Chapter 3 demonstrated a small pilot study of monocyte chemotaxis in STEMI patients in response to MCP-1. Interestingly there appeared to be an inhibitory effect in the plasma of patients sustaining STEMI and those patients with stable coronary artery disease, however this inhibitory effect was lost in patients who sustained a MACE therefore leading to an exponential rapid attraction of monocytes.

I then investigated the inflammatory role of monocytes in chapter 4, studying the effect of monocyte activated NFκB inflammatory pathway through assessment and measurement of IKKβ activity in STEMI patients. The method was validated in isolated CD14+ve monocyte. There was no correlation between monocyte subset and IKKβ levels. Even though the methodology has been validated, studying this pathway did not further our understanding of monocytes effect on this particular inflammatory pathway. This may be due to the interaction of several other inflammatory pathways at cellular level, e.g. effect of reactive oxygen species release and activation of heat shock proteins on inflammatory pathways. The complexity of the micro-environmental milieu at infarct level with various autocrine effects on multiple interconnected inflammatory pathways, makes it difficult to target a specific inflammatory pathway during infarct, as has been shown in clinical trials utilising immunosuppressant medication, with minimal benefit to improving patient outcome. In chapters 5 and 6 there was also no relationship between IKKβ and ventricular remodelling or clinical outcome respectively. In chapter 4 total monocyte count, Mon1, and Mon 2 as well as phagocytic activity of Mon 1 correlated with TNF-α and MMP- 9.

In chapter 5, I demonstrate that total monocyte counts, Mon 1, and Mon 2 are independent predictors of ventricular remodelling in STEMI patients, this was indicated in both the univariate and multivariate linear regression models. The monocyte quartiles were negatively associated with EF with higher counts of total monocytes, Mon 1 and Mon 2 being associated with lower EF.

In this study I report for the first time Mon 1, Mon 2 and phagocytic activity of Mon 1 as being independent predictors of MACE. Kaplan Meier survival curve indicated that patients who had

higher quartiles counts of total Monocyte count, Mon 1 and Mon 2 developed a MACE event earlier than patients who were in the lower quartile groups with 80% of patients who sustained a MACE had monocyte counts in the 3rd and 4th quartile of total monocyte count, as well as Mon 1, and Mon 2.

In summary, in this study I describe that Mon 1 and Mon 2 are predictive of negative ventricular remodelling, MACE and earlier mortality. Phagocytosis appears to be the functional mechanism through which monocytes exert their action. Results from this study suggest that despite surface expression of VCAM and ICAM, Mon 2 has a similar inflammatory role to Mon 1. This is in contrast to the angiogenic and fibrotic role (similar to that of Mon3) that Mon2 were previously thought to effect. It might be that it is time to consider the nomenclature of monocyte subsets, and start referring to Mon 1 as phagocytic monocytes, rather than inflammatory monocytes as more and more data becomes available from human subjects.

7.2 Limitations

This was a well powered study that achieved all its endpoints. The lack of fate mapping studies of the three different monocyte subsets was a drawback of this study. Investigating the role of reactive oxygen species in STEMI and their association with monocyte activity could have helped us to establish another functional role of monocyte subsets.

Immunological imaging of monocytes journey during an infarct would add more information on the function of these subsets. Iron based tagging of monocytes was also not available in our local facilities and hence were not possible to perform. The number of patients completing two echocardiogram scans was 60% of the recruited study however as the study was well powered this still met targets for assessing LV remodelling.

This study did not record the glucose level of patients at the point of admission and there was no subgroup analysis of diabetic patients.

7.3 Future Direction

This project adds functional information to the descriptive data that are already available on the possible inflammatory role of the three monocyte subsets and particularly on the intermediate monocyte subpopulation. It provides more evidence supporting an inflammatory role for Mon 2 but very little information is available on this population's transit time before becoming macrophages. It also comes in contrast to a recently published data by Lu et al. investigating 70 patients with NSTEMI and diabetes and concluding that Mon2 has a beneficial effect on cardiac remodelling (Lu et al., 2014). Besides a much bigger sample, our project investigates all comers and not just diabetic patients. As discussed, the admission blood glucose levels were not studied. Future studies need to investigate diabetic patients separately.

This study raises multiple questions for future study: the lifespan of Mon2 subpopulations and fate mapping experiments are the second natural step following on from my research. Addressing the question of whether monocyte sub population 2 matures into macrophages 1 or 2 would be important to answer as the resulting macrophages are likely to be resident in myocardial tissue for longer duration compared to monocytes thus allowing for targeted medical intervention. It is now an accepted fact that full eradication of resident macrophages could be detrimental to patient outcome hence partly accounting for the failure of anti-inflammatory medication to show, as yet, any promise in reducing adverse patient outcome. Finally, studying the role of monocyte mRNA in humans might be able to shed more light onto the differentiating capacity of monocyte subsets.

8 Appendices

I- Patient recruitment:

Patients with STEMI admitted to Heartlands Hospital, Queen Elizabeth Hospital and Sandwell and West Birmingham Hospitals (SWBH) NHS Trust. Patients who met the study criteria were recruited between March 2011 and December 2012. Details of patients recruited are included in the appendix A. from both the City Hospital and Sandwell General Hospital sites within the Trust. The cardiology Consultants within the Trust work at both sites and undertake PCI for management of STEMI patients. All operators adhere to the Trust policy on management of patients with STEMI, and hence there is little variation in practice and management of patients with STEMI.

For the longitudinal study, two hundred and nine patients with ST elevation myocardial infarction (STEMI) were recruited. Patients were approached in 5 teaching hospitals (City, Sandwell District General Hospital, Birmingham Heartlands Hospital–serving a population catchment of 1.2 million overall. Patients were recruited within 24 hours post-admission.

ST elevation myocardial infarction is defined by well-established clinical grounds, with a history of typical chest pain and concurrent electrocardiographic (ECG) changes, as defined using the European Society of Cardiology guidelines. All recruited patients had undergone a PCI as the default reperfusion strategy. As part of the standard clinical care, patients will have received high dose aspirin and another antiplatelet therapy (namely clopidogrel, prasugrel or ticagrelor). They will have also received standard cardiovascular prevention drug therapies (statins, ACE inhibitors, beta-blockers as well as antiplatelet therapy). Informed consent has been obtained from all patients.

II- Control Subjects

Data from control subjects were utilised to compare counts in a cross sectional study with the main STEMI population patients. Hence 20 stable coronary artery disease patients were recruited.

These are identified as patients with stable coronary artery disease, confirmed during elective coronary angiography, with no hospital admission for ≥ 9 months. This group was recruited from cardiology outpatients or cardiac rehabilitation programmes.

Healthy controls are identified as subjects who had no apparent risk factors for ischaemic heart disease and who did not suffer from vascular, metabolic, neoplastic or inflammatory disease. This was confirmed by careful history, examination and routine laboratory tests. They were recruited from a general practice facility (Baldwins Lane Surgery, Hall Green, Birmingham). The patients satisfying the inclusion criteria were approached in surgery during a pre-planned visit. If patients consented for participation they were recruited and a single peripheral venous sample was venesected from the antecubital fossa.

III- Statistical methods

III-i Power Calculation

Power calculations were based on the results by Larose et al. who have shown that 20% of ACS patients have cardiovascular events during one year (Larose et al., 2010). This was performed by resident statistician prior to the study commencing. Briefly, in order to achieve 80% statistical power to detect the difference in the MACE (death, new diagnosis of heart failure and recurrent ACS) (with $p<0.05$) in count of the monocyte subsets, at least 35 MACE events were needed. Thus, at least 175 patients were to be recruited to provide additional confidence and to manage potential drop-out, we aimed to recruit 200 patients for this part of the study. The impact of monocyte subsets and their functional characteristics on cardiac remodelling were assessed by the differences in LV ejection fraction and volumes between tertiles of monocyte parameters. In order to detect 0.5 standard deviation differences in parameters of cardiac remodelling, at least 35 patients were analysed for each quartile (i.e. at least 105 patients) for the echocardiographic study. However 120 patients were recruited for further confidence. Similarly, at least 20 patients were be recruited for each group of healthy individuals and patients with stable coronary artery disease in the cross-sectional part of the study

VI- List of published abstracts:

Ghattas A, Griffiths H, Lip GYH, Shantsila E. Monocyte subpopulations and associations with global longitudinal strain and clinical outcome in ST elevation myocardial infarction patients with normal ejection fraction. ESC 2014. European Heart Journal, 2014; 35: 488

Ghattas A, Griffiths H, Lip GYH, Shantsila E. Monocyte subpopulations counts and associations with global longitudinal strain in ST elevation myocardial infarction patients with normal ejection fraction. BCS 2014.

Ghattas A, Griffiths H, Lip GYH, Shantsila E. Monocyte subpopulations counts and associations with ejection fractionin ST elevation myocardial infarction patients. BCS 2013. London, UK. Abstract number: 043

Ghattas A, Griffiths H, Lip GYH, Shantsila E. Monocytes Functional characteristics of monocyte subpopulations in STEMI patients and their predictive role for major cardiac events. AHA 2012.

Ghattas A, Griffiths HR, Devitt A, Lip GYH, Shantsila E. Functional Characteristics of Monocytes Subsets in the Acute and Healing Phases of ST Elevation Myocardial Infarction and their Predictive Role for Major Adverse Cardiac Events. Circulation 2012; 126: A12560.

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Ghattas A, Griffiths H, Lip GYH, Shantsila E. Predictive role of specific monocyte subsets for major adverse cardiac events after ST-elevation myocardial infarction . ESC 2012. European Heart Journal, 2012; 33: 919-920.

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